Pergamon 0031-9422(95)00542-0 Phytochemistry, Vol. 41, No. 1, pp. 97–104, 1996
Elsevier Science Ltd
Printed in Great Britain.
0031-9422/96 $15.00 + 0.00

IMMUNOQUANTITATIVE ANALYSIS OF ARTEMISININ FROM *ARTEMISIA ANNUA* USING POLYCLONAL ANTIBODIES*

JORGE F. S. FERREIRA† and JULES JANICK

Department of Horticulture, Purdue University, 1165 Horticulture Building, West Lafayette, IN 47907–1165, USA

(*Received in revised form* 8 *June* 1995)

Key Word Index—*Artemisia annua*; Asteraceae; ELISA; sesquiterpene lactone; Artemisinin; HPLC; tissue culture.

Abstract—Artemisinin was derivatized to dihydroartemisinin carboxymethylether in three steps, without disturbing the peroxide bridge, and then linked to either thyroglobulin (TGB) or bovine serum albumin (BSA). The artemisinin–TGB and –BSA conjugates were injected in female New Zealand rabbits but only the artemisinin–TGB conjugate generated polyclonal antibodies. An enzyme-linked immunosorbent assay (ELISA) was developed and the specificity of the antibodies was confirmed by comparison with pre-immune serum and by competitive assays using different dilutions of artemisinin standards. Although anti-artemisinin antibodies cross-reacted with artemisitene and dihydroartemisinin at all dilutions used, cross-reaction with deoxyartemisinin, artemisinic acid, and arteannuin B occurred only at high concentrations. ELISA successfully detected artemisinin from crude extracts in concentrations as low as 1.5 ng ml^{-1}; and was *e* 400-fold more sensitive than the HPLC-EC. The ELISA successfully detected and quantified artemisinin in different organs of greenhouse-grown plants and in eight clones of *Artemisia annua* grown in tissue culture but artemisinin was overestimated owing to cross-reactivity of the antibodies with artemisinin-related compounds present in the samples. Despite overestimation of artemisinin content, the correlations between ELISA and HPLC-EC were $r = 0.92^{**}$ when samples were diluted 100 times, and $r = 0.90^{**}$ when samples were diluted 500 times, indicating that ELISA is a potential tool for screening large *A. annua* populations.

INTRODUCTION

Artemisinin (qinghaosu), an endoperoxide sesquiterpene lactone (Fig. 1) produced by aerial parts of *Artemisia annua* L., is effective against sensitive and multidrug resistant strains of *Plasmodium*, the malarial agent, with little or no toxicity to humans. Artemisinin and related compounds have been detected by a number of conventional methods, which include TLC [1–6], HPLC with UV detection [6–10] and with electrochemical detection (HPLC-EC) [11–14], gas chromatography (GC) [15, 16] and GC combined with mass spectrometry [17–19], and MS/MS [20]. Unconventional techniques include radioimmunoassay (RIA) [21] and enzyme-linked immunosorbant assay (ELISA) [22]. The conventional methods, in most cases, have been specific for artemisinin but are not sensitive enough to detect levels in small samples of plant tissue (<0.3 g dry wt) from young seedlings and from cell or tissue cultures. Although RIAs are more sensitive than conventional methods, the use of radioactive compounds presents a series of problems, including special acquisition and use requirements, uncertain stability, high cost, health hazards, and disposal difficulties. Enzyme immunoassays and immunodetection kits are becoming increasingly popular for use with Western blot, Southern blot, and detection of herbicides and plant secondary metabolites. ELISA is at least as sensitive as RIA, safer, and can be a faster and more sensitive method for detection and quantification of artemisinin and related compounds than conventional methods. The purpose of this study was to raise polyclonal antibodies against artemisinin, to develop an ELISA able to detect and quantify artemisinin in crude plant extracts with minimal sample preparation and to compare ELISA with HPLC-EC for quantification of artemisinin and for its efficiency as a screening method.

RESULTS AND DISCUSSION

Derivatization and conjugation

Artemisinin was derivatized in three steps to dihydroartemisinin carboxymethylether (**4**) (Fig. 2), as determined by NMR and HPLC-EC. The peroxide moiety is reported to be critical for antibody specificity [21, 22]. Compound **4** contained the desired reactive carboxyl group in the C_{12} position. The amino groups of lysine residues of the carrier proteins bovine serum albumin (BSA) and thyroglobulin (TGB) (Fig. 2, step D) were

*Journal paper No. 14553 of Purdue Agricultural Research Programs.

†Author for correspondence and reprints.

successfully linked to **4** through its carboxyl group via the carbodiimide (for TGB) and the mixed anhydride (for BSA) reaction (Fig. 2). The conjugate **4**-BSA, obtained by the mixed anhydride reaction, was used as the coating antigen on ELISA plates, but conjugation of **4**-BSA, by the carbodiimide reaction, was unsuccessful (see next section).

Polyclonal antibodies

Four female rabbits were injected with the artemisinin–protein conjugates. Rabbits T and U, injected with **4**-BSA conjugate, obtained by the carbodiimide reaction, did not produce specific antibodies. We believe that this was due to lack of success in linking **4** to this carrier protein. Rabbits V and W, inoculated with **4**-TGB obtained by the carbodiimide reaction, provided antibodies suitable for detection of artemisinin, but antibodies from rabbit V discriminated better between samples with close artemisinin contents (Fig. 3) and were used in quantification assays. The dilution curves for anti **4**-TGB antibodies showed high titres for all bleeds after the third booster injection. The antibodies were sensitive to artemisinin without purification of the G immunoglobulins. A typical ELISA standard curve with an enclosed logit plot is presented in Fig. 4. Artemisinin standard samples were dissolved in DMSO. Final DMSO concentrations in the standard solutions were 10% or lower and had no influence on the sensitivity of the assay.

