SUPPLEMENTARY MATERIAL

Assessment of Artemisinin and Antioxidant Activities of three wild Artemisia species of Algeria.

Hamza Ahmed Laloui[1]*, Hadjer Zaak[2], Abderrahman Rahmani[1], Imad Kashi[3], Smain Chemat[4], Mohamed Djamel Miara[6], Nora Cherb[5] and Mouna Derdour[3].

[1] *Animal Biotechnology Laboratory, Biotechnology and Agriculture Division, Biotechnology Research Center (C.R.Bt), Ali Mendjeli, New city UV 03, BP E73, Constantine, Algeria.*

[2] *Food Biotechnology Division, Biotechnology Research Center (C.R.Bt), Ali Mendjeli, New city UV 03, BP E73, Constantine, Algeria.*

[3] *Industry Biotechnology Division, Biotechnology Research Center (C.R.Bt), Ali Mendjeli, New city UV 03, BP E73, Constantine, Algeria.*

[4]*Extraction & Separation Techniques Team, Centre de Recherches Scientifique et Technique en Analyses Physico-Chimiques (C.R.A.P.C.), BP 248 Alger RP 16004, Algiers, Algeria.*

[5] *Environment Biotechnology Division, Biotechnology Research Center (C.R.Bt), Ali Mendjeli, New city UV 03, BP E73, Constantine, Algeria.*

[6] *Department of Nature and life Science, Faculty of Nature and life Science, Ibn Khaldoun University, Tiaret, Algeria.*

**Corresponding author: hamzavet21@gmail.com*

Abstract

Artemisinin, a natural product, has received considerable attention in the last few years as a potent antimalarial drug.

This study reports the presence of Artemisinin in three Algerian wild Artemisia species assessed by HPLC method: A. herba-alba (AH), A.campestris subsp.glutinosa (AC), and A.judaica subsp sahariensis (AJ).

The HPLC analysis of the hexane extracts, showed a difference in artemisinin content in studied species with a yield of 0.64%, 0.34% and 0.04% for AC, AH and AJ, respectively. Moreover, the level of artemisinin obtained in A. campestris was better than those found in A. sieberi and A. annua. This rate has been reported for the first time.

Furthermore, the antiradical activities of methanolic extracts of plants were also tested. There was a remarkable antioxidant capacity found in all Artemisia methanolic extracts analysed.

Keyword: *Artemisia, Artemisinin, Biological activities, Crystallization, HPLC analysis.*

Experimental section

Plant Material

Whole leaves of the selected *Artemisia* species originating in Oued ezarzi region, near the city of Tamanrasset in South of Algeria "Hoggar", locally named "Om nefsa" or "Dgouft" for *A. campestris subsp.glutinosa*. "Teheregele" or "Bahetseran" for *A. judaica subsp sahariensis* and "Chika", "ifsi" or "Zezzaré" for *A. herba-alba*, were kindly provided by one of the residents of the area (October 2018). Plants were identified by MIARA Mohamed Djamel botanist and a voucher specimen of each was deposited at the Herbarium of Department of Biology, Faculty of Nature and Life Science, University Ibn Khaldoun, Tiaret 14000, Algeria. The whole plant of A. judaica; voucher specimen (FNLS.T-AJ-34/21), A. herba-alba; voucher specimen (FNLS.T-AH-35/21) and A. campestris; voucher specimen (FNLS.T-AC-36/21). The identification of the plants species was confirmed by a botanical taxonomist (Quézel and Santa 1962, Sahki and Sahki 2004).

Chemical reagents

Artemisinin standard (99%) in the form of a white crystalline powder was kindly supplied by the Scientific and Technical Research Center (CRAPC)-Algiers of Algeria. Hexane and all chemicals used in this work were of analytical-grade.

Preparation of Artemisia extracts

The harvested plants were washed and dried at room temperature out of direct sunlight in a ventilated area, and then crushed using an electric grinder (MF 10 basic micro grinder).

Hydomethanolic extracts were obtained by macerating 50 g of the crushed plant in 300 mL of hydromethanolic solvent (70:30, v/v), for 10 days with magnetic stirring at room temperature. During this period 200 mL of the same solvent was added for a total volume of 500mL. The maceration extraction was repeated this time for 24 hours. The recovered solution is filtered, under vacuum on a graded filter (Wattman paper), to get rid of large particles, and then on a hydrophilic Millipore Millex-HV filter (0.45µm). Subsequently, the filtrates brought together and concentrated by evaporation, using a rotary evaporator (BUCHI R210) with a vacuum pump. The resulting extract is then stored at 4°C until used for different biological activities assays (Figure S1.).

