



**Artemisia and Madagascar: An Assessment and Technical Assistance  
Deliverable 1:  
Chemonics International Inc.  
Business and Market Expansion Program  
(Madagascar Business and Market Expansion (BAMEX) MOBIS No. GS-23F-9800H,  
Task Order #687-M-00-04-00212-00.**

**Submitted by Rutgers University  
Jim Simon<sup>1,4</sup>, H. Rodolfo Juliani<sup>1</sup>, Qing-Li Wu<sup>1</sup>, Chung Park<sup>1</sup>,  
Benjamin Neimark<sup>1</sup>, Ramu Govindasamy<sup>2,4</sup>, Linda Gavin<sup>3</sup> and  
Geetha Ghai<sup>3</sup>**

**<sup>1</sup>New Use Agriculture and Natural Plant Products Program,  
<sup>2</sup>Department of Agricultural Food Economics and Marketing,  
<sup>3</sup>Center for Advanced Food Technology, and  
<sup>4</sup>The Rutgers Food Policy Institute**

## CONTENTS

Project Background	3
Scope of Work Background and Purpose	3
Specific Tasks for this Deliverable, Focus on Artemisia were to provide	4
Approach and Results	4
Specific Task 1: Assessment of the production system now in place in the Malagasy commercial introduction of <i>Artemisia annua</i>	5
Specific Task 2: Recommendations in the harvesting and post-harvest handling of <i>Artemisia annua</i> , including extraction techniques and processing strategies	11
Specific Task 3: Two trainings in the (1) production and (2) processing and chemical analysis of <i>Artemisia annua</i>	19
The Potential as a Standardized Tea for Malaria vs. Developing a Standardized Extract from the Plant to Serve as the Artemisinin Source within an ACT	23
Selected Literature of Relevance to the Artemisia Community in Madagascar including patents	25
Draft: Artemisia Date Collection Guide	121
<i>Artemisia annua</i> Crop Budget Records:	124
Analysis of artemisinin in <i>Artemisia annua</i> field-grown in Madagascar, A Technical Report from NUANNP, Rutgers University, 2005a	125
Analysis of artemisinin from field-grown <i>Artemisia annua</i> , Madagascar, A Technical Report from NUANNP, 2005b	129
Analysis of artemisinin from 77 samples of field-grown <i>Artemisia</i> <i>annua</i> plants, Madagascar, 2005. A Technical Report from NUANNP, Rutgers University, 2006	133
<i>Artemisia annua</i> : A Primer for Production	138
Appendix.	
I. Selected List of Our Published Works on <i>Artemisia annua</i>	151
II. A New Novel Technology Being Promoted by a Canadian Company for the Extraction of Artemisinin	154

## **Project Background**

The purpose of BAMEX is to increase market-based, private sector-led growth. The project aims to increase trade and revenues from selected Malagasy products in domestic, regional and international markets.

The objectives of the program are to: (i) strengthen linkages along product chains that have strong market potential; (ii) build local capacity to assess and respond to changing economic conditions affecting investment and trade; and (iii) support the improvement of economic and trade policies that encourage investment and exports. The program will identify and promote creative, informed, and practical market-based approaches to introduce more productive business practices and techniques.

Specific interventions undertaken in this program will be selected based on their ability to address three basic criteria: (i) potential for a significant contribution to economic growth; (ii) contribution to poverty reduction; and (iii) complementarity with rural development activities and biodiversity conservation/natural resource management.

## **Scope of Work Background and Purpose**

This work is to assist Chemonics in increasing the number of successful Malagasy businesses in the natural products sector, by helping increase income, employment and development. To achieve these goals, the Subcontractor will implement an environmentally and socially conscious sustainable production of high quality, healthful natural products for local regional and overseas markets.

The aim of this Subcontract is to help Chemonics build capacity for the development of sustainable natural plant product businesses in a socially and environmentally sensitive manner. Rutgers' initial focus will be on reviewing and assessing the commercial development of artemisinin, a natural plant product from the anti-malarial plant, *Artemisia annua*.

Artemisinin is in short supply with demand expected to continue to far exceed current production availability. Several approaches are being intensively examined among the myriad of research groups around the world working on this issue, ranging from plant breeding (by increasing significantly the amount of artemisinin in the plant) to increased efficiency in the processing of the plant once harvested via varying extraction technologies. Members of the Rutgers team have conducted basic and applied research on the biology of this *Artemisia annua* since 1986.

Rutgers' focus has been in the field production of this species for both its anti-malarial compounds and its aromatic essential oils; in the genetic improvement of this plant, in the seed production of this plant, in the processing of this plant species, and in the standardization and chemical screening for artemisinin and related nonvolatile sesquiterpenes. Due to the complexities of processing, the variability of yield, the limited availability of seed and limited number of high yielding lines, and the shortage of supply, intervention and technical back-up is needed by the current private sector now working in Madagascar to commercialize this crop and natural phytopharmaceutical. Significant losses in artemisinin can occur from harvesting and

during post-harvest prior to extraction. Few studies have undertaken the needed studies to assess the best interventions and develop a Good Agricultural Practice QA system for *A. annua* that could reduce in a practical and low cost way, post-harvest and storage losses of artemisinin, thus improving the economic sustainability of the small growers, the processors and increase the supply of artemisinin, the main starting material for the pharmaceutical industry in the manufacturing of ACT and other artemisinin-based anti-malarials to improve human health and reduce the devastating impact of malaria.

**Specific Tasks for this Deliverable, Focus on Artemisia were to provide:**

1. Assessment of the production system now in place in the Malagasy commercial introduction of *Artemisia annua*;
2. Recommendations in the harvesting and post-harvest handling of *Artemisia annua*, including extraction techniques and processing strategies; and
3. Two trainings in the (1) production and (2) processing and chemical analysis of *Artemisia annua* (this was accomplished via telephone conference calls and meetings and a workshop held during the site visit in Madagascar)
4. Prepare a draft production guideline using our experiences and over time to merge it and modify it to meet with the Madagascan experiences.
5. Provide Technical Support to BIONNEX as needed and requested.

**Approach and Results:**

*General:* Since project inception, the Rutgers team has been in contact with BAMEX-Madagascar; and with BIONNEX-Madagascar. Since project inception, Rutgers has provided BIONNEX with strategic advise and counsel relative to artemisinin production, extraction, post-harvest handling, and processing. Rutgers has shared with BAMEX for distribution analytical protocols and SOP for the analysis of artemisinin. Rutgers has shared with BIONNEX the scientific papers, publically available patents and other information as requested and a fuller set of such literature is included in this report. Rutgers has also work close with BIONNEX in testing and screening some of their plant samples to better understand time of harvest and other production-related issues. To date, and for in support of BIONNEX and BAMEX, we have chemically tested and analyzed three sets of plant materials shipped to us from Madagascar which included over 130 samples of *Artemisia annua* field-grown in Madagascar for artemisinin (our service for fee rate for this test to outside groups or non-collaborating partners ranges from \$100 to \$150/sample, the amount dependent upon the number of samples submitted for analysis). Three technical reports providing the results are included in this report. Rutgers has also introduced into Madagascar seed of a new hybrid commercially available from Brazil to test and screen under Madagascan conditions. This seed was provided to BAMEX staff in December, 2005 for distribution to those interested commercial growers and researchers. Rutgers has also provided to BIONNEX a list of commercial companies and entities involved in or seeking to become in artemisinin processing and extraction. One such example is also included at the end of this report as an illustrative example of linking BIONNEX with other potential companies with specific technologies of potential interest.

A field visit to Madagascar in December by Simon (Rutgers) permitted the review of the field production that is being undertaken by BIONNEX, a tour of some facilities that could work with commercial artemisia producers for testing of artemisinin and QC issues; and other potential facilities that could be used to commercially process the artemisinin from the harvested and dried plant materials. During this site visit, several trainings were held between Simon and BIONNEX staff and personnel; and BAMEX organized and hosted a ½ day workshop focused on artemisia production and processing with the private and public setor players interested in this sector in attendance.

Ongoing discussion directly with BIONNEX continues and our intervention has assisted the private sector in Madagascar in achieving the goal of successfully producing artemisinin in Madagascar.

Specific issues addressing each of the objectives will be reviewed.

*Specific Task 1: Assessment of the production system now in place in the Malagasy commercial introduction of Artemisia annua;*

Simon toured the nursery and selected field production sites of BIONNEX during the December field trip. Overall impression was quite positive. BIONNEX has assembled an excellent team. It is clear that Madagascar can produce *Artemisia annua* and has a history with over a decade in producing it under small scale commercial and research trials. Lack of investment in this sector previously to this time had limited the commercial states of production now being sought. It is also clear that the Artemisia produced in Madagascar can accumulate high concentrations of artemisinin, with the upper end likely being dependent upon the germplasm used, the management and environmental conditions of the season and soil.

Upon reviewing in detail each of the steps from seed germination, to nursely production, to field production, we can conclude that BIONNEX is doing an excellent job and is producing the seedlings and transplants under GAP, though this review team has not seen the record keeping aspects, but have discussed the SOPs and GAP issues with the team. The serious investment by BIONNEX under very difficult conditions is quite noteworthy. The typical problems seen in the field were not unexpected but rather I had expected to see even more problems. A key challenge for BIONNEX in this first season of production was to raise significant biomass to ascertain serious and realistic crop yields of the total biomass, and of the amount of artemisinin that can be extracted/recovered by unit of land (e.g. total artemisinin/ha). To accomplish much of those objectives and to ensure that ample biomass was available, significant acreage needed to be produced. This was a high risk venture, that was purposefully conducted, and in my “opinion” fully justified and appropriate. However, moving so fast, several issues emerged that will need to be addressed in upcoming seasons.

The issues that need to be addressed in more detail are listed below and our focus is on these rather than the larger overall production that was progressing well. The issues plus recommended solutions for the issues, which are not listed in order of priority, now follow:

1.1 Water stress and low yields in some of the field sites, which will reduce yields significantly;

*Recommended action:*

For those field sites, already selected, irrigation systems need to be put into place and a greater number of systems need to be purchased and organized. An irrigation crew should be trained just to focus on irrigation to ensure the best rotation when using irrigation equipment that moves from field to field as needed. A greater number of pumps may be needed over time to ensure easier hook-up for the irrigation systems. BIONNEX is aware of this situation and given the acreage and the rapid pace in which they have moved, they need to be commended for making as much progress as possible. Having said that, great investment in both irrigation equipment as well as water management tools to record and monitor rainfall are still needed and recommended. In addition, irrigation schedules need to be developed for the larger and core fields and those decisions need to be based upon the soil type and stage of plant development as *Artemisia annua*, as many other plants do have critical stages under which water stress will have a greater impact than during other stages.

There are inexpensive tools that can be placed in the fields to monitor rainfall and/or amount of irrigation that has been applied so the grower can use the ‘checkbox method of water balance’ for irrigation scheduling. This technique is the least expensive and easiest and basically records water input, and given the soil type and plant state of growth can then provide the basic recommendation of how much water is needed/week or biweekly to ensure adequate water (regardless of whether it comes from rainfall and/or irrigation). These tools and techniques can be provided by us, locally by irrigation suppliers or both whereby the irrigation supplier provide the equipment and monitoring tools and we can work with BIONNEX to develop the scheduling and records needed to be taken.

In the future, soil selection and access to water will be critical elements to consider in establishing new *Artemisia* fields in current and/or new areas. Those soils that are heavily sandy with moisture holding capacity will need higher and more regular water input than soils of higher loam and/or clay content. Establishing fields particularly during the dry season, should only be considered in areas where irrigation is possible and water sources are available.

1.2 Premature chlorosis or yellowing of the bottom section of the canopy prior to flowering, which would reduce yields significantly, likely due to heat stress and water deficit;

*Recommended action:*

We suggest that the intense yellowing of the lower leaves and loss of the lower canopy of many of the fields was due to water stress. This can lead to either a pre-mature flowering or even a delayed flowering, despite it seems contradictory to see that both events can occur. *Artemisia annua* does have the capacity to undergo heavy water stress and survive and when water becomes available, the plants will ‘wake-up’ (metaphorically speaking) and continue to grow, with a concurrent greening of the new leaves and new growth. However, during that time period of intense stress and leaf loss, we suggest results in yield loss and should be avoided to the extent possible. In many trials, the lower canopy of *Artemisia* (somewhat dependent upon the plant density) contains the highest amount

of biomass (in weight or as a rel. % of the total arial parts of the plant). Thus, the loss of that bottom canopy is significant. And, furthermore the premature chlorosis of those bottom leaves also leads to another loss in recoverable biomass that can be collectd during manual harvesting. The greater the dry down of the bottom leaves in the field, the higher the actual breakage and physical loss of those leaves and stems when the plants are cut and collected. That is, the greater amount of leaves will be seen on the soil surface and all that falling to the soil surface when added together represents a loss of yield and recoverable artemisinin.

### 1.3 The timing of transplanting Artemisia into the fields.

#### *Recommended action:*

The timing of the best stage for field survival and rapid growth from the nusery into the field will be highly dependent upon the age and growth of the transplant and the availability of soil moisture. This is a very critical stage of growth for Artemisia and water stress should be avoided if possible during this initial transplanting and field establishment stage. During the first campaign, water was indeed observed to be quite limited. The timing of the seedlings and the transplanting into the fields should be adjusted as much as possible to the natural rainfall making it easy on irrigation at this point in time, but in anycase, improved growth and field establishment will occur under ample moisture conditions at this time.

### 1.4 The impact of stress on flowering and scheduling for harvest time. The key is to maximize biomass production and have a more predictable harvest schedule.

#### *Recommended action:*

Given the size of the crews and the different fields, and the fact that the dryer units can only hold a certain capacity at any given time, the avoidance of long-term stress will allow for better prediction of harvest times for each field. During Simon's visit, this was a major disussion point as when the rains would return, the additional needed regrowth of many of the fields would lead to a longer time in the field per transplanting date and those the maturation of too many fields at the same time puts additional pressure on the post-harvest handling and processing system.

### 1.5 Rapid expansion not allowing for a thorough critical analysis of interventions required to maximize yield;

#### *Recommended action:*

This is a largely a function of time, and the need to review the yields/field, the production costs per site and at each operational stage, the record keeping and the post-harvest handling systems. We recommend that all such inputs be compiled together and the team meet prior to and during the next campaign of production to minimize costs, direct the new expenses to the areas of highest priority all for the achievement of maximizing yields.

### 1.6 Soil Testing for pH and Nutrient Analsis:

#### *Recommended action:*

We recommend that all fields be tested for soil pH and macro and micronutrient analysis. Folders on the history of each and every field need to be constructed including but not

limited to prior year's crop, if any. If none, a description of the naturalized plants growing in the field. A history of any agro-chemicals is also beneficial.

A better understanding of the soil with such soil testing services will permit a better plan of action relative to the needed interventions for that particular campaign in that particular field. Such testing will also identify those soils that could be considered more difficult to manage. In this project, soils with very low pHs will be encountered and could be problematic if soil pH is not adjusted satisfactorily (See the next point below for more in this subject).

The Madagascar lab conducting the soil testing should also provide interpretation and recommendations for each field, and until we have a better understanding of the nutrient needs of *Artemisia anna*, we suggest asking the testing lab to provide the recommendations for non-leguminous vegetables as a comparative species, since nutrient recommendations are crop specific.

Such testing will also identify any micronutrients that may be deficient in the soil and would need to be applied either during pre-planting or post, with costs generally lower if applied prior to the transplanting of *Artemisia*.

- 1.7. Land rental vs. land ownership becomes an issue relative to the inputs a producer wishes to invest into a particular land area.

*Recommended action:*

One of the major challenges for BIONNEX was to identify and secure fields to produce *Artemisia*. Thus, given the enormous time that itself requires, it is not a surprise that several issues would later arise. Here, the one issue alluded to is the issue of excessive soil acidity at some site(s) and the need for significant lime in at least one site (e.g. 4-6 tonnes/ha). We are at one site that will be allocated to *Artemisia* whose soil pH was reported to be <4.0. This type of acid soil needs to be adjusted by liming and the faster the soil pH is needed to be adjusted (for that upcoming growing season), the finer the lime particles are needed, the more expensive the lime costs. Soil testing would also tell whether dolomitic lime (Mg-containing lime) is needed and even available in Madagascar. Such low soil pH could lead to aluminum toxicity, manganese toxicity, and an imbalance or deficiency of magnesium. We have worked with other crops under such acidic conditions and typically, significant yield losses occur when growing crops under this low soil pH. The actual soil pH surrounding the roots may even show up to less than the general soil pH that comes from soil testing. This could potentially lead to yield reduction and imbalance of nutrient uptake and availability.

Soil testing, proper application of lime and potential arrangements with the land owners to be charged less for the rental/use of the land is warranted and may help to justify the investment in the higher amounts of lime needed. Given the total lime requirement of that one field, it is too much to apply in one given season particularly just prior to planting. Typically, the soil pH and liming is done months prior to planting during field preparation. Use of non-acidifying nitrogen fertilizer, such as calcium nitrate, is also recommended but this material or others like it may not be available in Madagascar and if

available generally cost more than other forms of nitrogen fertilizer which generally act to further acidify the soils to some extent.

Recommend bringing on board a soil scientist either on a short term consultancy to get the plans and actions on-line and to provide overall strategic detailed advise and/or collaborate with a soil scientist or agronomic group at the university, or a fertilizer company to develop a detailed plan of action upon which to build.

Use the soil tests and recommendations for the planning of the fertilizer applications. We also recommend applying all or  $\frac{3}{4}$  of the nitrogen and fertilizer on pre-plant during the dry season, with a side-dress application coupled with irrigation (or rainfall) when the plant is undergoing its rapid vegetative growth and about one month prior to flowering. During the rainy season, it may be advisable to apply less as a pre-plant, due to the washing away of the nutrients beyond the root zone of the young transplants and return to the fields 2x for side-dressing applications. Use of standard ways of fertilizer application in Madagascar are needed and are easily found via discussion with fertilizer companies. Care not to damage the roots of Artemisia during mechanical and hand weeding as well as kinning any side-dress fertilizer into the soil is important.

#### 1.9. Insect and diseases.

##### *Recommended action:*

All plant samples exhibiting signs of diseases must be collected (the plant leaves plus surrounding soil when relevant) and outside expertise brought in to confirm the identification of the cause for the disease. Greater clarity of whether a symptom as observed is due to nutrient imbalance or a disease is important. Prior assumptions of causal agents should not be relied upon, as it is easy enough to culture in Madagascar any such causal fungal and/or bacterial organisms and be using standard Kochs postulates to confirm the cause of the symptom(s). There appeared to be some uncertainty in this area. Linkage to those labs and university experts would provide a valuable asset to BIONNEX and also bring in the university expertise to bear in plant diagnosis problems early on. Fees for such services are inexpensive.

#### 1.10 Plant Nutrition, soil fertility and Nutritional Stress.

##### *Recommended action:*

It was difficult to ascertain whether the plants observed were suffering any nutrient deficiency, though several plants appeared to be nitrogen deficient but this was only a visual observation and judgement. We already discussed issues and the importance of soil testing and using the recommendations from a soil test to plan for the fertilizer applications. Coupled to soil testing, we recommend taking some foliar samples for plant tissue testing. There is at present no standard way to sample Artemisia for nutrient analysis and know that the sampling time is reflective of the entire growing season. This type of background work simply has never been done before with Artemisia. As such, we recommend sampling from the same set of leaves used for artemisinin sampling but to remove 2-3 times the quantity for mineral analysis.

We recommend doing this only for a few samples that come from the most productive fields and the fields with the highest reported artemisinin level. Then we also recommend taking samples from the least productive (growth and vigor) fields and also from the plants that are at the same phenological stage of development (growth and maturity points) but whose chemical analysis showed the lowest level of artemisinin.

We only need samples from these three “observations”. There is capability to conduct such mineral analysis (testing macro and micronutrients) in Madagascar, but if needed, we at Rutgers could arrange to get these tissue tests done as well as this is routine and the cost not expensive. We recommend this to begin to better understand the nutrient levels that are associated with the highest yielding plants/fields (for biomass and artemisinin) so that future recommendations can be made with greater clarity as we would also have the soil profiles as well. Given all the fields are grown with the same seedline, this would be easy to accomplish and provide excellent data as the goal is to maximize yields. This would be one of several interventions that can be done to better understand the conditions associated with the best yields, and these answers can then be used in other fields and seasons to try to emulate by adjusting the water management schedules, the fertility, the soil pH etc.

#### 1.11 Greater communications needed between all team leaders in the field with central office in Antananarivo.

*Recommended action:*

Part of this is due to lack of wireless/cell phone service availability, but the difficulty in easily reaching each farm and each team member created more difficulties than needed and resulted in higher production costs and management time thus reducing the efficiency in scheduling, timing and application of field interventions on a more timely basis. Given this phytopharmaceutical, there needs to be weekly, if not more often daily contact with all fields during the production stages and this becomes more pronounced during harvesting, post-harvest handling and processing through the dryer stages. This issue is well recognized by BIONNEX and they already have plans to improve the communication. One of the challenges we face at Rutgers is in the maintenance of more regular, eg. Monthly conference calls and computer discussions. We recommend the use of desk-top video/phone conferencing to provide an easy and inexpensive solution that will encourage greater and more regular contact.

1.12 The commitment by BIONNEX to eventually involve small-scale growers under contract to grow Artemisia is excellent. The lessons learned by BIONNEX will go a long way in helping to develop production systems most appropriate for them. The poster and manual for Artemisia production for small-growers produced by them is excellent graphical representation of all the steps that such growers would need to understand.

In summary, the production is going well, but there needs to be a concerted effort to maximize biomass and artemisinin yield/ha over time. The issues addressed above are specifically for that purpose- as the difference between profitability and loss is often in the

management and in controlling those factors that are possible. The issues raised above all should be considered as normal issues during any new commercialization venture and in reality in general, are issues that face many of our commercial growers of traditional as well as non-traditional crops. The challenge with Artemisia is to look at this crop as a large-scale agronomic crop that can and will be grown in both large and smaller-acreage by both BIONNEX as well as by many small-scale outgrowers, yet in all cases the goal is to both maximizing yields as well as profitability to all the growers as that leads to the greatest chance of the sustainability of the crop. Developing and refining the production systems to achieve the highest goals possible thus must remain in the forefront of this venture. A crop budget template is included in this report and can be modified to fit any/all additional inputs that are needed. Building this template into an excel spreadsheet is recommended for use and later reviews.