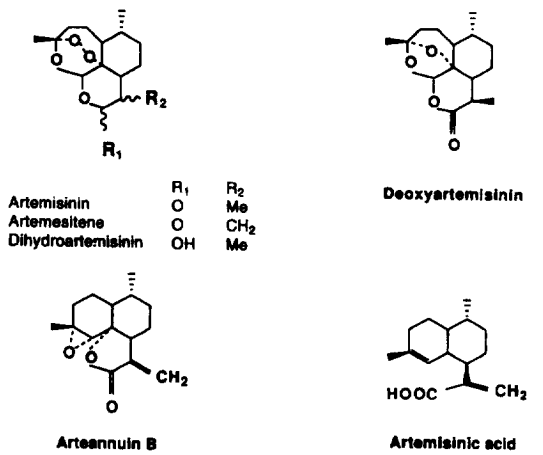

Fig. 1. Artemisinin and derivatives with and without the peroxide bridge. Artemisinin, artemisitene, dihydroartemisinin, deoxyartemisinin, arteannuin B, and artemisinic acid.

Affinity, specificity, and sensitivity of the antibodies

Affinity of the antibodies obtained from rabbits V and W was confirmed by comparing them with pre-immune serum, which had no reaction against artemisinin (data not presented). Antibody specificity was evaluated by cross-reactivity assays using several artemisinin-related compounds (Fig. 1). Anti-artemisinin polyclonal antibodies had a similar response to artemisitene and dihydroartemisinin at all dilutions (Fig. 5), but only cross-reacted with deoxyartemisinin, arteannuin B, and artemisinic acid at the highest concentrations (1 430 and 286 ng $50\,\mu l^{-1}$). The cross-reactivity of the anti-artemisinin antibodies to artemisitene and dihydroartemisinin was expected as they all contain the peroxide bridge and are structurally similar. Cross-reactivity was reported for other artemisinin-derived molecules containing the peroxide bridge, such as dihydroartemisinin, artemether, artesunate (100%), and deoxyartemisinin (<1%), with only one oxygen in the bridge, when these compounds were analysed with anti-artemisinin polyclonal antibodies in a radioimmunoassay [21]. Jaziri *et al.* [22] reported that deoxyartemisinin showed no cross-reaction with their anti-artemisinin antibodies. Although our antibodies cross-reacted with the higher

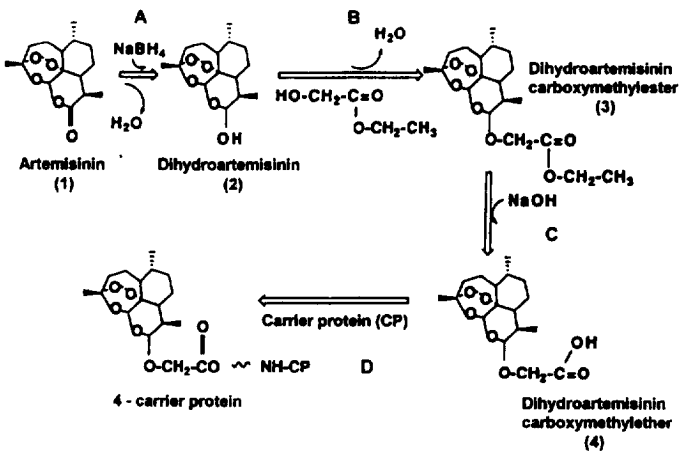

Fig. 2. Scheme of the synthesis of artemisinin–carrier protein conjugates. Artemisinin was linked to the carrier proteins. A, reduction of artemisinin (**1**); B, addition of an ester moiety to dihydroartemisinin (**2**); C, hydrolysis of the ester moiety of dihydroartemisinin carboxymethylester (**3**) to dihydroartemisinin carboxymethylether (**4**); D, conjugation of **4** to carrier proteins.

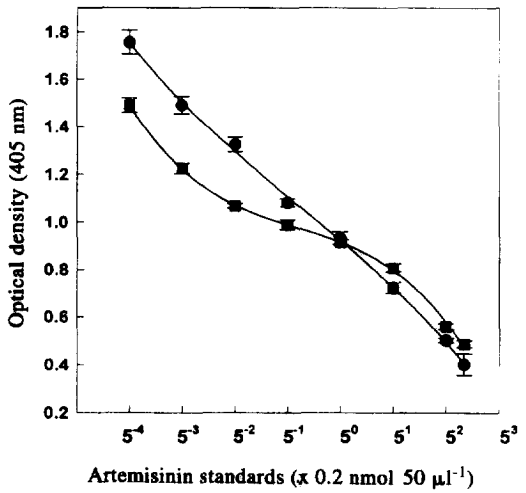

Fig. 3. Standard curves for polyclonal antibodies obtained from rabbits V(●) and W(■). Primary antibodies were used at a final dilution of 1:12000 and optical densities were read after a 30-min incubation of the secondary antibodies (at 1:3000) with *p*-nitrophenyl phosphate.

