Cytotoxicity of Ethanolic Extracts of *Artemisia annua* to Molt-4 Human Leukemia Cells

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**Key words**

- *Artemisia annua* 
- Asteraceae 
- Ethanolic extracts 
- Antioxidant 
- Flavonoids 
- Synergism 
- Leukemia 
- MOLT-4

**Abstract**

Although dihydroartemisinin (DHA) and other artemisinin derivatives have selective toxicity towards cancer cells, *Artemisia annua* (*A. annua*) extracts containing artemisinin have not been evaluated for their anticancer potential. Our main goal was to assess the anticancer effect of ethanolic leaf extracts of *A. annua* from Brazilian and Chinese origins (with DHA as a comparison) on normal and cancer cells. Leukocytes and leukemia (Molt-4) cells were counted at 0, 24, 48, and 72 hr after treatment with extracts having artemisinin concentrations of 0, 3.48, 6.96, and 13.92 µg/mL. Also, we assessed the antioxidant capacity of these extracts using the oxygen radical absorbance capacity (ORAC) test. Both extracts had high antioxidant capacity and toxicity towards Molt-4 cells. DHA was significantly more potent (*p* < 0.05) in killing Molt-4 cells than Brazilian extract at 48 and 72 hr and Chinese extract at 72 hr. In Molt-4 cells, LD50 values for Brazilian and Chinese extracts were comparable at all time points and not significantly different from DHA at 24 hr. In leukocytes, DHA, Chinese extract, and Brazilian extract had LD50 values of 760.42, 13.79, and 28.23 µg/mL of artemisinin, respectively, indicating a better safety index for the Brazilian extract compared to that of the Chinese extract at 24 hr. However, at 48 and 72 hr, the toxicity in leukocytes for any of the treatment groups was not significantly different. These experiments suggest that these extracts may have potential application in cancer treatment.

**Introduction**

*Artemisia annua* Linnaeus, family Asteraceae (*A. annua*), has been used for at least 1600 years in traditional Chinese herbal medicine to treat symptoms associated with malaria. *Herba Artemisiae annuae* (leaves + stems) was cited in the *Classified Materia Medica* as a food supplement related to longevity [1], a characteristic probably associated with its antiparasitic and antioxidant properties. *A. annua* is still the sole commercial source of artemisinin.

Dihydroartemisinin (DHA), a semisynthetic derivative of artemisinin, kills cancer cells in vitro [2, 3] and in vivo [4–6], leading to most anticancer research currently performed with DHA [7–9]. No evaluation of the potential anticancer properties of *A. annua* crude extracts has been published. Although ethanolic, aqueous, and other plant extracts are commercially available [10], the concentration of their bioactive metabolites and the origin of their plant source are undisclosed.

Chloroform crude extracts of *A. annua* are rich in methoxylated flavonoids that synergized artemisinin antiparasitic activity [11]. However, the nonpolar (lipophilic) nature of these extract makes their testing in vitro challenging. On the other hand, hydrophilic (polar) extracts are easy to dissolve in aqueous medium. In addition, the lyophilized ethanolic (70% EtOH) leaf extract used in this study had hydrophilic oxygen radical absorbance capacity (ORAC) of 2535 µmoles TE/g DW [12]. Considering that antioxidant phenolics (including flavonoids such as quercetin and casticin) present in *A. annua* leaves have anticancer activity of their own [13], we hypothesize that ethanolic leaf extracts of *A. annua* have similar anticancer effects as DHA. In addition, *Artemisia* flavonoids may synergize with artemisinin, increasing its biological activity [14]. To our knowledge, this is the first study in which *A. annua* crude ethanolic extracts have been assessed for their anticancer activity. Our goal was to evaluate the anticancer activity of two ethanolic *A. annua* extracts (from Chinese and Brazil-
ian origins), using DHA as a comparison (in concentrations similar to those that have been previously used) against Molt-4, a cancer cell line derived from human lymphoblastoid leukemia cells.

