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개똥쑥 약초차 제조에서 아르테미시닌의 전기화학적 측정과 차를 만드는 최적화로의 접근법

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Electrochemical Determination of Artemisinin in *Artemisia annua* L Herbal Tea Preparation and Optimization of Tea Making Approach

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요 약. 가끔 먼 지역 거주자들은 현대 의약품이나 의학 서비스에 있어서 불충분하거나 접근할 수 없다. 그들은 개똥쑥의 선택된 품종을 경작하고 차 제조의 적절한 방법에 따라 식물로부터 차나 달인즙을 만드는 것에 의해 말라리아에 대항한 치료의 관점에서 이익을 얻을 수 있다. 아르테미시닌에 대한 최대 추출 효율을 위해, 개똥쑥의 차제조의 다른 방법들은 발달된 DPP방법을 적용하여 연구되었고 이 논문에 서술되었다. 차는 시간을 다르게 하여 3가지 다른 방법으로 제조된다(굽기, 섞거나 섞지 않으면서 굽지 않기 그리고 마이크로 웨이브 오븐). 결과로부터, 아르테미시닌의 더 높은 농도(84.7%)는 15분 동안 섞으면서 굽지 않는 차 제조법에 의해 도달될 수 있다는 것을 발견했다(R.S.D. 2.34%). 아르테미시닌의 농도는 마이크로 웨이브 오븐에서 1.5분 이상 구울 때 감소한다. 최대한도의 추출(88.9%)은 증류수에서 5%에탄올과 함께 섞는 추출방법에서 가능했다(R.S.D. 2.28%).

주제어: 아르테미시닌, 개똥쑥, 차, 펄스차이폴라로그래피(DPP)

ABSTRACT. Sometimes inhabitants in remote areas have inadequate or no access to modern medicines or medical services. They can get benefit in term of the treatment against malaria by cultivating selected breeding of *A. annua* and making teas or decoctions from the plant materials following the proper way of tea preparation. In order to have the maximum extraction efficiency for artemisinin, different ways of tea preparations of *A. annua* were investigated by applying the developed DPP method and described in this article. Tea was prepared by three different ways (cooking, without cooking with/without shaking and microwave oven) with different times. From the results, it has been found that higher concentration of artemisinin (84.7%) can be attained by following the approach for tea preparation without cooking with shaking for 15 minutes (R.S.D. 2.34%). The concentration of artemisinin decreases with cooking more than 1.5 min in microwave oven. The utmost extraction (88.9% of artemisinin) is possible to extract by shaking with boiled 5% ethanol in distilled water (R.S.D. 2.28%).
Keywords: Artemisinin, *Artemisia annua* L, Tea, Differential pulse polarography (DPP)

INTRODUCTION

Malaria is an endemic infectious disease in the world. Every year about 300-500 million people are infected by it and between 1.5-2.7 million infected people die from which most of them are children.[1] Maximum deaths are caused by the most aggressive species *falciparum* having rapid rate of asexual reproduction that can progress rapidly to severe malaria like cerebral malaria. New non-alkaloidal antimalarial drugs artemisinin and its derivatives have become increasingly important in consequence of the fast developing resistance of the malaria parasite *Plasmodium falciparum* to currently used alkaloidal drugs such as quinine and chloroquine. Artemisinin (*Fig.* 1), discovered by Chinese scientists in 1970 and originally isolated from the herb of the Chinese medicinal plant *Artemisia annua*,[2] or Qing Hao, is a potent antimalarial drug against the resistant strains of *P. falciparum*.[3] Clin-

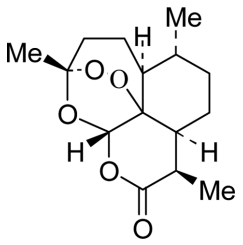

***Fig.* 1.** Chemical structure of Artemisinin.

ical studies showed that artemisinin is an exceptional antimalarial agent with negligible toxicity and high efficacy against all forms of the parasites, including *P. falciparum*.[4]

