

Determination of Artemisinin Content in *Artemisia annua L.*

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Abstract

Artemisinin from dry leaves of *Artemisia annua L.* was extracted by supercritical fluid extraction using CO₂ as the solvent and Microwave-assisted extraction by different solvents as ethanol, chloroform, n-hexane and toluene. Extracts were analyzed in GC-MS system gas chromatograph, by comparison of the retention time with that of the standard artemisinin. Determination of arteannuin B, artemisinic acids were conducted by comparison of the spectrum with that of a library. The content of artemisinin was determined as 0.29-0.85% of dry weight for microwave-assisted extraction, and 0.32% for supercritical fluid extraction, for arteannuin B as 0.18-1.23% and 1.18%, for artemisinic acid as 0.32-2.33% and 0.66%, respectively. The yield of total extract obtained by supercritical fluid extraction was 1.70%, by microwave-assisted extraction - 1.33-8.62%. Best solvents for the artemisinin extraction were toluene, chloroform and ethanol.

Key words: artemisinin, supercritical extraction, arteannuin B, artemisinic acids

Introduction

Artemisia annua L. is the herb of the family Asteraceae, which known as Annual Wormwood is native in Asia. It has been used in Chinese traditional medicine for treatment of fever from ancient times. Artemisinin is naturally formed in *A. annua* and has mainly been detected in the aerial parts of the plant, particularly in the leaves, stems, buds and flowers. Its amount ranges from 0.01% to 1.54% (Charles & Simon, 1990; Ferreira *et al.*, 1995) of dry weight according to various factors such as the plant's origin, stage of development and the cultivation condition. Artemisinin is a naturally occurring peroxidic sesquiterpene. Systematically, it is named [3R-(3a, 5ab, 6b, 9a, 12b, 1aR)]-octahydro-3, 6, 9-trimethyl-3, 12H-pyrano [4, 3-*j*]-1, 2-benzodioxepin-10(3H)-one (Avery *et al.*, 2003).

The mechanism of action of artemisinin is still non conclusive. Experimental and theoretical studies suggest existence of several processes involving artemisinin, as forming carbon free radicals which alkylate specific malarial proteins causing lethal damage to parasites (Teja-Isavadharm *et al.*, 2004), inhibition of heme polymerization by breaking hemozoin (crystalline heme aggregate, malaria pigment,

hematin) (Ziegler *et al.*, 2001) into heme units, or specifically inhibiting the parasite membrane Ca²⁺-transporting ATP-ases (sarcoplasmic reticulum Ca²⁺-transporting ATP-ase or SERCA) (Klayman *et al.*, 1984). Artemisinin and its derivatives are the only group of compounds that is still effective against drug-resistant *P. falciparum* strains, and has the ability to quickly lower parasite level (Krishna *et al.*, 2004).

Materials and Methods

Plant material. The plant sample of *A. annua* was collected in the vicinity of Ulaanbaatar city, Mongolia in August 2006, and air dried. The leaves were separated from the other parts of plants and for artemisinin extraction.

Supercritical fluid extraction. The supercritical fluid extraction (SFE) method uses CO₂ as a solvent. In comparison with the Soxhlet extraction, this method has many advantages such as lower solvent consumption and lower working temperature. This method offers other advantages related to the favorable properties of supercritical fluids: low viscosity, high solute diffusivity, improved mass transfer and reduction of extraction time. The appearance of the SFE extracts depends on the operational condition used during the extraction. Extraction process of

artemisinin was carried out in supercritical fluid extractor.

Liquid carbon dioxide (food grade, 99.8% of minimum purity) was fed through a 2- μm filter to an ISCO 260D high-pressure pump. The CO_2 was compressed and pumped into a preheating coil contained in a temperature controlled water bath. Thermal equilibrium was achieved in the preheating coil and the fluid then passed into an extraction column packed with solute. The 1.92 g of the plant material was placed inside. A cotton wool plug was placed at both sides of the extraction column in order to avoid the drag of small particles by the solvent. The solute laden CO_2 leaving the cell was directed through a 0.5- μm filter to prevent physical entrainment. The filter was then flashed to atmospheric pressure through a regulating valve resulting in the precipitation of solute within both the valve and a 0.5- μm filter. The gas released through the regulating valve was passed through a water saturator and a wet test meter for volume determination. A static period of 1 hour was used to allow the contact between the particles and the supercritical solvent. Experiment was performed using 150 bar pressure and 40°C temperature. These mild operating conditions avoid degradation of the analyzing material and give rise to clean plant extract. The CO_2 was allowed to flow into the extraction column during 50 minutes. The CO_2 mass flow rate was 10 ml/sec. After extraction the regulating valve and filter were washed with 8.5 ml of chloroform to recover the extract deposited on. The total extract mass from plant sample was determined gravimetrically as 1.698%.