**Materials and Methods**

*Plant material and growing conditions*

Artemisia annua of both Brazilian (University of Campinas, Brazil, cultivar 3 M) and Chinese (Chongqing Holley Pharma Company, Chongqing, China) origins were cultivated in soil type Gilpin silt loam (fine-loamy, mixed, mesic Typic Hapludult) in Beaver, West Virginia, USA, at 37°45′ N, 80°59′ W (945 m altitude). Seedlings were greenhouse-grown under a 16-hr photoperiod and field transplanted when 20 cm high in June. Plants had a 1 plant·m⁻² density and received 45 kg N, 20 kg P, and 37 kg K per hectare at planting. Both Brazilian and Chinese cultivars were test-cultivated in West Virginia for three years (2005-2007) and produced peak artemisinin between late August and the first week of September [15], with artemisinin concentrations ranging from 0.7–1.5%. Both cultivars were identified by one of the authors (JFSF), an expert on the species, and were confirmed as Artemisia annua L. (Asteraceae). In addition, although three species out of approximately 400 have been reported to produce artemisinin, only selected A. annua, such as the cultivars used here, produce artemisinin at levels high enough to be of commercial interest.

**Materials**

Ethanol, acetonitrile, and petroleum ether were all HPLC-grade (Fisher Scientific). Artemisinin (Sigma-Aldrich, 98% pure), dihydroartemisinic acid (92.2% pure), and artemisinic acid (> 90% pure) both from Amyris, as well as deoxyartemisinin (96.2% purity, kind gift of Dr. Mitchell Avery) were used as standards to identify and quantify compounds from plant extracts. For the ORAC analysis, fluorescein sodium salt, AAPH [2,2′-azobis(2-methylpropionamide)] dihydrochloride, Trolox (a vitamin E water soluble analog, over 98% pure), K₂HPO₄ dibasic, and NaH₂PO₄·H₂O monobasic were purchased from Fisher Scientific or Sigma-Aldrich. The ORAC assay was performed according to a preestablished method [16]. For the in vitro cell culture experiment, unless mentioned otherwise, all chemicals were purchased from Sigma-Aldrich.

**Preparation of ethanolic extracts and HPLC-UV analysis**

Leaves of Brazilian and Chinese cultivars were dried in a forced-air oven at 45°C for 48 hr and then ground in a cyclone grinder to 2-mm size particles and kept at ~15°C until extraction. Ground dry leaves (25.0 g) of each cultivar were extracted using 150 mL of 70% aqueous ethanol (70:30 ethanol:water) at 60°C with stirring for 2 hr. Extracts were sonicated for 30 min and filtered through Whatman #2 filters. Extraction and sonication were repeated, extracts combined, rotary evaporated and sonicated in pure ethanol to remove all extract from the evaporation flask. No trace of ethanol remained after lyophilization. Extracts were concentrated under nitrogen and freeze-dried. This resulted in 8.37 g (33.5% yield) of green crystals for the Brazilian Artemisia and 6.44 g (25.74% yield) for the Chinese Artemisia. Crystals were powdered using mortar and pestle before in vitro studies and antioxidant analysis.

To test if 70% EtOH extracts the major A. annua sesquiterpenes as commercial nonpolar (hexane or petroleum ether) extraction, another Brazilian clone (3 M-39), which produces 1.5% artemisinin, was extracted and analyzed (HPLC-UV) by a validated procedure [17]. Ethanolic extracts were prepared by adding 10 mL of acetonitrile to 100 mg of each lyophilized extract. These solutions were sonicated for 30 min, filtered and analyzed. The chromatograms (Fig. 1) are for comparing phytochemical profiles between hexane extracts and lyophilized ethanolic extracts, but not for concentration comparison due to biomass differences generated by those two extraction methods.