To maximize yields:

- \* Recommend greater attention be addressed to soil pH, soil/plant nutrition and fertility inputs, and irrigation management.

- \* Additional newer lines (hybrids and OP lines) should be tested in Madagascar. Toward this end, Rutgers has provided BAMEX in 2005 with fresh seed of a potentially promising new hybrid to trial. BAMEX will distribute the seeds to BIONNEX and other commercial growers to trial. Rutgers will analyze the plants for artemisinin.

- \*In addition, In December 05, 2005, BIONNEX and Rutgers signed a Biological Use Agreement permitting Rutgers to send to BIONNEX, using proper importation permits, etc. advanced selections of non-commercially available lines that could be screened for biomass yield and artemisinin content. This sharing of the genetic materials could help determine the relative vigor and yield of the existing lines developed in Madagascar from some of the original Vietnamese plants that came into Madagascar over 10 years ago.

- \*Production results, as well as costs of production and input interventions need to be reviewed in context of improving the current and future campaigns. Drafts of crop budget templates are included in this report.

- \*Mechanical interventions vs. Manual labor. This is one topic we discussed in detail during the December visit, but is not highlighted in this deliverable report. Mechanical intervention is recommended not only in land preparation as is being done with soil preparation and fertilizer and liming application but will also be quite important relative to harvesting and transport of harvested materials. Mechanical weed control and harvesting technologies can significantly reduce the labor allocation toward these tasks and allowing the labor crews under larger-scale production to be shifted into other interventions in where they are needed.

*Specific Task 2: Recommendations in the harvesting and post-harvest handling of Artemisia annua, including extraction techniques and processing strategies:*

2.1. To review the post-harvest handling practices and tour the planned drying facilities and drying operations for *Artemisia annua*;

The topic of harvesting and post-harvest handling practices will be an ongoing one for several seasons/campaigns to ensure the best systems are employed. Both are key components within a GAP system and feed directly into the processing systems to be employed. At this stage it is premature to make conclusions, so the following are observations, comments and a few recommendations, all subject to modification as we collectively with BIONNEX learn from each campaign.

2.1.2. Harvesting and Post-Harvest Handling: The goal is to maximize the biomass and artemisinin accumulation in the field, and during harvest to retain all the artemisinin that has been accumulated, to reduce or eliminate any losses, and to ensure traceability of the harvested materials to the original fields from which they were harvested.

Toward that end, the actual time of harvest needs to be based upon the recovery of the most biomass and tempered by the rise and potential fall of artemisinin levels. There are two 'schools of thought'. In East Africa, they harvest the plants just prior to flowering and are convinced that under their conditions this leads to the highest artemisinin yields. In our experiences, it typically is best to harvest the plant at full flowering as that is when maximum artemisinin in both the leaves and flowers are reached. This is however, under the presumption that there are no major stresses facing the plants (e.g. water and heat stress). As both approaches would result in a significant time difference, which is correct?

As it turns out, studies have shown that both approaches are not in contradiction with each other as pointed out by Morales, Charles and Simon (See: 1993. Seasonal accumulation of artemisinin in *Artemisia annua* L. Acta Horticulturae 344: 416-420) who showed that indeed different chemotypes or varieties may simply maximize their artemisinin at different times (e.g. prior to flowering, others in mid or full flowering). In general, studies have shown that under greenhouse and field studies, *Artemisia* biomass continues to grow after the onset of flowers, and that artemisinin continues to accumulate during flowering; and that the flowers themselves are rich sources of artemisinin. In short, under most conditions and given the actual line or germplasm used grows in this manner, higher agricultural yields will be realized by waiting until flowering commences for harvesting.

However, under stressful conditions in which water is limited and the bottom leaves are beginning to lose green color and become chlorotic and/or actually begin to drop off, then significant yield loss would occur. As such, we postulate that in some regions, harvesting prior to flowering is recommended to ensure the collection of the massive amounts of the lower or bottom leaf canopy. Higher plant densities will also lead to earlier leaf drop from the bottom canopy. Thus, under both of these scenarios, it is safer to harvest earlier such as prior to flowering, as ultimately yield will be based upon the amount of recoverable yield.

We would suggest the following guidelines be used as that would provide the best option for Madagascar.

- Weekly observation of the field and state of the plants. Are the plants still rapidly growing? Are all the leaves, including those from the lower parts of the canopy still green

(which they should be even at the onset of flowering) and still intact? If so, let the plants continue to grow;

- Weekly screening for artemisinin content. Are the levels still increasing or at least level? If so, let the plant continue to grow. Levels of artemisinin need not always continue to grow. Even if the levels (on a rel. % dry wt basis) remain the same but the plant continues to grow the overall yields increase.
- From just prior to flowering to full open flowering is the harvest window.
- Based harvesting time on the availability of crews, drying space, and the scheduling needs of all the fields as there will be a reality check in that not all fields can come out at the same time anyway.
- Use the artemisinin analysis and the overall growth of the plants- and color of the plants to help judge time of harvest.
- The plants can be cut manually or mechanically. In these campaigns, the manual harvesting of the plants are to be used and this works well.
- Cutting several plants and then stacking the plants in an upright fashion with each resting on the other to keep the plants off the ground can work well (barring heavy winds and adverse weather).
- The plants should be cut off just above the soil line with care to retain all the leaves and branches.
- Depending upon the weather, the plants can be left in the field for several days, but if it rains, if adverse weater comes into fields after the plants are cut during this ‘natural curing or dry-down’, significant leaf loss may occur and a decrease in recoverable artemisinin may be noted.
- In the future, or under a small trial in the next campaign, we suggest that a mechanical system of cutting the plants be trialed. This can be done by ‘rigging-up a cutting bar’ that can slice through the plants making a clean single slice and without ‘crimping the plants’. A canvas conveyor and/or other collector can be fitted to behind and under the cutter to collect and move the material to behind the harvesting without allowing the plants to touch the ground. Crews can follow the mechanical harvester keeping the plants from falling onto the ground and/or then arranging the plants into the ‘stacks’ in the field.
- Once the plants are partially dried, they can be physically picked-up and carried to a transport system (a flat-bed truck, or another) where they are stacked and brought over to the larger drying facility.
- Either prior to or after drying, the main stem can be removed as the main stem contains no artemisinin and simply adds weight and space to the extraction system. However, some places may not bother reovming the main stem pers but rather can cut up the plant and place the cut-up parts into the solvent extractor. The removal of the main stems, and the retention of all the dried leaves will be more functional, and take up less space in the drying and storage facility.
- Plants sun-dried only normally do not dry down sufficiently to be held for long term till processing. Insufficient drying will lead to mold and other potential adverse quality effects in storage. However, initial sun drying will reduce the costs and time in using artificial drying.
- In all cases, the harvested materials need to be labeled as the are removed from the field, so that a label and code can follow the harvested crom from the field it where it

originated all the way through storage and processing, thus allowing traceability back to the field and a better understanding of which fields provided specific artemisinin yields.

### *2.1.3. Drying and Storage:*

- We recommend that the plants then be subjected to forced warm air that continually passes through the plant material to hasten the drying process and minimize both color loss, loss due to microbial spoilage and loss of natural products. The color loss is an example of a factor not relevant to the extraction of artemisinin
- Temperatures within the stack of plants should not exceed 35°C to 40°C. Artemisinin, the most biologically active sesquiterpene is relative unstable and the integrity of the peroxide bridge must be maintained for the chemical to retain its anti-malarial activity. Studies have indicated that even higher drying temperatures can be used without the breakdown of this compound, but to date, those studies from our understanding have not been re-confirmed and verified. At Rutgers, we are conducting these studies right now to identify the breakdown temperature point or the thermal stability of artemisinin within the plant matrix and under similar drying units. As we get these numbers we will share them with BAMEX.
- In all cases, thermometers and temperature probes are needed to be installed at the entrance, exit and side areas of the drying unit to see how uniform the ‘newly constructed dryers are’. Getting uniform drying temperatures throughout the dryer is not easily achieved and largely based upon the dryer design and air flow. Simon and BIONNEX had active discussions on dryer design, with BIONNEX taking the lead in the designing and construction of the systems.
- Final rel. % moisture should be reduced to 12-14%. Several systems to test moisture are available using the older fashion oven drying technique to use of moisture analyzers and moisture probes as used in the agronomic industry.
- In all cases, the product coming into the dryer needs to be labeled as to the field it originated from thus allowing traceability back to the field.
- Storage is a major issue and a real logistical and cost issue for this project. Plants materials can be stored in clean bags that are breathable and more easily packed, stored, moved and transported to site of processing. Maximum bag size is related to availability of bag options in Madagascar, ability of a single worker to pick up and move the bag and cost. The breathability of the bags is important as the dried materials will normally re-absorb some degree of moisture from the ambient rel. humidity. Each bag needs to be labeled as to the origin of the plant material for traceability purposes.
- Storage units should be clean and enclosed and protected from rodents, insects etc. Major problems in this arena are not expected but recommendations are to keep everything clean and sanitary are important.
- Caution must be recognized relative to the flammability of Artemisia in storage. The terpenes of this plant are highly flammable, and without adequate air circulation and with excessive heat, the vapor density of the terpenes surrounding the plants in enclosed space can spontaneously combust creating a fire hazard. Recognition of this can allow proactive plans to circumvent and avoid this issue.

2.2. To visit the local potential processors of Artemisia in order to see their facilities and assess their capabilities.

During the December trip, Simon had the opportunity to meet with many potential processors and to visit some facilities (e.g. generic drug company). The only facility in operation that could rapidly handle the commercial extraction and processing of artemisinin is ENDINA. More of this is discussed below under 2.3. The extraction and processing of artemisinin section. There are many facilities including the generic drug manufacturer that has potential of running pilot scale and even larger processing, but the condition of the plant are nebulous, the future ownership and direction unclear and the GMP conditions would need to be re-visited and modernized. Alternative longer-term solutions are to purchase and introduce into Madagascar new technologies- in a turn-key fashion, and this too is discussed in greater detail in the next section.

2.3. *The extraction and processing of artemisinin.*

### **2.3.1 Analytical Approaches for Artemisinin Quantitation.**

We first address the differences between LC/MS and TLC as well as some other approaches.

The HPLC (High-Performance Liquid Chromatography) based analytical approaches are currently the most commonly used method for quality control of many pharmaceutical and botanical products for the property of its sensitive or high separated resolution. HPLC could combine with different detectors, e.g. Ultraviolet detector (HPLC/UV), Evaporative Light Scattering Detectors (HPLC/ELSD), Electrochemical Detector (HPLC/ECD) and Mass Spectrometer detector (HPLC/MS, so called LC/MS).

Although using UV detector is currently the most commonly available detector coupled with HPLC through out industrial areas and many academic institutions. However, the best and most appropriate analytical technique to use must be based upon the knowledge of the molecule of interest- and not all react in the same way. Thus, by understanding the properties of the targeted molecule, in this case, artemisinin, one can predict which methods and which detectors would be most sensitive. As a molecule, artemisinin lacks the “property” to serve as a strong UV absorber, and as a consequence is generally analyzed using HPLC with MS (LC/MS), ELCD and ECD detectors. An MS detector, for its inherent exclusive selectivity and great sensitivity, plus the high separated resolution achieved by HPLC, is currently considered as the most reliable approach. However, the use of an LC/MS system is often too expensive (>\$300k/unit), and most labs and private companies, except the very big ones cannot afford it even though it may be the unit of choice. We have this one in our lab. As the ‘next best choices’, we then look and consider HPLC/ELSD and HPLC/ECD for their promising sensitivity for the non-UV compound of Artemisinin, and the price of these equipments is quite reasonable (\$50-60k/unit). We also have these in our lab, but we normally choose not to use them as they are not as fast

or as sensitive as our other in-house options, which we use now for your current samples. However, these systems would be the systems of choice by you for your standardization needs and QC both for field plant sampling, and sampling during your processing and extraction.

As for TLC (Thin Layer Chromatography) method, it's a very old approach once introduced over 30 years ago. For its lower separated resolution (compared to HPLC), it's very hard to ensure that the target component can be totally separated with others (for natural products in plant are very complex). So cross-reactivity is often occurred and results can lead to an overestimation of actual "yield" or quantitation. Sometimes if the plant sample is "overloaded on TLC plate" (only part of the target component can be pushed to the appropriate position by mobile phase), you can have an underestimation of the yield, all while the pure concentration standard curve still looks fine as a calibration curve and achieving a linearity in range desired with pure standard set over a concentration range would naturally look better than the amount within a plant matrix. Also the uniformity of the TLC plates and other uncontrolled conditions could affect the final results. Anyway TLC method is a very rough approach compared to the HPLC based methods. The upside is that it is the least expensive method to use and sample analysis is less costly and given the automated TLC systems a reasonable number of samples can be run in a day. It can also be applied as an alternative for ESTIMATION of natural compounds, if no HPLC available. Given that MediPlant and IMRE use this technique it could have application in selection and as crude indicators within a field or even with processing could be acceptable but we do not recommend this technique as your analytical method of choice except for 'ease and availability' until a better more robust one is purchased. In short, we do not recommend this technique to be used as part of your QV program once you get involved in the extraction and processing of artemisinin.

We strongly recommend that BIONNEX purchase it's own unit as the multitude of samples, the expertise and the controls needed in this upcoming phase is rather important to have under control and always available.

Non-chromatographic Immunoassay is also a very sensitive method. Because of the high throughput screening and relatively low cost per sample, it is particularly suitable for large amount of Artemisia sample assays. One disadvantage of this approach is also the possible cross-reactivity over the similar compounds and overestimation of analytes could be occurred.

Lastly, a newer novel approach that also appears quite interesting is the use of NIR (Near-Infrared Spectroscopy). This is a newer yet well used technology for other medicinals and NPs, and which may be quite effective and simple to rapidly screen for Artemisinin. We have used it for other products (see Juliani et al. 2006) but not yet tried it for artemisinin. Theoretically, it should work and its advantages include: low sampling and analytical time, easy to use, and the instrument not as sensitive to environmental controlled conditions as more expensive scientific chromatography equipment, and easier to maintain. The main disadvantage is the initial cost to purchase the instrument, which is

high but similar to the purchase and set-up of a good LC system. In addition, the system would need to be calibrated and the algorithm curves developed using the system on site to build the models for artemisinin quantitation, but that is something either the company can do or we can do on behalf of our private sector BAMEX partner. There are many other papers on this technology, even handbooks, but our recent paper on other African NPs should illustrate its usefulness. Though not in the contract, we have contacted the manufacturer of this technology and asked that they work with us to see if it can be used for artemisinin analysis.

Further information on a NIR spectrometer can also be obtained directly by: LabSpec Pro; Analytical Spectral Devices, Boulder, CO, USA.

**Sampling for Artemisinin:** With regard to sampling, we recommend weekly sampling of the fields as it gets close to harvest, but using larger sampling or leaf tissue units to get a better accurate assessment of the artemisinin contents. We also recommend using one method, and a second lab such as the NUANPP at Rutgers for cross lab validation. We also urge the same quantitation method used in field sampling as will be used by the processor. A different method for crude estimations in the field is acceptable as long as they are recognized as estimations only and estimations on a comparative basis for that particular field. We have already discussed the recommended methodologies. With regard to sampling, more work needs to be done to confirm the reliability and consistency with the final selected equipment and protocols. We can provide, as we have been already, the protocols used in all the LC and GC methods. The smaller the leaf samples, the greater plant to plant variation and higher the variability relative to the final prediction of yield. The use of small leaf sampling to probe the fields as a pre-determinant for harvest time is fine, but each week, larger plant samples- using several branches should be removed from the same spot on the plant (same distance downward from the tip of the plants growing point). While significant progress has been achieved in this area, this issue will need to be continued during the next deliverables to develop a rapid and reliable system. This topic is of such significance it is worthy to continue to address.

### **2.3.2. Extraction of Artemisinin: Processing and Purification**

The extraction of the artemisinin can be accomplished by a variety of methods. Each extraction method has their associated strengths and weaknesses. Traditional solvent extraction is the lowest cost, now used around the world to extract artemisinin. The strengths include it is the lowest cost, relatively easy and introduces a moderate specialty technology. The weakness in the use of this technology is that the processing technology can be done without following GMP and thus the final product may not reach a certifiable standard. Other concerns however relate to the safety, handling and disposal of the traditional solvents now used in the extraction of artemisinin, and possible exposure to workers, their families and the facility and the potential for environmental contamination and difficulty in final storage and disposal unless handled properly. It is possible to use solvent extraction in a manner that overcomes the current concerns in the

solvent extraction of artemisinin. Some of the solvents used include but are not limited to hexane, petroleum ether, and ethyl acetate.

Alternative processes can also be employed in the extraction of artemisinin and the one presented below involves the process of super critical fluid technology (SCFT) using CO<sub>2</sub> as the solvent. The advantage of this system is its high degree of control, its high efficiency, and that it lends itself to use of GMP. The system avoids the concerns listed above with petroleum solvent extraction, and the system is environmentally friendly and the products accepted as high quality. The disadvantage of this system is its initial high cost; despite that small custom tailored CO<sub>2</sub> units can be purchased and moved into a new location. The system would require a much higher level of trained management and staff. This technology would be exciting to introduce into production regions as one can develop additional products from *A. annua* as well as use the same system for a wide range of other medicinal and food products. Thus, the recovery of additional value-added products from this plant and the high quality of the final product and the environmentally friendly processing system are among its major advantages.

To address in greater detail, the procedures for purification of artemisinin, several protocols have been published as scientific publication and/or US patents. We include the associated patents and papers in this report. See references #263 to #267, and this still may be incomplete as there are several additional Chinese patents on the extraction, but we are awaiting copies of these patents. In summary, the dry materials are first extracted using hexane and then participated between acetonitrile/water and hexane following with chromatographic separation; or the dry material are first extracted using polar solvent e.g. ethanol and then participated between water and hexane following recrystallization. You may read the references carefully and select and optimize a suitable approach depend on your facilities.

Practical Recommendations for the processing of artemisinin in Madagascar. We recommend and have counseled BIONNEX to collaborate in some fashion with ENDENA, whom we find as the only large-scale processor in Madagascar that has the experience, the facilities and capability for large-scale commercial artemisinin procurement. As the BIONNEX extraction will likely be done in concert with ENDENA, located in Fianarantoa, in Madagascar, they have the expertise and facilities to conduct the trial runs, the information provided in this report should be of additional assistance to both groups. ENDENA's experience is limited to extracting natural products from *Prunus africanus* (Madagascan Pygium). We recommend using solvent extraction as the technology for artemisinin extraction.

However, there are several other competing technologies that should also be noted and considered over time. This includes, but is not limited to, Super Critical Fluid Technology (SCFT). Several groups since the early 1990's have used CO<sub>2</sub> extraction for the procurement of artemisinin. The first patents for the extraction of artemisinin was awarded to a Chinese group, and a couple of years later a similar patent was awarded to Tom Chapman and co-workers, the latter receiving a patent on this technology for use in both the UK and the USA (again see more detailed information in the patent section, particularly Reference number 267).

Another newer technology, which we have forwarded over to BAMEX last summer, 2005, was from a company, called

**Conversion of Artemisinin into Artesunate:**

Research groups since the early 1980's, in part led by the pioneering work at the Walter Reed Hospital of Experimental Therapeutics, US Army, had begun researching a variety of ways to derivative the artemisinin molecule yet maintain the endoperoxide bridge, considered the most chemically reactive moiety of artemisinin. Catalytic hydrogenation of the molecule yields deoxyartemisinin, an epoxide exhibiting no anti-malarial action. When artemisinin is reduced by sodium borohydride (NaBH<sub>4</sub>), the lactone of the molecule is converted into a lactol, and this new compound as dihydroartemisinin, and with an intact peroxide bridge. When dihydroartemisinin is crystalline, the beta form is present, but when in solution, the alpha and beta epimers are present. In late 1987, work by Luo and Shen first reported that dihydroartemisinin can be converted into esters and ethers. Reduction of artemisinin with NABH<sub>4</sub>, using boron trifluoride as a catalyst and dry tetrahydrofuran as the solvent, yields 71% conversion into dooxoartemisinin. In 1990, that compound was later reported to be >7 times more active against chloroquinine-resistant malaria in vitro than artemisinin. Since the 1990's, a wide range of published and non-published standard and creative chemical reactions using both catalysts and solvents have been able to convert artemisinin into Artesunate with varying degrees of conversion efficiency. Toward this end, we refer to you to the references and the patents provided in the later sections of this report.

*Specific Task 3: Two trainings in the (1) production and (2) processing and chemical analysis of Artemisia annua (this was accomplished via telephone conference calls and meetings and a workshop held during the site visit in Madagascar):*

The trainings took place through three different routes. (1) Telephone conference calls; (2) A workshop held in Antananarivo, Madagascar in December; and (3) field tour to production sites of Artemisia annua and potential processing facilities.

3.1. Rutgers University conducted several informal trainings with BIONNEX via telephone conference calls and discussions focused on providing BIONNEX with technical information, technical assistance and strategic counsel on production, sampling, harvesting, processing and analysis. Further discussion is provided below. Four such telephone conference calls were conducted.