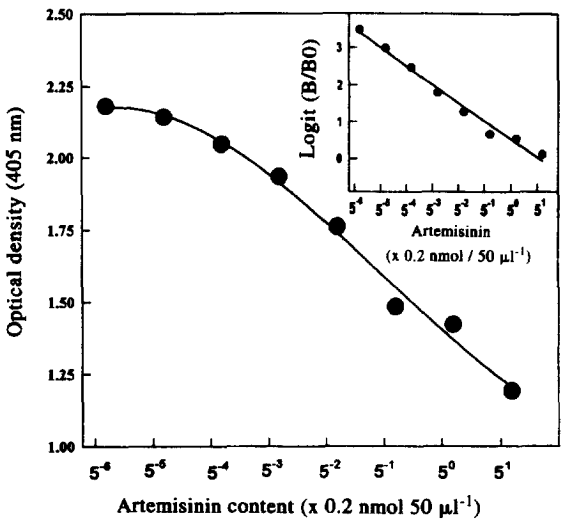

Fig. 4. ELISA standard curve for artemisinin. 4-TGB antibodies at 1:1500 mixed 1:1 with different dilutions of artemisinin standards originally dissolved in DMSO. The insert plot is a Logit-log plot obtained from ln[(B/Bo)/(1 − B/Bo)]. Standard error at each point was lower than 0.025.

concentrations of deoxyartemisinin used in the standard curves, lower doses showed a flat response, similar to that produced by the controls (Fig. 5). Deoxyartemisinin has only one oxygen in the bridge, but it is otherwise identical to artemisinin, and caused cross-reactivity in this study, despite the fact that anti-artemisinin polyclonal antibodies have not been reported to cross-react with deoxyartemisinin. The same was true for arteannuin B and, to a lesser extent, for artemisinic acid. It appears that structure, in addition to the peroxide moiety, affects antibody specificity when the compounds are present at high con-

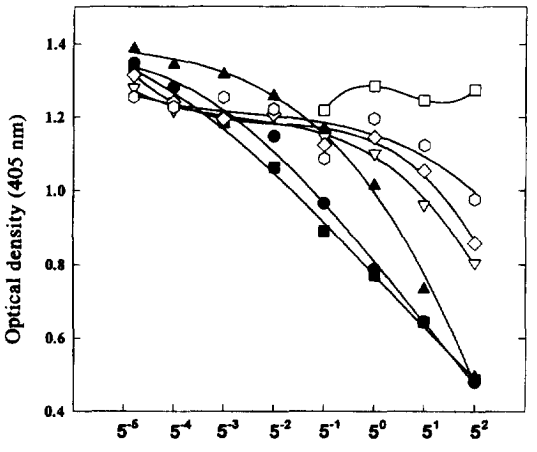

Fig. 5. Cross-reactivity study of anti-artemisinin antibodies. Antibodies were tested against artemisinin (●), artemisitene (■), dihydroartemisinin (▲), deoxyartemisinin (▽), arteannuin B (◇), and artennuic acid (⬡). Controls (□) are antibodies diluted to 1:18000 and optical density was read after 60 min incubation of secondary antibodies (at 1:3000), labelled with alkaline phosphatase, with *p*-nitrophenyl phosphate. Standard errors at each point were lower than 0.026 for $n = 4$.

centrations. This observation is supported by Zhao *et al.* [21] who reported that their polyclonal antibodies had less than 0.4% cross-reactivity with 'yinghaosu A', which has the peroxide bridge found in artemisinin but has major structural differences. The antibodies also reacted to shoot samples of *A. absinthium* and root extracts of *A. annua* when samples were diluted only 10 and 20 times (Fig. 6), falsely indicating the presence of artemisinin in shoots of *A. absinthium* as well as more artemisinin in roots than in main stems of *A. annua*, which contrasts with previous HPLC-EC results. However, when samples from this experiment were diluted 500-fold (Fig. 7), the results of the ELISA were consistent with results of HPLC-EC. Despite the 500-fold dilution, shoot samples of *A. absinthium* still produced some cross-reactivity with the antibodies compared with the control (optical density 1.26 versus 1.31, respectively).

Cross-absorption assays, in which antibodies were diluted in gelatin buffer (G-PBS) containing root extracts of *A. annua*, produced a twofold effect: (1) a decrease in optical density indicating cross-reactivity caused by the presence of compound(s) in the root extract recognized by the antibodies as artemisinin; and (2) a decrease in the discrimination of the assay between the shoots of *A. absinthium* and different organs of *A. annua*. Thus, the presence of the root extracts acted to quench the specificity of the assay. However, even in the presence of root extract the assay indicated that artemisinin content in *A. annua* was highest in flowers, less in leaves, and least in side stems (Fig. 7), which confirmed our previous HPLC-EC analyses.