**Oxygen radical absorbance capacity (ORAC) assay**

ORAC assay for hydrophilic components (flavonoids and phenolic acids) was performed for both Brazilian and Chinese ethanolic extracts as follows: 100 mg of each lyophilized extract was mixed with 10 mL of acetone:water:acetic acid (70:29.5:0.5). Solutions were vortexed (K-550-G; VWR Scientific) for 30 sec and...
sonicated (FS60H; Fisher Scientific) for 10 min at 37 °C. Solutions were vortexed for 15 sec and centrifuged (Sorval Evolution RC; Thermofisher) at 3000 rpm for 7 min. The supernatant from each centrifuge tube was transferred to a 25-mL volumetric flask. Extraction, sonication, and centrifugation steps were repeated, supernatants combined, and the final volume was brought to 25 mL using the acetonite:water:acetic acid solution described above. Extracts were diluted 120 times with phosphate buffer, and 40 µL was transferred to a 48-well Multiwell™ polypropylene plate (Becton Dickinson Labware). To each well, 400 µL of fluorescein and 40 µL of AAPH in phosphate buffer were dispensed automatically. Plates were read in a fluorescence reader (FLUOSTar Optima; BMG Labtech) set at 485 nm excitation and 520 nm emission wavelengths. Samples were reacted with AAPH (an oxidizer) and fluorescein at 37 °C and read in 45 cycles, at 3.35-min intervals for 150 min. Antioxidant activity of the extracts was evaluated by their neutralization of the oxidizer AAPH, thus preventing fluorescein oxidation. Antioxidant capacity was calculated by differences between the areas under the curve (AUC) generated by the samples, blank, and standard curves (using AAPH as an oxidizer and Trolox as the antioxidant). Calculations were done by the MARS software (version 1.10, Build 1.10.1.28; BMG Labtech), and results were expressed in µmoles of Trolox equivalents per gram of sample dry weight (µmoles TE/g DW), according to a published procedure [16].

**Table 1** The antioxidant capacity of freeze-dried (FD) and oven-dried (OD) leaves of *A. annua* from a cloned Swiss cultivar and of Brazilian and Chinese *A. annua* leaf ethanolic (EtOH) extracts determined by the oxygen radical absorbance capacity (ORAC) assay. All plants were field-cultivated in Beaver, WV, USA. They are expressed in micromoles of Trolox equivalents per gram of dry weight (µmoles TE/g DW). Total antioxidant capacity (TAC) of FD and OD leaves is the summation of the hydrophilic fraction of the leaves (ORAC_{hydro}) and the lipophilic fraction (ORAC_{lipoph}). The ORAC_{lipoph} of the ethanol extracts was not determined (ND) and the TAC of the polar ethanol extracts is equal to the hydrophilic fraction ORAC (ORAC_{hydro}). Relative standard deviation (RSD) is shown in between parentheses.

<table>
<thead>
<tr>
<th>Organ/extract type</th>
<th>Hydrophilic ORAC_{hydro} (µmoles TE/g DW)</th>
<th>Lipophilic ORAC_{lipoph} (µmoles TE/g DW)</th>
<th>TAC ORAC_{hydro} + Lipo (µmoles TE/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves (FD)</td>
<td>1029.2 (15.8)</td>
<td>58.6 (23.0)</td>
<td>1088</td>
</tr>
<tr>
<td>Leaves (OD)</td>
<td>1041.7 (6.3)</td>
<td>39.6 (14.9)</td>
<td>1081</td>
</tr>
<tr>
<td>Brazilian EtOH extract</td>
<td>2535 (0.84)</td>
<td>ND</td>
<td>2535 (0.84)</td>
</tr>
<tr>
<td>Chinese EtOH extract</td>
<td>1960 (1.07)</td>
<td>ND</td>
<td>1960 (1.07)</td>
</tr>
</tbody>
</table>

FD = freeze-dried; OD = oven-dried; EtOH = 70% ethanol; ORAC = oxygen radical absorbance capacity; Hydro = hydrophilic fraction of sample submitted to ORAC assay; Lipo = lipophilic fraction of sample submitted to ORAC; TAC = total antioxidant capacity is the sum of ORAC_{hydro} plus ORAC_{lipoph}. Values are in µmoles of Trolox equivalents/gram of sample dry weight (µmoles TE/g DW).

**Molt-4 cell culture treatment and counting**

Cells used in these experiments were purchased from ATCC (American Type Culture Collection) and were validated to be Molt-4, a human lymphoblastoid leukemia cell line. These cells were grown in 100% humidity at 37°C in 5% CO2, in RPMI-1640 medium (Life Technologies) with 10% fetal bovine serum (HyClone). Each experiment was conducted three times using Molt-4 cells and leukocytes. Mean and standard deviation were calculated for each data point. Data were plotted as percentage, determined by dividing the mean cell count at 24, 48, or 72 hr by the mean cell count at 0 hr. Data were analyzed by one-way ANOVA, and differences between pairs of data points were compared by the Newman-Keuls Test. Statistical difference was considered significant when p < 0.05.