In 340 AD, *Artemisia annua* was recorded first as the treatment of fever in a medicinal book, "Zhou Hou Bei Ji Fang" (Handbook of Prescriptions for Emergency Treatment). *A. annua* seems to be the unique natural source of artemisinin. Other species of *Artemisia* have been examined but none of them contain artemisinin.[3] The plant is widely used in traditional herbal medicine in the countries of South East Asia. *Herba Artemisiae Annuae* or Qing Hao is contained in the Pharmacopoeia of the People's Republic of China. This plant is commonly used in China with a long history of use (for over 1000 years) as an antipyretic to treat alternate chill and fever symptoms of malaria and other "heat syndromes" in the traditional Chinese medical systems.[5-6] Traditionally the plant is used to prepare a drink as indicated in the compendium of treatments (Ben Cao Gang Mu), written in 1596 AD by Shizhen: "take a handful of qinghao, soak it in a sheng (liter) of water, and squeeze out the juice and drink it all."[7-8]

Artemisinin production in the *A. annua* varies with plant components, time from vegetative phase to full flowering, strain origin concerning plant profile etc which is usually in the range of 0.01% to 0.4% but some hybrids produce over 1%.[9,10] 1.4% artemisinin (dry weight) can be obtained from hybrid plant called "Artemis" which has been developed for commercial artemisinin production.[10] Analysis of artemisin in the traditional herbal tea preparations of *A. annua* is a challenging issue because the compound is thermolabile, the concentration of artemisinin in the plant is low and the intact molecule stains poorly. Moreover it is sensitive to acid base treatment and other compounds in the plant also interfere in its detection. Until now, various methods have been reported for the determination of artemisinin in the plant such as thin-layer chromatography,[11] HPLC with various detection system (UV, EC, ELSD, RI, DAD,[12-18] reversed-phase HPLC,[19] a liquid chromatography-mass spectrometry (LC-MS) method,[20] GC method with FID,[16,21] combined gas chromatography/mass spectrometry method.[22]

However, artemisinin and its derivatives contain an endoperoxy bridge (-O-O-) which is electrochemically active and can be therefore reduced at different electrodes.[23,24] In order to develop an analytical method for antimalarial endoperoxides, polarography is preferred because it is selective, well reproducible and durable. Furthermore it is not necessary to separate auxiliary materials with the result to be not time consuming and economical. In our previous report we have developed a method to estimate artemisinin in crude plants using differential pulse polarography (DPP) method.[25] As our continuous effort we have applied this method to analyse artemisinin in traditional herbal tea preparation of *Artemisia annua*. The purpose of the present study was to investigate the different ways of tea preparations to optimize the artemisinin in the tea solution by analyzing with the developed method of differential pulse polarography. As a result the population in isolated areas will be benefited by making teas or decoctions from the plant materials using appropriate methods of tea preparation to achieve a positive outcome against malaria.

EXPERIMENTAL

Apparatus, plant materials and chemicals

The polarographic investigations were carried out with a polarographic analyzer/stripping voltammeter model 264 A (EG&G, PARC, New Jersey, USA) in combination with a polarographic stand model 303 A SMDE (EG&G) and plotter model RE0150 (EG&G). This electrode stand consists of a dropping mercury electrode (DME) as working electrode, an Ag/AgCl (3M KCl) reference electrode and a platinum wire as an auxiliary electrode. The pH values of the solutions were adjusted employing a Metrohm pH meter Model 632 and a glass electrode model 6.0202.000 (Metrohm AG, Herisau, Switzerland). All measurements were carried out at room temperature. Hot plate/a domestic microwave oven and as a plant "Artemis" a hybrid plants were used for tea preparation. The plant was provided with as gift from Professor Heide of *Tübingen* University of Germany. Artemisinin (pure substance, 98%) was obtained from Acros Organics (Geel, Belgium). All reagents and solvents were of Suprapur and/or Proanalysis grade (Merck, Darmstadt, Germany). Distilled water was purified with a Milli-Q Nanopure® (Millipore, Bedford, MA, USA) system and was stored in Nalgene® containers. The nitrogen used was 99.9995% pure, while the mercury was 99.999% pure