On another hand, an ultrasound extraction method was used for its significance in increasing artemisinin yield compared to conventional extraction (Chemat et al. 2017). For this purpose,

20 g of dry plants material was mixed with a solvents mixture of hexane-ethyl acetate (95: 5) to biomass ratio (6: 1, v/w). The extraction was realised using vibra-cell 75186 w-130 sonifier fitted, with an immersible horn with an end surface equal to $1cm^2$ which emits sound vibrations at 20 kHz via a converter CV 18. Piezoelectric lead zirconate titanate crystals (32 mm diameter) dipped into the extraction medium as described in earlier studies (Chemat and Esveld 2013). A double-jacket flask (150 mL volume) connected to a water bath that ensures temperature control was used to enable contact between solvent and ultrasound horn. In this set of experiments, the output power of the ultrasound generator is set at amplitude of 35% and a temperature between 30°C and 40°C. The mixture is filtered through Whatman filter paper and the filtrate solution is evaporated to dryness in a rotary evaporator. The obtained extract was stored at 4°C until used for a liquid chromatography analysis (Figure S2.).

Determination of total phenolic, total flavonoid and tannins contents

TPC

Total Phenolic Content of the studied plant extracts was performed by using the Folin-Ciocalteu reagent (Singleton and Rossi 1965), according to a method described by Müller et *al*. (2010) (Müller et al. 2010). TPC was expressed as gallic acid equivalent (GAE) in mg/m of extract based on calibration curve of the standard (Gallic acid was used as standard).

TFC

Total flavonoid content was evaluated according to the method of Topçu et *al*. (2007) with minor modifications (Topçu et al. 2007). Quercetin was used as standard in this assay and the blank was prepared by replacing the reagents with methanol. TFC was calculated as quercetin equivalent (QE) in mg/g of extract based on the standard calibration curve.

TC

Proanthocyanidins were measured using the modified vanillin assay (Sun et al. 1998). To 50 µL of suitably diluted samples, 3 mL of methanol vanillin solution (4%) and 1.5 mL of concentrated sulfuric acid were added. The mixture was let to stand for 15 min before measuring the absorption at 500 nm. The amount of total condensed tannin was expressed as mg of catechin equivalent (CE) per gram of dried extract.

Antioxidant properties of Artemisia species

Four assays (DPPH, ABTS, CUPRAC, and β-carotene), widely used for the assessment of the antioxidant capacities of medicinal plants, were tested in this study on the selected *Artemisia* species.

Free radical scavenging activity (DPPH assay)

The free radical scavenging activity was determined spectrophotometrically by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Blois 1958). An aliquot of 40 µL of the sample (extracts and standards) at various concentrations was added to 160 µL of the methanolic solution of DPPH (0.1 mM) in a 96-well microliter plate. Blanks were prepared using the solvent in addition to the DPPH reagent. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 515 nm using a microplate reader. Butylhydroxyltoluene (BHT), butyl hydroxyanisole (BHA) and α-tocopherol were used as standards for activity comparison.

ABTS Cation Radical Decolourisation assay

The ABTS scavenging activity is determined by the method of Re et *al*. (1999) with slight modifications (Re et al. 1999). From ABTS (7 mM) and potassium persulfate (2.45 mM), The ABTS·+ was produced. The two products in aqueous solution were mixed and stored in the dark at room temperature for 12 to 16 hours. 40 µL of each dilution of the different plant extracts are used to fill a 96-well micro plate reader, then, 160 µL of ABTS·+solution were added to the sample. After 10 min, the percentage inhibition at 734 nm was calculated for each concentration relative to a blank absorbance . The blank is prepared in the same way, replacing the extract with the solvent used (methanol).

The scavenging capability of ABTS·+ was calculated using the following equation and the results were given as IC_{50} value (µg/mL)

$$ABTS^{\bullet+} \text{ scavenging activity (\%)} = [(A_{Control} - A_{Sample})/ A_{Control}] \times 100$$

Cupric reducing antioxidant capacity (CUPRAC assay)

The CUPRAC was determined according to the method described previously with minor changes (Apak et al. 2004). 50 µl of neocuproine solution (7.5 mM) and 60 µL of acetate ammonium solution ($AcNH_4$) were added to 50 µL of Cu solution ($CuCl_2$, $2H_2O$; 10 mM). 40µL of the sample solutions at different concentrations were added to the above mixture. After 60 min, the absorbance at 450 nm was recorded against a reagent blank using a 96-well micro plate reader. Blank was prepared by replacing the reagents with methanol.

The results were given as $A_{0.5}$ (µg/ml) indicating half absorbance intensity (corresponding to the concentration) and were compared with those of the standards BHA and BHT.

β-carotene linoleic acid and bleaching assay

1mg of ß-carotene was dissolved in 2 mL of chloroform and introduced into a flask containing 25 µL of linoleic acid and 200 mg of Tween 40 following (Miraliakbari and Shahidi 2008) with some modifications. The chloroform was completely evaporated using a vacuum evaporator. Then, 100 mL of hydrogen peroxide was added slowly with vigorous stirring.