The ELISA was sensitive to artemisinin concentrations as low as 1.5 ng ml^{-1} (Fig. 5) and the antibodies

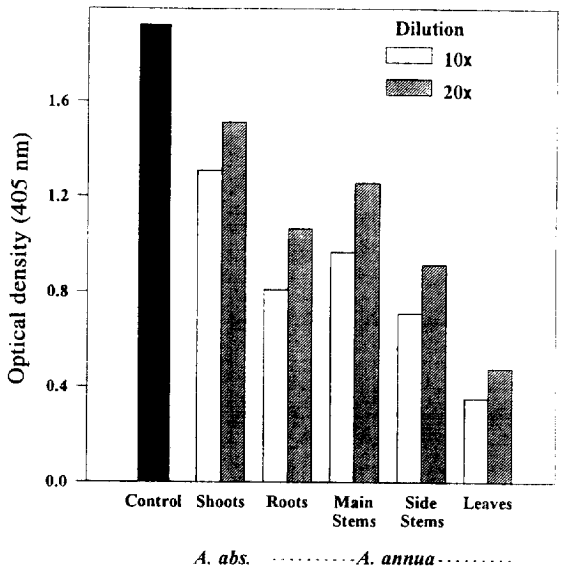

Fig. 6. Artemisinin immunodetection in crude samples of shoots of *Artemisia absinthium* and roots, main stems, side stems, and leaves of *A. annua*. Samples were diluted 10 (unfilled) or 20 times (shaded), primary antibodies were used at a final dilution of 1:12000, and optical densities were read after a 50 min incubation time of secondary antibodies (at 1:3000) with *p*-nitrophenyl phosphate. Antibodies diluted in PBS to 1:12000 were used as controls. Standard error, based on the controls ($n = 4$) was 0.013.

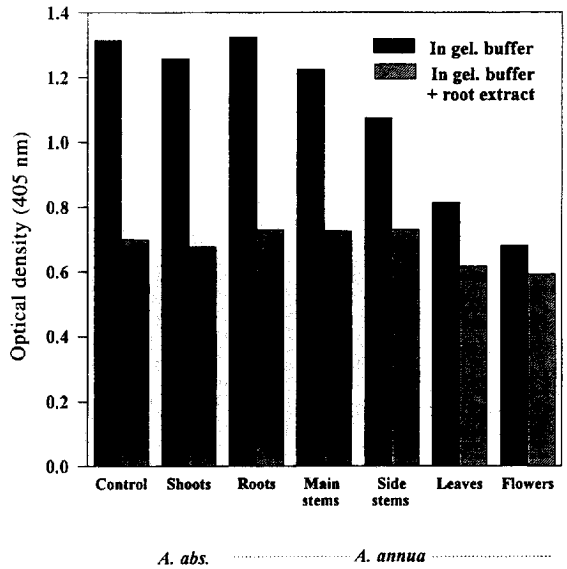

Fig. 7. Artemisinin immunodetection assay and cross-absorption study using crude extracts of shoots of *Artemisia absinthium* and roots, main and side stems, leaves, and flowers of *A. annua*. The control consisted of antibodies diluted to 1:18000 with gelatin buffer. Shoots of *A. absinthium* and roots of *A. annua* lack artemisinin and were considered as additional controls. The samples were diluted 500-fold in gelatin buffer and mixed 1:1 with anti-artemisinin antibodies at 1:9000, diluted in either gelatin buffer (black bars) or gelatin buffer plus root extracts at 10 mg dry wt l^{-1} (shaded bars) before they were incubated overnight, to evaluate cross-absorption. Optical densities were obtained 45 min after secondary antibodies labelled with alkaline phosphatase were incubated with *p*-nitrophenyl phosphate. Standard errors, based on the controls ($n = 4$) for gel. buffer and for gel. buffer + root extracts were 0.012 and 0.009, respectively.

were responsive to a titre of *c* 1:40000. Thus, artemisinin concentrations detected by ELISA were *c* 420 times lower than the minimal detection level provided by the HPLC-EC apparatus used in this study.

Artemisinin detection and quantification

Artemisinin quantification by ELISA from crude samples of a single plant diluted 20 or 40 times were highly correlated ($r = 0.99^{**}$) with HPLC-EC (data not shown). In contrast, quantification results of ELISA did not correlate with HPLC-EC when eight tissue-cultured clones of *A. annua* were evaluated at dilutions of 20- or 40-fold (data not presented). However, when samples (0.15 to 0.3 g dry wt) of these clones were diluted 100- or 500-fold, significant correlations ($r = 0.92^{**}$ or 0.90^{**}, respectively) between ELISA and HPLC-EC were obtained (Fig. 8). Artemisinin values from ELISA, however, were 2.6 to 10.8 times higher than HPLC-EC determinations when samples were diluted 100 times and 3.5 to 15.0 times higher when samples were diluted 500 times (Table 1).

Overestimation of artemisinin content by ELISA infers that impure crude plant extracts contain other compounds undetected by HPLC-EC but recognized as artemisinin by polyclonal antibodies. In view of the high correlations obtained between ELISA and HPLC-EC, those compounds recognized by the anti-artemisinin antibodies must be directly associated with artemisinin content. Cross-reactivity was also observed when 10- and 20-fold diluted samples of roots of *A. annua* and leaves of *A. absinthium* were analysed by ELISA (Fig. 6), but cross-reactivity was ameliorated by diluting samples up to 500 times (Fig. 7). Jaziri *et al.* [22] also found that their anti-artemisinin polyclonal antibodies cross-reacted with root extracts but did not identify the artemisinin-related compounds produced by the roots. We analysed root extracts of *A. annua* and shoots of *A. absinthium* by GC-mass spectrometry and compared to leaf and flower extracts of *A. annua*, but no common compounds were found that could directly explain the cross-reactivity. The high dilution of the samples was effective in achieving the screening of the clones, probably because the compounds that mimic artemisinin and react, at high concentrations, with the antibodies appear unreactive (or less reactive) at low concentrations. This can be seen in Fig. 5 where deoxyartemisinin, arteannuin B, and artemisinic acid are detected at high concentrations (286 and 1430 ng 50 μl^{-1}) but not lower concentrations. In contrast, the assay discriminated between different dilutions (up to 0.02 ng 50 μl^{-1}) of artemisinin, artemisitene, and dihydroartemisinin, when these compounds were present in lower concentrations in standard solutions.