(Oegussa, Graz, Austria). $(NH_4)_2SO_4$ (0.05 M) pH 4-8, KH_2PO_4 (0.1 M) pH 4-7, acetic acid/sodium acetate buffer (0.1 M) pH 3.5-5.5 and Britton Robinson buffer solutions (0.1 M) pH 2-10 were used as supporting electrolytes for fundamental polarographic tests. The proper pH value was adjusted with the addition of 0.5 M NaOH to the buffer solutions mentioned above. The construction of the calibration curve and the analysis of artemisinin in the herbal tea preparation were performed in a phosphate buffer (pH 5.5) mixed with methanol (7:3; v/v). This methanolic buffer solution has a pH value of 6.4. The aqueous buffer solution can be used for two weeks but the mixture with methanol was prepared freshly just before determination. Stock solution of artemisinin was prepared by transferring 15.3 mg artemisinin to a 50 mL volumetric flask, dissolving in methanol and bringing to volume. This solution contains 0.30 mg/mL of artemisinin and is stable for two weeks. Further standard artemisinin solutions were prepared freshly by diluting the stock solution with methanol.

Instrumental parameters for DPP and CV

For preparation of the calibration curve and analysis of the artemisinin in the tea, the analyser was operated under following parameters: Method: DPP; Drop size: M; Drop time: 1s; Potential range: +0.15 to -0.45 V; Scan rate: 5 mV/s; Pulse amplitude: 50 mV; Current sensitivity: 1-2 μA. The following apparatus parameters were set for cyclic voltammetric analysis: Drop size: M; Potential range: +0.15 to -0.35 V; Scan rate: 10-500 mV/s; Current sensitivity: 1 μA and Equilibration time: 15 s.

General procedure for DPP and CV measurements

10 mL of the appropriate buffer were transferred into the polarographic cell. After purging 8 min with nitrogen for deoxygenation, the blank solution was determined by using the above-mentioned instrumental parameters. Then a volume of 60 μL of the stock solution of artemisinin (0.3 mg/mL) for DPP and/or 250 μL for CV was added and then nitrogen was passed for another 30 s. The polarogramm and/or cyclic voltammogram was recorded and evaluated.

Optimisation of DPP method for the determination of artemisinin

Optimisation was carried out as our previous article.[23,25] For artemisinin, among different buffer systems phosphate buffer of pH 5.5-6 was the best, for this phosphate buffer (pH 5.5) with methanol and ethanol in different ratios were also tested. Polarographic analysis was carried out as described above.

Method validation

The method was validated according to ICH Guidelines Q2A and Q2B.

Calibration curves including linearity, range, LOQ and LOD: 10 mL of the mixture of phosphate buffer (pH 5.5) and methanol (7:3; v/v) were transferred to a polarographic cell and deoxygenated by purging 8 min with nitrogen. After determining the blank value, 6 aliquots (each 50 μL) of artemisinin stock solution were added successively and the cell was purged after each addition with nitrogen for another 30 sec. The polarogram was then recorded using the instrumental parameters described above. The data were evaluated by applying the tangent method, correlating for the increase in volume. Using suitable standard solutions it is possible to determine artemisinin in the concentration range of 0.18 - 9.00 μg/mL by using proper standard solutions.[25] The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated according to the Analytical Methods Committee.[26] LOD is defined as the mean value of the intercepts (blank mean y_B) of the calibration curves plus three times of the standard deviation of the intercepts (blank S_B). LOQ is estimated similarly to the LOD value, but: $y_B + 10\ S_B$.

Preparation of Tea

Method A: 100 g of boiling distilled water was added to 0.5 g of dried herb. The mixture was allowed to cool to room temperature, weighed and then the plant material was removed by filtration.