3.2. Rutgers University participated in and was a featured speaker in a Round Table on the Artemisia annua sector at BAMEX headquarters, Antananarivo, Wednesday, December 16, 2005. During this workshop, all the public and private sectors in Madagascar were invited to participate, and Jean Robert Estime, provided the introduction and set the context of the workshop and its intended purpose which was to bring together the players and actors in this sector and better identify what can be achieved in Madagascar. Simon, Rutgers University next provided a 2-hour PowerPoint presentation and overview on the botany, ecology,

horticultural production, processing, and extraction training. Charles Gibrain, BIONNEX presented an overview of their production to date, including over 90 ha of Artemisia being grown. Short presentations and introductions were made by AGRIEL, IMRA, CNARP, and LPN. A long engaged discussion on the development and implementation/actualization of the Artemisia sector in Madagascar concluded the workshop. The list of invited participants included:

Producers: BIONNEX, Charles Gibrain  
Societe AGRIEL: Raolison Gerald  
HOMEOPHARMA: Ratsimivony Jean-Claude  
President Association Koloharena: Jules Randrianarivelo  
Industry: ENDENA, Mario Del Fiore  
OFafa, Rajonson Lala Fidiso  
SOAM, Djaohasy, Charles  
Laboratories:  
ESSA, Jean Rasoarahona  
IMRA, Mme Rakoto Ratsimamanga  
CNARP, Docteur Etienne Rakotobe and Dr. Lalaso  
LPN, Marta Andriatsiferana  
CNRE, Razanaka Samuel

While there are other small-scale growers and other parties interested in production, the largest and most serious player as a producer at this stage appears to be BIONNEX. They are serving as a major driving force. Other serious players may be operating in the country, and there were suggestions to this effect, but we were not able to identify another party that has the investment, the current production and the team that BIONNEX has put together. It is our recommendation that the best option, particularly under the shorter-term, is for BIONNEX to partner with ENDENA to conduct the processing of Artemisia at their facility in Fianarantsoa. Madagascar can indeed become a potent force in the global Artemisia sector.

Each of the labs have strong scientific expertise and each a history of sorts with Artemisia annua, as this plant may be new as a commercial crop in Madagascar, but was introduced and grown in several areas of Madagascar for well over a decade. Indeed, the seed stock grown on the >90ha of BIONNEX's field come from locally produced and adapted seed. Upon review of the laboratories, and discussion, it is our recommendation that any large private sector company involved in the production, as the processing needs to have their own analytical equipment and on-site quality control capabilities using the correct analytical equipment.

The public and private sector labs in Antananarivo will be needed to provide strong back-up and conduct more in-depth research that can improve the production systems, the post-harvest handling systems, even the development of rapid screening methods, and toward that end forge a close and effective relationship with the private sector involved in the actual commercial production and processing. These labs can also focus on the production problems that will be encountered (insects, diseases, stress); and help to identify improved technologies and systems (drying, etc.).

3.3. Simon toured the nursery and several field production sites of BIONNEX and meet with their field production staff for an overview and discussion on comparative techniques in production and drying.

*Specific Task 4: Prepare a draft production guideline using our experiences and over time to merge it and modify it to meet with the Madagascan experiences:*

A preliminary production primer for *Artemisia annua* was developed and is also included in this report (See pages 138 to 151). This primer provides another perspective on the production of *Artemisia* that should be useful in reviewing production systems and when compiling the results of the current season's production and yield information in anticipation of next season, which is already underway.

*The Citation For this preliminary production primer* (full copy included in this report):

2005. Simon, J., C.H. Park. And R. Juliani. *Artemisia annua: A Primer for Production*. 13 pages.

*Specific Task 5: Provide Technical Support to BIONNEX as needed and requested:*

This task has been achieved in an ongoing manner through continual discussions with BIONNEX. Rutgers has conducted an extensive literature search covering the topics of agronomy and genetics (production concerns), chemistry, biochemistry, and extraction (processing concerns), pharmacognosy and biological activity indications, and patents (for artemisinin extraction and purification concerns as well as the potential to identify new value-added products and applications). This literature review covers over 270 references, all listed by topical area in this report. This literature had been reviewed and applicable parts presented to BIONNEX and/or used in the earlier above discussions.

BIONNEX requested assistance in identifying companies involved in the processing of artemisinin. Toward this end, we provided to them a number of private sector companies and organizations along with their contact information, and reviewed with them some of the critical aspects to consider. In some cases, we forged the linkage between the company and BIONNEX from which they can communicate directly. One such illustrative example is with the Canadian company, BIOEXX. We mention this as they have a rather unique technology, and it is a newer company. Our efforts brought our Madagascan private sector company then to be in contact with them to explore whether they can provide something better and most cost effective than other options available. In this report, we actually provide the BIOEXX's industry/company information **as an illustrative example**, of how we have assisted linking BIONNEX to other companies that could provide unique services and/or technologies. We provided the contact information, assisted in forging a link and then each party can discuss

and meet with each other. We assisted in making this type of information available to the private sector in Madagascar. This is not a recommendation to use BIOEXX but an example of our recommendation for BIONNEX to be engaged in discussions with any/all companies that are involved in this sector, thus enhancing their options and competitive decisions with which they should partner and with which companies could they and others in Madagascar work. Thus, this represents just one of several private sector companies. Our role was to provide assistance in the identification of such private sector companies and organizations, provide contact information to BIONNEX and others in Madagascar and then contact the companies outside of Madagascar when applicable to get the company information to Madagascar. This has been achieved and will need to be continued during the entire project phase as new issues arise in the processing and/or purification or semi-purification of artemisinin.

BAMEX requested assistance in developing a protocol of analysis for artemisinin. We have done this. We have sent BAMEX and BIONNEX, private sector in Madagascar analytical methods to rapidly screen and detect artemisinin.

In addition, BIONNEX has relied on us to analyze many of their samples (139 to date). Their first set of data reached us in late fall, 2005. The artemisinin contents in 28 *Artemisia annua* samples from Madagascar, were quantified by the LC/MS method developed by the NUAPP, Cook College/Rutgers. The artemisinin content in the 28 Madagascar samples varied from 0.379% to 1.038%. (See Wu et al. 2005, Technical Report of December 02, 2005, full copy of the report is included in this report, pages 125 to 128).

The second set of *Artemisia annua* samples reached us over the December holidays. The artemisinin contents in those 28 *Artemisia annua* samples from Madagascar, were quantified by the LC/MS method developed by our NUAPP, Cook College/Rutgers. The artemisinin content in the 28 Madagascar samples varied from 0.379% to 1.038%. dry wt artemisinin. (See Wu et al. 2006a, Technical Report of January 13, 2006, full copy of the report is included in this report, pages 129 to 132).

A third set which is deliverable 2, but reported here for ease of compilation, was received in late January, 2006. The artemisinin contents in these 77 *Artemisia annua* samples from Madagascar, were again quantified by the LC/MS method developed by us at NUAPP, Cook College, Rutgers University. The artemisinin contents in the 77 samples varied from 0.450% (Sample M197, O3) to 1.189%. (Sample M170, SO4). (See Wu et al. 2006b, Technical Report of February 08, 2006, full copy of the report is included in this report, pages 133 to 137).

The greatest challenge is in maintaining the continual dialogue and communication due to the long distances. We recommend monthly telephone conference calls.

*Citations For these Technical Reports* (whose full copies are included in this report) are:

2005a. Wu, Qing-Li, Chungheon Park, Diandian Shen, Rodolfo Juliani and James E. Simon. Analysis of artemisinin in *Artemisia annua* field-grown in Madagascar, 2005. A Technical Report from NUANNP, Rutgers University in Support of BAMEX. 4 pages.

2005b. Wu, Qing-Li, Chungheon Park, Diandian Shen, Rodolfo Juliani and James E. Simon. Analysis of artemisinin from field-grown *Artemisia annua*, Madagascar, 2005. A Technical Report from NUANNP, Rutgers University in Support of BAMEX. 3 pages.

2006. Wu, Qing-Li, Chungheon Park, Diandian Shen, Rodolfo Juliani and James E. Simon. Analysis of artemisinin from 77 samples of field-grown *Artemisia annua* plants, Madagascar, 2005. A Technical Report from NUANNP, Rutgers University in Support of BAMEX. 4 pages.

*Additional Tasks That Arose Prior to and During Field Trip to Madagascar:*

**Issue: Potential as a Standardized Tea for Malaria We Do Not Recommend, but We Do Suggest A Most Promising Economic Opportunity and Potential Medical Solution Resides in Developing a Standardized Extract from the Plant to Serve as the Artemisinin Source within an ACT:**

There was significant interest by one private sector company seeking to develop the dried *Artemisia* leaves as an anti-malarial tea. The concept is not new, in fact, this plant; a Chinese medicinal herb has traditionally been used to treat fevers and malaria for thousands of years and was prepared in tea form. However, given the recognition that not all fevers are malaria; not all plants of *Artemisia annua* contain significant concentrations of artemisinin and its natural derivatives, not all teas will be prepared in the identical fashion (heat, time of boiling) to ensure effectiveness, make this issue quite complex. In addition, if the public sees a tea, albeit standardized tea as effective, then the next logical step for many will be to 'grow' their own plants of *Artemisia annua* as a kitchen herb, and picking the leaves for use as an anti-malarial tea. This would lead to far greater complications again due to the chemical variability within *Artemisia* as a source of artemisinin. Our tests, and others, have shown that some *A. annua* plants and lines can be devoid or contain only trace amounts, others higher and most variable at best.

In short, while this concept is both intriguing and worthy of discussion, and there is clearly some clinical evidence showing its efficacy under some conditions as a tea, we do NOT recommend any herbal tea from *Artemisia annua* to be developed as an anti-malarial treatment.

In addition, any and all such discussions should be conducted in concert with the countries national health care and malarial boards.

We do however instead recommend that we should focus on developing, in concert with the health board in Madagascar a standardized extract (e.g. dried power in capsule, or

compress tablet) from the aerial parts of the plant based upon artemisinin content and provided in a gel or caplet form to be used as the ‘artemisinin source’ within a ACT. We believe this has not been done, and thus, we suggest this novel idea here as this approach would safeguard against the negative issues associated with the use as a tea (as discussed above) while still maintaining the quality control needed for any pharmaceutical products. To test this hypothesis, medical trials would be needed to be conducted in Madagascar to test the absorption, efficacy and safety of this delivery system. If successful, that could however make it far easier to produce locally, process locally and develop a pharmaceutical new product while still adhering to the strict pharmaceutical needs of quality, traceability and effectiveness. It would allow lead to greater local opportunities for product development and economic gain for Madagascar.

Our NUANPP Rutgers program would be very interested in working on the standardized botanical product that would be used in such trials. We do such botanical standardization work on other herbs. There are many precedents in the pharmaceutical industry that would justify this approach. We see this as a low technological approach that would significantly contribute to, if successful, lowering the cost of ACT’s and making more ACTs available in a shorter time period. The associated costs in both preparing such standardized extracts and in the associated clinical trials and safety trials would be minor in cost and time should there be interest.

We recommend that this concept be raised for discussion among the malarial and Artemisia community of researchers and industry, and government health care officials and others of Madagascar involved in public policy on malaria and that collectively, and if there is support, funding opportunities should be pursued in Madagascar. We would be excited to collaborate on such a project.

For further reading just on effectiveness of Artemisia tea and the recovery of the artemisinin through hot water extraction, we provide only one scientific paper on this topic for further discussion:

-See Reference # 38. Mueller MS, Karhagomba IB, Hirt HM, Wemakor E. The potential of *Artemisia annua* L. as a locally produced remedy for malaria in the tropics: agricultural, chemical and clinical aspects. *Journal of Ethnopharmacology* 2000, Volume 73, Issue 3, Pages 487-93. Source: Pubmed. The abstract of this paper is:

“ The plant *Artemisia annua* L. (Asteraceae) is listed in the Chinese pharmacopoeia as a remedy for various fevers including malaria, and contains the well-established antimalarial compound artemisinin. In this study, a hybrid form of *A. annua* was successfully cultivated in Central Africa. The aerial parts of the plant contained 0.63-0.70% artemisinin per dry weight, and approximately 40% of this artemisinin could be extracted by simple tea preparation methods. Five malaria patients who were treated with *A. annua* tea showed a rapid disappearance of parasitaemia within 2-4 days. An additional trial with 48 malaria patients showed a disappearance of parasitaemia in 44 patients (92%) within 4 days. Both trials showed a marked improvement of symptoms. In our opinion, these results justify further examinations of the antimalarial effect of *A. annua* preparations”.

## **Selected Literature of Relevance to the Artemisia Community in Madagascar:**

This section is divided into: agronomy, botany, genetics, chemistry, biochemistry, pharmacology and medicinal activities (antimicrobial and antiprotozoal) and patents and processing. Herein lists and describes the papers, as procured from the searched databases. We have pdf copies of many of the papers and have reviewed most of the literature listed below for this report. This search came from a variety of databases and is presented as technical information to support our review of the Artemisia literature within and for the the project team members, and not for re-distribution, re-sale or re-publication to ensure no violation of their databases. We provide over 270 references in this report. Full copies of most can be obtained upon request. Many papers ones on production, processing and extraction of Artemisia have already been shared with BAMEX and private and public sector partners in Madagascar both prior to and during the December trip by Simon to Madagascar, with follow-up discussions on the issues raised within the papers.

### Selected Literature on the Agronomy of *Artemisia annua*:

1. Artemisia annua multi-harvesting improves artemisinin yield.

*Drug Week*, 2/20/2004, p285, 2p

Database: Academic Search Premier

Presents the results of the investigation on the feasible cultivation method for the anti-malarial medicinal plant *Artemisia annua* in India. Effects of variation in the time of planting and harvest; Correlation between artemisinin yield and leaf yield and number of harvests; Average yields achieved by multi-harvesting.

2. Artemisia annua multi-harvesting improves artemisinin yield.

*Biotech Week*, 2/18/2004, p255, 2p

Database: Academic Search Premier

Examines the effects of variation in the time of planting and harvest and of the number of harvests of the yield of artemisinin and plant growth and development. Means of improving the yield of *Artemisia annua*; Correlation of the artemisinin yield with leaf yield and number of harvests; Growth of stem tissues.

3. Novel culture augments *Artemisia annua* artemisinin production.

*Drug Week*, 10/10/2003, p238, 2p

Database: Academic Search Premier

4. Novel culture augments *Artemisia annua* artemisinin production.

*Biotech Week*, 10/8/2003, p109-110

Database: Academic Search Premier

*TB & Outbreaks Week, 10/7/2003, p28-29.*

Database: Academic Search Premier

*Health & Medicine Week, 10/6/2003, p462.*

Database: Academic Search Premier

5. Abdin MZ, M. Israr, R.U. Rehman and S.K. Jain.

Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production.

*Planta Medica 2003, Volume 69, Issue 4, Pages 289-99.*

Database: Pubmed

Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge, has been isolated from the aerial parts of *Artemisia annua* L. plants. It is effective against both drug-resistant and cerebral malaria-causing strains of *Plasmodium falciparum*. The relatively low yield (0.01-0.8 %) of artemisinin in *A. annua* is a serious limitation to the commercialization of the drug. Therefore, the enhanced production of artemisinin either in cell/tissue culture or in the whole plant of *A. annua* is highly desirable. It can be achieved by a better understanding of the biochemical pathway leading to the synthesis of artemisinin and its regulation by both exogenous and endogenous factors. Furthermore, genetic engineering tools can be employed to overexpress gene(s) coding for enzyme(s) associated with the rate limiting step(s) of artemisinin biosynthesis or to inhibit the enzyme(s) of other pathway competing for its precursors. These aspects which may be employed to enhance the yield of artemisinin both in vitro and in vivo are discussed in this review.

6. Abdin, M. Z, Israr, M., Srivastava, P. S., Jain, S. K. In vitro production of artemisinin, a novel antimalarial compound from *Artemisia annua*. *Journal of Medicinal & Aromatic Plant Sciences. 22-23(4A-1A). October-March, 2000-2001. 378-384.*

Database: Biosis

Artemisinin, a non-conventional and highly potent antimalarial drug, is extracted from *Artemisia annua* L. It is a sesquiterpenoid lactone with an endoperoxide bridge and is highly effective even in drug-resistant malarial parasites as well as in cerebral malaria. The relatively low concentration of artemisinin (0.1-0.5%) in *A. annua* is, however, a serious limitation to the commercialization of the drug. This fact led us to search for an alternate route for the production of artemisinin. Being unstable at high temperature due to the presence of endoperoxide bridge, the chemical synthesis of this compound is relatively difficult and uneconomical. Consequently, a novel approach was used by us where the immediate precursors of artemisinin, i.e. artemisinic acid and arteannuin-B were easily converted into artemisinin with a high yield using cell free extract of plant leaves. The enzyme (s) involved in this conversion have been partially purified using (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and the maximum enzyme activity was found in 75-100% fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> with 8 fold purification of enzyme(s). SDS-PAGE profile of 75-100% fraction showed two major bands with the molecular weights of 12 KD and 14.5 KDa, respectively. These proteins, therefore, might be involved in the conversion of

simple precursors of artemisinin into this compound. Since artemisinic acid can be easily synthesized chemically, hence, the partially purified enzyme(s) can be used in a bioreactor to produce artemisinin from artemisinic acid. This approach is currently being pursued in our laboratory for the commercial production of artemisinin.

7. Bharel, S., Gulati, A., Abdin, M.Z., Srivastava, P.S., Vishwakarma, R.A., Jain, S.K. Enzymatic synthesis of artemisinin from natural and synthetic precursors. *Journal of Natural Products* 1998, Volume 61, Issue 5, Pages 633-636.  
Database: Agricola

To investigate the biosynthetic pathway of artemisinin, an assay system for the determination of activity of the enzymes involved in its synthesis has been developed. Results from these experiments have shown that HEPES provides a better buffer system than Tris-HCl. The enzyme(s) requires  $Mg^{2+}$  and/or  $Mn^{2+}$ , and the addition of ATP and  $NADPH+H^+$  significantly enhances the enzyme activity. A new substrate, dihydroarteannuin B, has been synthesized that can easily be radiolabeled with high specific activity. It is utilized by the enzyme system and is converted to artemisinin with the same efficiency as the natural substrates. This can be conveniently used as a precursor for elucidation of the pathway for artemisinin biosynthesis.

8. Cai G, Li G, Ye H, Li G. Hairy root culture of *Artemisia annua* L. by Ri plasmid transformation and biosynthesis of artemisinin. *Chin J Biotechnol.* 1995, Volume 11, Issue 4, Pages 227-35.

The hairy root culture system of the medical plant *Artemisia annua* L. was established by infection with *Agrobacterium rhizogenes* R1601. The transgenic state of transformed roots was confirmed by Southern blot hybridization with TL-DNA of pFw302. The expression of NPTII gene was confirmed by enzymic assay. The important secondary metabolites-artemisinin was obtained in the hairy root culture. The effects of various physical and chemical factors on the growth of the hairy roots and production of artemisinin were studied. Artemisinin could be detected in hairy roots cultures in the light. The optimum pH value of the medium was 5.4. Fast growth of the hairy roots and maximal production of artemisinin was observed in the presence of 3% sucrose. Low concentration of naphthylacetic acid (0.025 mg/L) enhanced the growth of the roots but inhibited the production of artemisinin. The growth and artemisinin production in hairy root cultures were greatly promoted by the addition of gibberellin (GA3) to the medium. Its optimum concentration was 4.8 mg/L.

9. Chalchat, J., Garry, R., Lamy, J. Influence of harvest time on yield and composition of *Artemisia annua* oil produced in France. *Journal of Essential Oil Research*, 1994. v. 6 (3) p. 261-268.  
Database: Agricola

The essential oils of *artemisia annua* were isolated and analyzed at different stages of flowering using a combination of GC and GC/MS. At the peak of flowering, the proportion of artemisia ketone (the most abundant component) exceeded 50% and the oil yield was 0.4-0.5%.

10. Chen HR. [Tissue culture of *Artemisia annua*. II. Some factors: effect on callus formed on *Artemisia annua* and green shoot regeneration]

[Article in Chinese]- not read by this team.

Zhong Yao Tong Bao. 1986 Feb;11(2):10-1.

**Database:** Pubmed

11. Chen, P.K., Lukonis, C., Go, L., Leather, G.R.

Increasing artemisinin production through biotransformation of precursors.

*Proceedings of the Plant Growth Regulator Society of America. 1991. p. 2-8*

Database: Agricola

12. Chenshu A, X. Wang, X. Yuan, B. Zhao, Y. Wang.

Optimization of cryopreservation of *Artemisia annua* L. callus.

*Biotechnology Letters, 2003, Volume 25, Issue 1, Pages 35-8.*

Database: Pubmed

Cryopreservation of callus tissue of *Artemisia annua* L. was optimized. Two lines of calli were precultured on MS medium with 5% (v/v) dimethyl sulfoxide, and protected by a cryoprotectant containing 15% (v/v) ethylene glycol, 15% (v/v) dimethyl sulfoxide, 30% (v/v) glycerol and 13.6% (w/v) sucrose. The highest survival rate of callus A201 reached 87% after it was pretreated at 25 degrees C, cryopreserved by liquid nitrogen, recovered in water bath at 25 degrees C and reloaded at 25 degrees C with 34% (w/v) sucrose solution, and that of callus A202 reached 78% after it was treated as callus A201, except pretreated at 35 degrees C, recovered at 35 degrees C and reloaded with 47.8% (w/v) sucrose solution.

13. Condon, Jessica L., Weathers,

A study of the relative activities of antioxidative enzymes in hairy roots grown in a nutrient mist bioreactor.

*Plant Biology (New York). 2000 2000. 69.*

Database: Biosis

14. De Jesus-Gonzalez, L. Tetraploid *Artemisia annua* hairy roots produce more

artemisinin than diploids *Plant Cell Reports, 2003, Volume 21, Number 8, Pages: 809 - 813*

Database: Academic Search Premier

Hairy root cultures of diploid *Artemisia annua* L. (clone YUT16) grow rapidly and produce the antimalarial sesquiterpene artemisinin. Little is known about how polyploidy affects the growth of transformed hairy roots and the production of secondary metabolites. Using colchicine, we produced four stable tetraploid clones of *A. annua* L. from the YUT16 hairy root clone. Analysis showed major differences in growth and artemisinin production compared to the diploid clone. Tetraploid clones produced up to six times more artemisinin than the diploid parent. This study provides an initial step in increasing our understanding of the role of polyploidy in secondary metabolite production, especially in hairy roots

15. De Jesus-Gonzalez, Larry, Weathers, Pamela. Effects of polyploidy on hairy root cultures of *Artemisia annua* L. *Plant Biology* (New York). 2001 72.  
Database: Biosis

16. Delabays N, Simonnet X, Gaudin M. The genetics of artemisinin content in *Artemisia annua* L. and the breeding of high yielding cultivars.  
*Current Medicinal Chemistry*, 2001, Volume 8, Issue 15, Pages 1795-801.  
Database: Pubmed

Artemisinin, the endoperoxide sesquiterpene lactone produced by the Chinese medicinal herb *Artemisia annua*, is very difficult to synthesise. Moreover, its production by mean of cell, tissue or organ cultures is very low. Presently, only its extraction from cultivated plants is viable. A large variation in artemisinin content has been observed in the leaves of plants from different origins. The genetic basis of this variation has been assessed and evidence for a quantitative inheritance of the artemisinin concentration presented. Additive genetic components were predominant, resulting in a high narrow-sense heritability estimate. Thus, goods results can be expected from mass selection for the breeding of lines of *Artemisia annua* rich in artemisinin. Yet, dominance variance is also present in the total genetic variability, indicating that crosses between selected genotypes should generate progenies with particularly high artemisinin content. As a matter of fact, selection and crossing, in wild populations, of genotypes with high artemisinin concentration resulted in hybrid lines containing up to 1.4 % artemisinin (on dry leaves basis).