The ELISA was considerably faster than HPLC-EC analysis for detection and screening of artemisinin, with

sensitivity as low as 1.5 ng ml^{-1} (*c* 400 times more sensitive than the HPLC-EC system used here). It was only possible to analyse a maximum of 25 samples per day with a single HPLC-EC apparatus, whereas a single technician should be able to analyse *c* 160 samples per day (two 96-well plates, including standards) with the ELISA—a greater than sixfold increase in efficiency —which makes it suitable for rapid screening of seedlings. Although cross-reactivity caused overestimation of artemisinin content, this problem could be ameliorated by dilution of the samples, enabling a successful artemisinin screening, confirmed by HPLC-EC. The antibodies should be useful for pharmacokinetic studies, in which artemisinin or its derived compounds are tested as antimalarial drugs. These drugs are tracked in blood or saliva to study compound stability [21]; however, additional investigations with the antibodies produced in this study will be required to determine their applicability for this use. Attempts should be made to generate monoclonal antibodies to try to overcome the cross-reactivity problems encountered when polyclonal antibodies are used in ELISA tests.

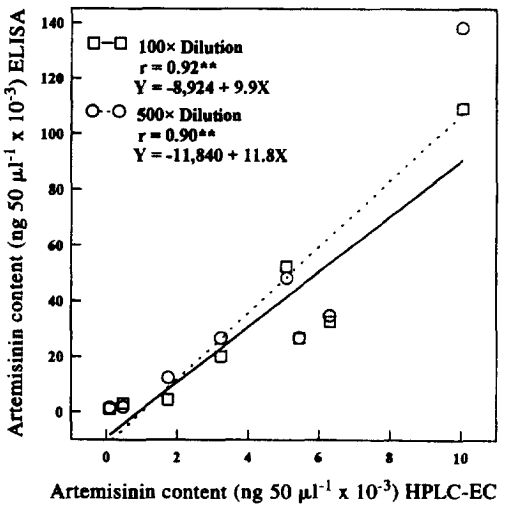

Fig. 8. Relationship of artemisinin content of samples (0.3 mg dry wt) of eight clones of tissue-cultured *Artemisia annua* estimated by ELISA and compared to the HPLC-EC analysis. Samples were diluted 100 and 500 times and antibodies were used at 1:18000. Optical density was taken after 45 min of incubation of secondary antibodies labelled with alkaline phosphatase with *p*-nitrophenyl phosphate. Standard errors at each point were lower than 0.019 for 100-fold and lower than 0.021 for 500-fold dilution.

EXPERIMENTAL

Plant material. Five-month-old *A. annua* and 1-year-old *A. absinthium* plants, grown in a greenhouse set at 27° (day)/25° (night), were used for testing the sensitivity of the assay and specificity of the antibodies for artemisinin detection. Different sample weights of a single plant and shoots of eight tissue-cultured clones were used in two studies to compare ELISA and HPLC-EC for quantification of artemisinin.

Reagents and materials. Artemisinin, boron trifluoride etherate, ethyl glycolate, isobutyl chloroformate, complete and incomplete Freunds adjuvant, BSA (Fraction V), porcine thyroglobulin (TGB), *p*-nitrophenyl phosphate, alkaline phosphatase-labelled goat antirabbit IgG, molecular sieve (3A), high-binding, flat, ELISA plates, carbodiimide hydrochloride (EDC), and *N*-hydroxysulphosuccinimide (Sulpho-NHS) were purchased from commercial sources. Dihydroartemisinin was obtained by reduction of artemisinin with $NaBH_4$. Artemisitene, deoxyartemisinin, arteannuin B, and artemisinic acid were provided by Dr A. J. Lin, Walter Reed Army Institute, Washington, DC, USA. All other chemicals and solvents used for derivatizing artemisinin and conjugating it to carrier proteins were reagent grade.

Artemisinin derivatization. Artemisinin (mol. wt 282) is too small to be immunogenic and must be conjugated to a large carrier protein that will function as the primary immunogen. However, artemisinin (Fig. 1) lacks a reactive group for linkage to a protein. To overcome this, artemisinin was derivatized in three steps (Fig. 2).

Table 1. Artemisinin content (ng 50 µl^{-1}) of eight clones of tissue-cultured *Artemisia annua* determined by HPLC-EC and estimated by ELISA with 100- and 500-fold dilution. Overestimation values of ELISA are based on HPLC-EC determination

	Artemisinin (ng 50 µl^{-1})			ELISA overestimation of artemisinin	
		ELISA			
Clones	HPLC-EC	100	500	100	500
1–4	100	1062	1505	10.6	15.0
31	479	2994	1698	6.2	3.5
8	1750	4464	12398	2.6	7.0
11	3232	20694	26700	6.4	8.3
87	5102	52406	48264	10.3	9.4
1–3	5455	26555	26825	4.9	4.9
129	6326	32567	34633	5.2	5.4
127	10050	109275	138230	10.8	13.8

Step A (reduction of artemisinin). Artemisinin (**1**), 500 mg (1.77 mmol), was dissolved in 40 ml of methanol at room temperature, cooled to 0–5°, and 300 mg (7.9 mmol) of $NaBH_4$, was added over a period of 30 min. The reaction was monitored by TLC (2% MeOH in $CHCl_3$) until artemisinin was completely reduced (2–3 hr). The reaction was stopped by lowering the pH to 5.0–6.0 with 2.6 ml of 30% HOAc in MeOH. Methanol was evapd under vacuum and the reduced compound (dihydroartemisinin = **2**) was washed three times with 50 ml of EtOAc. The washes were filtered through Whatman no. 1 filters, combined, and refiltered. The clear EtOAc solution was evapd under vacuum, and a final yield of 84% of **2** was obtained.