Method B: 100 g of boiling distilled water was added to 0.5 g of dried herb. After addition of the boiling water, the mixture was briefly stirred for 5 min. Then the container was covered for 10 minutes, weighed and subsequently the plant material was removed by filtration.

Method C: 100 g of boiling distilled water was added to 0.5 g of dried herb. The mixture was boiled for 5 to 30 minutes. After cooling to room temperature, weighed and the plant material was removed by filtration.

Method D: 100 g of boiling distilled water was added to 0.5 g of dried herb. The mixture was kept in microwave oven (1 to 3.5 min) at 750 W. Then the container was covered for 10 minutes, weighed and subsequently the plant material was removed by filtration.

Using water and 5% ethanol as solvents

Following method C normal water, distilled water, nanopure and 5% ethanol was used as solvent for tea prep-

aration. In the case of normal water, distilled water and nanopure 100 g of water was added to the 0.5 g of dried herb. For 5% ethanol as solvent, 4 grams of ethanol (about 5 mL) was added first to 0.5 g of dried herb (Artemis) and then 96.0 g of boiling water was added to the mixture.

Extraction of artemisinin from tea preparation

50 g tea was divided into 3 portions. Each portion was extracted twice with petroleum ether at 40-60 °C (1:1 ratio). The organic phases of 3 portions were dried with sodium sulphate and the solvent was evaporated. The residue was dissolved in 6 mL of 50% methanol and filtered with 0.45 μm filter in order to get a clear solution for analysis.

Analysis of artemisinin from tea extract

10 mL of a mixture of phosphate buffer (pH 5.5) and methanol (7:3; v/v) were transferred to the polarographic cell and purged with nitrogen for 8 min. After determination of the blank value, 50 μL of the tea extract (mean linearity range) was added and purged with nitrogen for another 30 sec. The polarogram was then recorded using the instrumental parameters described above. The content of artemisinin was determined applying the standard addition method by adding 50 μL stock solution three times (15 μg artemisinin/50 μL). The peak height was evaluated using the tangent method.

RESULTS AND DISCUSSION

The development of a DPP method for analysis of artemisinin and validation

Fig. 2 illustrates a typical cyclic voltammogram of artemisinin in phosphate buffer of 5.5 that exhibited a very distinct cathodic peak at a potential of -0.05 V vs Ag/AgCl and no anodic signal which indicates that the electrode reaction is irreversible. A differential pulse polarographic method was developed as this method was selected as one of most sensitive method among the electrochemical procedure. The method was optimized, the peak current of artemisinin was observed optimum using a mixture of phosphate buffer of pH 5.5 and methanol (7:3, v/v). The optimised DPP method for the determination of artemisinin was validated following ICH Guidelines Q2A and Q2B concerning linearity, LOQ, LOD, precision, specificity, recovery studies and robustness. After validation this method was applied to the analysis of artemisinin in the traditional herbal tea preparation.

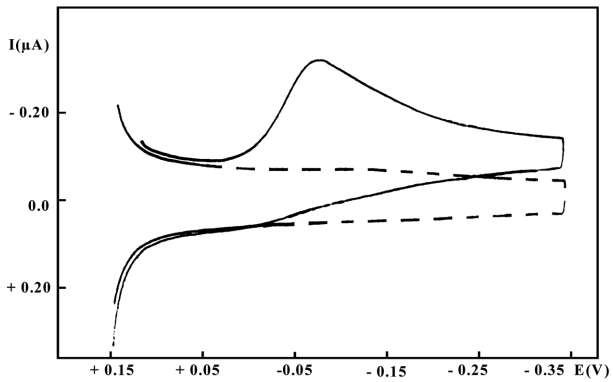

***Fig.* 2.** Cyclic voltammogram of 2.6×10^{-5} M artemisinin in a phosphate buffer of pH 5.5. Scan rate 100 mV/s. A dashed line shows the supporting electrolyte.