17. De Magalhaes, P. M.; Pereira, B.; Sartoratto, A. Yields of antimalarial *Artemisia annua* L. species. *Acta Horticulturae* (2004), 629(*Proceedings of the XXVI International Horticultural Congress, 2002*), 421-424.  
Database: Scifinder Scholar

Since the development of the selection program for *Artemisia annua* L. hybrid lines of high artemisinin content and agricultural improvement within the program at CPQBA-UNICAMP, new selections have been annually evaluated for biomass yields, rates between leaves and stem, artemisinin content, and essential oil (compn. and yields). Among the genotypes were evaluated during the period of Nov., 2000, to Mar., 2001, at CPQBA-UNICAMP, in Campinas-SP, Brazil, artemisinin ranged from 1.69 to 2.01 g/m<sup>2</sup>.

The essential oil yield and compn. of a population cultivated in large-scale exhibited variations by phenol. stages: at booming, 0.40%; in mid flowering, 0.30%; and close to senescence stage 0.21%. In the same phenol. stages, some of the major compds. of essential oil also varied, resp.: 1, 8 cineole, 17.06%, 21.88%, and 28.6%; camphor: 28.44%, 14.89% and 30.87%. The results provide parameters to develop raw material with antimalarial applications as well as to characterize the essential oil obtained from large-scale cultivation.

18. Ferreira, Jorge F. S., Janick, Jules. Production of artemisinin from in vitro cultures of *Artemisia annua* L Nagata, T. [Editor], Ebizuka, Y. [Editor]. *Biotechnology in Agriculture and Forestry. Medicinal and aromatic plants XII. 2002. 1-12. Series Information: Biotechnology in Agriculture and Forestry. Vol. 51.*  
Database: Biosis.

19. Galambosi, B.  
Results of cultivation trials with *Artemisia annua* L.  
*Herba Hungarica. 1982. v. 21 (2/3) p. 119-125.*  
Database: Agricola

20. Geldfre, E. van., Vergauwe, A., Eeckhout, E. van den.  
State of the art of the production of the antimalarial compound artemisinin in plants.  
*Plant Molecular Biology, 1997, Volume 33, Issue 2, Pages 199-209.*  
Database: Agricola

For more than three centuries we have relied on the extracts of the bark of *Cinchona* species to treat malaria. Now, it seems we may be changing to the leaves of a Chinese weed. *Artemisia annua*, and its active compound artemisinin. Artemisinin-derived drugs have been proved particularly effective treatments for severe malaria, even for multidrug-resistant malaria. However, this promising antimalarial compound remains expensive and is hardly available on a global scale. Therefore, many research groups have directed their investigations toward the enhancement of artemisinin production in *A. annua* cell cultures or whole plants in order to overproduce artemisinin or one of its precursors. This article provides a brief review of the state of art of the different aspects in *A. annua* research.

21. Giri, Archana, Ravindra, Sarish T., Dhingra, Vikas, Narasu, M.  
Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua*  
*Current Science (Bangalore). 81(4). 25 August, 2001. 378-382.*  
Database: Biosis

Different strains of *Agrobacterium rhizogenes*, viz. A4, 15834, K599, LBA 9402, 9365 and 9340, were evaluated for induction of transformed hairy roots in *Artemisia annua* using shoot-tip meristem explants. Incorporation of acetosyringone in bacterial culture and co-cultivation medium increased the frequency of hairy root induction in *A. annua*.

Different strains of *A. rhizogenes* varied in their virulence for induction of hairy roots. Growth kinetics of transgenic hairy roots induced by different strains indicated a similar pattern of growth, with maximum growth occurring between 14 and 16 days. Transformed hairy root cultures showed significant differences in artemisinin content. A hairy root line induced by strain 9365 was found to contain highest amount of artemisinin (0.23%). Artemisinin content of different hairy root lines was also found to be growth-related.

22. Haider, Flora, Dwivedi, Premdutt, Singh, S., Naqvi, Ali Arif, Bagchi, Gurudas  
Influence of transplanting time on essential oil yield and composition in *Artemisia annua* plants grown under the climatic conditions of sub-tropical North India.  
*Flavour & Fragrance Journal*. 19(1). January-February 2004. 51-53.  
Database: Scifinder scholar

The yield and composition of the essential oil obtained by hydrodistillation of inflorescences from different populations of *Artemisia annua* L. (Asteraceae), transplanted during different months, was analysed using a combination of GC and GC-MS. Oil yield was found to range from 0.5 to as high as 1.6% (w/v), in which 24 constituents were identified, representing 84.4-92.2% of the total oil. Camphor was identified as the most abundant component in all the samples examined, followed by 1,8-cineole, except in June-transplanted plants, where beta-caryophyllene was found to be the second most abundant compound. Camphor and 1,8-cineole alone constituted 44.7-65% of the total oil. The percentage occurrence of rest of the compounds was found to vary in different populations.

23. He, X.C., Zeng, M.Y., Li, G.F., Liang, Z. Callus induction and regeneration of plantlets from *Artemisia annua* and changes of qinhausu contents.  
*Chih Wu Hsueh Pao*. [Peking : K'o hsueh Ch'u pan she] Jan 1983. v. 25 (1) p. 87-90.  
Database: Agricola

24. Kawamoto, H., Sekine, H., Furuya, T., Production of artemisin and related sesquiterpenes in Japanese *Artemisia annua* during a vegetation period.  
*Planta Medica*, 1999, Volume 65, Issue 1, Pages 88-89.  
Database: Agricola.

25. Khafagi, Ishrak; Dewedar, Ahmed; Amein, Mahamoud.  
Opportunities of Finding Novel Anti-Infective Agents from Plant Cell Cultures  
*Current Medicinal Chemistry - Anti-Infective Agents*, 2003, Vol. 2 Issue 3, p191-211.  
Database: Academic Search Premier

Higher plants have evolved into efficient biochemical defense mechanisms, which comprised of a wide variety of secondary compounds including alkaloids, flavonoids,

terpenoids and saponins. Induction of secondary plant metabolism in cells, tissues and organ cultures do provide a potential alternative to the use of the whole plant or total synthesis of the active components. In vitro manipulation of plant secondary products offers biotechnological techniques to exploit cultured plant cell metabolism. In spite of the fact that very few plant cell processes are operating commercially, the most successful commercial pharmaceuticals produced from undifferentiated cell cultures are antibiotic compounds. The naphthoquinone shikonin from *Lithospermum erythrorhizon* is an anti-inflammatory, the isoquinoline alkaloid berberine from *Coptis japonica* is an intestinal antiseptic and the oral antimicrobial benzo-phenanthridine alkaloid sanguinarine from *Papaver bracteatum* are well known examples. Moreover, intensive research studies is currently devoted to produce the anti-leukaemic alkaloids vinblastine and vincristine from *Catharanthus roseus*, the antimalaria artemisinin from *Artemisia annua* and the anti-ovarian cancer taxol from *Taxus brevifolis*. The scope of this review is to analyze the successful in vitro production examples of antimicrobial agents, to give examples of novel chemical structures produced by plant cell cultures and not reported from the corresponding parent plants and to highlight the potential of biotransformation, elicitation and transformation techniques for the production of antiinfective agents from plant cell, tissue and organ cultures.

26. Kim YJ, Weathers PJ, Wyslouzil BE.

Growth dynamics of *Artemisia annua* hairy roots in three culture systems.

*Biotechnology and Bioengineering*, 2003, Volume 83, Issue 4, Pages 428-43.

Database: Pubmed

The transient growth of *Artemisia annua* hairy roots was compared for cultures grown in shake flasks and in bubble column and mist reactors. Instantaneous growth rates were obtained by numerically differentiating the transient biomass measurements. Specific sugar consumption rates showed good agreement with literature values. From the growth rate and sugar consumption rate, the specific yield and maintenance coefficient for sugar were determined for all three culture systems. These values were statistically indistinguishable for roots grown in shake flasks and bubble columns. In contrast, the values for roots grown in bubble columns and mist reactors were statistically different, suggesting that sugar utilization by roots grown in these two systems may be different. By measuring respiration rates in the bubble column reactor we also determined the actual biomass yield and maintenance coefficient for O<sub>2</sub> and CO<sub>2</sub>. Together with an elemental analysis of the roots, this allowed us to obtain a reasonable carbon balance.

27. Kim YJ, Weathers PJ, Wyslouzil BE. Growth of *Artemisia annua* hairy roots in liquid- and gas-phase reactors. *Biotechnology and Bioengineering*, 2002, Volume 80, Issue 4, Pages 454-64.

Database: Pubmed

*Artemisia annua* hairy roots were grown in liquid-phase bubble column and gas-phase nutrient mist reactors. In most cases the bubble column reactor accumulated more biomass than the mist reactor; the highest final biomass concentrations observed were

15.3 g DW/L in the bubble column reactor and 14.4 g DW/L in the mist reactor. Further analysis showed that the average specific growth rate in the mist reactors was essentially constant and independent of the biomass concentration at the beginning of the mist mode. In contrast, at low packing densities the average growth rate in the bubble column reactors was higher than in the mist reactors, decreasing to comparable rates at high packing densities. Finally, an aerosol deposition model was used to compare the volume of medium captured by the root bed in the mist reactor to the volume of medium required to maintain a specified growth rate. The results suggest that under the current operating conditions, lower growth rates in the mist reactor may be due to insufficient nutrient availability.

28. Kim YJ, Weathers PJ, Wyslouzil BE

A comparative study of mist and bubble column reactors in the in vitro production of artemisinin

*Plant Cell Reports*. 20(5). July, 2001. 451-455.

Database: Biosis

*Artemisia annua* hairy roots grown in nutrient mist reactors produced nearly three times as much artemisinin as roots grown in bubble column reactors, 2.64 mug/g DW and 0.98 mug/g DW, respectively.

29. Kumar, Sushil; Gupta, S.K.; Singh, Poorinima; Bajpai, Pratima; Gupta, M.M.; Singh, Digvijay; Gupta, A.K.; Ram, Govind; Shasany, A.K.; Sharma, Srikant.

High yields of artemisinin by multi-harvest of *Artemisia annua* crops

*Industrial Crops and Products*, 2004, Volume 19, Issue 1, Pages 77-90.

Database: Academic Search Premier.

Several field crop experiments were carried out on the annual determinate anti-malarial medicinal plant *Artemisia annua* over three annual cropping periods in the subtropical agroclimate of Indo-Gangatic plains. The experiments examined the effects of variation in the time of planting and harvest and of number of harvests on the yield of artemisinin and plant growth and development characters related to it. The observations showed that the artemisinin yield was positively correlated with leaf yield and number of harvests. High yields of artemisinin were realized when crop produced artemisinin-rich leaves accompanied by least possible growth of stem tissue, attained by multiple harvesting in early planted full time grown crops. The crops grown for  $\geq 30$  weeks and harvested three and four times gave average yields of  $44.1 \pm 14.2$  and  $74.2 \pm 15.6$ , respectively, much higher than the maximum yield of  $25 \text{ kg ha}^{-1}$  reported for *A. annua* in earlier studies. It was concluded that for obtaining artemisinin in high yields the *A. annua* crop should be ratooned/multi-harvested four times.

30. Laughlin JC. Agricultural production of artemisinin--a review.

*Trans R Soc Trop Med Hyg*. 1994, Volume 88, Suppl 1, Pages :S21-2.

Source: Pubmed

Artemisinin (qinghaosu) is extracted from the plant *Artemisia annua* (qinghao). Wild stands of these plants contain between 0.01 and 0.5% (w/w), with the highest concentrations in the leaves just before flowering. A review of recent agricultural techniques to improve yields from cultivated plants is presented.

31. Linden JC, Haigh JR, Mirjalili N, Phisaphalong M.

Gas concentration effects on secondary metabolite production by plant cell cultures.

*Adv Biochem Eng Biotechnol.* 2001, Volume 72, Pages 27-62.

Database: Pubmed

One aspect of secondary metabolite production that has been studied relatively infrequently is the effect of gaseous compounds on plant cell behavior. The most influential gases are believed to be oxygen, carbon dioxide and other volatile hormones such as ethylene and methyl jasmonate. Organic compounds of interest include the promising antimalarial artemisinin (known as "qing hao su" in China where it has been a folk remedy for centuries) that is produced by *Artemisia annua* (sweet wormwood) and taxanes used for anticancer therapy that are produced by species of *Taxus* (yew). The suspension cultures of both species were grown under a variety of dissolved gas conditions in stoppered culture flasks and under conditions of continuous headspace flushing with known gas mixtures. An analysis is presented to show the culture conditions are such that equilibrium between the culture liquid and gas head-space is assured. The growth rate of the cells and their production rates of artemisinin and paclitaxel were determined. These and other parameters are correlated as functions of the gas concentrations. Interdependence of ethylene and methyl jasmonate is also explored with respect to regulation of secondary metabolite formation.

32. Liu, Chun-Zhao; Guo, Chen; Wang, Yu-Chun; Ouyang, Fan.

Comparison of various bioreactors on growth and artemisinin biosynthesis of *Artemisia annua* L. shoot cultures

*Process Biochemistry*, 2003, Vol. 39 Issue 1, p45-49.

Database: Academic Search Premier

Shoot cultures of *Artemisia annua* L. were cultivated in three different bioreactors: a modified airlift bioreactor, a multi-plate radius-flow bioreactor and an ultrasonic nutrient mist bioreactor. The shoots cultivated in the multi-plate radius-flow bioreactor and nutrient mist bioreactor showed excellent growth; however, hyperhydrated shoots were observed in the totally immersion cultivation in the modified airlift bioreactor. The dry weight increase (45 times) of shoot cultures in the mist bioreactor was higher than those (29 times and 36 times) in both the modified airlift bioreactor and the multi-plate radius-flow bioreactor. Furthermore, artemisinin production of shoot cultures in the nutrient mist bioreactor was 1.4- and 3.3-fold higher than those in both the multi-plate radius-flow bioreactor and the modified airlift bioreactor, respectively. The mist bioreactor was found to be advantageous for *A. annua* L. shoot cultures

33. Liu, Chun-zhao, Guo, Chen, Wang, Yu-Chun, Ouyang, Fan

Factors influencing artemisinin production from shoot cultures of *Artemisia annua* L. *World Journal of Microbiology & Biotechnology*. 19(5). July 2003. 535-538.  
Database: Biosis

Artemisinin, an anti-malarial drug isolated from the annual wormwood *Artemisia annua* L., has a marked activity against chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum*, and is useful in treatment of cerebral malaria. Shoot cultures of *Artemisia annua* L. were established on Murashige and Skoog basal medium which contained (per litre) 30 g sucrose, 0.5 mg 6-benzyladenine and 0.05 mg naphthaleneacetic acid. Using an optimized combination of sucrose (30 g/l), nitrate (45 mM), inorganic phosphate (200 mg/l), gibberellic acid (7 mg/l) and the ratio of  $\text{NH}_4^+\text{-N}$  to  $\text{NO}_3^{--}\text{-N}$  of 1:3, artemisinin production reached 26.7 mg/l after 30 days. This procedure provides a potential alternative for production of artemisinin from in vitro tissue cultures.

34. Liu, Chun-zhao, Guo, Chen, Wang, Yu-Chun, Ouyang, Fan. Effect of light irradiation on hairy root growth and artemisinin biosynthesis of *Artemisia annua* L. *Process Biochemistry*, 2002, vol. 38, issue 4, p 581  
Database: Academic Search Premier

The effects of light irradiation on growth and production of artemisinin were studied in hairy root cultures of *Artemisia annua* L. When the hairy roots were cultured under illumination at 3000 Lux for 16 h using several cool-white fluorescent lamps, and then darkness for 8 h, the dry weight and artemisinin concentration reached 13.8 g/l and 244.5 mg/l, respectively. The results obtained showed that the growth and artemisinin accumulation increased on improving the conditions of light irradiation, which affected metabolite formation conspicuously. Under optimal conditions, kinetics of growth, artemisinin accumulation and nutrient uptake were studied

35. Liu B, Ye H, Li G, Chen D, Geng S, Zhang Y, Chen J, Gao J. Studies on dynamics of growth and biosynthesis of artemisinin in hairy roots of *Artemisia annua* L. *Chin J Biotechnol*. 1998, Volume 14, Issue 4, Pages 249-54.  
Database: Pubmed

Seven hairy root lines with the properties of fast growth and high artemisinin contents were selected from 747 hairy roots induced by transformation of *Artemisia annua* L. strain 025 with *Agrobacterium rhizogenes* ATCC15834. The differences of growth rates and artemisinin contents among the 7 selected hairy root lines were extremely significant, of which HR-9 gave the highest yield of artemisinin, reaching 33.25 mg/y.L. The differences of growth rates and artemisinin contents among hairy roots, untransformed roots and callus were also significant. There were no obvious lag phase in batch culture of hairy roots of *Artemisia annua* L. The exponential growth phase was 7 approximately 15 days after inoculation. The growth rate reached the highest on the 11th day, and the cultures reached a stationary phase on the 20th day. The properties of artemisinin content of hairy roots were obviously "related to growth". The artemisinin content decreased slowly during the exponential phase, increased while the growth rate slowed down and

remained consistent after the growth stopped. The optimum culture time for hairy roots of *Artemisia annua* L. was 21 days in our system.

36. Marchese, J. A., Rehder, V. L. G. The influence of temperature in the production of artemisinin in *Artemisia annua* L. *Revista Brasileira de Plantas Medicinai*s. 4(1). Outubro, 2001. 89-93.

Database: Biosis

*Artemisia annua* is a rich source of artemisinin, a sesquiterpene lactone proven to be effective in the control of the resistant strain of the malaria agent *Plasmodium*. Secondary metabolism compounds, such as artemisinin, show changes in their contents when the plants come under the influence of environmental stress and variations. The scope of this work was to study the influence of temperature in the production of artemisinin in plants of hybrid CPQBA 2/39X1V of *A. annua*. Plants of 88 days of age were cultivated in growing chambers, under two temperature levels, 11/20°C and 18/28°C, night and day, for 16 days. The artemisinin content in dry leaf mass was determined through high performance liquid chromatography technique using ultraviolet detection. Other determining parameters were dry leaf and stem mass, and artemisinin production by plant and the leaf stem relation. The average results were compared by t-student test. The test at 18/28°C accumulated significantly more artemisinin than the 11/20°C treatment (p=0.003). Significant differences were not found between the 18/28°C and 11/20°C treatments relating to dry leaf matter and artemisinin production by plant parameters. In plants submitted to the 18/28°C level, there was observed a significant reduction in dry stem mass (p=0.024), and consequently, a significant increase in the leaf/stem relation (p=0.008), when compared with the 11/20°C treatment plants. A greater leaf/stem relation is desirable in the *A. annua* industrial processing, in that the stem contains waxes which make more difficult the artemisinin isolation and purification.

37. Mueller MS, Karhagomba IB, Hirt HM, Wemakor E. The potential of *Artemisia annua* L. as a locally produced remedy for malaria in the tropics: agricultural, chemical and clinical aspects. *Journal of Ethnopharmacology* 2000, Volume 73, Issue 3, Pages 487-93. Source: Pubmed

The plant *Artemisia annua* L. (Asteraceae) is listed in the Chinese pharmacopoeia as a remedy for various fevers including malaria, and contains the well-established antimalarial compound artemisinin. In this study, a hybrid form of *A. annua* was successfully cultivated in Central Africa. The aerial parts of the plant contained 0.63-0.70% artemisinin per dry weight, and approximately 40% of this artemisinin could be extracted by simple tea preparation methods. Five malaria patients who were treated with *A. annua* tea showed a rapid disappearance of parasitaemia within 2-4 days. An additional trial with 48 malaria patients showed a disappearance of parasitaemia in 44 patients (92%) within 4 days. Both trials showed a marked improvement of symptoms. In

our opinion, these results justify further examinations of the antimalarial effect of *A. annua* preparations.

38. Nair MS, Acton N, Klayman DL, Kendrick K, Basile DV, Mante S. Production of artemisinin in tissue cultures of *Artemisia annua*.

*Journal of Natural Products*, 1986, Volume 49, Issue 3, Pages 504-7.

Database: Pubmed

39. Nicolas Delabays, Xavier Simonnet and Myriam Gaudin. The Genetics of Artemisinin Content in *Artemisia annua* L. and the Breeding of High Yielding Cultivars.

*Current Medicinal Chemistry*, 2001, Vol. 8 Issue 15, p1795, 7p

Database: Academic Search Premier

Artemisinin, the endoperoxide sesquiterpene lactone produced by the Chinese medicinal herb *Artemisia annua*, is very difficult to synthesise. Moreover, its production by mean of cell, tissue or organ cultures is very low. Presently, only its extraction from cultivated plants is viable. A large variation in artemisinin content has been observed in the leaves of plants from different origins. The genetic basis of this variation has been assessed and evidence for a quantitative inheritance of the artemisinin concentration presented. Additive genetic components were predominant, resulting in a high narrow-sense heritability estimate. Thus, goods results can be expected from mass selection for the breeding of lines of *Artemisia annua* rich in artemisinin. Yet, dominance variance is also present in the total genetic variability, indicating that crosses between selected genotypes should generate progenies with particularly high artemisinin content. As a matter of fact, selection and crossing, in wild populations, of genotypes with high artemisinin concentration resulted in hybrid lines containing up to 1.4 % artemisinin (on dry leaves basis).

