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Agrotechnological aspects of the anti-malarial plant *Artemisia annua* and its potential use in animal health in Appalachia

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SUMMARY

Haemonchus contortus is the most detrimental gastrointestinal parasite in small ruminants worldwide. The problem is increasing due to the developing resistance of the parasite to commercially available anthelmintics. Medicinal plants with their biologically-active compounds may provide a viable alternative to synthetic anthelmintics, but sound scientific evaluation of such compounds is lacking. *Artemisia annua* is an effective antimalarial drug used currently in over 50 countries as the first line of treatment against chloroquine-resistant malaria. Its mode of action against malaria and cancer indicates that the plant and its main active ingredient (artemisinin) may also be used as an alternative way to control gastrointestinal parasites in small ruminants. The plant has naturalized in several areas of the world, but wild plants are photoperiod sensitive and low in artemisinin. New varieties developed in Switzerland and Brazil are late-flowering, have artemisinin concentrations varying from 0.5 to 1.0% w/w, and are more suitable to tropical areas afflicted by malaria. First-year trials in West Virginia have indicated that these new cultivars have potential for biomass accumulation and artemisinin production in the Appalachian region, and cultivars will be tested for their anthelmintic potential in goats. Research is ongoing to determine artemisinin fate and stability in ruminants, its effect on rumen microflora and dry matter digestibility, and artemisia herbage palatability to ruminants. This paper reviews past work with *Artemisia* spp. in small ruminants and presents preliminary results of our research with *Artemisia annua* in goats. If *A. annua* proves to be as valuable an anthelmintic as it is an anti-malarial, meat-goat (*Capra hircus*) farmers in Appalachia and other regions affected by haemonchosis will have a natural way to control gastrointestinal parasites, boosting the growth of the meat goat industry.

1- ARTEMISININ AND MALARIA

The term malaria (*paludisme*) first entered the English medical literature in 1827, introduced by John Mucculloch (1). The word was derived from the Italian *mala* (bad) *aria* (air) due to the association of the disease with swamps (*palus*). Among infectious diseases, malaria is only second to AIDS, costing Africa approximately $12 billion/year in lost gross domestic product (GDP). Its control in Africa would cost US$ 2 billion/year (2). In recent years, the malaria epidemic has at best stabilized in African countries. Each year, there are 300-500 million clinical cases worldwide, killing from one to three million children (3). Over 90% of these cases occur in the sub-Saharan Africa, but large areas of Asia, Central, and South America also have high incidences of the disease (4). Out of 37 countries and territories, which are members of the Pan American Health Organization (PAHO), 21 still have active malaria transmission (5). Artemisinin is effective against all four *Plasmodium* species that cause malaria.

Unfortunately, there are few success stories of malaria eradication, but in Jamaica and Taiwan, economic growth accelerated after the parasite was eliminated in 1958 and 1961, respectively (6). In 1969, the Chinese army found that a diethyl ether extract of *Artemisia annua* L. (**Fig. 1**) had an excellent effect against malaria and, in 1972, artemisinin (**Fig. 1**) was identified as the main active ingredient (7). Artemisinin is considered one of the principal discoveries in recent medicinal plant research, and its isolation and characterization have increased the interest in *A. annua* worldwide. Increased production of artemisinin may also allow utilization of its recently established anti-cancer

attributes (8). Artemisinin is the base compound for the semi-synthesis of antimalarial drugs that are more potent and stable than artemisinin itself.

Current alternatives to natural artemisinin for control of malaria include OZ277, a synthetic triloxane peroxide developed through a multi-organizational approach launched in 1999, headed by the Medicines for Malaria Venture, and involving academia, pharmaceutical companies, and research institutes. The drug is effective, affordable, and reported to last longer in the body than artemisinin (9, 10), but is still at least five years away from being commercially available. This year, the *in vitro* production of an artemisinin precursor, artemisinic acid, in yeast was achieved with the promise of more affordable artemisinin in the near future (11), but the process has not reached commercial production capacity yet. Although the new technologies offer hope of affordable artemisinin, or a substitute, in the near future, the loss of a child every 40 seconds (3) prompts us to increase the supply of plant-produced artemisinin.