Estimation of artemisinin in tea preparations

Artemisinin content in *A. annua* L "Artemis": The artemisinin content of the dried plant material of *A. annua* "Artemis" was determined as 1.4%, using extraction with petroleum ether and employing DPP method.[25]

Extraction of artemisinin from tea and analysis: The current Pharmacopoeia of the People's Republic of China officially lists the dried herb of *A. annua* as a remedy for fever and malaria. The daily dose is specified as 4.5 to 9 grams of dried herb to be prepared as a tea infusion with boiling water. Artemisinin itself is poorly soluble in water, presence of other plant constituents with amphiphilic properties (e.g., flavonoids or saponins) might be helpful for solubilisation. The tea preparation was done on the basis of dosage recommendations of the current pharmacopoeia of the People's Republic of China (5 g herbal drug in 1L/day for five days). According to this guideline 100 mL of tea was prepared from 0.5 g of herbal drug using boiling water and this hot water extract was applied directly to the cell for the analysis of artemisinin by DPP method and the polarogram is presented in *Fig.* 3. The optimised condition (phosphate buffer: methanol = 7:3; v/v) has been used in order to analyze artemisinin in tea preparation. The peak current was not good reproducible as the concentration of artemisinin is low in the tea. For this reason, it was necessary to extract artemisinin by an organic solvent from tea. Several solvents have been used for optimising extraction step and among these solvents petroleum ether (40-60 °C) was the most selective one and therefore it was considered to be the solvent of choice for extraction of artemisinin from the tea preparation. Using petroleum ether the extraction procedure was also optimised and the best result was obtained when the herbal tea

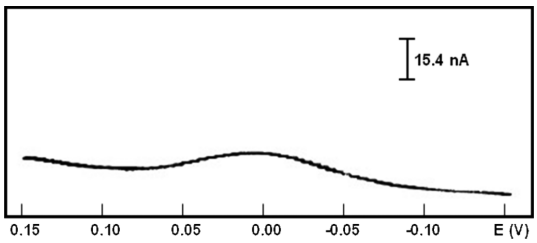

***Fig*. 3.** Differential pulse polarogram of artemisinin in the tea preparation (hot water extraction) without extraction by petroleum ether.

was extracted twice with petrol ether by following petroleum ether/tea ratio 1:1. After extraction by petroleum ether the organic phase was collected and evaporated at reduced pressure; the residue was dissolved in 50% methanol in water (v/v) that was optimised. Standard addition method was used for the determination of artemisinin in the tea of *A. annua* plant.

After determining the blank value, 50 µL of the methanolic plant extract (mean linearity range) was added and purged with nitrogen for another 30s. The polarogram was then recorded and evaluated using the tangent method. The content of artemisinin was determined by applying the standard addition method with the addition of 3×50 µL stock solution as shown in *Fig.* 4. This figure shows that there is no interfering component present in the tea extract between this measuring potential ranges which is particularly suitable for the analysis of artemisinin in tea.

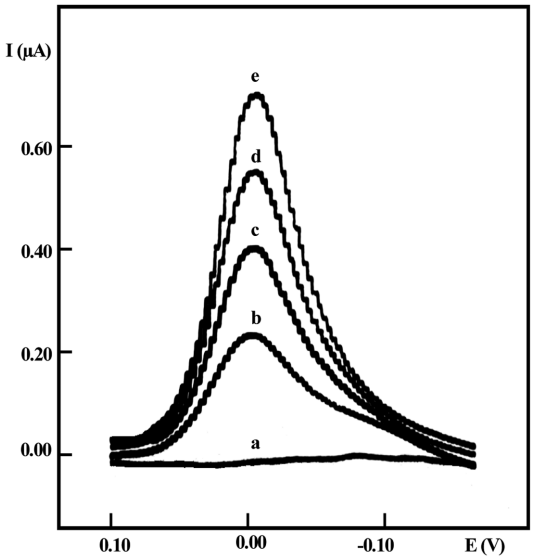

***Fig.* 4.** Determination of artemisinin in tea extraction (method B) using phosphate buffer of pH 5.5/methanol solution (7:3 v/v). Differential pulse polarograms of (a) blank, (b) 50 µL tea extraction and (c-e) addition of artemisinin stock solution, 50 µL each.