40. Ouyang Jie, Wang Xiao-Dong, Zhao Bing, Wang Yu-Chun. Effects of Ag-carrying zirconium phosphate on the kinetics of growth of the roots of culture *Artemisia annua*.

*Acta Botanica Sinica*. 45(2). February 2003. 136-139.

Database: Biosis

41. Paniego, N., Maligne, A.E., Giulietti, A.M. *Artemisia annua* (Quing-Hoa): in vitro culture and the production of artemisinin. *Biotechnology in Agriculture & Forestry*, 1993. (24) p. 64-78.

Database: Agricola

42. Prasad, A., Kumar, D., Anwar, M., Singh, D., Jain, D. Response of *Artemisia annua* L. to soil salinity. *Journal of Herbs, Spices, & Medicinal Plants*, 1997, Volume 5, Issue 2, Pages 49-55.

Database: Agricola

43. Ram, M., Gupta, M., Kumar, S., Singh, Rajbir, Roy, S. K. Chemical and integrated weed management in *Artemisia annua*. *Journal of Medicinal & Aromatic Plant Sciences*. 23(2). June, 2001. 36-43.

Database: Biosis

A field study was conducted on *Artemisia annua* L. during the winter-summer season of 1996-97 at Lucknow, India (26.5degree N latitude, 80.5degree E longitude and 120 m altitude), representing semiarid-subtropical climate. Twelve treatment combinations consisting of 3 planting densities (2.22, 0.71 and 0.56 X 10<sup>5</sup> plants/ ha-1) and 4 methods of weed control (pendimethalin at 0.5 and 1.0 kg a.i. ha<sup>-1</sup> two hand weedings at 30 and 50 days after planting and weedy check) were tested in a factorial randomised block design to evaluate the effect of different method of weed control and planting densities on weed management and yield of artemisinin and essential oil. Results revealed that the critical weed crop competition was observed up to 60 days after transplanting of the *A. annua* crop. The crop planted at the highest plant density of 2.22X10<sup>5</sup> plants ha<sup>-1</sup> significantly reduced the weed dry matter production from 90 days of planting than the crop planted at the smallest plant densities. Pre-planted application of pendimethalin at 1.0 kg ha<sup>-1</sup> was found to be most effective in reducing the weed infestation from 30 days of planting, followed by hand weeding from 60 days onwards. In order to improve the productivity of the crop of 2.22 X 10<sup>5</sup> plants ha<sup>-1</sup> density, the pre-plant application of pendimethalin at 1.0 kg a.i. ha<sup>-1</sup> and two hand weedings at 30 and 50 days after planting allowed the crop plants to produce 68 and 97% higher dry matter, respectively over weedy check, with respective increases in the artemisinin yield of 62 and 58%. The respective treatments increased the essential oil yield by 71% and 78%. The chemical weed control had no deleterious effect on the synthesis of artemisinin in the plant. The quality of essential oil of the crop did not change by the use of chemical and hand weeding methods of weed control in varying plant populations. It is considerable that the weeds in *A. annua* field crops could be at a low level with respect to their density and growth by maintaining weed free conditions up to 60 days planting either by the use of chemical weed control or hand weeding methods; chemical weed control proved to be more economical than manual weeding due to cost effective factor. Adoption of the chemical weed control method in the high density plots of *Artemisia annua*, permitted more than 70% increase in the yield of essential oil and artemisinin under the sub tropical conditions of north Indian plains.

44. Ram, M., Gupta, M., Naqvi, A., Kumar, S. Effect of planting time on the yield of essential oil and artemisinin in *Artemisia annua* under subtropical conditions. *Journal of Essential Oil Research: 1997. Volume 9, Issue 2, Pages 193-197.*

Database: Agricola

A field experiment was conducted during 1994-95 at the Research Farm of the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India to study the yield potential of *Artemisia annua* planted at different times on sandy loam soil in a subtropical environment. Results revealed that the crops planted between September to

December produced significantly higher herbage yield measured directly, or after drying compared with that of February planted crop. September planted crop yielded the highest amount of artemisinin. Therefore, the plants established during pre-winter (August-September) weather and allowed to grow through the entire winter synthesized and accumulated more artemisinin than plants established during early (October-November) and late (February) winter periods. The crop planted in November (or the beginning of winter) was found to be significantly superior to a crop planted in August, January or February, both in herbage production and oil yield. The September planted crop yielded relatively less oil than the November planted crop but the artemisia ketone content of the oil of the former was better. It is concluded that the artemisinin content is dependent on the weather conditions, and high yields of artemisinin do not necessarily depend on high dry matter production by the crop. Under northern Indian plains conditions, the *Artemisia annua* crop should be planted in September forgetting higher artemisinin yield with superior quality of oil as measured by the artemisia ketone content, and in November for obtaining maximum oil yield.

45. Shock, C.C., Stieber, T.

Productivity of *Artemisia annua* in the Treasure Valley.

*Special Report - Oregon State University, Agricultural Experiment Station. June 1987. (814) p. 115-123.*

Database: Agricola

46. Singh, Munnu. Effect of nitrogen, phosphorus and potassium nutrition on herb, oil and artemisinin yield of *Artemisia annua* under semi-arid tropical condition.

*Journal of Medicinal & Aromatic Plant Sciences. 22-23(4A-1A). October-March, 2000-2001. 368-369.*

Database: Biosis

A field experiment was conducted at Bangalore, India to study the effect of levels of nitrogen (0, 50 and 100 kg ha<sup>-1</sup>), phosphorus (0 and 50 kg ha<sup>-1</sup>) and potassium (0 and 50 kg ha<sup>-1</sup>) on herb, oil and artemisinin yields of *Artemisia annua* under semi-arid tropical conditions. Herb, essential oil and artemisinin yields increased significantly with the application of 50 kg N/ha compared with 0 kg N/ha (control) and were at par at 100 kg N/ha. Application of 50 and 100 kg N/ha increased herb, oil and artemisinin yields by 26.2% and 40.1% respectively, compared with control. There was no response to phosphorus (P) and potassium (K). Oil content and artemisinin content were not influenced by N, P and K nutrition.

47. Souret FF, Y. Kim, B.E. Wyslouzil, K.K. Wobbe, P.J. Weathers.

Scale-up of *Artemisia annua* L. hairy root cultures produces complex patterns of terpenoid gene expression. *Biotechnology and Bioengineering, 2003, Volume 83, Issue 6, Pages 653-67.*

Database: Pubmed

Hairy roots grow quickly, reach high densities, and can produce significant amounts of secondary metabolites, yet their scale-up to bioreactors remains challenging. *Artemisia annua* produces a rich array of terpenoids, including the sesquiterpene, artemisinin, and transformed roots of this species provide a good model for studying terpenoid production. These cultures were examined in shake flasks and compared with cultures grown in two types of bioreactors, a mist reactor and a bubble column reactor, which provide very different environments for the growing roots. Mist reactors have been shown previously to result in cultures that produce significantly more artemisinin per gram fresh weight of culture, while bubble column reactors have produced greater biomass. We have compared expression levels of four key terpenoid biosynthetic genes: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), and farnesyl diphosphate synthase (FPS) in the three culture conditions. In shake flasks we found that although all four genes showed temporal regulation, only FPS expression correlated with artemisinin production. Light also affected the transcription of all four genes. Although expression in reactors was equivalent to or greater than that of roots grown in shake flasks, no correlation was found between expression level within six different zones of each reactor and their respective oxygen levels, light, and root-packing density. Surprisingly, transcriptional regulation of HMGR, DXS, DXR, and FPS was greatly affected by the position of the roots in each reactor. Thus, relying on a single reactor sample to characterize the gene activity in a whole reactor can be misleading, especially if the goal is to examine the difference between reactor types or operating parameters, steps essential in scaling up cultures for production.

48. Teoh KH, Weathers PJ, Cheetham RD, Walcerz DB. Cryopreservation of transformed (hairy) roots of *Artemisia annua*. *Cryobiology* 1996, Volume 33, Issue 1, Pages 106-17. Database: Pubmed

The antimalarial drug artemisinin has been found in transformed (hairy) roots of *Artemisia annua*. A protocol was developed to preserve *A. annua* hairy roots in liquid nitrogen. Root tips were excised from 7-day-old cultures and held on solid White's medium for 24 h prior to cryoprotection. They were then treated with a cryoprotecting mixture containing 8% (v/v) dimethyl sulfoxide (Me<sub>2</sub>SO) and 20% (w/v) sucrose at 25 degrees C for 1 h followed by cooling at 0.09 degrees C/min to 4 degrees C then cooling to -35 degrees C at 0.72 degrees C/min. Vials containing the root tips were then plunged into liquid nitrogen. After thawing in a water bath to 37 degrees C, root tips were held in the cryoprotecting mixture for 10 min before it was diluted to 25% of its original concentration. Root tips were washed once with fresh liquid White's medium and held for 1 h prior to culturing on White's medium with 0.2% Gelrite and 3% (w/v) sucrose. Regrowth of root tips averaged 65%. Independent variables in this study included 1) Me<sub>2</sub>SO concentration; 2) the type and concentration of cosolutes; 3) cooling rate(s); 4) the temperature at which the sample is transferred to liquid nitrogen; 5) the age of the culture from which root tips are taken; 6) the recovery period between root tip excision

and immersion in cryoprotectant; and 7) the amount of Gelrite used in the postthaw plating medium.

49. Teixeira da Silva, Jaime A. Anthemideae: Advances in tissue culture, genetics and transgenic biotechnology. *African Journal of Biotechnology*. 2003. 547-556.  
Database: Agricola

Members of the Anthemideae include important floricultural (cut-flower) and ornamental (pot and garden) crops, as well as plants of medicinal and ethno-pharmacological interest. Despite the use of many of these plants (over 1400 species) in the extraction of important secondary metabolites and essential oils, the greatest emphasis has been on their in vitro tissue culture and micropropagation. Few studies have been conducted on genetic transformation, with those primarily focused on increasing yield of compounds in plants. This review, the first and only available for plants within this Family, highlights all the available literature that exists on Anthemideae (excluding ornamental chrysanthemums) in vitro cell, tissue and organ culture, micropropagation and transformation.

50. Towler M.J., P.J. Weathers. Adhesion of plant roots to poly-L-lysine coated polypropylene substrates. *Journal of Biotechnology* 2003, Volume 101, Issue 2, Pages 147-55.  
Database: Pubmed

The ability to immobilize plant tissue in a bioreactor is an important process tool. We have shown that roots of several species rapidly attach to poly-L-lysine coated polypropylene mesh in a liquid environment. Using transformed roots of *Artemisia annua* as a model, the attachment process was found to be enhanced by sheep serum, but not BSA and inhibited by excess Mn(2+), but unaffected by Ca(2+) or Mg(2+). Attempts to characterize the molecule(s) responsible for binding using lectins and antibodies showed that the binding site does not appear to be glycosylated or vitronectin-like. This method of rapid attachment should prove useful for controlled immobilization of roots in bioreactors.

51. Usha, R., Swamy, P. M. Influence of nutrient deficiency and sucrose concentrations on the production of artemisinin by cultured cells of sweet wormwood (*Artemisia annua* L). *Indian Journal of Plant Physiology*. 7(2). April-June, 2002. 159-162.  
Database: Biosis

Sweet wormwood (*Artemisia annua* L) produces an antimalarial drug artemisinin. Callus cultures of *A. annua* were subjected to nitrate, phosphate deficiency and sucrose concentrations to study their effects on the callus growth and production of artemisinin content. Nitrate deficiency in the medium caused 1.13 fold increase in the artemisinin. Phosphate and osmotic deficiency resulted in the reduction of artemisinin content. Under conditions of either nitrate deficiency or osmotic stress, growth of callus decreased with a

concomitant increase in the artemisinin content. Phosphate deficiency caused increased growth and decreased artemisinin content. However, 2% sucrose concentration in the medium caused maximum increase in the artemisinin content. The results indicated permissive role of carbon, nitrogen and phosphorus in the callus growth and production of artemisinin.

52. Van Geldre E, Vergauwe A, Van den Eeckhout E. State of the art of the production of the antimalarial compound artemisinin in plants. *Plant Mol Biol.* 1997, Volume 33, Issue 2, Pages 199-209. Source: Pubmed

For more than three centuries we have relied on the extracts of the bark of *Cinchona* species to treat malaria. Now, it seems we may be changing to the leaves of a Chinese weed, *Artemisia annua*, and its active compound artemisinin. Artemisinin-derived drugs have been proved particularly effective treatments for severe malaria, even for multidrug-resistant malaria. However, this promising antimalarial compound remains expensive and is hardly available on a global scale. Therefore, many research groups have directed their investigations toward the enhancement of artemisinin production in *A. annua* cell cultures or whole plants in order to overproduce artemisinin or one of its precursors. This article provides a brief review of the state of art of the different aspects in *A. annua* research.

53. Wang Jian, Xia Zhong-Hao, Tan Ren-Xiang. Elicitation on artemisinin biosynthesis in *Artemisia annua* hairy roots by the oligosaccharide extract from the endophytic *Colletotrichum* sp. B501. *Acta Botanica Sinica.* 44(10). October 2002. 1233-1238. Database: Biosis

The oligosaccharide elicitor from the mycelial wall of an endophytic *Colletotrichum* sp. B501 promoted the production of artemisinin in *Artemisia annua* L. hairy root culture. When hairy roots of 22-day-old cultures (later growth phase) were exposed to the elicitor (20 mg/L) for 4 d, the maximum content of artemisinin reached 1.15 mg/g, a 64.29% increment over the control. The electron X-ray microanalysis disclosed the rapid accumulation of Ca<sup>2+</sup> in the elicited cortical cells of hairy root. The electronic microscope observation revealed the high electron density area in vacuole of elicited cells. During the first day of elicitation the peroxidase activity of hairy roots was improved sharply. Some cellular morphological changes including cell shrinkage, condensation of cytoplasm and nuclear fragmentation, coincident with the appearance of DNA ladders, were observed after the third day of elicitation. It was suggested that the oligosaccharide elicitor triggered the programmed cell death, which may provide the substance or chemical signal for artemisinin biosynthesis.

54. Wang, Jian, Kong, Fang Xiang, Tan, Ren Xiang. Improved artemisinin accumulation in hairy root cultures of *Artemisia annua* by (22S, 23S)-homobrassinolide. *Biotechnology Letters.* 24(19). October 2002 2002. 1573-1577. Database: Biosis

When (22S, 23S)-homobrassinolide (SSHB) was added at 1  $\mu\text{g l}^{-1}$  to hairy root cultures of *Artemisia annua*, the production of artemisinin reached to 14  $\text{mg l}^{-1}$ , an increment of 57% over the control. SSHB treatment led concomitantly to an increased biomass production of 15  $\text{g l}^{-1}$ . A stimulatory activity of SSHB on nucleic acids and soluble protein content in hairy roots was also observed at the growth stage.

55. Wang, Jian Wen, Tan, Ren Xiang. Artemisinin production in *Artemisia annua* hairy root cultures with improved growth by altering the nitrogen source in the medium *Biotechnology Letters*. 24(14). July, 2002. 1153-1156. Database: Biosis

Murashige and Skoog medium was modified for enhancing artemisinin production in *Artemisia annua* hairy root cultures by altering the ratio of  $\text{NO}_3^-/\text{NH}_4^+$  and the total amount of initial nitrogen. Increasing ammonium to 60 mM decreased both growth and artemisinin accumulation in hairy root cultures. With  $\text{NO}_3^-/\text{NH}_4^+$  at 5:1 (w/w), the optimum concentration of total initial nitrogen for artemisinin production was 20 mM. After 24 days of cultivation with 16.7 mM nitrate and 3.3 mM ammonium, the maximum artemisinin production of hairy roots was about 14  $\text{mg l}^{-1}$ , a 57% increase over that in the standard MS medium.

56. Wang, Yuchun, Zhang, Haoxian, Zhao, Bing, Yuan, Xiaofan. Improved growth of *Artemisia annua* L hairy roots and artemisinin production under red light conditions *Biotechnology Letters*. 23(23). December, 2001. 1971-1973. Database: Biosis

Hairy root cultures of *Artemisia annua* L were cultivated for 30 days under either white, red, blue, yellow or green light. Red light at 660 nm gave the highest biomass of hairy roots (5.73 g dry wt cells  $\text{l}^{-1}$  medium) and artemisinin content (31 mg artemisinin g $^{-1}$  dry cells) which were, respectively, 17% and 67% higher than those obtained under white light.

57. Wang, Jian Wen, Zhang, Zhen, Tan, Ren Xiang. Stimulation of artemisinin production in *Artemisia annua* hairy roots by the elicitor from the endophytic *Colletotrichum* sp. *Biotechnology Letters*. 23(11). June 1, 2001. 857-860. Database: Biosis.

Artemisinin content in hairy roots of *Artemisia annua* was increased from 0.8  $\text{mg g}^{-1}$  dry wt to 1  $\text{mg g}^{-1}$  dry wt by using elicitor treatment of mycelial extracts from the endophytic fungus *Colletotrichum* sp. The increase of artemisinin was dependent on the growth stage of hairy roots as well as on the dose of the elicitor applied. When hairy roots of 23-day-old cultures (later growth phase) were exposed to the elicitor at 0.4  $\text{mg total sugar ml}^{-1}$  for 4 days, the maximum production of artemisinin reached to 13  $\text{mg l}^{-1}$ , a 44% increase over the control. This is the first report on the stimulation of artemisinin production in hairy roots by the elicitor from an endophytic fungus of *A. annua*.

58. Wang Hong; Ye He-Chun; Li Guo-Feng; Liu Ben-Ye ; Chong Kang. Effects of fungal elicitors on cell growth and artemisinin accumulation in hairy root cultures of *Artemisia annua*. *Acta Botanica Sinica*. 42(9). Sep., 2000. 905-909.

Database: Biosis

The artemisinin accumulation in the hairy root cultures of *Artemisia annua* L. was enhanced via a treatment of three fungal elicitors separately (*Verticillium dahliae* Kleb., *Rhizopus stolonifer* (Ehrenb. ex Fr.) Vuill and *Colletotrichum dematium* (Pers.) Grove). Among these three elicitors, *V. dahliae* had the highest inducing efficiency, but none of them manifests any noticeable effects on the cell growth of the hairy root cultures. The artemisinin content of the hairy root cultures treated with *V. dahliae* elicitor was 1.12 mg/g DW, which was 45% higher than the control (0.77 mg/g DW). The results showed that elicitation was dependent on the elicitor concentration, the incubation period and the physiological stage at which the hairy root cultures were treated. In addition, the authors found that for *V. dahliae*, the optimum concentration was 0.4 mg carbohydrate per millilitre medium, the strongest response of *A. annua* hairy root cultures to the elicitation was at the late exponential growth stage, and the highest artemisinin content of the hairy root cultures was on the 4th day post treatment.

59. Weathers, P. J., Souret, F. F., Kim, Y. J., Wyslouzil, B. E., Wobbe, K. K. Heterogeneity in terpenoid gene expression in transformed roots of *Artemisia annua* L. grown in bioreactors. *In Vitro Cellular & Developmental Biology-Plant*. 39(Abstract). Spring 2003. 22-A.

Database: Biosis

60. Weathers, Pamela J., Kim, Yoo Jeong. Transformed roots of *Artemisia annua* exhibit an unusual pattern of border cell release. *In Vitro Cellular & Developmental Biology-Plant*. 37(4). July-August, 2001. 440-445.

Database: Biosis

Border cells from *Artemisia annua* were examined from hairy roots grown in shake flasks, culture plates, a bubble column reactor, and a nutrient mist (aeroponic) reactor. When well-hydrated roots were subjected to shear, border cells were first released as an agglomerate and did not disperse for several hours. Staining with neutral red and fluorescein diacetate (FDA) showed that both agglomerates and dispersed cells were alive. It was determined that FDA is cleaved by pectin methylesterase (PME) and that PME may not be particularly active in the released agglomerates until the border cells disperse. Untransformed roots isolated from *A. annua* plants showed no border cell agglomerate formation and border cells readily dispersed. These results suggest that our hairy root clone is deficient in border cell release perhaps resulting from the transformation process.

61. Wyslouzil BE, Waterbury RG, Weathers PJ. The growth of single roots of *Artemisia annua* in nutrient mist reactors. *Biotechnology and Bioengineering*, 2000, Volume 70, Issue 2, Pages 143-50. Database: Pubmed

To better characterize the development and growth of hairy roots in a mist-fed root bed, a single root aerosol reactor was developed. Growth kinetics studies were conducted on hairy roots of *Artemisia annua* as a function of the mist cycle, carrier gas, and nutrient compositions. Sustained rapid growth was only observed when conditioned medium was fed to the roots. The presence of 1% CO<sub>2</sub> in the carrier gas did not enhance the growth kinetics but it did prevent necrosis of the tissue at the highest mist cycle.

62. Wyslouzil BE, Whipple M, Chatterjee C, Walcerz DB, Weathers PJ, Hart DP. Mist deposition onto hairy root cultures: aerosol modeling and experiments. *Biotechnol Prog.* 1997, Volume 13, Issue 2, Pages 185-94. Database: Pubmed

We analyzed the applicability of the standard models for aerosol deposition in randomly packed fibrous filter beds to mist deposition across a bed of hairy roots in the nutrient mist bioreactor. Although the assumptions inherent in the models are met on a local level, the overall structure of the root bed introduces some uncertainty into the correct choice of root packing fraction and gas velocity required by the model. For reasonable parameter values, the minimum in the deposition efficiency curves is close to the peak in the mist number and mass distributions, and good penetration of the root bed is possible. We then measured the deposition of mist across a packed bed of *Artemisia annua* transformed roots as a function of droplet size, bed length, and gas flow rate at a root packing fraction  $\alpha = 0.5$ . We compared the experimental measurements with the predictions of the aerosol deposition model and found good agreement between the measured and predicted values for the diameter where the deposition efficiency across the bed is 50%, D<sub>0.5</sub>. Agreement between the model and the experiments broke down when the flow rate was increased to the point where the creeping flow assumptions were no longer valid.