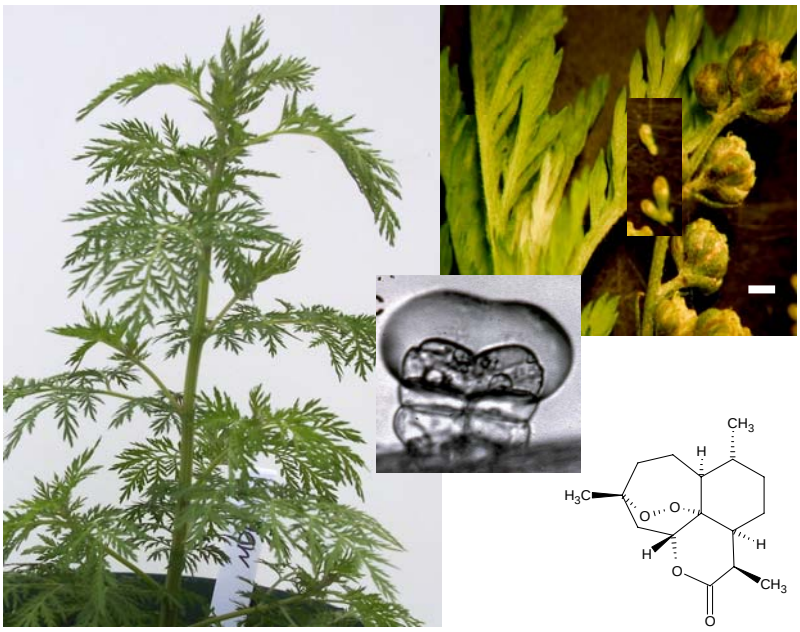

Figure 1. Exomorphology of *Artemisia annua*. Left column: two-month old *Artemisia annua* plant (grown from Artemis®[1] seeds, Mediplant, Switzerland[1]). Right column : upper insert –details of leaves, capitula, and hermaphroditic florets (bar size = 2 mm) before anthesis, central inset – Capitate, 10-celled, glandular trichome found in leaves and flowers, which when mature accumulate essential oils and artemisinin in the upper subcuticular space; lower insert - Artemisinin molecule, a sesquiterpene lactone with a peroxide bridge that is effective against malaria and cancer (12).

[1] *Trade names and companies are used throughout this manuscript for the convenience of the reader, and do not imply endorsement by the USDA over comparable products or competitors*.

Administration of artemisinin with other anti-malarial drugs (artemisinin-based combination therapy, ACT) has been recommended by the World Health Organization since 2001 wherever resistance to conventional anti-malarial drugs has been observed (13). From 2001 to 2005, 53 countries in Africa, Asia, and South America have adopted ACT as the first- or second-line antimalarial treatment where chloroquine is no longer effective (13). This recommendation caused the demand for artemisinin to increase, leading to supply shortages up to 2004 (14). To meet the artemisinin demand in Africa, East African Botanicals has expanded area planted with *A. annua* from 200 to more than 1,000 hectares in Kenya, Tanzania, and Uganda (15). In China, Holley Pharmaceuticals[1], working with associated farmers, has announced its intent to increase annual production of artemisinin from 14 to 40 metric tons per year (12). It is estimated that the worldwide area needed to meet the current WHO-estimated demand for 266 million ACTs for 2006 is between

10,000-11,000 hectares, based on the estimate that 1 ha of artemisia produces enough artemisinin for approximately 25,000 adult courses of ACT (16).

Due to the steady spread of chloroquine-resistant malaria, and the lack of affordable and efficient vaccines or alternative drugs at the moment, the search for effective, safe, and affordable antimalarial drugs is one of the most pressing health priorities worldwide (17). Producing artemisinin from *A. annua* is currently the most economical alternative given the low yield, complexity, and high cost of synthetic artemisinin.

2- ARTEMISIA AGROTECHNOLOGY

2.1- Environmental conditions

Artemisia is believed to be originally from Sichuan, a Chinese province with temperate climate, where it grows naturally at altitudes above 1,000 meters. *Artemisia annua* is a C3 plant (18), which partly explains the problem in cultivating the plant close to the equator. Ironically, the plant is not naturally suited to the tropical latitudes of Africa, which have hot weather and short photoperiod, and where there is high demand for artemisinin, with malaria morbidity at its highest. While the critical photoperiod to keep artemisia from flowering was reported to be over 13.5h for a Chinese cultivar (19), new cultivars developed by the Swiss company Mediplant[1] (Artemis®)[1] and by the University of Campinas, Brazil (A3) have been successfully grown in Kenya, Tanzania, and Nigeria. In tropical locations, planting artemisia at high altitudes improves plant adaptation and allows for further selection on those latitudes. A recent review (12) focuses on other aspects of *A. annua* cultivation and genetics, and can be obtained at no cost from the USDA-ARS, AFSRC website: http://199.133.10.189/pandp/docs.htm?docid=9649, posted with the consent of Plant Genetic Resources[1].