2.5 mL of this new solution was transferred into tubes and 40 µL of each extract was added (1 mg mL^{-1} in methanol); three repetitions were performed for each extract. The test tubes were incubated in dark at laboratory temperature. Two control tubes were also prepared by the same procedure, one containing an antioxidant reference BHT (positive control) and the other without antioxidant (negative control), where the sample was replaced by methanol. Absorbance was immediately measured at 490 nm. Other readings were measured at different time intervals (0, 30, 60, 90, and 120 min). The relative antioxidant activity (RAA) of the extracts after 120 min was calculated as:

$$AA\ (\%) = [1- (A_{H0} - A_{Ht}) / (A_{C0} - A_{Ct})] \times 100$$

Where

AA (%): Antioxidant activity;

A_{H0}: absorbance value of β-carotene in the presence of the extract measured at t = 0;

A_{C0}: absorbance value of β-carotene in the presence of negative control measured at t = 0;

A_{Ht}: absorbance value of β-carotene in the presence of the extract measured at t = 120 min

A_{Ct}: absorbance value of β-carotene in the presence of negative control measured at t = 120.

Purification of Artemisinin crystals

Artemisia herba-alba was chosen in the experiment. Silica gel and activated charcoal were used as adsorbents. In order to eliminate pigments such as chlorophyll, charcoal is added to the extract in a ratio (1:100, w/v) and left for 30 min under magnetic stirring. After filtration, silica gel is added to remove fatty material in a ratio (2:100 w:v) and the solution is mixed under magnetic stirring for another 30 min. When filtred, the obtained solutions are evaporated to a volume of 10 mL using a rotavapor and stored overnight at 4°C to favour crystallisation of artemisinin. The next day, crystals are recovered by filtration through a nylon membrane filter (0.2 µm pore size), dissolved in acetonitrile and checked for purity by HPLC (Chemat, Aissa, Boumechhour, Arous and Ait-Amar 2017, Numonov et al. 2019).

Quantitative analysis of Artemisinin by HPLC

For the artemisinin extraction from plants, liquid solvent extraction is the current used method and several miscellaneous solvents are tested such as toluene (Kohler et al. 1997), n-hexane (ElSohly et al. 1987, Peng et al. 2006), petroleum ether (Klayman et al. 1984, Qian et al. 2005), and chloroform (Woerdenbag et al. 1991). In our study, n-hexane was used effectively for the extraction of artemisinin.

In order to determine the maximum artemisinin amount that can be recovered from the selected *Artemisia* species, an HPLC analysis was carried out. For this purpose, five milligrams of each hexane-dried extracts were dissolved in 20 mL of acetonitrile. The suspensions were sonicated for 20 min and filtered through 0.2 µm membrane to eliminate precipitate before being submitted for HPLC analysis which was achieved using HP-Agilent Technologies 1100 HPLC system equipped with a UV–visible detector. Column Hypersil 120Å ODS 3; (250×5.4 mm; id: 4.6 µm), set at a temperature of 40°C, is used with an acetonitrile:water (65:35 v/v) mobile phase at an isocratic mode 0.8 mL min^{-1} flow rate where detection is set at wavelength of 220 nm. The artemisinin standard was used as external standard for plotting the calibration curve of the extract at different concentrations varying from 0.125 to 5 mg/mL, and each analytical experiment was repeated at least three times.

Statistical analysis

Means and standard deviations (SD) of the samples were calculated. Each determination was carried out with three replicates and all data are expressed as the mean ± SD.

References

Apak R, Güçlü K, Özyürek M, Karademir SE. 2004. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. Journal of agricultural and food chemistry.52:7970-7981 https://doi.org/10.1021/jf048741x.

Blois MS. 1958. Antioxidant determinations by the use of a stable free radical. Nature.181:1199-1200 https://doi.org/10.1038/1811199a0.

Chemat S, Aissa A, Boumechhour A, Arous O, Ait-Amar H. 2017. Extraction mechanism of ultrasound assisted extraction and its effect on higher yielding and purity of artemisinin crystals from Artemisia annua L. leaves. Ultrasonics sonochemistry.34:310-316 https://doi.org/10.1016/j.ultsonch.2016.05.046.

Chemat S, Esveld EDC. 2013. Contribution of microwaves or ultrasonics on carvone and limonene recovery from dill fruits (Anethum graveolens L.). Innovative Food Science & Emerging Technologies. 2013/01/01/;17:114-119 https://doi.org/10.1016/j.ifset.2012.12.002.