63. Xie, Deyu, Wang, Lianhui, Ye, Hechun, Li, Guofeng. Isolation and production of artemisinin and stigmasterol in hairy root cultures of *Artemisia annua* *Plant Cell Tissue & Organ Culture.* 63(2). 2000. 161-166. Database: biosis

Scaled-up hairy root culture of *Artemisia annua* L. was established in three-liter Erlenmeyer flask. Both artemisinin and stigmasterol that derive from the common precursors of isopentenyl diphosphate and farnesyl pyrophosphate were isolated from hairy roots. The production rate of artemisinin isolated by column chromatography from hairy root cultures was 0.54% (mg.gDW<sup>-1</sup>). Stigmasterol was identified by mass spectrometry and nuclear magnetic resonance analysis. The production of stigmasterol isolated by column chromatography from hairy root cultures was 108.3 % (mg.gDW<sup>-1</sup>). In hairy root cultures, the production rate of stigmasterol was estimated to be 201 times greater than that of artemisinin. Our results suggest that investigation of secondary metabolites may provide a new insight to study artemisinin production in hairy root cultures.

64. Xie, Deyu, Zou, Zhuorong, Ye, Hechun, Li, Guofeng, Guo, Zhongchen. Selection of hairy root clones of *Artemisia annua* L. for artemisinin production. *Israel Journal of Plant Sciences*. 49(2). 2001. 129-134.

Database: Biosis

Hairy roots were induced from two kinds of explants and selection of hairy root clones was studied for artemisinin production. Leaf blade pieces and petiole segments of *Artemisia annua* plantlets were infected with *Agrobacterium rhizogenes* strain 1601. The efficiency of leaf blade pieces forming hairy roots was higher than that of petiole segments. Light promoted hairy root induction and branching. Two hundred and twenty hairy root clones showed considerable variations in capacity of growth and branching. Six hairy root clones were established in suspension culture and showed obvious differences in their biomass and artemisinin content. Among six clones, clone 1601-L-3 produced the highest biomass, more than 70 times the inoculum, while clone 1601-L-1 gave the lowest biomass. The artemisinin content of clone 1601-L-1 was the highest, 1.195 mg/g DW, and this line yielded the highest artemisinin level of 9.08 mg/L.

65. Xun, Xiaohong; Jiang, Taiwen; Peng, Xiaoying; Zhang, Liangbo; Jiang, Daosong. Studies on the ways of shoot regeneration and the technique for inducing polyploid of *Artemisia annua*. *Hunan Nongye Daxue Xuebao* (2003), 29(2), 115-119

Database: Scifinder Scholar

To establish the tech. system of variety improvement and micropropagation of *Artemisia annua* L. with its leaves and stems used as explants, this expt. is an attempt to study the ways of shoot regeneration and the polyploid induction of it. The results showed that loose calli emerge from explants cultured in the medium with 6-BA (6-benzylaminopurine) and KT (kinetin), the embryoids which will grow into shoots come from these calli. Compact calli emerge from explant cultured in the medium with 2,4-D (2,4-dichlorophenoxyacetic acid) and 6-BA, and they would grow into shoots after transplanting into the medium with cytokinins. While stem with axillary buds are cultured in the medium with 6-BA and KT, many shoots would emerge, and micropropagation system can be established. The polyploid of *Artemisia annua* L. is obtained using calli with buds treated with 0.05% colchicine soln. for 48 h. The inducing percentage reaches 60% and the chromosomal no. is  $2n=4x=36$ . In addn., some other problems such as: medium prepn., hormonal regulation and the double method for prepg. polyploid of *Artemisia annua* L. are also discussed.

#### **Selected Botanical Literature on *Artemisia annua*:**

66. Duke, Mary V., Paul, Rex N. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L.

*International Journal of Plant Sciences*, 1994, Vol. 155 Issue 3, p365-342.

Source: Academic Search Premier

Determines the tissue localization of the antimalarial sesquiterpenoid compound artemisinin in annual wormwood (*Artemisia annua* L.) by differential extraction of a glanded biotype and glandless biotype. Result of the extraction of all detectable artemisitene, an artemisinin analog; Implication for artemisinin production.

67. Ferreira, Jorge F.S., Janick, Jules. Floral morphology of *Artemisia annua* with special reference to trichomes. *International Journal of Plant Sciences*, 1995, Vol. 156 Issue 6, p807, 9p. Source: Academic Search Premier

Describes the floral morphology of *Artemisia annua* L. using light and scanning electron microscopy. Artemisinin analysis; Floral trichomes; Glandular trichomes

68. Gupta, Shiv K. Morphogenetic variation for artemisinin and volatile oil in *Artemisia annua*. *Industrial Crops & Products*, 2002, vol. 16, issue 3, p 217  
Source: Academic Search Premier, Agricola.

Seeds of *Artemisia annua* cv. Jeevanraksha were sown in the nursery in the middle of December in 1997, 1998 and 1999. About 1-month old seedlings were transplanted in the field having sandy loam soil in the subtropical agroclimate of Lucknow, India. The plant tops were sampled fortnightly for leaves during vegetative phase and for leaves and capitula during post-flowering stages for the estimation of artemisinin content. The *A. annua* plants continued to grow logarithmically in height from the end of rosette phase at about 9 weeks to the pre-flowering stage at about 44 weeks age and attained a height of 3.4 m. The artemisinin content of the leaves was observed to be high from 0.8 to 1.0% in May and 0.8 to 1.3% through late July to late September. Subsequently, plants entered the reproductive phase. While in the vegetative phase, 90% of artemisinin was in the leaves, in the mature plants, about 30% of the artemisinin was in the leaves and 40% was in capitula. In the vegetative stage plants the younger leaves born on the tops of secondary and higher order branches were richer in the artemisinin than the older leaves. The tops of *A. annua* plants in their vegetative growth phase possessed low levels of essential oil at about 0.2% as compared to 1.2% of essential oil in the full blooming stage plants. The extraction of artemisinin from leaves is more economic than from the mixture of leaves and capitula on account of higher levels of lipids in the extract of the latter. Since *A. annua* plants grew logarithmically all through vegetative phase from March to late September and artemisinin content in the leaves was high in May and from late July to late September, it is suggested that under the subtropical agroclimates, *A. annua* crops may be harvested more than once. The ratooning is expected to reduce losses in artemisinin yield resulting from senescence caused dropping of old leaves and favour preponderance of young leaves found richer in artemisinin content.

69. Ishmuratova, M. Yu., Egeubaeva, R. A., Adekenov, S. M. Ontogenesis of *Artemisia annua* L. grown up in Karaganda (the central Kazakhstan). *Rastitel'Nye Resursy*. 38(2). 2002. 74-78. Database: Biosis.

Ontogenesis of *Artemisia annua* L. - annual plant grown up in Karaganda is investigated. Three periods - dormant, virgin and generative as far as six age states are recorded. The duration of ontogenesis is 6 months.

70. Nabi-Zade, L.I., Baeva, R.T., Karryev, M.O. Artemisinin from *Artemisia annua* growing in Turkmenistan. *Izvestiia Akademii nauk Turkmenskoi SSR. Seriya biologicheskikh nauk*. 1986. (2) p. 72. Database: Agricola

71. Singh, V. P., Singh, Man, Singh, Kambod, Naqvi, A. A., Saini, P. Influence of harvest time on the yield and composition of *Artemisia annua* essential oil in North Indian plains. *Journal of Medicinal & Aromatic Plant Sciences*. 24(2). June 2002. 390-392. Database: Agricola

A field experiment was carried out at CIMAP, Lucknow during 1993-94 to find out the optimum harvesting time of *Artemisia annua* L. for maximum essential oil production. The herb yield, essential oil content and artemisia ketone content in the *A. annua* oil increased with the advancement of crop age, reaching the maximum at the time of full flowering. The maximum fresh herb (183 q/ha) and oil (49.4 kg/ha) yield with highest 62.5% artemisia ketone (the most abundant constituent) and lowest, 9.8% artemisia alcohol content were obtained from *A. annua* harvested at full flowering. The 1, 8-cineole content in essential oil was not influenced by harvest time. Harvesting of *A. annua* at full flowering stage is suggested for higher oil production with highest artemisia ketone and lowest artemisia alcohol content.

72. Zivkovic, T; Vajs, V.; Djokovic, D.; Glisic, O.; Stevanovic, B. Variation in composition and content of essential oils of *Artemisia* species in relation to environmental conditions. *Bulgarian Journal of Plant Physiology.(Special Issue)*. 1998. 325. Database: Biosis

### **Selected Literature on the Genetics of *Artemisia annua*:**

73. Kreitschitz, Agnieszka, Valles, Joan. New or rare data on chromosome numbers in several taxa of the genus *Artemisia* (Asteraceae) in Poland. *Folia Geobotanica*. 38(3). 2003. 333-343. Database: Agricola

Chromosome counts in 16 populations of five *Artemisia* species from Poland are presented in this paper. Those of *A. annua* ( $2n=18$ ) and *A. dracunculus* ( $2n=90$ ) are reported for the first time in Polish populations. The decaploid level ( $2n=90$ ) is described for the first time in non-cultivated populations of *A. dracunculus*, and several cases of aneusomy (intraindividual aneuploid variations in chromosome number:  $2n=87$ , 88 and 89) have been detected in this species. In addition to the already known diploid number ( $2n=18$ ), the tetraploid level ( $2n=36$ ) has been detected in *A. absinthium*. The same two numbers have been recorded in *A. abrotanum*, which represents the first tetraploid count

in populations of this taxon occurring outside botanical gardens. Finally, the chromosome number of *Artemisia campestris* subsp. *sericea* (tetraploid,  $2n=36$ ) is reported for the first time. The relevance of polyploidy for the evolution of the genus and other cytotoxic or cytobiogeographical aspects are briefly discussed.

74. Mengistu, Lemma W., Christoffers, Michael J., Kegode, George O. Genetic diversity of biennial wormwood. *Weed Science*. 52(1). January-February 2004. 53-60. Database: Biosis.

Biennial wormwood is native to North America and has become an important weed problem in soybean and dry bean fields of North Dakota, South Dakota, and Minnesota in the United States and in the prairie provinces of Canada. Intersimple sequence repeat (ISSR) markers were used to study the genetic diversity among six biennial wormwood and one annual wormwood populations. Deoxyribonucleic acid (DNA) sequences from internal transcribed spacer (ITS1 and ITS2) regions of ribosomal DNA and morphological diversity among the biennial and annual wormwood populations were also studied. High levels of genetic diversity were evident with Nei's gene diversity statistic ( $h$ ) = 0.40 for biennial wormwood and  $h = 0.36$  for annual wormwood. Total diversity of six biennial wormwood populations was  $HT = 0.40$ , and 22% of this diversity was among populations ( $GST = 0.22$ ). Estimated gene flow among biennial wormwood populations was low ( $Nm = 0.9$ ), and high levels of differentiation may be due in part to low levels of genetic exchange among biennial wormwood populations. Although biennial wormwood behaves more like an annual than a biennial, the ISSR, ITS, and morphological studies show that the two species are dissimilar.

75. Sangwan, R. S., Sangwan, N. S. Molecular markers of putative association with chemotypic characters in medicinal and aromatic plants: Progress towards identification of sequence tagged QTLs in *Artemisia annua*. *Journal of Medicinal & Aromatic Plant Sciences*. 22-23(4A-1A). October-March, 2000-2001. 297-299. Database: Biosis.

Detection and appraisal of genetic variation in natural and founder populations of many modern economic plants has been made more precise and reliable with the application of DNA-based techniques. Among them, RAPDs besides being simple and time saving primary choice of gauging the extent of genetic variations, also provide multi locus profiling of gene sequence differences across the whole genome. The technique has been applied to anti-malarial plant *Artemisia annua* to measure the gene diversity in the present day population derived from the founder accessions in India. Also an attempt of analysis of the polymorphic markers individually to look for their association with the economic characters like essential oil content and concentration of artemisinin is urgently required. Accordingly, the progress towards identification of putative sequence tagged quantitative trait loci (QTLs) deserving tests of scrutiny in *A. annua* is stated. The protocol is primarily search for nonrandom association of sorted DNA markers.

76. Sangwan RS, Sangwan NS, Jain DC, Kumar S, Ranade SA. RAPD profile based genetic characterization of chemotypic variants of *Artemisia annua* L. *Biochem Mol Biol Int.* 1999, Volume 47, Issue 6, Pages 935-44. Source: Pubmed

The annual herbaceous plant, *Artemisia annua* L., belonging to family asteraceae, is the natural source of the highly potent antimalarial compound, artemisinin, besides producing valuable essential oil. The plant is at present the sole commercial source for artemisinin production since all the chemical syntheses are non-viable. Therefore, economic and practical considerations dictate that plants with maximum content of artemisinin be found and/or ways to increase their artemisinin content be sought. The key to this selection and breeding is a comprehension of chemical and genetic variability and suitable selection(s) of elites from within the available population. In the present study, RAPD analyses of selected chemotypes from a decade old introduced population in India were carried out using arbitrary primers. The RAPD data clearly indicate the distinction amongst these plants. Further, the detection of highly polymorphic profiles (97 polymorphic markers out of a total of 101 markers) suggests the existence of very high levels of genetic variation in the Indian population despite geographical isolation and opens out a strong possibility of further genetic improvement for superior artemisinin content. UPGMA analyses of RAPD and phytochemical trait data indicate that the wide phytochemical diversity is included within the genetic diversity. These results further support the prospects for selection and breeding of superior artemisinin containing lines.

77. Torrell, M., Cerbah, M., Siljak-Yakovlev, S., Valles, J. Molecular cytogenetics of the genus *Artemisia* (Asteraceae, Anthemideae): fluorochrome banding and fluorescence in situ hybridization. I. Subgenus *Seriphidium* and related taxa. *Plant Systematics & Evolution.* 239(1-2). July 2003. 141-153. Database: Biosis

The distributional pattern of AT- and GC-rich regions and the physical mapping of ribosomal DNA (location of 18S-5.8S-26S and 5S rDNA) in the chromosomes of seven *Artemisia* species have been established by means of fluorochrome banding and fluorescence in situ hybridization (FISH). This is the first study in the large genus *Artemisia* using FISH. Five species (*A. barrelieri*, *A. caerulescens* subsp. *gallica*, *A. fragrans*, *A. herba-alba* subsp. *valentina*, *A. herba-alba* subsp. *herba-alba*) belong to the subgenus *Seriphidium*, one of the most homogeneous in the genus; one (*A. tridentata* subsp. *spiciformis*) belongs to the small subgenus *Tridentatae*, classically included in *Seriphidium*; and one (*A. annua*) belongs to the subgenus *Artemisia*, but shows some affinities with *Seriphidium*. Genome organization is relatively constant in all the species studied. AT- and GC-rich DNA is predominantly terminal, but some intercalary and centromeric bands also exist. The rDNA loci are also most often terminal and usually located in GC-rich regions. 5S rDNA sites are present in a lower number than 18S-5.8S-26S sites, and are always collocated with some of them. In the light of these cytogenetic features, subgenus *Seriphidium* is clearly placed within the genus *Artemisia*, so that it does not make sense to segregate it as a genus; on the other hand, subgenus *Tridentatae* must not be classified within *Seriphidium*, but kept as an independent subgenus.

78. Valles, J.; Torrell, M.; Garnatje, T.; Garcia-Jacas, N.; Vilatersana, R.; Susanna, A. The genus *Artemisia* and its allies: phylogeny of the subtribe Artemisiinae (Asteraceae, Anthemideae) based on nucleotide sequences of nuclear ribosomal DNA internal transcribed spacers (ITS). *Plant Biology (Stuttgart, Germany)* (2003), 5(3), 274-284 Database: Scifinder Scholar.

Sequences of the internal transcribed spacers (ITS1 and ITS2) of nuclear ribosomal DNA were analyzed for 44 *Artemisia* species (46 populations) representing all the five classical subgenera and the geog. range of the genus, 11 species from 10 genera closely related to *Artemisia*, and six outgroup species from five other genera of the Anthemideae. The results definitely support the monophyly of the genus *Artemisia* in its broadest sense (including some taxa segregated as independent genera, like *Oligosporus* and *Seriphidium*). Eight main clades are established in this mol. phylogeny within *Artemisia*; they agree in part with the classical subdivision of the genus, but they also suggest that some infrageneric groups must be redefined, esp. the subgenus *Artemisia*. The subgenera *Tridentatae* and *Seriphidium* are independent from each other. Some of the satellite genera are clearly placed within *Artemisia* (*Artemisiastrum*, *Filifolium*, *Mausolea*, *Picrothamnus*, *Sphaeromeria*, *Turaniphytum*), whereas some others fall outside the large clade formed by this genus (*Brachanthemum*, *Elachanthemum*, *Hippolytia*, *Kaschgaria*). Our results, correlated to other data such as pollen morphol., allow us to conclude that the subtribe Artemisiinae as currently defined is a very heterogeneous group. Affinities of the largest genus of the subtribe and tribe, *Artemisia*, and of other genera of the subtribe to some genera from other subtribes of the Anthemideae strongly suggest that subtribe Artemisiinae needs a deep revision and redefinition. Phylogenetic utility of region trnL-F of the plastid DNA in the genus *Artemisia* and allies was also evaluated: sequences of the trnL-F region in *Artemisia* do not provide phylogenetic information.

79. Wallaart TE, Pras N, Beekman AC, Quax WJ. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. *Planta Medica* 2000, Volume 66, Issue 1, Pages 57-62. Source: Pubmed

The time course of the levels of artemisinin, its biosynthetic precursors and the biosynthetically related sesquiterpenes was monitored during a vegetation period of *Artemisia annua* plants of different geographical origin. Considerable differences in contents of artemisinin and its direct precursors artemisinic acid and dihydroartemisinic acid were found between these *A. annua*'s. For the first time the *A. annua* plants of different geographical origin were found to belong to different chemotypes. A chemotype with a high artemisinin level was found to have also a high dihydroartemisinic acid level but a relatively low artemisinic acid level. Reversibly, a chemotype with low levels of artemisinin and dihydroartemisinic acid contained a high artemisinic acid level. Artemisinic acid is considered to be the direct precursor of dihydroartemisinic acid in the biosynthetic pathway of artemisinin. The observed accumulation of artemisinic acid in one of the *A. annua* chemotypes may indicate the presence of a rate-limiting step in the

biosynthetic pathway of artemisinin. The enzymatic reduction of artemisinic acid into dihydroartemisinic acid is probably a "bottle neck" in the biosynthetic pathway of artemisinin in varieties with high artemisinic acid and consequentially low artemisinin levels. After a night-frost period, the level of artemisinin was increased, in the Vietnamese *A. annua* plants, while the dihydroartemisinic acid level was decreased. This phenomenon is in accordance with our hypothesis that stress triggers the conversion of dihydroartemisinic acid to artemisinin. It is suggested that the presence of high levels of dihydroartemisinic acid may be an adaptation to stress conditions (e.g., night-frost), during which relatively high levels of  $1O_2$  are formed. Dihydroartemisinic acid gives the plant protection by reacting with these reactive oxygen species yielding artemisinin as stable end-product.

80. Wang, Hong; Ge, Lei; Ye, He-Chun; Chong, Kang; Liu, Ben-Ye; Li, Guo-Feng: Studies on the Effects of *fpf1* Gene on *Artemisia annua* Flowering Time and on the Linkage between Flowering and Artemisinin Biosynthesis  
*Planta Medica*, Apr2004, Vol. 70 Issue 4, p347-352.  
Source: Academic Search Premier

The flowering promoting factor1 (*fpf1*) from *Arabidopsis thaliana* was transferred into *Artemisia annua* L. via *Agrobacterium tumefaciens*. The *fpf1* gene was firstly inserted in the binary vector pBI121 under the control of CaMV 35S promoter to construct the plant expression vector pBI<sub>fpf1</sub>, then leaf explants of *A. annua* were infected with *A. tumefaciens* LBA4404 containing pBI<sub>fpf1</sub>, and induced shoots. Transgenic plants were obtained through the selection with kanamycin. PCR, PCR-Southern and Southern blot analyses confirmed that the foreign *fpf1* gene had been integrated into the *A. annua* genome. RT-PCR and RT-PCR-Southern analyses suggested that the foreign *fpf1* gene had expressed at the transcriptional level. Under short-day conditions, the flowering time of *fpf1* transgenic plants was about 20 days earlier than the non-transformed plants; however, no significant differences were detected in artemisinin content between the flowering transgenic plants and the non-flowering non-transgenic plants. These results showed that flowering is not a necessary factor for increasing the artemisinin content, furthermore, there may be no direct linkage between flowering and artemisinin biosynthesis.

81. Zhao Yujun; Ye Hechun; Li Guofeng; Chen Dahua; Liu Yan. Cloning and enzymology analysis of farnesyl pyrophosphate synthase gene from a superior strain of *artemisia annua* L. *Chinese Science Bulletin*; Jan2003, Vol. 48 Issue 1, p63-67.  
Source: Academic Search Premier.

A cDNA(Af1) encoding farnesyl pyrophosphate synthase AaFPS1 (FPS, EC2.5.1.1/EC2.5.1.10) from a high yield *Artemisia annua* 025 has been cloned from its cDNA library. Sequence analysis showed that the cDNA encoded a protein of 343 amino acid (aa) residues with molecular weight of 39 kD. Deduced a sequence of the cDNA was similar to FPS from other plants, yeast and mammals, containing 5 conserved domains found in both prenyl transferase and polyprenyl synthase. The expression of the cDNA in

*Escherichia coli* showed measurable specific activity of FPS in vitro. The enzyme was purified by ion exchange chromatography and its kinetics was measured. These results would further promote the molecular regulation of artemisinin biosynthesis

### **Chemistry Literature on *Artemisia annua*:**

82. Acton, N., Roth, R.J. Synthesis of epi-deoxy- and deoxyarteannuin B. *Phytochemistry*. 1989. v. 28 (12) p. 3530-3531. Database: Agricola

83. Acton, N., Klayman, D.L. Conversion of artemisinin (qinghaosu) to iso-artemisitenone and to 9-epi-artemisinin. *Planta Medica* 1987. v. 53 (3) p. 266-268. Database: Agricola

84. Ali, Mohd., Siddiqui, Nasir A. Volatile oil constituents of *Artemisia annua* leaves *Journal of Medicinal & Aromatic Plant Sciences*. 22(1B). March, 2000. 568-571. Database: Biosis.