2.2- Nutrition and pH

Although the US Agency for International Development is working in partnership with WHO to increase artemisia cultivation, especially in Africa (20), there is little published information on individual nutrient requirements for artemisinin production (12). Increasing field N supply to a Turkish *A. annua* cultivar did not significantly affect artemisinin content or yield (21). In Madagascar, applying 97 kg/ha N to a field crop with three plants/m^2 increased dry leaf production from 2420 kg/ha (control) to 4690 kg/ha, while concentration of artemisinin dropped from 1.11% to 0.87%. The higher leaf production caused by surplus N increased artemisinin total yield from 27 kg/ha to 41 kg/ha (22). Also, a mean 4.7 t/ha increase (19%) in total fresh plant biomass, cultivated in densities varying from 27.8 to 111.1 thousand plants/ha, was reported in Indiana (23) with the addition of 67 kg of N/ha, but artemisinin was not analyzed. Under greenhouse conditions, boron deficiency inhibited flowering and decreased artemisinin concentration by approximately 50%, while a decrease of 25-30% in artemisinin concentration was reported for plants deficient in Fe, Mn, Zn, and Cu (24). In general, nutrient deficiencies or addtions have been reported to decrease the concentration (g/100g) of artemisinin. However, when a Swiss *A. annua* cultivar (Artemis®)[1] was grown under greenhouse conditions in a low fertility Appalachian soil (Gilpin fine-loamy, mixed, mesic Typic Hapludults), at a pH of 5.8, it produced significantly more (75%) artemisinin (g/100g) under potassium deficiency than when potassium was supplied, but total yield of artemisinin (g/plant) under potassium deficiency was no difference from the control. Although there was no difference between N- or P-deficient treatments in the concentration (g/100g) of artemisinin, the total yield of artemisinin (g/plant) of those treatments was significantly lower than the control yield (25). In a previous study using the same soil as above, corrected to pH 5.8, K and N were less limiting to leaf biomass production than low pH and P deficiency (26). Artemisia has been reported to develop well with pH above 5.5 or higher (25, 27). On an Appalachian soil (Gilpin silt loam – fine-loamy, mixed, mesic Typic Hapludults) near Beaver, WV (37° 45’ N, 80° 59’ W, 945 m altitude) a cloned *A. annua* provided with 45 kg of N, 20 kg of P, and 37 kg of K per hectare, twice during the three-month period (June to August 2005), produced 0.69% (g/100g) of artemisinin, 0.25% of dihydroartemisinic acid, and 0.04% of artemisinic acid, with an average dry leaf biomass yield of 453 g/plant (25).

3- POTENTIAL USE IN ANIMAL HEALTH

Because of the success of artemisinin in the treatment of malaria and its promising results in the control of cancer and *Schistosoma* sp. (28-30), and its low toxicity to mammals (31), it is possible that this compound, and the plant, could be effective in fighting blood-feeding gastrointestinal (GI) parasites in small ruminants. In sheep (*Ovis aries*) or meat goat (*Capra hircus*) production systems, the most detrimental GI parasite is the barber pole worm (*Haemonchus contortus*). The few commercially available anthelmintics (e.g., Avermectins) recommended to eliminate *Haemonchus* from small ruminants have become less effective due to the development of GI parasite resistance to such drugs. Even when effective, residues of Avermectins and other classes of anthelmintics can be detected in muscles, fat, and milk if the withdrawal periods (36-72 hours) are not observed (32). Consumer awareness about potential chemical residues in the food chain and the increased animal mortality caused by GI parasites resistant to synthetic anthelmintics has stimulated research on alternative approaches to control GI parasitism in small ruminants (33).