ElSohly HN, Croom E, ElSohly M. 1987. Analysis of the antimalarial sesquiterpene artemisinin in Artemisia annua by high-performance liquid chromatography (HPLC) with postcolumn derivatization and ultraviolet detection. Pharmaceutical Research.4:258-260 10.1023/A:1016472531527.

Klayman DL, Lin AJ, Acton N, Scovill JP, Hoch JM, Milhous WK, Theoharides AD, Dobek AS. 1984. Isolation of Artemisinin (Qinghaosu) from Artemisia annua Growing in the United States. Journal of Natural Products. 1984/07/01;47:715-717 10.1021/np50034a027.

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. 1997/09/01;8:223-227 https://doi.org/10.1002/(SICI)1099-1565(199709/10)8:5<223::AID-PCA370>3.0.CO;2-A.

Ma N, Zhang Z, Liao F, Jiang T, Tu Y. 2020. The birth of artemisinin. Pharmacology & Therapeutics. 2020/12/01/;216:107658 https://doi.org/10.1016/j.pharmthera.2020.107658.

Miraliakbari H, Shahidi F. 2008. Antioxidant activity of minor components of tree nut oils. Food Chemistry. 2008/11/15/;111:421-427 https://doi.org/10.1016/j.foodchem.2008.04.008.

Müller L, Gnoyke S, Popken AM, Böhm V. 2010. Antioxidant capacity and related parameters of different fruit formulations. LWT-Food Science and Technology.43:992-999 https://doi.org/10.1016/j.lwt.2010.02.004.

Numonov S, Sharopov F, Salimov A, Sukhrobov P, Atolikshoeva S, Safarzoda R, Habasi M, Aisa HA. 2019. Assessment of Artemisinin Contents in Selected Artemisia Species from Tajikistan (Central Asia). Medicines.6:23.

Peng CA, Ferreira JFS, Wood AJ. 2006. Direct analysis of artemisinin from Artemisia annua L. using high-performance liquid chromatography with evaporative light scattering detector, and gas chromatography with flame ionization detector. Journal of Chromatography A. 2006/11/10/;1133:254-258 https://doi.org/10.1016/j.chroma.2006.08.043.

Qian GP, Yang YW, Ren QL. 2005. Determination of Artemisinin in Artemisia annua L. by Reversed Phase HPLC. Journal of Liquid Chromatography & Related Technologies. 2005/02/23;28:705-712 10.1081/JLC-200048890.

Quézel P, Santa S. 1962. Nouvelle flore de l'Algérie et des régions désertiques méridionales: Editions du Centre National de la recherche scientifique.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999//;26.

Sahki A, Sahki R. 2004. Le Hoggar: promenade botanique: Atelier ésope 2903420262.

Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American journal of Enology and Viticulture.16:144-158.

Sun B, Ricardo-da-Silva JM, Spranger I. 1998. Critical Factors of Vanillin Assay for Catechins and Proanthocyanidins. Journal of Agricultural and Food Chemistry. 1998/10/01;46:4267-4274 10.1021/jf980366j.

Topçu G, Ay M, Bilici A, Sarıkürkcü C, Öztürk M, Ulubelen A. 2007. A new flavone from antioxidant extracts of Pistacia terebinthus. Food Chemistry.103:816-822 https://doi.org/10.1016/j.foodchem.2006.09.028.

Woerdenbag HJ, Pras N, Bos R, Visser JF, Hendriks H, Malingré TM. 1991. Analysis of artemisinin and related sesquiterpenoids from artemisia annua L. by combined gas chromatography/mass spectrometry. Phytochemical Analysis. 1991/11/01;2:215-219 https://doi.org/10.1002/pca.2800020507.

Table. S1. Total phenolic (TPC), flavonoid (TFC) and Tannin (TC) contents of *Artemisia sp* extracts.

Assay	Extract		
	A. herba Alba	*A.campestris*	*A.judaica*
TPC (mg GAE/g)	480.41±2.63	189.49±2.21	692.82±16.94
TFC (mg QE/g)	260.03±2.05	73.3±2.47	132.87±3.22
TC (mg CE /g)	62.15±1.67	52.53±0.32	74.81±1.46

Table. S2. Antioxidant capacity of the plant extracts.

Extract	IC_{50} (µg/mL)			$A_{0.5}$ (µg/mL)
	DPPH	**ABTS**	**β-carotene**	**CUPRAC**
A. herba-alba (AH)	72.07±1.25	27.19±4.10	58.64±2.48	21.31±0.36
A.campestris (AC)	40.00±0.88	14.22±0.98	<12.5	15.03±0.21
A.judaica (AJ)	21.92±0.94	11.01±0.99	45.22±4.30	28.66±1.92
BHA	5.73±0.41	5.98±0.10	0.91±0.01	3.64±0.19
BHT	1.94±0.41	1.68±0.30	1.05±0.03	1.75±0.01

Table. S3. Artemisinin amount in *Artemisia* species.