Analysis of the isolate by GLC and GC-MS resulted in isolation of 59 constituents in which 36 components comprising 73.1% of the total volatiles were identified. Quantitatively, the volatile oil was characterized by high amount of fifteen monoterpenes (Ca 43.6%); 1,8-cineole (12.8%) being prominent was followed by trans-sabinene hydrate (7.1%), p-cymene (5.46%); beta-pinene (3.0%); alpha-thujene (3.3%), myrcene (2.9%), terpinene-4-ol (2.6%) and camphor (1.1%), and the total of monoterpene hydrocarbons being 38.7%. Among the 16 sesquiterpenes and the completely identified constituents (17.4%) gamma-cadinene (3.5%) was prominent component followed by aromadendrene epoxide (3.1%), caryophyllene (2.1%), artemissin (1.3%) and ledene epoxide (1.2%), and the total of sesquiterpenic hydrocarbons being 6.9%. The sesquiterpenes epiglobulol, dehydrosebinen, gamma-gurjunene, globulol, beta-selinene, beta-gurjunene, isogermacrene epoxide and beta- elemene occurred in trace amounts, 4-methylfuran (0.5%), 4-methyl-2,3 dihydrofuran (6.7%), p-cymene (5.4%) and eugenyl isovalerate (1.1%) were the aromatic constituents or their derivatives. N-heneicosane (1.3%) was the only hydrocarbon identified in the essential oil. Thirteen volatile components, comprising 16.3%, were characterized partially.

85. Baeva, R.T., Nabizade, L.I., Zapesochneya, G.G., Karryev, M.O. Flavonoids of *Artemisia annua*. *Chemistry of Natural Compounds*. 1988. v. 24 (2) p. 256-257. Database: Agricola.

86. Bhattacharya, Asish K., Pal, Mahesh, Jain, Dharam C., Joshi, Bhawani S., Roy, Raja Rychlewska, Urszula, Sharma, Ram P. Stereoselective reduction of arteannuin B and its chemical transformations. *Tetrahedron*. 59(16). 14 April, 2003. 2871-2876. Database: Biosis.

Absolute stereochemistry of dihydroarteannuin B 5 obtained by the reduction of arteannuin B 3 with Ni<sub>2</sub>B, NaBH<sub>4</sub> or CdCl<sub>2</sub>-Mg-MeOH-H<sub>2</sub>O has been established by 2D NMR and single crystal X-ray diffraction studies. Some experiments aimed at the

synthesis of dihydrodeoxyarteannuin B (C-4, 5 double bond isomer of 11) are also discussed.

87. Bhonsle, J.B., Pandey, B., Deshpande, V.H., Ravindranathan, T. New synthetic strategies towards (+)-artemisinin. *Tetrahedron Letters*, 1994, Volume 35, Issue 30, Pages 5489-5492. Database: Agricola

Starting from (-)-menthol, two useful precursors for the formal total synthesis of (+)-Artemisinin, involving novel OH-assisted chemo and stereoselective C-H functionalisation and subsequent acid/base induced chemo-selective ringopening as key steps, have been synthesised.

88. Brown, Geoffrey D., Sy, Lai-King. Synthesis of labelled dihydroartemisinic acid. *Tetrahedron*. 60(5). 26 January, 2004. 1125-1138. Database: Agricola

(15-<sup>13</sup>C<sub>2</sub>H<sub>3</sub>)-Dihydroartemisinic acid (2a), (15-C<sub>2</sub>H<sub>3</sub>)-dihydroartemisinic acid (2c) and (15-<sup>13</sup>CH<sub>3</sub>)-dihydroartemisinic acid (2c) have been obtained in food yield and high isotopic enrichment by a reconstructive synthesis from artemisinin. These labelled compounds were designed to be used in biosynthetic experiments to determine the origins of artemisinin and other sesquiterpene natural products from *Artemisia annua*.

89. Brown, Geoffrey D.; G.Y. Liang and L.K.Sy. Terpenoids from the seeds of *Artemisia annua*. *Phytochemistry*, 2003, Volume 64, Issue 1, Pages 303-323. Database: Academic Search Premier.

Fourteen sesquiterpenes, three monoterpenes and one diterpene natural product have been isolated from the seeds of *Artemisia annua*. The possible biogenesis of some of these natural products are discussed by reference to recently reported experimental results for the autoxidation of dihydroartemisinic acid and other terpenoids from *Artemisia annua*.

90. Chen HL, Wang KT, Pu QS, Chen XG, Hu ZD. On-line conversion and determination of artemisinin using a flow-injection capillary electrophoresis system. *Electrophoresis* 2002, Volume 23, Issue 17, Pages 2865-71. Database: Pubmed

A novel, rapid, and simple capillary electrophoresis (CE) method has been developed for the determination of antimalarial artemisinin by on-line treatment with alkaline. By on-line reaction, artemisinin was automatically and reproducibly converted to the strongly UV-absorbing compound, Q292, by treating it with 0.20 mol/L NaOH solution for 3 min at 40 degrees C. Analysis was carried out in less than 12 min after conversion of artemisinin in a flow injection (FI) system that was coupled to CE equipment via a split-flow interface cell, and a sampling frequency of 8 h<sup>-1</sup> is achievable. The on-line conversion method has been applied to the determination of artemisinin in the traditional Chinese herbal drug *Artemisia annua* L., and the results are satisfactory.

91. Chorki, Fatima, Grellepois, Fabienne, Crousse, Benoit, Hoang, Vu Dinh, Van Hung, Nguyen, Bonnet-Delpon, Daniele, Begue, Jean-Pierre. First synthesis of 10 $\alpha$ -(trifluoromethyl)deoxoartemisinin. *Organic Letters*. 4(5). March 7, 2002. 757-759. Database: Biosis.

A novel, nonacetal (trifluoromethyl)deoxoartemisinin was prepared with good stereoselectivity. This compound was obtained by debromination of the 10 $\alpha$ -CF<sub>3</sub>-10-bromodeoxoartemisinin in the presence of tributyltin at reflux in toluene without alteration of the endoperoxide bridge. It presented a reasonable antimalarial activity.

92. Christen, P., Veuthey, J.-L. New Trends in Extraction, Identification and Quantification of Artemisinin and its Derivatives. *Current Medicinal Chemistry* 2001, 8, 1827-1839. Database: Pubmed.

Artemisinin, a sesquiterpene lactone endoperoxide, and a number of its precursors, metabolites and semisynthetic derivatives have shown to possess antimalarial properties. Several methods have been reported for the measurement of artemisinin and its main derivatives in plant material and biological fluids. However, most of them are either not sufficiently sensitive and do not offer reliable results, or are difficult to apply in routine analyses. Therefore, new methods for the determination of these compounds, such as supercritical fluid extraction and chromatography, pressurized solvent extraction, microwave-assisted extraction, high-performance liquid chromatography coupled to mass spectrometry or evaporative light scattering detection, will be presented. Applications to plant material, pharmaceutical formulations and biological fluids will also be reviewed

93. Dost, Kenan. Analysis of artemisinin by a packed-column supercritical fluid chromatography-atmospheric pressure chemical ionisation mass spectrometry technique. *Analyst*, 2003, vol. 128, issue 8, Pages 1037- 1042. Database: Academic Search Premier

A packed-column supercritical fluid chromatography-atmospheric pressure chemical ionisation mass spectrometry method was studied for the determination of artemisinin from *Artemisia annua* L. extracts. The technique does not require any kind of derivatisation prior to the analysis. All samples were simply dissolved in methanol and injected into the mobile phase. Detection was achieved by using mass spectrometry with atmospheric pressure chemical ionisation. The ionisation technique is relatively soft and provides protonated molecular ion and informative structural fragmentation for the compound. Benzophenone was used as a chromatographic standard for the determination of the analytical reproducibility. The supercritical carbon dioxide mobile phase used in the system was modified by 10% methanol. The average absolute retention time was 3.54 min with a standard deviation of 0.017 min and a relative standard deviation of 0.4% with respect to benzophenone for the procedure. The correlation coefficient was 0.998 and detection limit 370 pg on column.

94. Elford BC, Roberts MF, Phillipson JD, Wilson RJ. Potentiation of the antimalarial activity of qinghaosu by methoxylated flavones. *Trans R Soc Trop Med Hyg.* 1987. 81(3):434-6. Database: Pubmed

Interaction between the flavones casticin and artemetin and the antimalarial activity of chloroquine and qinghaosu (QHS) was examined using an in vitro growth assay based on [3H]hypoxanthine incorporation in synchronized cultures of a cloned line of *Plasmodium falciparum*. Casticin, and to a lesser extent artemetin, selectively enhanced the inhibition of growth by QHS, but had little effect on the activity of chloroquine. The findings suggest that flavones indigenous to *Artemisia annua*, from which QHS is isolated, might significantly alter the clinical potential of this novel antimalarial drug in the treatment of chloroquine-resistant malaria.

95. Ferreira JF, Janick J. Immunoquantitative analysis of artemisinin from *Artemisia annua* using polyclonal antibodies. *Phytochemistry.* 1996, Volume 41, Issue 1, Pages 97-104. Database: Pubmed

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<sup>-1</sup>; and was epsilon 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.

96. Grellepois, Fabienne; Bonnet-Delpon, Daniele; Begue, Jean-Pierre. Ring-contracted artemisinin derivatives: Stereoselective reaction of anhydrodihydro-artemisinin towards halogenating reagents. *Tetrahedron Letters.* 42(11). 11 March, 2001. 2125-2127. Database: Biosis.

Reaction of anhydrodihydroartemisinin 1 with halogenating reagents and further rearrangement of adducts into ring-contracted aldehydes has been revisited. The stereochemistry of reactions with iodine is opposite to that with bromine, and allowed the preparation of the new aldehyde 4 with conservation of the configuration of starting artemisinin at C-9.

97. Haynes RK, Vonwiller SC. Extraction of artemisinin and artemisinic acid: preparation of artemether and new analogues. *Trans R Soc Trop Med Hyg.* 1994, Issue 88, Suppl 1, Pages S23-6. Database: Pubmed

The preparation of artemether from artemisinin is reviewed. Firstly, the extraction of artemisinin from *Artemisia annua* is described and an estimation of the yield per hectare based on literature data is given. Artemisinin is reduced with sodium borohydride to produce dihydroartemisinin as a mixture of epimers. The mixture is treated with methanol and an acid catalyst to provide artemether. Increasing demand for use of artemether places pressure on the supply of artemisinin, and an alternative means of preparing the drug from artemisinic acid, an abundant constituent of *A. annua*, which could triple current yields, is described. In anticipation of problems of drug resistance emerging with the continued use of artemether and artesunate to treat malaria, development of new derivatives of artemisinin which have enhanced stability is required. Examples of such derivatives which have been prepared in our laboratories, or proposed, are described.

98. Hethelyi, E., Cseko, I., Grosz, M., Mark, G., Palinkas, J.J. Chemical composition of the *Artemisia annua* essential oils from Hungary. *Journal of Essential Oil Research*, 1995, Volume 7, Issue 1, Pages 45-48. Database: Agricola

Chemical composition of the essential oils of 85 individuals of *Artemisia annua* L. was determined by GC. The plant material was cultivated in Budaors (near Budapest). The essential oil content varied between 0.48-0.81%. The main components of the oil obtained from fresh flowering shoots were artemisia ketone (33-75%) and artemisia alcohol (15-56%). Five other components of the oils were identified by GC/MS. Four different types of *A. annua* oils were identified: (a) 41% of the individuals contained artemisia ketone (75%) and artemisia alcohol (15%); (b) a decreasing ratio of artemisia ketone and an increasing ratio of artemisia alcohol (38% of the individuals); (c) Artemisia ketone (50%) and artemisia alcohol (45%) (14% of individuals); and (d) Artemisia ketone (33%) and artemisia alcohol (56%) (7% of individuals).

99. Jain, N., Srivastava, S.K., Aggarwal, K.K., Kumar, S., Syamasundar, K.V. Essential oil composition of *Artemisia annua* L. 'Asha' from the plains of northern India. *Journal of Essential Oil Research*. July/Aug 2002., Volume 14, Issue 4, Pages 305-307. Database Agricola.

*Artemisia annua* L. 'Asha' was grown in agro-climatic conditions of north Indian plains in Lucknow. The aerial parts on hydrodistillation gave 0.53% of an oil on a fresh weight basis. GC and GC/MS analysis of the oil resulted in the identification of 64 constituents, representing 95.4% of the oil. Artemisia ketone (52.9%), 1,8-cineole (8.4%) and camphor (6.0%) were the major constituents. It is suggested that *A. annua* 'Asha' can be grown as an economically viable crop in the northern plains of India.

100. Jeremic, D., Stefanovic, M., Dokovic, D., Milosavljevic, S. Flavonols from *Artemisia annua* L. *Glasnik Hemijskog Drushtva*. 1979. v. 44 (9/10) p. 615-618. Database: Biosis.

101. Kasymov, Sh.Z., Ovezdurdyev, A., Yusupov, M.I., Sham'yanov, I.D., Malikov, V.M. Lactones of *Artemisia annua*. *Chemistry of Natural Compounds*. 1987. v. 22 (5) p. 599. Database Agricola.

102. Kohler M, Haerdi W, Christen P, Veuthey JL. Extraction of artemisinin and artemisinic acid from *Artemisia annua* L. using supercritical carbon dioxide. *J Chromatogr A*. 1997, Volume 785, Issue 1-2, Pages 353-60. Database: Pubmed

Artemisinin (an antimalaric compound) and its major precursor artemisinic acid, isolated as the active principles of the medicinal plant *Artemisia annua* L., were extracted by supercritical fluid extraction (SFE) and analyzed by supercritical fluid chromatography (SFC) using a capillary column, coupled with a flame ionization detector (FID). With optimized operating conditions, artemisinin and artemisinic acid were quantitatively extracted at a flow-rate of 2 ml min<sup>-1</sup> in less than 20 min. The supercritical fluid was composed of carbon dioxide and 3% methanol with temperature and pressure fixed at 50 degrees C and 15 MPa, respectively. From the kinetic curves, it appears that the extraction of artemisinin is not limited by the diffusion of the analyte from the plant into the extraction fluid but rather by the elution process. These conditions avoided degradation of the analyte and gave clean extracts ready to be analyzed by SFC. The SFE-SFC-FID method was successfully applied to six samples of *A. annua* containing various concentrations of artemisinin and artemisinic acid. Results were compared with two conventional liquid solvent extraction processes.

103. Klayman DL, Lin AJ, Acton N, Scovill JP, Hoch JM, Milhous WK, Theoharides AD, Dobek AS. Isolation of artemisinin (qinghaosu) from *Artemisia annua* growing in the United States. *Journal of Natural Products*, 1984, Volume 47, Issue 4, Pages 715-7. Database Biosis.

104. Kumar, T., Khanuja, S., Jain, D., Srivastava, S., Bhattacharya, A., Sharma, R., Kumar, S. A simple microbiological assay for the stereospecific differentiation of alpha and beta isomers of arteether. *Phytotherapy Research*, 2000. Volume 14, Issue 8, Pages 644-646. Database: AGRICOLA

105. Lakhtin, V.M., Magazova, N.S., Kunaeva, R.M., Mosolov, V.V. Use of lectins for separation of wormwood beta-glycosidases. *Applied Biochemistry & Microbiology*, 1989. v. 25 (1) p. 45-50. Database: AGRICOLA.

106. Lee, Seokjoon, Oh, Sangtae. A simple synthesis of C-10 substituted deoxoartemisinin and 9-epi-deoxoartemisinin with various organozinc reagents *Tetrahedron Letters*. 43(16). 15 April, 2002. 2891-2894. Database: Biosis

A direct substitution reaction of 10 $\alpha$ - or 10 $\beta$ -benzenesulfonyl dihydro-artemisinin, prepared from dihydroartemisinin with thiophenol in the presence of BF<sub>3</sub>Et<sub>2</sub>O and consecutive oxidation with H<sub>2</sub>O<sub>2</sub>/urea complex, with organo zinc reagents derived from allyl, benzyl, phenyl, vinyl and n-butyl Grignard reagents stereoselectively produced C-10 substituted deoxo-artemisinins in good to moderate yields. The same reaction of 10 $\beta$ -benzene sulfonyl-9-epi-dihydroartemisinin gave corresponding 9-epi-deoxoartemisinin derivatives.

107. Libbey, L.M., Sturtz, G. Unusual essential oils grown in Oregon. II. *Artemisia annua* L. *Journal of Essential Oil Research*: 1989. v. 1 (5) p. 201-202. Database: AGRICOLA

108. Li, Ying, Wu, Jin-Ming, Shan, Feng, Wu, Guang-Shao, Ding, Jian, Xiao, Dong, Han, Jia-Xian, Atassi, Ghanem, Leonce, Stephane, Caignard, Daniel-Henri, Renard, Pierre. Synthesis and cytotoxicity of dihydroartemisinin ethers containing cyanoarylmethyl group. *Bioorganic & Medicinal Chemistry*. 11(6). 20 March, 2003. 977-984. Database: Biosis

A new type of ether of dihydroartemisinin containing cyano and aryl groups was prepared and tested for cytotoxicity to A549, P388, L1210 and HT29 cells using the MTT assay. 12k and 12l were the most cytotoxic compounds. 13 lacking the peroxy group showed a 1000-fold less potency than 12l. Similarly, the inactive compound 14 indicated that the position of cyano groups was also important. Flow cytometry data showed that the compounds caused an accumulation of P388 cells in the G1-phase of the cell cycle.

109. Liu, H.J., Yeh, W.L., Chew, S.Y. A total synthesis of the antimalarial natural product (+)-qinghaosu. *Tetrahedron Letters: the International Organ for the Rapid Publication of Preliminary Communications in Organic Chemistry*, 1993. v. 34 (28) p. 4435-4438. Database: AGRICOLA

Starting from (-)-beta-pinene, an efficient total synthesis of the title antimalarial agent has been accomplished using an intermolecular Diels-Alder approach

110. Macreadie, P. I., Taylor, D. K., Avery, T. D., Greatrex, B. W., Macreadie, I. G., Humphries, A., Fox, E., Klonis, N., Tilley, L. Novel endoperoxide antimalarials: Synthesis, heme-binding, membrane, and antimalarial activity. *Experimental Parasitology*. 105 (1). 2003. 62. Database: Agricola

111. Marchese, J. A., Rehder, V. L. G., Sartoratto, A. A comparison of thin layer chromatography and high performance liquid chromatography for artemisinin analyses. *Revista Brasileira de Plantas Mediciniais*. 4(1). Outubro, 2001. 81-87. Database: Biosis

*Artemisia annua* L., a native of China and adapted to Brazilian climate, is a rich source of artemisinin, a sesquiterpene lactone proven to be effective against the malaria agent *Plasmodium*. The scope of this work was to compare the method of thin layer chromatography (TLC) using densitometric detection with high performance liquid chromatography (HPLC) using ultraviolet detection for the analytical quantification of the artemisinin contents. The results show that the TLC method does not have the same selectivity and sensibility necessary to be a useful alternative method to HPLC. Under experimental conditions the TLC method over-estimated the artemisinin contents, compared to those determined by HPLC.

112. Misra LN, Ahmad A, Thakur RS, Lotter H, Wagner H. Crystal structure of artemisinic acid: a possible biogenetic precursor of antimalarial artemisinin from *Artemisia annua*. *Journal of Natural Products*, 1993, Volume 56, Issue 2, Pages 215-219. Database: Pubmed.

Artemisinic acid, a possible biogenetic precursor of the antimalarial artemisinin, was isolated from the hexane extract of *Artemisia annua*. X-ray crystallography of the dimer of artemisinic acid shows that the cyclization during intermolecular hydrogen bonding occurs by the opposite orientation of the alpha, beta-methylene group in each molecule. Complete spectroscopic data of 1 are also given.

113. Misra LN, Ahmad A, Thakur RS, Jakupovic, J. Bisnor-cadinanes from *Artemisia annua* and definitive <sup>13</sup>C NMR assignments of beta-artether. *Phytochemistry* 1993. v. 33 (6) p. 1461-1464. Database: Agricola.

The hexane extract of the aerial part of *Artemisia annua* yielded two bisnor-cadinanes, norannuic acid and qinghaosu I. The structures were elucidated by 400 Mhz H NMR, 2D NMR and mass spectrometry. The corrective <sup>13</sup>C NMR assignments of beta-artether, the potent artemisinin derivative, were performed on a 400 Mhz instrument using HETCOR.

114. Nair MS, Basile DV. Bioconversion of arteannuin B to artemisinin. *Journal of Natural Products*, 1993, Volume 56, Issue 9, Pages 1559-66. Database: Pubmed.

Arteannuin B, which co-occurs with artemisinin, the potent antimalarial principle of the Chinese medicinal herb *Artemisia annua* (Asteraceae), has been converted to the latter using crude and semi-purified cell-free extracts of the leaf homogenates of the plant. Detection procedures to quantitate this bioconversion, including one that is novel which uses gcms, are detailed.

115. Pandey, Shilpi, Malik, Heetika, Singh, Chandan. Chemistry of 1,2,4-trioxanes: Reaction with aromatic amines. *Medicinal Chemistry Research*. 12(6-7). 2003. 374-375. Database: Biosis.

116. Pham, Gia Dien. Modification process of component contents in the essential oil of *Artemisia annua* L. in Vietnam. *Tap Chi Duoc Hoc* (2003)  
Database: Scifinder Scholar.

The content change of compds. in the *Artemisia annua* essential oils of Viet Nam and Bulgaria, depending on the period of its growth, was investigated. It was found that the quantity of the main compds. such as 1,6- cineole, artemisia cetone, camphor, - cubebene in blooming period of *Artemisia annua* were almost the highest.

117. Pham, Gia Dien. Chemical components of the essential oil of *Artemisia annua* L. in Vietnam and Bulgaria. *Tap Chi Duoc Hoc* (2003), (4), 11-12  
Database: Scifinder Scholar.