Step B (addition of an ester moiety to 2). All glassware was first oven-dried overnight at 100° and cooled in a desiccator. CH_2Cl_2 was dried by distillation over calcium hydride and stored in a dark flask containing dried molecular sieve beads until needed. Anhydrous diethyl ether could substitute for CH_2Cl_2 with the same or increased efficiency. The reaction was initiated by dissolving 0.42 g (1.48 mmol) of **2** in 25 ml of dry CH_2Cl_2 or anhydrous Et_2O and 3.0 g of dry molecular sieve and 7.4 mmol of ethyl glycolate (700 μl) were added to the mixture. The soln was then cooled to 0°, and 3 drops of a 50% soln of boron trifluoride etherate in anhydrous Et_2O were added. The reaction was stirred under nitrogen and monitored by TLC (20% EtOAc in petroleum ether) until no signs of **2** could be detected on the silica plates (2–3 hr). An additional 15 ml of CH_2Cl_2 was added during the reaction. The molecular sieve was filtered out of the soln and the CH_2Cl_2 evapd to a final yellowish oily compound. This compound mixture was washed with petroleum ether, and the yellowish fraction sepd. The petroleum ether was evapd to obtain the epimers (α and β) of dihydroartemisinin carboxymethylester (**3**).

Step C (base-catalysed hydrolysis of the ester to obtain the carboxyl group). One equivalent (2.86 mmol) of NaOH (0.114 g) was dissolved in 50 ml of 95% EtOH and added to **3**, obtained from Step B. This soln was allowed to stand overnight at room temp to produce the sodium salt of the ester. EtOH was evapd under vacuum, and the salt mixture was washed with EtOAc and filtered to separate the NaCl resulting from the reaction. Ethyl acetate was evapd, and the remaining compound was dissolved in water and treated with 6 N HCl to pH 4.0–5.0. The resulting ppt (*c* 230 mg) was a crude epimeric mixture (α and β) of the desired carboxylic acid (dihydroartemisinin carboxymethylether, **4**), which contained the desired reactive carboxyl group (Fig. 2). Compound **4** was then purified by CC with a mobile phase of 40% EtOAc, 59% hexane, and 1% HOAc. The compounds obtained from steps A through C were purified by CC. The stationary phase was silica, and the mobile phase used was the same as for the monitoring TLCs. All compounds were analysed and their identity was confirmed by at least two of the following methods: IR, NMR, HPLC-EC, and GC-MS.

Conjugation to carrier proteins. After **1** was derivatized into **4**, which had the desired reactive carboxyl group, it was attached to BSA or TGB (Fig. 2, step D). Two approaches were followed for this conjugation: (1) the mixed anhydride method [21], and (2) a milder reaction using a water-soluble carbodiimide (EDC) and sulpho-NHS, with slight modifications from the reaction described in [23].

4-BSA (mixed anhydride method). A sample of 40 mg (0.117 mmol) of **4** was dissolved in 2 ml of anhydrous dioxane and kept on ice. Then, 100 mg of BSA (0.0015 mmol) was dissolved in 5 ml of double-distilled (dd) H_2O, adjusted to a pH of 9.5, and cooled to 10°. Anhydrous dioxane, 2 ml, was added to this aqueous BSA soln and kept on ice. To the solution of **4**, 50 μl of tri-*n*-butyl amine was added and stirred for 30 min at 10° under nitrogen. Then, 30 μl of isobutyl chloroformate was added, and the soln was stirred continually at 10° for 20 min under N_2. After another 2 ml of anhydrous dioxane was added to this solution, it was added dropwise to the ice-cold stirring soln of BSA in H_2O:dioxane (5:2) at pH 7.4. After the two solutions were mixed, pH declined to 6.9 but was brought to 7.4 with 1 M $NaHCO_3$. This final solution was stirred overnight in a cold room. The next day, it was centrifuged at 13 000 ***g*** 5 min, and the supernatant was dialysed in water in a cold room for 3 days (two changes of 1000 ml day^{-1}). This conjugate was used as coating antigen on ELISA plates.

4-TGB (carbodiimide method). The first step was to dissolve 10 mg (0.03 mmol) of **4** in DMSO. Then, 70 mg of TGB was dissolved in 5 ml of ddH_2O, soln stirred, 192 mg EDC (1.0 mmol) was added, followed by 14 mg Sulpho-NHS (0.064 mmol). Compound **4** in dimethyl sulphoxide (DMSO) was then added to this EDC–sulpho-NHS/protein mix and stirred overnight at room temperature. The reaction was stopped the next day by adding it to 80 ml of ice-cold 10% trichloroacetic acid (TCA) and keeping the mixture on ice for 20 min for the conjugate to ppt. Excess TCA was poured off and the protein conjugate sepd from the remaining TCA by centrifuging at 16 000 ***g*** for 20 min. Trichloroacetic acid was discarded, then the conjugate was redissolved with cold ddH_2O and dialysed against water for 3 days in the cold room (2×1000 ml day^{-1}). All conjugates were lyophilized after dialysis and kept at $-70°$ until needed. This procedure was also used to link **4** to BSA. Both conjugates were injected in rabbits to raise polyclonal antibodies (see Results and Discussion).