Extract	***Artemisinin sample RT(min)***	***Artemisinin standard RT (min)***	***Artemisinin amount (mg/mL)***	***Yield %***	***Formula***	***R^2***
A. herba-alba	7.492	7.46	5.4421 e^{-1}	0.34	y=626.07 x +17.11	0.99983
A.campestris	8.166	8.041	7.7838 e^{-1}	0.65	y=512.63 x -9.84	0.99854
A.judaica	7.47	7.46	1.8632 e^{-1}	0.04	y=626.07 x +17.11	0.99983

RT: retention time in minutes.

R^2: Pearson's linear coefficient of determination

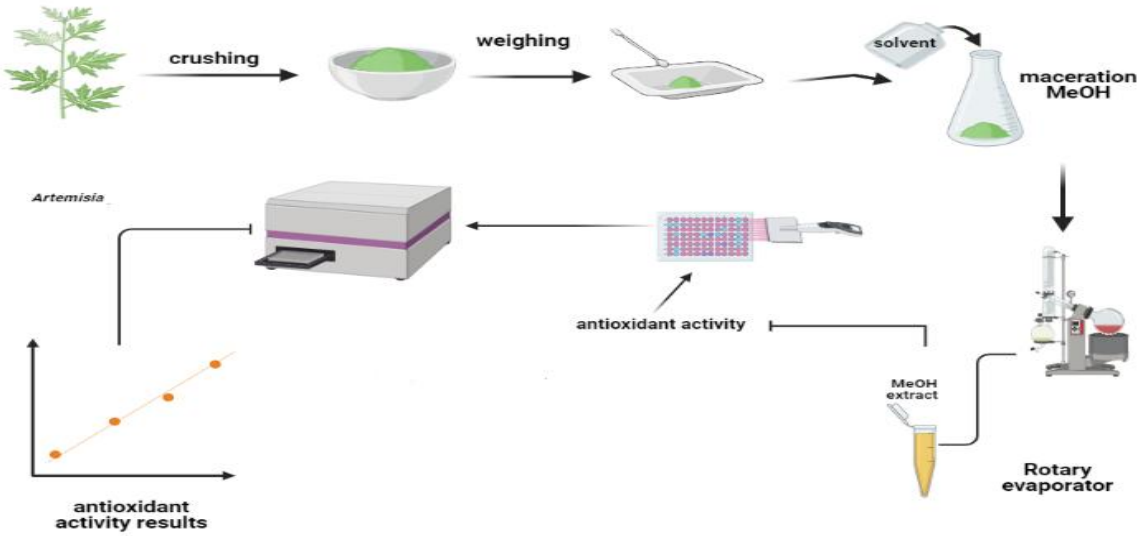


Fig. S1. Preparation of Artemisia extracts for different biological activities assays.

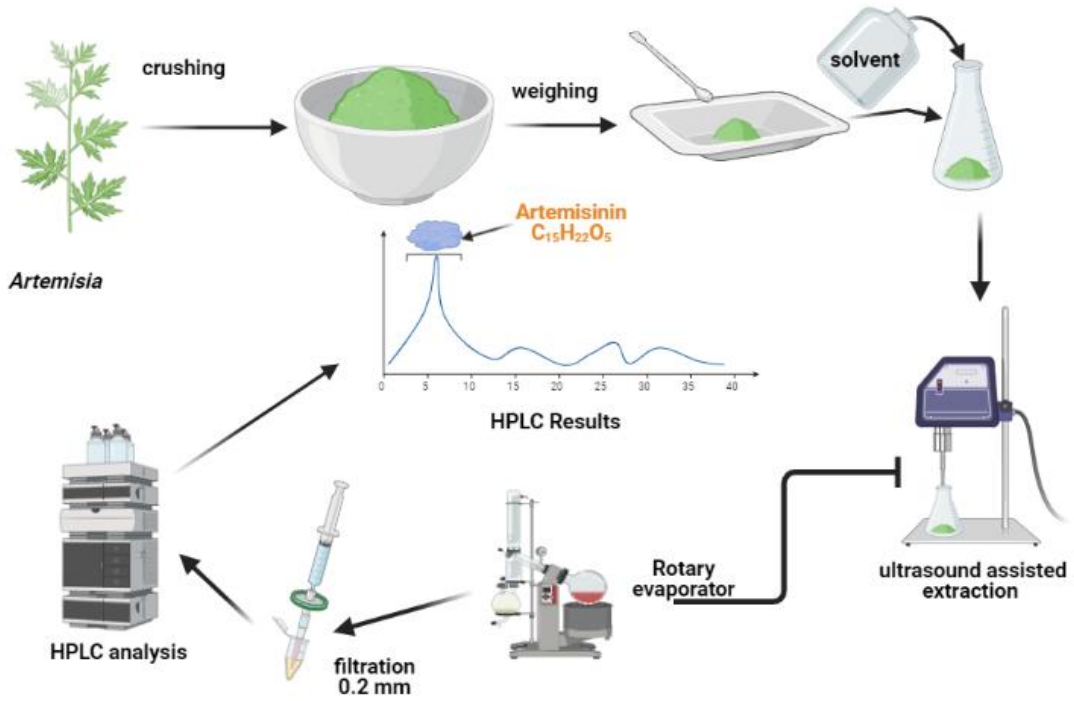
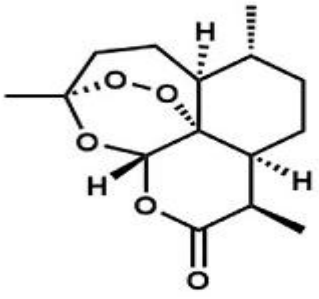