Artemisinin concentration in different herbal tea preparations: In this study, tea preparations of the Chinese traditional medicinal plant *A. annua* L in different ways in order to have the maximum extraction efficiency were investigated and the concentration of artemisinin was determined in the tea preparations by the developed DPP method. Four different methods (infusion and decoction) with different times of tea preparations were studied. In method A, after adding boiling water the mixture was left to cool to room temperature and then plant material was removed by filtration. In method B, after adding boiling water the mixture was briefly stirred and covered the container for 10 minutes. In the case of infusions (without further boiling) the highest content of artemisinin was extracted from method B with 84.7% where as from method A with 81% (*Table* 1).

In the case of decoction (method C), it is observed that there exists a great difference in content of artemisinin from cooking 5 minutes and 30 minutes. Artemisinin is known to be thermally unstable compound and *Table* 1 shows that tea prepared by adding boiling water to the leaves without further heating yields higher artemisinin concentration than if the leaves are boiled for more than 5 minutes (Method C). Extended boiling (30 minutes) reduced the yield of artemisinin probably owing to the known chemical lability of artemisinin that was also observed by Professor Heide and co-workers.[12] In method D cooking in domestic microwave oven with varying times up to five minutes was used and the results were compared. Same results as method C were obtained in method D i.e. artemisinin concentration decreases with cooking times (*Table* 1). No great differences were observed between method D (using microwave oven not more than 1.5 min) and method B. Microwave oven can also represent an alternative method to extract artemisinin from *A. annua*. In addition the investigations were also carried out using different

***Table* 1.** Analysis of artemisinin from tea using different methods

Preparation method	Mean amount of artemisinin (mg) per gm of sample	R.S.D%	Extraction efficiency
Method A	5.70	±1.28	81.4%
Method B	5.93	±2.34	84.7%
Method C: 5 min	5.85	±3.57	83.6%
: 30 min	3.73	±1.96	53.3%
Method D: 1.5 min	5.77	±3.86	82.4%
: 2.0 min	5.56	±1.33	79.4%
: 3.5 min	4.54	±2.93	64.9%

n = 6

***Table* 2.** Analysis of artemisinin from tea (tea preparation using different solvents)

Measurement	Normal water	Nano pure	Distill. water	5% Ethanol
	Artemisinin (mg)	Artemisinin (mg)	Artemisinin (mg)	Artemisinin (mg)
Mean	5.77	4.33	5.93	6.22
S.D	±0.171	±0.066	±0.139	±0.142
R.S.D.	±2.96%	±1.52%	±2.34%	±2.28%
Extraction efficiency	82.4%	61.9%	84.7%	88.9%

types of water (normal, distilled and nanopure water) and 5% ethanol in distilled water as solvents of tea preparations. Different contents of artemisinin was found in the tested extracts, as reported in *Table* 2 concerning the percentage of artemisinin in the water and 5% ethanol extracts. It has been appeared that artemisinin could be extracted up to 89% by using 5% ethanol as solvent of tea preparation and only 62% of the total artemisinin using nanopure water. There is no significant difference between distilled or normal water as media for tea preparations. However, 5% ethanol extract showed a high power of extraction for artemisinin of *A. annua* if compared with infusions and decoctions.

CONCLUSION

The developed DPP method can be applied straightforwardly to analyze artemisinin content in herbal tea preparation without using any special separation technique and any molecular breakdown or derivatization of artemisinin. Moreover no intervention was observed in the range of potential window used in the developed method to estimate artemisinin. The developed method is not so expensive and time consuming. The relative standard deviation (R.S.D) below 4% indicates an excellent reproducibility of the method. To get the optimum therapeutic effect against malaria from the herbal tea of *A. annua*, the suggested best solvent for tea preparation is 5% ethanol in distilled water and following the method B (without further cooking after the addition of boiled solvent with stirring).