Various plants have been evaluated as potential GI parasite controls, but specific compounds or fractions responsible for the reported anthelmintic activity have not been clearly identified. Compound isolation and identification is a major undertaking and is not feasible to a laboratory with limited resources. I*n vitro* studies are more common because they involve much less time and resources than *in vivo* studies. However, *in vitro* studies are mainly performed with free-living stages rather than parasitic stages of the nematode, making it questionable whether the *in vitro* assays are relevant to *in vivo* conditions (33). *In vivo* studies and compound (or at least fraction) isolation would bring advanced knowledge on the biological activity of medicinal plants in ruminant species. Currently, no specific compounds from a medicinal plant source have been linked to the control of an individual GI parasite, but the genus Artemisia has been cited as a potential source of compounds that might be effective against GI parasites. There are no studies involving artemisinin pharmacokinetics in ruminants. In whole-plant studies, goats (2 per treatment) artificially infected with *Haemonchus contortus* were fed *Artemisia herba-alba* in doses of 2, 5, and 10 grams dry weight (DW), for one (2 g total), two (10 g total), and three days (30 g total), respectively (34). The results indicated that *A. herba-alba* reduced fecal egg counts (FEC) at all doses with no side effects on animals. However, there were no follow-up *in vivo* studies. Sheep (4 animals/treatment) naturally infected with a mixture of GI parasites including *Haemonchus contortus,* and fed *Artemisia brevifolia* for 14 days at 3.0 g/kg of body weight (BW) of the crude power (CP) or 3.0 g/kg BW as crude aqueous extract (CAE) equivalent (of plant tissue used as CP) had 62.1% and 67.2% reduction of FEC for CP and CAE, respectively. Crude methanolic extracts (CME), used at 1.0 to 3.0 g/kg BW had results similar to the untreated control (35). When CP, CAE, and CME were tested in an *in vitro* study using adult *Haemonchus* kept in phosphate-buffered saline solution (PBS), CME killed *Haemonchus* and CAE paralyzed them, but the paralyzing effects of the CAE disappeared after the worms were transferred to fresh PBS. In this study, the effective component or components were present in both the aqueous and methanolic fraction, but the aqueous fraction was more effective than the methanolic fraction *in vivo*. Alcoholic extracts of *Punica granatum* and *Artemisia silversiana* inhibited development from eggs to larvae stage L3 when added (at 100 to 1000 µg/g) to a feces/charcoal mixture. *Punica granatum* effects were dose-dependent, and inhibited 79% of egg to larvae development *in vitro* at 1000 µg/g. In the same study, *Artemisia silversiana* was reported to be more effective than *P. granatum* but specific results were not presented (36).

4- CURRENT APPROACH IN EVALUATING POTENTIAL ANTHELMINTIC PLANTS

Selection of potential anthelmintic plants for animals, based on the previous use (ethnobotany) of the plant as an anthelmintic source in humans (monogastric), is not always effective due to the polygastric nature of ruminant animal targets. Also, the intrinsic nature of the GI parasite and its feeding habits must be taken into consideration. For example, *Chenopodium ambrosioides* has a long history of use as an anthelmintic in humans and non-ruminants, but it was ineffective in eliminating *H. contortus* from goats in a short-term study (37). In another study involving the use of *Chenopodium* plant material and oil in lambs, there was a reduction in the number of trichostrongyle eggs per gram

of feces, but treated lambs continued to release eggs in their feces, and their dry matter intake decreased considerably.

Meat goat production in Appalachia is growing each year, but GI parasite resistance to Imidazothiazole and Benzimidazole classes of dewormers is also on the rise (38). We intend to evaluate potential anthelmintic plants that are originally from the area or that can be adapted to Appalachian climate and soil conditions so that they can be produced where they are needed. We have developed several criteria for screening potential medicinal plants to treat GI parasite infestations in small ruminants in Appalachia, as follows: 1) ease in germinating and propagating potential plants, 2) rapid development and abundant production of biomass in one growing season, 3) average to high concentration of the potential anthelmintic compounds, 4) low toxicity of such compounds, 5) plant adaptability and persistence at zones 5-6 if perennials, or successful reseeding if annuals, and 6) suitable palatability in order to not cause reduced food intake. Ideally, plants should provide, besides the anthelmintic effect, an alternative source of nutrition for animals. Artemisia species (*annua, vulgaris, absinthium*) are considered candidates for testing, and perhaps could be used in combination to provide a synergistic effect. The goal is not to provide 100% parasite elimination, but instead is to keep parasite infection below the economic threshold, and to improve the immunity of the host, or decrease host exposure to the parasite (39). Impacts of using medicinal plants for GI parasite control can be measured by: 1) increased weight gain, 2) improved feed conversion, 3) decreased host mortality, 4) reduced use of commercial anthelmintics, 5) decreased FEC, 6) reduced L3 larvae counts in coprocultures, etc. (39). We have used FEC and packed-cell volume to evaluate results.