**Results**

The antioxidant capacity of both Chinese and Brazilian extracts, determined by the ORAC assay, established that both extracts had high antioxidant capacity. However, the antioxidant capacity of the Brazilian ethanolic extract was approximately 30% higher than that of the Chinese cultivar. The total antioxidant capacity (TAC) of oven-dried Brazilian *A. annua* leaves (used to make the ethanolic extracts tested) was similar to the TAC of the freeze-dried subsamples (1081 and 1088 µmoles TE/g), with the hydrophilic fraction of the oven-dried Brazilian *A. annua* accounting for 96% of the total leaf antioxidant capacity, while lipophilic extracts accounted for only 4% (1041 µmoles TE/g DW vs. 39.6 µmoles TE/g DW, @ Table 1). While the TAC of the Brazilian ethanolic extract is higher than that of the Chinese extract, the concentration of artemisinin in the Chinese extract (2.7% in g/100 g DW) was approximately 2.2 times higher than in the Brazilian (1.26%) extract, although both were extracted in 70% ethanol in the same way.
The HPLC-UV chromatogram shows that the ethanolic extracts had most peaks (except the peak at 14 min) present in *A. annua* commercial extracts made with nonpolar hexane or petroleum ether (Fig. 1). The Chinese ethanolic extract also contained 1.6 times more dihydroartemisinic acid (1.46% vs. 0.9%, respectively), and 2.4 times more artemisinic acid (0.24% vs. 0.1%, respectively) than the Brazilian ethanolic extract (in g/100 g DW).

Fig. 2A and B show the cytotoxicity of various doses of DHA and *A. annua* extracts, as a percent cell count from time zero, in Molt-4 cells and leukocytes, respectively at 24, 48, and 72 hr time points. The results established that DHA and various doses of extracts are significantly effective (p < 0.01) in killing Molt-4 cells compared to extract-free DMSO controls.

As shown in Table 2, the relative toxicity (LD$_{50}$ values) of each treatment on normal versus cancer cells was analyzed based on the mean of the cell count. At 48 hr of treatment, DHA had 1.26 times higher anticancer activity than the Brazilian ethanolic extract (p < 0.05) in killing Molt-4 cells. Moreover, at 72 hr of treatment, DHA was 1.66 times more potent than the Chinese extract (p < 0.01) and 1.49 times more potent than the Brazilian extract (p < 0.01). On the other hand, data from leukocytes showed that both Brazilian and Chinese extracts were more toxic to normal cells than DHA at 24 hr of treatment (p < 0.05), but the difference in toxicity between DHA and the extracts decreased considerably at 48 hr. Overall, no significant effect was observed between the two *Artemisia* extracts at all time points on Molt-4 cells and leukocytes.

### Discussion

The Chinese ethanolic extract was 68% more concentrated in artemisinin than the Brazilian extract. This reflects the genotypic variation of seed-generated plants and between cultivars, which agrees with reports of different chemotypes for *A. annua* [18]. However, the commercial-type (petroleum ether) extract from a Brazilian plant had a similar phytochemical profile as both ethanolic extracts (Fig. 1) indicating that 70% ethanol can extract almost all components extracted by nonpolar solvents, including dihydroartemisinic acid and artemisinic acid. These compounds may synergize with artemisinin increasing its anticancer effects. It is also true that polar fractions, extracted in 70% acetone, usu-
ally retain 95% or more of the total antioxidant capacity compared to nonpolar fractions extracted in hexane [12] (Table 1). Thus, ethanolic extracts have higher concentrations of antioxidant flavonoids than hexane or petroleum ether extracts, increasing the likelihood of synergism for ethanolic extracts. Data also showed that the oven-dried leaves used to make the ethanolic extracts tested on Molt-4 cells had similar antioxidant capacity (hydrophilic ORAC) as freeze-dried subsamples, and that hydrophilic fractions contained over 95% of the total antioxidant capacity (TAC), while lipophilic antioxidant components of the extract (such as vitamin E) accounted for less than 4% of the TAC (Table 1). These ORAC results and the HPLC–UV data (Fig. 1) suggest that 70% ethanol is a perfect medium to extract most of the antioxidant components and artemisinin from A. annua leaves.