Fig. S2. Ultrasound extraction method and HPLC analysis



artemisinin

Molecular formula: $C_{15}H_{22}O_5$

Melting point: 156 ~ 157 °C

$[\alpha]_D^{17}$ = + 66.3° (C=1.64, $CHCl_3$)

m/e 282.1472 M^+

Fig. S3. Structure and physical & chemical properties of artemisinin (Ma et al. 2020).

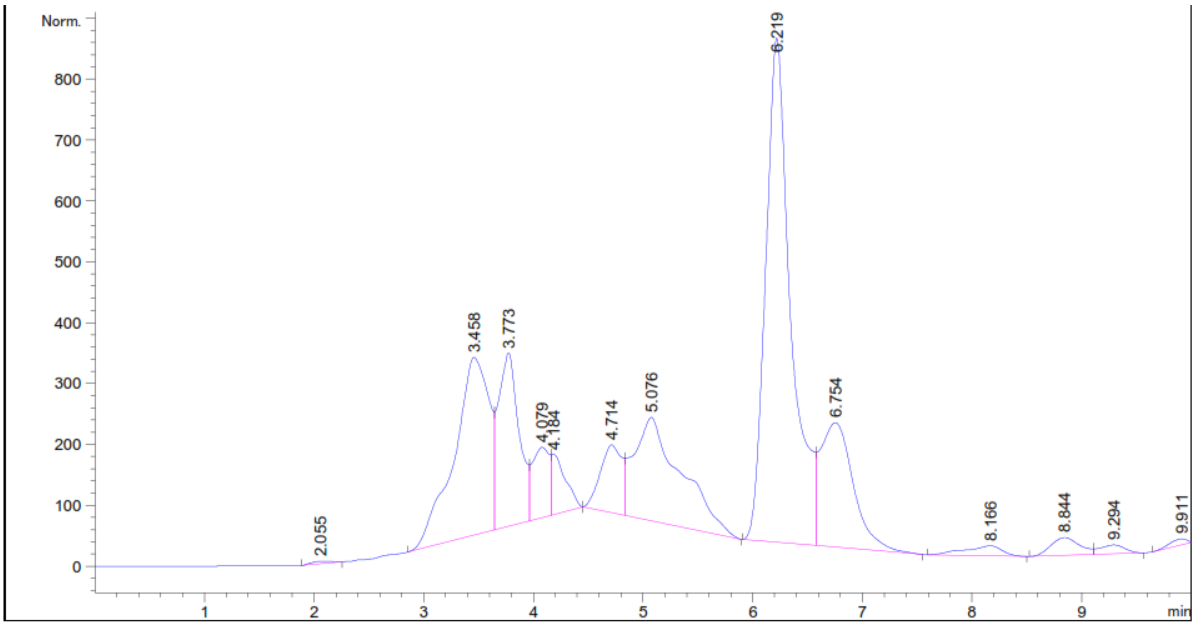


Fig. S4. HPLC chromatogram of standard artemisinin and extract from *Artemisia campestris*.