5- PRELIMINARY RESULTS ON THE USE OF ARTEMISININ IN GOATS

Artemisia annua was successfully cultivated in West Virginia in 2005, with seed-generated plants achieving an average of 300 g of leaf dry weight (DW)/plant and clones of a Swiss cultivar (Artemis®)[1] achieving an average of 450 g of leaf DW/plant. Goats artificially infected with *Haemonchus* and administered artemisinin at doses of 11.6 to 15.8 mg artemisinin/kg BW, had no reduction on FEC (12). These artemisinin doses may have been too low because they were based on those used in malaria studies (20-30 mg/kg BW). Recent investigations with mice indicate that the doses to control blood parasites need to be at least 200 mg/kg of BW (28).

To investigate if the doses chosen for the previous study would be sufficient for artemisinin to reach the blood, 5 goats were given doses similar to the ones received by human subjects with malaria (33 mg/kg of BW) in a single dose in a time-course study (0, 1, 2, 4, 8, 12, 24 hours). At these doses, artemisinin and dihydroartemisinin (α and β epimers) were detected in their blood plasma by HPLC-MS as early as 4 hours after treatment. Quantification of dihydroartemisinin is currently underway to try to establish pharmacokinetics and metabolic fate of artemisinin in small ruminants. Current results on the use of artemether to control multicellular parasites such as *Schistosoma* in mice required doses as high as 200 mg/kg BW (30), about 7 times higher than the doses we used in the goat study, and better results were obtained *in vitro* when artemether was used with hemin than when artemether was used alone (29). In our project, upcoming studies will be directed first at determining appropriate doses and delivery systems in an in vivo system using gerbils artificially infected with *Haemonchus contortus* before proceeding to goat *in vivo* studies. Artemisinin stability in rumen fluid has been partially studied.

Artemisinin has been successfully recovered (70 to 94%) from bovine rumen fluid incubated for 24 h at 39 °C. Based on these preliminary *in vitro* results, one could assume that if artemisinin is delivered orally in pills or as *A. annua* forage, it would successfully pass through the rumen, and reach the lower gut where it might be absorbed into the blood. Although artemisinin, its related compounds, and the essential oils produced by *A. annua* might have an effect on adult GI parasites in the abomasum, or on the parasite eggs eliminated with the feces, our belief is that best results can be obtained on the control of adult GI parasites in the abomasum after artemisinin and other biologically-active plant compounds are transferred to the parasites through the blood.

Regarding the effect of *Artemisia annua* and artemisinin on rumen fermentation, leaves containing 1.4% artemisinin were incubated *in vitro* with bovine rumen fluid in different proportions with alfalfa (*Medicago sativa* L.), and did not affect dry matter digestibility even in the treatment

containing 100% artemisia leaves (12). *Artemisia annua* may provide some nutritional value to livestock (Table 1). However, feeding studies have not addressed this issue.

Table 1 : Nutritive value parameters of sweet wormwood (*Artemisia annua* L.) leaves and whole plant alfalfa (*Medicago sativa* L.). Source: (12)

Item	***A. annua* (Leaves)**	**Alfalfa (Whole Plant)**
OM, %	90.3	90.3
NDF, %	23.3	32.7
ADF, %	12.8	27.3
IVOMD, %	63.2	66.8
Artemisinin (g/100g)	1.4	Not Detected

N, nitrogen; OM, organic matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; IVOMD, *in vitro* organic matter disappearance.

A preference trial involving goats offered coarsely ground dried leaves of alfalfa, oregano (*Oreganum vulgare*) and artemisia from a 45-minute feeding period indicated that those animals preferred oregano and alfalfa over *A. annua* (unpublished data). It is not know if the amount of artemisia consumed was sufficient to produce an anthelmintic effect or whether consumption of artemisia might have been higher if it had been presented fresh or mixed with another forage or medicinal herb. Although *A. annua* is undeniably a safe and effective antimalarial, the effect of artemisinin or *A. annua* forage on the control of GI parasites such as *Haemonchus contortus* and *Ostertagia* sp. in small ruminants remains unproved. Past results (28, 29, 34-36, 40) indicate that *Artemisia* spp. and artemisinin-derived drugs have the potential to reduce burden of GI parasite infestation in ruminants, but the right amounts of herbage, dose of the drug, and duration of the treatment are still to be determined in small ruminants.

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