Microwave-assisted extraction. Microwave-assisted extraction (MAE), also called as microwave extraction which combines microwave and traditional solvent extraction. It is a method, which uses the energy of microwave radiation to heat solvents quickly and efficiently. By using a closed system, extraction can be performed at higher temperatures and extraction time can be reduced. MAE has many advantages, such as extremely short extraction times (15 to 30 minutes), high sample throughput (8-16 samples in one extraction run), very low solvent consumption, exact reaction control by temperature and pressure sensors, possibility of automation, higher extraction rate and better product with lower cost. MAE was carried out

using microwave extractor (CEM Corporation, Model Mars 5, USA). Dry leaves of *A. annua* were placed into the fluid extractor (FE) closed vessels and were extracted by 10 ml of different solvents (ethanol, chloroform, n-hexane and toluene) in the microwave extractor at the maintaining temperature (35°C and 50°C for n-hexane and 50°C for the other solvents) for 15 minutes. Microwave power of 300W-600W per minute was given according to the number of vessels in the apparatus. After cooling for 15 minutes the extracts were filtered through a 0.45 micron filter and stored at +4°C. Microwave extraction gave the yellow extract for toluene, dark green for ethanol, light yellow for n-hexane and dark yellow for chloroform.

Identification of artemisinin by GC-MS. All extracts were analyzed in GC-MS system (Perkin Elmer, USA), fitted with mass selective detector, with a silica capillary column (HP5MS 30m x 0.25 μm x 0.25mm, cross linked 5% PH ME Siloxane, Hewlett Packard, USA) by direct probe injection. Helium was used as the carrier gas at a flow rate of 1 ml/min with the velocity of 36.2 cm/sec, maintained constant for analysis. The temperature of the injector was 240°C. The column was heated to 50°C for 5 min and programmed at 5°C/min to 280°C, heated for 5 min. One micro liter of the extract was injected. For the ethanol extract filtration was repeated before injection, because of precipitate formation. All analyses were carried out in duplicate. The identification of the major components was done by comparison of the retention time with that of the standard artemisinin (98%, CAS number 63968-64-9, Aldrich, USA).

Quantification of the components was performed using the linear calibration graph for the standard artemisinin (Fig.1). Standard solutions with three different concentrations were prepared by solving the standard artemisinin in ethanol.

Results

Mass spectral analyses of standard artemisinin was identical to that of library and showed that except of artemisinin peak also the peaks of decomposition compounds are present (Fig. 2, compounds A, B and C).

Artemisinin is stable up to 150°C, it but

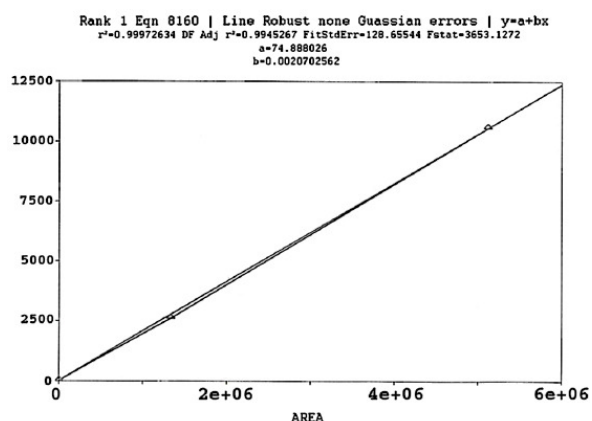


Figure 1. Linear calibration graph for the standard artemisinin.

degrades into number of products when heated at 180-200°C. Therefore, gas chromatographic analyses measure artemisinin indirectly by detection of degradation compounds. This fact was taken into the consideration for following calculations. Standard artemisinin had its retention time at about 35 minutes.

Determination of arteannuin B, artemisinic acids were conducted by comparison of the

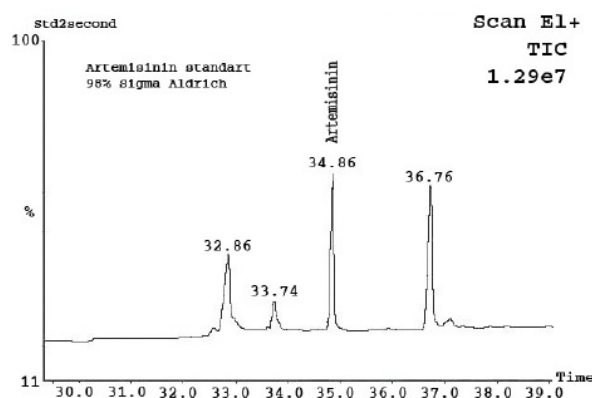


Figure 2. Mass spectra of standard artemisinin.

spectrum with that of a library. Retention time of artemisinic acid was at about 30 minutes, and arteannuin B was at 34 minutes for all solvents, correspondingly.

The content of artemisinin was determined as 0.29-0.85% of dry weight for MAE and 0.32% for SFE, for arteannuin B as 0.18-1.23% and 1.18%, for artemisinic acid as 0.32-2.33% and 0.66%, respectively (Table 1).