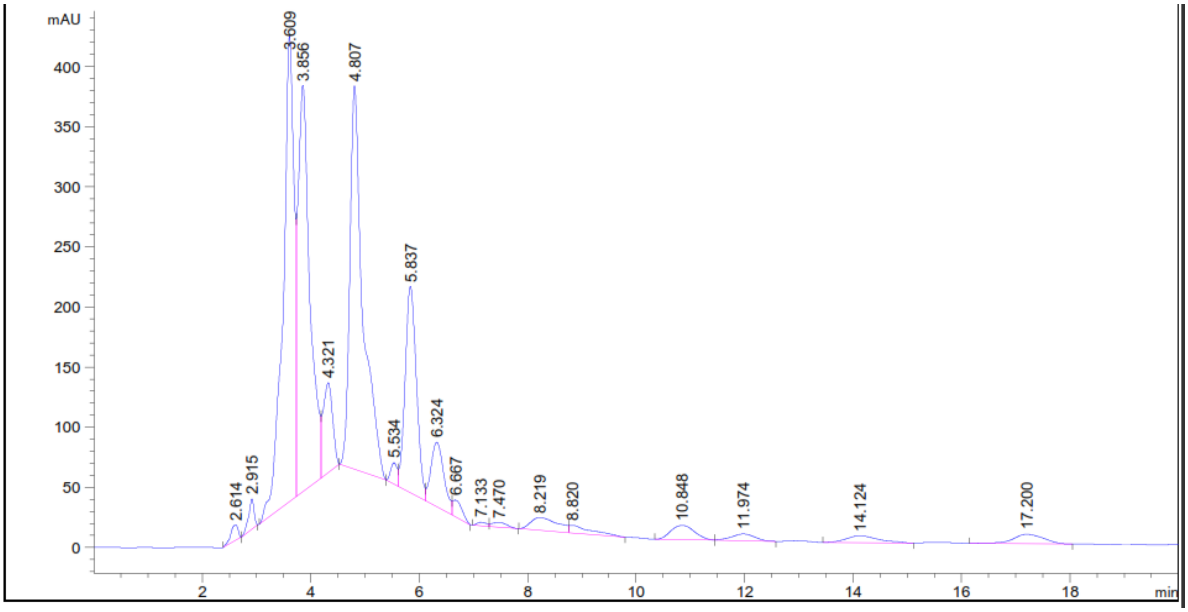


Fig. S5. HPLC chromatogram of standard artemisinin and extract from *Artemisia judaica.*

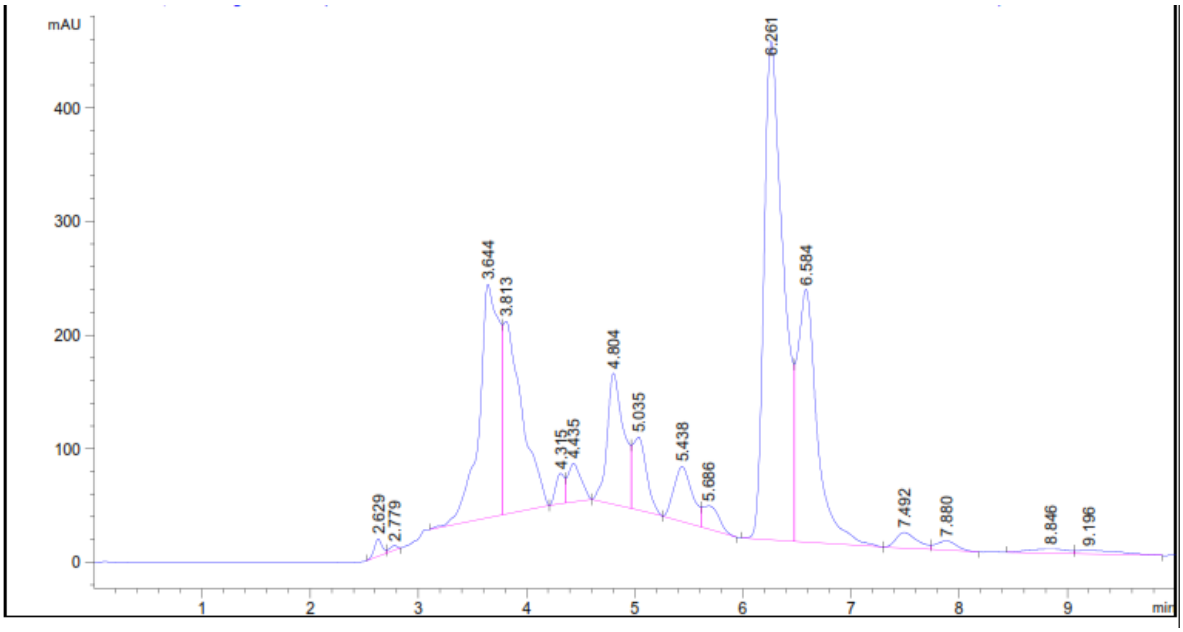


Fig. S6. HPLC chromatogram of standard artemisinin and extract from *Artemisia herba-alba*.