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REFERENCES

1. World Health Organization 1996. *The World Health report-Fighting disease,* Fostering Development, WHO: Geneva, Switzerland.
2. Klayman, D. L.; Lin, A. J.; Acton N.; Scovill, J. P.; Hoch J. M.; Milhous, W. K.; Theoharides, A. D. *J. Nat. Prod.* **1984**, *47*, 715.
3. Klayman, D. L.; *Science* **1985**, *228*, 1049.
4. Anon. *China Pharmaceutical Bulletin* **1979**, *2*, 49.
5. Charles, D. J.; Cebert, E.; Simon, J. E. *J. Ess. Oil Res.* **1991**, *3*, 33.
6. Anon. *Lancet* **1992**, *339*, 649.
7. Dhingra, V.; Rao, K. V.; Narasu, M. L. *Life sciences* **2000**, *66,* 279.
8. Li, Y.; Wu, Y. L. *Medecine Tropicale* **1996**, *58,* 9.
9. Delabays, N.; Benakis, A.; Collet, G. *Hort.* **1993**, *330*, 203.
10. Delabays, N.; Simonnet, X.; Gaudin, M. *Curr. Med. Chem.* **2001**, *8*, 1795.
11. Marchand, E.; Atemnkeng, M. A.; Vanermen, S.; Vercammen1 J. P. *Biomed. Chromatogr.* **2008**, *22*, 454.
12. Raeth, K.; Taxis, K.; Walz, G.; Gleiter, C. H.; Li, S. M.; Heide, L. *Am. J. Trop. Med. Hyg.* **2004**, *70,* 128.
13. Zhang, R. S.; Wang, Y.; Zhao, M. *Linchan Huaxue Yu Gongye* **2009**, *9*, 115.
14. Vandenberghe, D. R.; Vergauwe, A. N.; Montagu, M. V.; Vandeneckhout, E. G. *J. Nat. Prod* **1995**, *58*, 798.
15. Liu, C. Z.; Zhou, H. Y.; Zhao, Y. *Anal. Chim. Acta* **2007**, *581*, 298.
16. Congyue, A. P.; Jorge, F. S.; Ferreira, A. J. W. *J. Chromatog. A* **2006**, *1133*, 254.
17. Celeghini, R. M. S.; Sousa, I. M. O.; Silva, A. P.; Rodrigues, R A. F.; Foglio, M. A. *Quimica Nova* **2009**, *32*, 875.
18. Bilia, A. R.; Malgalhaes M. P.; Bergonzi, M. C.; Vincieri, F.F. *Phytomed.* **2006**, *13,* 487.
19. Qian, G.-P.; Yang, Y.-W.; Ren, Q.L. *J. Liq. Chromatogr. Related Technol.* **2005**, *28*, 705.
20. Wang, M.; Park, C. H.; Wu, Q.; Simon, J. E. *J. Agric. Food Chem.* **2005**, *53*, 7010.
21. Sipahimalani, A. T.; Fulzele, D. P.; Heble, M. R. *J. Chromatogr.* **1991**, *538*, 452.
22. Ranasinghe, A.; Sweatlock, J. D.; CooKs, R. G. *J. Nat. Prod.* **1993**, *56,* 552.
23. Debnath, C.; Haslinger, E.; Likussar, W.; Ortner, A. *J. Pharm. Biomed. Anal.* **2006**, *41*, 638.
24. Zhou, Z. J.; Yang, P. H.; Feng, D. X.; Zhu, Y. T.; Zhang, M. Y. *Yunnan Daxue Xuebao* **2003**, *25*, 144.
25. Debnath, C.; Haslinger, E.; Ortner, A. *NPC* **2006**, *1*, 487.
26. Analytical Methods Committee. *Analyst* **1987**, *112*, 199.