Immunization, boosting, and bleeding procedures. Preimmune serum was collected from the four rabbits used in this study before immunization. Conjugates were dissolved in PBS ($1 \times$), mixed and emulsified with complete or incomplete Freund's adjuvant to a total volume of 0.6 ml. Six to seven dorsal injections were made with 23G1 needles. The first injection was 600 μg 0.6 ml^{-1} of **4**-carrier protein (BSA or TGB) mixed and emulsified at a 1:1 ratio with complete Freund's adjuvant per rabbit. Three booster injections were made in biweekly intervals with 600 μg of conjugate mixed 1:1 with incomplete Freund's adjuvant. Bleedings started 1 week after the third booster injection (seven bleedings total). Booster injections and bleedings were then performed weekly.

The bleedings were performed with a razor blade at the lower marginal vein of the ear, and 50–60 ml of blood per rabbit were collected. The blood was coagulated at room temp, clots were cut in squares, and stored at 4° overnight for better serum sepn. After samples were centrifuged, 13000 *g* for 20 min, sera were sepd and treated with 0.1% NaN_3. Subsamples of 50 μl sera were sepd for analysis, and the bulk of the serum was stored at −20°.

Immunoassay

Buffers. Coating carbonate buffer (for 500 ml) consisted of 0.53 g Na_2CO_3 (0.01 M), 1.47 g $NaHCO_3$ (0.035 M), 0.25 g NaN_3 (stored at 4°). PBS buffer (for 1 l): 8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4.12H_2O$, and 0.2 KCl, pH adjusted to 7.4. Blocking solution: 0.25% gelatin in PBS (G-PBS) (stored at 4°). Washing buffer (for 1 l): 8.76 g NaCl, 0.264 g $NaH_2PO_4.H_2O$, 0.115 g Na_2HPO_4 (anhydrous), 0.5 g NaN_3, and 0.5 ml Tween 20 (PBS-TZ) (stored at room temp). Developing buffer (for 500 ml): 48.5 ml diethanolamine, 0.2 g NaN_3 in 400 ml ddH_2O, pH adjusted to 9.8 with 6.0 N HCl, brought to final volume, and stored in a dark bottle at 4°.

Sample preparation. Samples (0.3 g dry wt) of roots, leaves, main and side stems, and flowers of *A. annua*, and leaves of *A. absinthium* plants grown in the greenhouse, and *c* 0.15 g dry wt from tissue-cultured clones were air-dried and extracted with petroleum ether at 45° for 24 hr. After evaporation of petroleum ether, samples reconstituted in 200 μl DMSO were then diluted 100 or 500 times with buffer (G-PBS). Samples were then mixed 1:1 with polyclonal antibodies diluted with G-PBS to 1:6000 or 1:9000, incubated overnight at 4°, and submitted to the ELISA next day. Samples of *A. absinthium* leaves and *A. annua* roots were used as negative controls for antibody specificity.

ELISA procedure. Conjugate **4**–BSA, obtained by the mixed anhydride reaction, was used as coating antigen for ELISAs, (TGB conjugate obtained by the carbodiimide reaction was not completely soluble, making it difficult to achieve the desired dilution for coating the plates). Plates were incubated overnight at 4° with 25 ng 50 μl^{-1} of **4**–BSA coating antigen in carbonate buffer. Coating antigen was discarded next day, plates were washed three times with PBS-TZ, and excess buffer removed. Triple washing and drying procedure was repeated between each step of the ELISA. Each well was blocked for 1–2 hr at room temp with 175 μl of G-PBS; 50 μl of primary antibody (polyclonals against **4**–TGB), diluted to 1:12000 or 1:18000 with G-PBS + samples or standards diluted in G-PBS (see Sample Preparation section above) was added to wells. After incubation, 1 hr room temp, primary antibodies were discarded. Secondary goat antirabbit antibody labelled with alkaline phosphatase and diluted to 1:3000 in G-PBS was added (50 μl) to the wells, and plates were incubated 1–2 hr at room temp. Secondary antibodies discarded, 100 μl of fresh substrate solution was added: Sigma-104 tablets (5 mg 6 ml^{-1}) dissolved in developing buffer. Colour developed for 30–50 min, and plates were read on a microplate reader set at 405 nm. Colour development stopped by adding 50 μl $well^{-1}$ 3 M NaOH. In each 96-well plate, three wells were filled with antibodies brought to final dilution with G-PBS (no artemisinin or derivatives) as a control, two wells with coating antigen but no primary antibodies (anti-artemisinin), two wells with primary but not secondary antibodies (goat anti-rabbit), and eight wells with different dilutions of artemisinin standards mixed 1:1 with primary antibodies and incubated overnight. These eight wells constituted the artemisinin standard curve which was run in each plate of every assay. Samples with unknown artemisinin content were analysed in the remaining 80 wells.