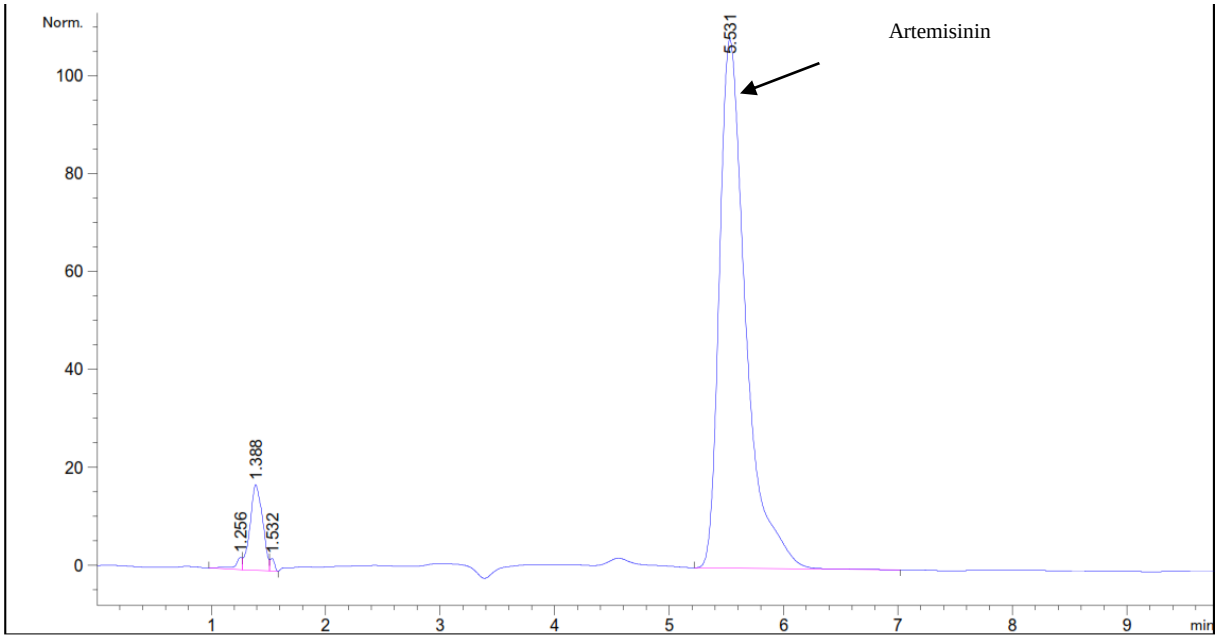


Fig. S7. HPLC chromatograms of artemisinin standard.

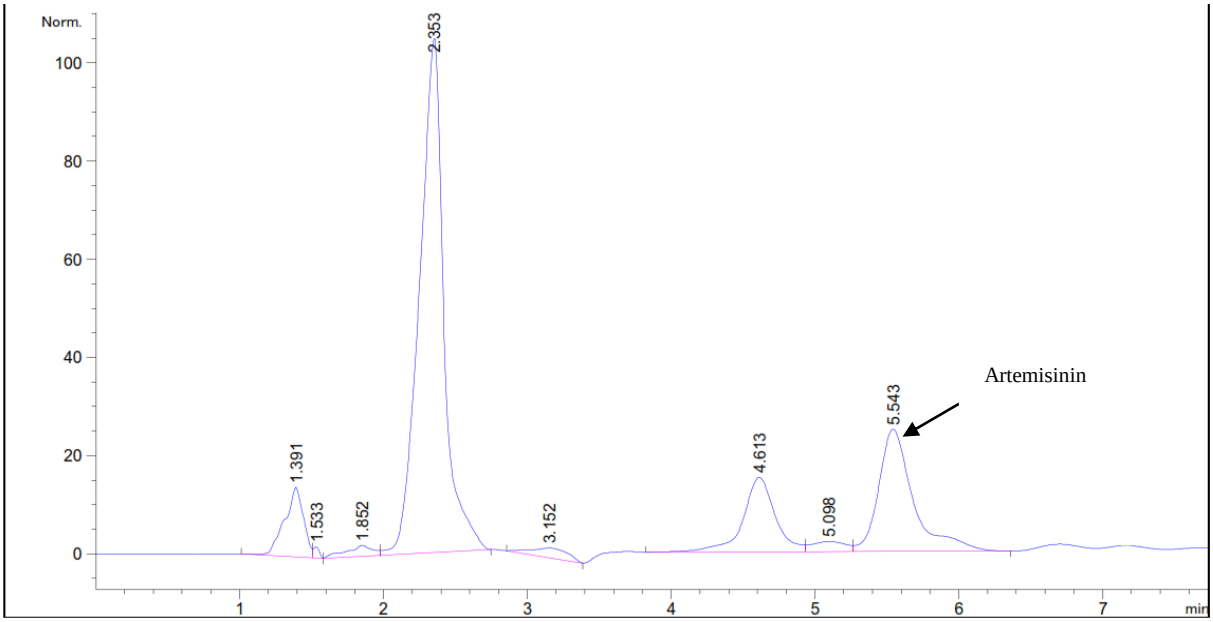


Fig. S8. HPLC chromatograms of the purified extract of *A. herba-alba* using adsorbents.