Table 1. Active compounds (artemisinin, arteannuin B and artemisinic acid), extracted by MAE and MFE methods

Extraction method	Solvent	Artemisinin, %	Arteannuin B, %	Artemisinic acid, %
microwave	ethanol	0.40	0.37	1.10
microwave	toluene	0.85	1.23	2.33
microwave	chloroform	0.62	0.68	0.32
microwave	n-hexane	0.29	0.18	0.49
supercritical CO ₂		0.32	0.84	0.77

By SFE many peaks of hydrocarbons with odd number of carbon atoms in molecule were obtained at the area of determination and double peak about artemisinin. It means that for this method we have to use additional purification step after extraction before gas chromatography. The experiment was repeated under the same condition by changing the flow rate of CO₂ from 10 to 133 ml/sec. In this case few peaks were observed, which means that for supercritical fluid extraction method the flow

rate of CO₂ might be more selective for artemisinin extraction. As for MAE, there were no double peaks determined for artemisinin, arteannuin B and artemisinic acid and fewer peaks were observed in the area of determination. It may be related to the selectivity of microwave extraction method. Artemisinin and artemisinic acid contents in *A. annua* were compared with results from literature for extraction of artemisinin by MAE method (Table 2).

Table 2. Artemisinin and artemisinic acid content in *A. annua* extracted by MAE using different solvents

Solvents	Artemisinin, %			Artemisinic acid, %	
	Our result	Kohler <i>et al</i> 1997	Hao <i>et al.</i> , 2002	Our result	Kohler <i>et al.</i> , 1997
Ethanol	0.400	0.140	0.111-0.115	1.100	0.072
Toluene	0.850	0.557	-	2.330	0.057
n-hexane	0.290	0.800	-	0.490	0.188

It showed that contents of artemisinin, especially of artemisinic acid in our sample was higher than that determined by other researchers. The SFE content of artemisinin was lower in comparison with previous results (0.32% in comparison with 0.62-0.7%, according to data of Hao *et al.* (2002).

The yield of total extracted product (1) and the yield of artemisinin were determined (2) after solvent evaporation at room temperature.

$$\text{Yield of total extract} = \frac{\text{quantity of extract}}{\text{quantity of raw material}} 100\% \quad (1)$$

$$\text{Yield of artemisinin} = \frac{\text{quantity of artemisinin in extract}}{\text{quantity of raw material}} 100\% \quad (2)$$

Table 3. Yield of artemisinin extract by different solvents and comparison with available data

Extraction method	Solvent	Yield of extract, %		Yield of artemisinin, %	
		Our result	Hao <i>et al.</i> , 2002	Our result	Hao <i>et al.</i> , 2002
microwave	ethanol	8.62	12.3	3.42	-
microwave	chloroform	6.04	-	3.75	-
microwave	n-hexane	1.33	3.37	0.38	0.11
microwave	toluene	6.45	-	5.48	-
supercritical CO ₂		1.70	-	0.54	1.13*

*(Kohler *et al.*, 1997)

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References

- Avery, M. A., Muraleedharan, K. M., Desai, P. V., Bandyopadhyaya, A. K., Furtado, M. M. & Tekwani, B. L. 2003. Structure-activity relationship of the antimalarial agent artemisinin 8. Design, synthesis and CoMFA studies toward the development of artemisinin-based drugs against leishmaniasis and malaria. *Journal of Medical Chemistry*, 46: 4244-4258.
- Charles, D. J. & Simon, J. E. 1990. Germplasm variation in artemisinin content of *Artemisia annua* using an alternative method of artemisinin analysis from crude plant extract. *Journal of Natural Products*, 53(1): 157-160.
- Ferreira, J. F. S., Simon, J. E. & Janick, J. 1995. Developmental studies of *Artemisia annua*: flowering and artemisinin production under greenhouse and field conditions. *Planta Medica*, 61(2): 167-170.
- Hao, J., Han, W., Huang, S., Xue, B. & Deng, Z. 2002. Microwave assisted extraction of artemisinin from *Artemisia annua* L. *Separation and Purification Technology*, 28: 191-196.
- Klayman, D. L., Lin, A. J., Acton, N., Sconill, P. J., Hoch, J. M., Milhous, W. K. & Throharifes, A. D. 1984. Isolation of artemisinin (qinghaosu) from *Artemisia annua* growing in the United States. *Journal of Natural Products*, 47 (4):715-717.
- Krishna, S., Uhlemann, A. & Haynes, R. K. 2004. Artemisinins: mechanisms of action and potential for resistance. *Drug Resistance Updates*, 7: 233-244.
- Kohler, M., Haerdi, W., Christen, P. & Veuthey, J-L. 1997. Supercritical fluid extraction and chromatography of artemisinin and artemisinic acid. An improved method for the analysis of *Artemisia annua* samples.

- Phytochemical Analysis*, 8: 223-227.
- Teja-Isavadharm, P., Peggins, J. O., Brewer, G., White, N. G., Webster, K. & Kyle, D. E. 2004. *Plasmodium falciparum*-Based Bioassay for Measurement of Artemisinin Derivatives in Plasma or Serum. *Antimicrobial Agents Chemotherapy*, 48(3): 954-960.
- Ziegler, J., Linck, R. & Wright, D. W. 2001. Heme aggregation inhibitors: antimalarial drugs targeting an essential biomineralization process. *Current Medical Chemistry*, 8: 171-189.

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