Antibody specificity. Competitive assays were carried out to determine the best antibody to use in the quantification assays and to establish a standard curve for artemisinin. Competitive assays for the crossreactivity study included artemisitene, dihydroartemisinin, deoxyartemisinin, arteannuin B, and artemisinic acid, in addition to artemisinin. Affinity of polyclonal antibodies was evaluated by comparing them with pre-immune sera and specificity was evaluated through cross-reactivity tests with artemisitene, dihydroartemisinin, deoxyartemisinin, arteannuin B, and artemisinic acid. A cross-absorption study was performed to investigate the possible presence of nonspecific antibodies mixed with the anti-artemisinin polyclonal antibodies: antibodies were diluted with G-PBS to which root extract of *A. annua* at 1 g dry wt 10 ml^{-1} of G-PBS was added. Replicate samples of the second study (see HPLC-EC versus ELISA Analyses section below) were used.

ELISA versus HPLC-EC analyses. Three studies compared crude extracts from different sources to test the efficiency of ELISA compared with an HPLC-EC method previously described [14]. Values obtained from plate readings (405 nm) after 30–50 min colour development were used to calculate the amount of artemisinin (ng 50 μl^{-1}) in each sample, compared with the artemisinin standard curve in the plate. Artemisinin contents obtained by HPLC-EC and ELISA were compared by linear regression. First study consisted of samples of 50, 100, 200, 400, 600, and 800 mg (dry wt) of leaves and flowers of *A. annua*. Second study compared artemisinin content from shoots of *A. absinthium* (no detectable artemisinin by HPLC-EC) and roots, main and side stems, leaves, and flowers of a greenhouse-grown *A. annua* plant. A third study compared the shoot artemisinin content from eight tissue-cultured clones of *A. annua*.

Acknowledgements—We thank Drs Zhen-Yu Song, Institute of Materia Medica, China, Mondher Jaziri, University of Brussels, and Peter Heinstein for valuable advice during the course of this research. We thank Dr Charles Woloshuk for critically reviewing this manuscript. We acknowledge the help of the Purdue University Cancer Center in using its animal facilities. Thanks are due to the Brazilian National Research Council (CNPq) and the Research Enterprise of Minas Gerais (EPAMIG), Brazil, for their financial support.

REFERENCES

1. Tu, Y-Y., Ni, M-Y., Zhong, Y. R., Li, L-N., Cui, S-L., Zhang, M-Q., Wang, Y. Z., Ji, Z. and Liang, X-T. (1982) *Planta Med.* **44**, 143.
2. Klayman, D. L., Lin, A. J., Acton, N., Scovill, J. P., Hoch, J. M., Milhous, W. K. and Theorides, A. D. (1984) *J. Nat. Prod.* **47**, 715.
3. Luo, X. D. and Shen, C. C. (1987) *Med. Res. Rev.* **7**, 29.
4. Roth, R. J. and Acton, N. (1989) *J. Nat. Prod.* **52**, 1183.
5. Tawfiq, N. K., Andersson, L. A., Roberts, M. F., Phillipson, J. D., Bray, D. H. and Warhurst, D. C. (1989) *Plant Cell Rep.* **8**, 425.
6. Pras, N., Visser, J. F., Batterman, S., Woerdenbag, H. J. and Malingré, T. M. (1991) *Phytochem. Anal.* **2**, 80.
7. Zhao, S. S. and Zeng, M. Y. (1985) *Planta Med.* **51**, 233.
8. Acton, N. and Klayman, D. L. (1985) *Planta Med.* **51**, 441.
9. Laughlin, J. C. (1994) *Trans. Royal Soc. Trop. Med. Hyg.* **88** (Suppl. 1), 21.
10. Singh, A., Vishwakarma, R. A. and Husain, A. (1988) *Planta Med.* **54**, 475.
11. Acton, N., Klayman, D. L. and Rollman, I. J. (1985) *Planta Med.* **51**, 445–446.
12. Zhou, Z., Huang, Y., Xie, G., Sun, X., Wang, Y., Fu, L., Jian, H., Guo, X. and Li, G. (1988) *J. Liq. Chrom.* **1**, 1117.
13. Melendez, V., Peggins, O., Brewer, T. G. and Theorides, A. D. (1991) *J. Pharm. Sci.* **80**, 132.
14. Ferreira, J. F. S., Charles, D. J., Wood, K., Simon, J. E. and Janick, J. (1994) *Phytochem. Anal.* **5**, 116.
15. Fulzele, D. P., Sipahimalani, A. T. and Heble, M. R. (1991) *Phytother. Res.* **5**, 149.
16. Sipahimalani, A. T., Fulzele, D. P. and Heble, M. R. (1991) *J. Chrom.* **538**, 452.
17. Banthorpe, D. V. and Brown, G. D. (1989) *Phytochemistry* **28**, 3003.
18. Woerdenbag, H. J., Pras, N., Bos, R., Visser, J. F., Hendriks, H., and Malingré, T. M. (1991) *Phytochem. Anal.* **2**, 215.
19. Woerdenbag, H. J., Bos, R., Salomons, M. C., Hendriks, H., Pras, N., and Malingré, T. M. (1993) *Flav. Frag. J.* **8**, 131.
20. Ranasinghe, A., Sweatlock, J. D. and Cooks, R. G. (1993) *J. Nat. Prod.* **56**, 552.
21. Zhao, K. C., Liu, C. X., Liang, X. T., Mingguang, Y. and Song, Z. Y. (1986) *Proc. Chinese Acad. Med. Sci.* **1**, 213.
22. Jaziri, M., Diallo, B., Vanhaelen, M., Homes, J., Yoshimatsu, K. and Shimomura, K. (1993) *Phytochemistry* **33**, 821.
23. Staros, J. V., Write, R. W. and Swingle, D. M. (1986) *Analyt. Biochem.* **156**, 220.