To evaluate the anticancer potential of A. annua extracts, we assessed the cytotoxicity of both dihydroartemisinin (DHA) and the plant extracts. DHA (an analog of artemisinin but not produced by the plant A. annua) was used primarily as an index and confirmation that our system of testing cytotoxicity was reliable. We were unable to use artemisinin as a marker because it is poorly soluble, making it unsuitable for this use, alongside liquid extracts. Interestingly, the LD50 value in Molt-4 cells for the Brazilian ethanolic A. annua extract was 3.5-fold lower than for DHA and 1.7-fold lower than for the Chinese extract at the 24 hr time point, while Brazilian and Chinese extracts were more toxic to leukocytes than DHA. The Brazilian extract was used at approximately twice the concentration of the Chinese extract to maintain the artemisinin equivalence in both extracts. The Brazilian extract was relatively more toxic to cancer cells than the Chinese extract, and both extracts were more toxic to normal cells than DHA (Table 2). Although our in vitro toxicity data indicated that both Chinese and Brazilian extracts were significantly more toxic to leukocytes than DHA (Table 2), animal studies showed that both extracts presented no toxicity when fed to gerbils by oral gavage at 100 mg/mL, for 5 consecutive days [19]. The dose used in that study was approximately 100–200-fold (100 mg/mL) higher than the maximum concentrations of the Chinese (516 µg/mL) and Brazilian (1074 µg/mL) extracts used here. Several factors may be responsible for the significantly different effects of DHA and Brazilian ethanolic A. annua extract on cancer cells. While there are few studies comparing A. annua extracts with its synthetic derivatives (none for cancer), previous research on other natural compounds may elucidate this effect. It has been shown that isolating and using a pure active compound may do more harm than good since isolated components may lead to increased side effects as compared with whole plant extracts [20, 21]. Moreover, Olsson and collaborators [22] reported that pure vitamin C and purified compounds from whole fruit and berry extracts were not as effective in killing breast (MCF-7) and colon cancer (HT29) cells in vitro as crude extracts. These extracts had natural vitamin C and a higher antioxidant activity than pure vitamin C, indicating synergism between vitamin C and other compounds from the whole extracts [22].

The work of others [11] showed that dichloromethane extracts from the same Brazilian cultivar had more methoxylated flavonoids than a Chinese cultivar. They reported that these flavonoids were also found in hexane extracts and that they enhanced the reaction between artemisinin and hemin, synergizing artemisinin as antimalarial. If some of these flavonoids were also extracted in 70% EtOH at 60°C, they could also have synergized the anticancer effect of artemisinin in the extract. This potential synergism has been recently reviewed for several flavonoids present in A. annua [23].

Although flavonoids have anticancer effects, they also carry some degree of toxicity to normal cells [24, 25]. This toxicity may be considerably lower in vivo than in vitro due to detoxification mechanisms not present in isolated cells. Potential benefits in the use of a flavonoid-rich extract include suppression of CYP450 enzymes, such as CYP2B6 and CYP34A, which degrade artemisinin, thus increasing artemisinin bioavailability [26]. Artemisinin alone has anticancer effects in vitro by suppressing nitric oxide synthesis through the inhibition of the nuclear factor NF-κB [27]. Thus, the potent anticancer activity of the ethanolic extracts could be due to artemisinin plus various compounds in the extract synergizing the artemisinin effect. Since flavonoids have been hypothesized to trigger internal antioxidants (such as glutathione) and blockage of cellular inflammatory responses instead of exerting a direct antioxidant activity, a plant extract may provide longer lasting changes than the direct effect of artemisinin alone. Furthermore, extracts may be effective in smaller doses, with fewer side effects [28]. Flavonoids have also been shown to suppress P-glycoproteins (ABC transporters involved in the multidrug resistant pumps found in cancer cells and in some parasites), which may explain why there are no records of resistance to whole plant extracts [29].

Because different extraction solvents and procedures may result in different contents of artemisinin and other anticancer agents, it is important to optimize the extraction of anticancer compounds from A. annua leaves and to standardize these extracts before further anticancer tests in vivo. Once extract preparation is optimized, a method of delivery with appropriate and safe solvents may lead to a plant extract with similar, or better anticancer efficacy than currently available chemotherapies, and with significantly less toxicity to normal cells. The results presented here warrant further research focused on elucidating the natural compounds responsible for the antioxidant capacity, cytotoxicity, and synergistic anticancer activities of A. annua ethanolic extracts.

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Conflict of Interest

The authors declare no conflict of interest. Mention of trade names or commercial products in this publication is solely for the convenience of the reader and does not imply endorsement of the U.S. Department of Agriculture over similar products. The USDA is an equal opportunity provider and employer.
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