The chemical composition of the *Artemisia annua* essential oil of Viet Nam and Bulgaria has been investigated by gas chromatog. method. The main difference between two oils is the absence of artemisiaketone in *Artemisia annua* oil of Viet Nam in comparison with its notable amt. in the oil of Bulgaria.

118. Ranasinghe A, Sweatlock JD, Cooks RG. A rapid screening method for artemisinin and its congeners using ms/ms: search for new analogues in *Artemisia annua*. *Journal of Natural Products*, 1993, Volume 56, Issue 4, Pages 552-563. Database: Pubmed.

A rapid screening method based on tandem mass spectrometry (ms/ms) is described for artemisinin-related compounds present in complex matrices. These compounds produce abundant ammonium adducts,  $[M + NH_4]^+$ , using ammonia desorption chemical ionization (dci), and dissociation of the mass-selected adducts yields the protonated molecules,  $[M + H]^+$ , which subsequently eliminate characteristic neutral molecules ( $H_2O$ ,  $CO$ ,  $HCO_2H$ ,  $HOAc$ ). Neutral loss ms/ms scans which are selective for different elimination reactions were used in order to screen for groups of related analogues present

in a crude hexane extract of *Artemisia annua*. Comparison of ms/ms product spectra of known *Artemisia* compounds with those of the new analogues provided information on the functional groups and the molecular weights of the new compounds present in the plant, and tentative structures are suggested.

119. Roth, R.J., Acton, N. A simple conversion of artemisinic acid into artemisinin. *Journal of Natural Products*. 1989. v. 52 (5) p. 1183-1185. Database: Agricola

120. Roth, R.J., Acton, N. Isolation of epi-deoxyarteannuin B from *Artemisia annua*. *Planta Medica*. [Stuttgart, W. Ger. : Georg Thieme Verlag] 1987. v. 53 (6) p. 576. Database: Agricola.

121. Rucker, G., Mayer, R., Manns, D. Isolation of quinghaosen from *Artemisia annua* of European sources. *Planta Medica*. 1986. (3) p. 245. Database: Agricola

122. Shilin, Y., Roberts, M.F., Phillipson, J.D. Methoxylated flavones and coumarins from *Artemisia annua*. *Phytochemistry*. 1989. v. 28 (5) p. 1509-1511. Database: Agricola

Chinese grown *Artemisia annua* contained 14 known methoxylated flavonoids and three new naturally occurring compounds: quercetagenin-3-methyl ether, 5,2'4'-trihydroxy-6,7,5'-trimethoxyflavone and 5,7,8,3'-tetrahydroxy-3,4'-dimethoxyflavone. Four known commonly occurring coumarins were also isolated.

123. Singh, Chandan, Tiwari, Pallavi. A one-pot conversion of artemisinin to its ether derivatives. *Tetrahedron Letters*. 43(40). 30 September, 2002. 7235-7237. Database: Biosis.

A one-pot preparation of artemether, arteether and related antimalarial compounds from artemisinin, using NaBH<sub>4</sub>/ Amberlyst-15, is reported.

124. Singh, Chandan, Gupta, Nitin, Puri, Sunil K. Photo-oxygenation of geraniol: Synthesis of a novel series of hydroxy-functionalized anti-malarial 1,2,4-trioxanes *Bioorganic & Medicinal Chemistry Letters*. 12(15). 5 August, 2002. 913-1916. Database: Biosis.

Photo-oxygenation of geraniol 2, an abundantly available allylic alcohol, furnished a mixture of mono- and di-hydroperoxy products; the latter have been used for the preparation of a novel series of hydroxy-functionalized anti-malarial 1,2,4-trioxanes (7a-d, 8a-d).

125. Singh, B. L., Singh, D. V., Verma, R. K., Gupta, M. M., Jain, D. C., Kumar, Sushil

Simultaneous determination of antimalarial drugs using reversed phase high-performance liquid chromatography: Diode-array detection. *Journal of Medicinal & Aromatic Plant Sciences*. 22-23(4A-1A). October-March, 2000-2001. 17-20. Database: Biosis

A simple and rapid reversed phase HPLC method using photodiode array detection has been developed for simultaneous quantitation of alpha-arteether (3a), beta-arteether(3b), its chemical precursor artemisinin (2) and intermediate precursors alpha-dihydroartemisinin (DHA) (1a) and beta-dihydroartemisinin (1b). The method was capable of separating two isomeric forms of DHA (alpha,beta), arteether(alpha,beta) and artemisinin in a single run.

126. Singletary, Jeananne; Dudley, Gregory. Progress toward the total synthesis of (+)-artemisinin based on a novel ozonide rearrangement. *Abstracts, 55th Southeast Regional Meeting of the American Chemical Society, Atlanta, GA, United States, November 16-19, 2003 (2003)*. Database: Scifinder.

Artemisinin, from the Chinese plant *Artemisia annua*, is a promising and potent antimalarial drug that meets the challenges posed by the increasingly drug-resistant vector parasite *Plasmodium falciparum*. This sesquiterpene owes its success to the endoperoxide bridge, which is cleaved by Fe<sup>2+</sup> to generate carbon-centered radicals that damage the parasite. This presentation will describe the development of our route to obtain the tricyclic core, and highlight the proposed ozonide rearrangement leading to the potent peroxy-ketal substructure.

127. elSohly HN, Croom EM, elSohly MA. Analysis of the antimalarial sesquiterpene artemisinin in *Artemisia annua* by high-performance liquid chromatography (HPLC) with postcolumn derivatization and ultraviolet detection. *Pharm Res*. 1987;4(3):258-60. Database Biosis.

128. Sy LK, Brown GD. The mechanism of the spontaneous autoxidation of dihydroartemisinic acid *Tetrahedron*. 58(5). 28 January, 2002. 897-908. Source: Biosis.

Dihydroartemisinic acid undergoes slow spontaneous autoxidation to artemisinin and other natural products, which have been reported from the medicinal plant *Artemisia annua*. The mechanism of this complex transformation is shown to involve four steps: (i) initial reaction of the DELTA4,5-double bond of dihydroartemisinic acid with molecular oxygen, (ii) Hock cleavage of the resulting tertiary allylic hydroperoxide; (iii) oxygenation of the enol product from Hock cleavage; and (iv) cyclization of the resulting vicinal hydroperoxyl-aldehyde to the 1,2,4-trioxane system of artemisinin.

129. Sy LK, Brown GD. Deoxyarteannuin B, dihydro-deoxyarteannuin B and trans-5-hydroxy-2-isopropenyl -5-methylhex-3-en-1-ol from *Artemisia annua*. *Phytochemistry*. 2001, Volume 58, Issue 8, Pages 1159-66. Database: Pubmed.

The amorphane sesquiterpenes, deoxyarteannuin B and dihydro-deoxyarteannuin B, were isolated from *Artemisia annua* and their structures mainly determined by two-dimensional NMR spectroscopic analyses. The irregular monoterpene, trans-5-hydroxy-2-isopropenyl-5-methylhex-3-en-1-ol, was also characterized in the same way, and its structure was confirmed by synthesis from lavandulol. All of these natural products are suggested to be formed by autoxidation reactions. Full assignments of the <sup>1</sup>H and <sup>13</sup>C resonances for the known natural products epideoxyarteannuin B and isoannulide, determined by the same methodology, are also reported.

130. Sy, Lai-King, Cheung, Kung-Kai, Zhu, Nian-Yong, Brown, Geoffrey D. Structure elucidation of arteannuin O, a novel cadinane diol from *Artemisia annua*, and the synthesis of arteannuins K, L, M and O. *Tetrahedron*. 57(40). 1 October, 2001. 8481-8493. Database: Biosis

The novel cadinane diol, arteannuin O (1), has been obtained from *Artemisia annua* and its structure has been established by 2D NMR and X-ray crystallography. A reconstructive synthesis of arteannuin O from artemisinin is described, which also yields the natural products arteannuin K and arteannuin L. Mechanistic considerations have led to the conclusion that the stereochemistry of the 5-hydroxyl group was wrongly assigned when arteannuins K, L and M were first reported as natural products. This was confirmed by derivatization of synthetic arteannuins K, L and M as their Mosher esters.

131. Sy, Lai-King, Zhu, Nian-Yong, Brown, Geoffrey D. Syntheses of dihydroartemisinic acid and dihydro-epi-deoxyarteannuin B incorporating a stable isotope label at the 15-position for studies into the biosynthesis of artemisinin *Tetrahedron*. 57(40). 1 October, 2001. 8495-8510. Database: Biosis.

(15-<sup>13</sup>C<sub>2</sub>H<sub>3</sub>)-Dihydroartemisinic acid (3a) and (15-<sup>13</sup>CH<sub>3</sub>)-dihydro-epi-deoxyarteannuin B (7b), intended for evaluation in vivo as biosynthetic precursors to artemisinin, have been obtained from a reconstructive synthesis. The decalenone acid 8 from acid degradation of artemisinin (1) serves as a common intermediate: following addition of labeled methyl Grignard reagent to 8, either labeled precursor can be prepared in good yield by varying the work-up conditions employed. It is shown that both compounds are prone to autoxidation on storage and that the products of such oxidation and subsequent rearrangement reactions might be confused with bona fide metabolites when using these labeled precursors in feeding experiments designed to determine the biosynthetic route to artemisinin in *Artemisia annua*.

132. Torok, D.S., Ziffer, H. Synthesis and reactions of 11-azaartemisinin and derivatives. *Tetrahedron Letters*, 1995, Volume 36, Issue 6, Pages 829-832. Database: Agricola

11-Azaartemisinin was prepared in 45% yield in a one pot, two-step sequence from the reaction of artemisinin with excess ammonia followed by acid treatment. Analogous

reactions of artemisinin with primary alkylamines gave N-alkylzaartemisinins in similar yields.

133. Tu, Y.Y., Ni, M.Y., Zhong, Y.R., Li, L.N., Cui, S.L., Zhang, M.Q., Wang, X.Z., Ji, Z., Liang, X.T. Studies on the constituents of *Artemisia annua*. II. *Planta Medica; Journal of Medicinal Plant Research* 1982. v. 44 (3) p. 143-145. Database: Agricola

134. Vandenberghe, D., Vergauwe, A., Montagu, M. van, Eeckhout, E.G. van den. Simultaneous determination of artemisinin and its bioprecursors in *Artemisia annua*. *Journal of Natural Products*, 1995, Volume 58, Issue 5, Pages 789-803. Database: Agricola

135. Vishwakarma RA, Mehrotra R, Tripathi R, Dutta GP. Stereoselective synthesis and antimalarial activity of alpha-artelinic acid from artemisinin. *Journal of Natural Products*, 1992, Volume 55, Issue 8, Pages 1142-1144. Database: Pubmed.

alpha-Artelinic acid, a potent, stable, and water-soluble antimalarial agent, has been synthesized from artemisinin, the sesquiterpene lactone endoperoxide isolated from *Artemisia annua*. The blood schizontocidal antimalarial activity of alpha-artelinic acid evaluated against *Plasmodium knowlesi* is also reported.

136. Wallaart TE, Pras N, Quax WJ. Isolation and identification of dihydroartemisinic acid hydroperoxide from *Artemisia annua*: A novel biosynthetic precursor of artemisinin *Journal of Natural Products*, 1999, Volume 62, Issue 8, Pages 1160-2. Database: Pubmed.

Dihydroartemisinic acid hydroperoxide was isolated for the first time as a natural product from the plant *Artemisia annua* in a 29% yield. Its structure was identified by H and C NMR spectroscopy. Compound 2 is known as an intermediate of the photochemical oxidation of dihydroartemisinic acid leading to artemisinin. The presence of 1 and 2 in the plant and the conditions under which 1 can be converted into 2, which can very easily oxidize to 3, provide evidence for a nonenzymatic, photochemical conversion of 1 into 3, in vivo, in the plant.

137. Wallaart TE, van Uden W, Lubberink HG, Woerdenbag HJ, Pras N, Quax WJ. Isolation and identification of dihydroartemisinic acid from *Artemisia annua* and its possible role in the biosynthesis of artemisinin *Journal of Natural Products*, 1999, Volume 62, Issue 3, Pages 430-433. Database: Pubmed.

Dihydroartemisinic acid was isolated as a natural product from *Artemisia annua* in a 66% yield, and its structure was confirmed by <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy. Compound 2 could be chemically converted to artemisinin under conditions that may also be present in

the living plant. The results suggest that the conversion of 2 into 4 in the living plant might be a nonenzymatic conversion.

138. Weiss, E., Ziffer, H., Ito, Y. Use of countercurrent chromatography (CCC) to separate mixtures of artemisinin, artemisitene, and arteannuin B. *Journal of Liquid Chromatography & Related Technologies*. 23(6). March 2000. 909-913.  
Database: Biosis.

A series of solvent systems was developed for CCC to separate mixtures containing variable amounts of artemisinin, artemisitene, and arteannuin B. To purify multigram quantities of artemisinin by CCC it was necessary to maximize the amount of a mixture that could be dissolved in a fixed quantity of the solvent mixture

139. Yadav, J. S., Babu, R. Satheesh, Sabitha, G. Stereoselective total synthesis of (+)-artemisinin. *Tetrahedron Letters*. 44(2). 6 January 2003. 387-389.  
Database: Biosis.

The total synthesis of the novel antimalarial drug, a sesquiterpene endoperoxide, (+)-artemisinin is described. The approach is flexible and stereoselective. The use of an intermolecular radical reaction on an intermediate iodolactone and a Wittig reaction on a ketone were employed for the synthesis.

140. Yusupova, I.M., Tashkhodzhaev, B., Mallabaev, A. Molecular structure of the sesquiterpene lactone arteannuin B. *Chemistry of Natural Compounds*. 1987. v. 22 (6) p. 733-735. Database: Agricola.

141. Zhang, Yan; Zhang, Ji; Yao, Jian; Wang, Lai; Huang, Ai-lun; Dong, Li-na. Studies on the chemical constituents of the essential oil of *Artemisia annua* L. in Xinjiang. *Xibei Shifan Daxue Xuebao, Ziran Kexueban* (2004)  
Database: Scifinder scholar.

The essential oil from *Artemisia annua* L. in Xinjiang was extd. by steam distn. and the chem. constituents were analyzed by capillary GC-MS method. The relative content of each component was calcd. by area normalization, 48 peaks were sepd. and all of them were identified. The main chem. components were 2,5-dihydro-3-methyl-furan(68.48%); beta-myrcene (10.13%); 1R, 3Z, 9S-4,11,11-trimethyl-8-methylenebicyclo [7,2,0] undec-3-ene (7.74 %); 7,11-dimethyl-3-methylene-1,6,10-dodecatriene (2.42 %); eucalyptol (1.68 %); santolina triene(1.52%), etc. The study provided solid and scientific proof for the further exploitation and utilization of *Artemisia annua* L. in Xinjiang.

142. Zhang XQ, Xu LX. Determination of qinghaosu (arteannuin) in *Artemisia annua* L. by pulse polarography. [Article in Chinese]. *Yao Xue Xue Bao*. 1985 May;20(5):383-6.  
Database: Pubmed. Not reviewed by team.

143. Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry* 2001, Volume 49, Issue 11, Pages 5165-70. Database: Pubmed

The antioxidant capacities (oxygen radical absorbance capacity, ORAC) and total phenolic contents in extracts of 27 culinary herbs and 12 medicinal herbs were determined. The ORAC values and total phenolic contents for the medicinal herbs ranged from 1.88 to 22.30 micromol of Trolox equivalents (TE)/g of fresh weight and 0.23 to 2.85 mg of gallic acid equivalents (GAE)/g of fresh weight, respectively. *Origanum x majoricum*, *O. vulgare* ssp. *hirtum*, and *Poliomintha longiflora* have higher ORAC and phenolic contents as compared to other culinary herbs. The ORAC values and total phenolic content for the culinary herbs ranged from 2.35 to 92.18 micromol of TE/g of fresh weight and 0.26 to 17.51 mg of GAE/g of fresh weight, respectively. These also were much higher than values found in the medicinal herbs. The medicinal herbs with the highest ORAC values were *Catharanthus roseus*, *Thymus vulgaris*, *Hypericum perforatum*, and *Artemisia annua*. A linear relationship existed between ORAC values and total phenolic contents of the medicinal herbs ( $R = 0.919$ ) and culinary herbs ( $R = 0.986$ ). High-performance liquid chromatography (HPLC) coupled with diode-array detection was used to identify and quantify the phenolic compounds in selected herbs. Among the identified phenolic compounds, rosmarinic acid was the predominant phenolic compound in *Salvia officinalis*, *Thymus vulgaris*, *Origanum x majoricum*, and *P. longiflora*, whereas quercetin-3-O-rhamnosyl-(1 → 2)-rhamnosyl-(1 → 6)-glucoside and kaempferol-3-O-rhamnosyl-(1 → 2)-rhamnosyl-(1 → 6)-glucoside were predominant phenolic compounds in *Ginkgo biloba* leaves.

144. Zhou, W.S., Xu, X.X. The structures, reactions and syntheses of arteannuin (Qinghaosu) and related compounds. *Studies in natural products chemistry / edited by Atta-ur- Rahman.. New York Elsevier, 1988. p. 495-527.* Database: Agricola.

145. Zhao, S.S., Zeng, M.Y. Spectrometric high pressure liquid chromatography (HPLC) studies on the analysis of Qinghaosu. *Planta Medica. 1985. (3) p. 233-237.* Database : Agricola.

### **Biochemistry Literature on *Artemisia annua*:**

146. Abdin, M.Z. Artemisinin, a Novel Antimalarial Drug: Biochemical and Molecular Approaches for Enhanced Production. *Planta Medica, 2003, Vol. 69 Issue 4, p289-299.* Database: Academic Search Premier

Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge, has been isolated from the aerial parts of *Artemisia annua* L. plants. It is effective against both drug-resistant and cerebral malaria-causing strains of *Plasmodium falciparum*. The relatively low yield (0.01 - 0.8 %) of artemisinin in *A. annua* is a serious limitation to the commercialization of the drug. Therefore, the enhanced production of artemisinin either

in cell/tissue culture or in the whole plant of *A. annua* is highly desirable. It can be achieved by a better understanding of the biochemical pathway leading to the synthesis of artemisinin and its regulation by both exogenous and endogenous factors. Furthermore, genetic engineering tools can be employed to overexpress gene(s) coding for enzyme(s) associated with the rate limiting step(s) of artemisinin biosynthesis or to inhibit the enzyme(s) of other pathway competing for its precursors. These aspects which may be employed to enhance the yield of artemisinin both in vitro and in vivo are discussed in this review.

147. Akhila, A., Thakur, R.S., Popli, S.P. Biosynthesis of artemisinin in *Artemisia annua*. *Phytochemistry*. 1987. v. 26 (7) p. 1927-1930. Database: Agricola

The isotope ratios ( $^3\text{H}$ : $^{14}\text{C}$ ) in arteannuin B and artemisinin biosynthesized in *Artemisia annua* from [ $4R$ - $^3\text{H}_1$ , $2$ - $^{14}\text{C}$ ]-, [ $5$ - $^3\text{H}_2$ , $2$ - $^{14}\text{C}$ ]- and [ $2$ - $^3\text{H}_2$ , $2$ - $^{14}\text{C}$ ]( $3RS$ )- mevalonate have revealed that two specific 1,2-hydride shifts take place during the oxidation and lactonization of the germacrane skeleton to yield dihydrocostunolide. The *gem*-methyls of DMAPP retain their identity until the final steps of artemisinin biosynthesis. Arteannuin B is considered to be a late precursor of artemisinin and the following biosynthetic sequence is suggested: farnesylpyrophosphate  $\rightarrow$  germacrane skeleton  $\rightarrow$  dihydrocostunolide  $\rightarrow$  cadinanolide  $\rightarrow$  arteannuin B  $\rightarrow$  artemisinin.

148. Bhakuni, R. S., Jain, D. C., Sharma, R. P., Kumar, S. Secondary metabolites of *Artemisia annua* and their biological activity. *Current Science (Bangalore)*. 80(1). 10 January, 2001. 35-48. Database: Biosis

*Artemisia annua* synthesizes and accumulates a variety of secondary metabolites. Some of the biologically active secondary metabolites substantiate the claim made in traditional system of medicine. The present review summarizes the information available on the secondary metabolites isolated from *A. annua*.

149. Bouwmeester HJ, Wallaart TE, Janssen MH, van Loo B, Jansen BJ, Posthumus MA, Schmidt CO, De Kraker JW, Konig WA, Franssen MC. Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. *Phytochemistry* 1999, Volume 52, Issue 5, Pages 843-54. Database: Pubmed.

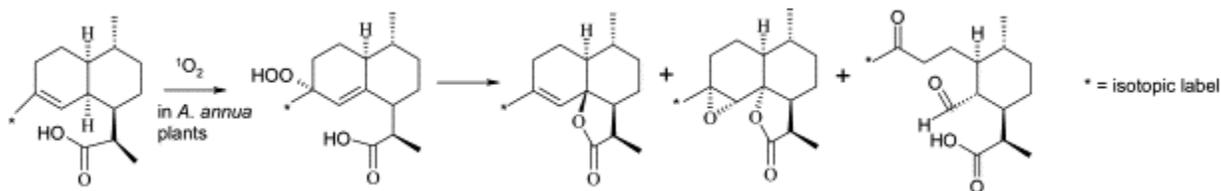
The endoperoxide sesquiterpene lactone artemisinin and its derivatives are a promising new group of drugs against malaria. Artemisinin is a constituent of the annual herb *Artemisia annua* L. So far only the later steps in artemisinin biosynthesis--from artemisinic acid--have been elucidated and the expected olefinic sesquiterpene intermediate has never been demonstrated. In pentane extracts of *A. annua* leaves we detected a sesquiterpene with the mass spectrum of amorpha-4,11-diene. Synthesis of amorpha-4,11-diene from artemisinic acid confirmed the identity. In addition we identified several sesquiterpene synthases of which one of the major activities catalysed

the formation of amorpho-4,11-diene from farnesyl diphosphate. This enzyme was partially purified and shows the typical characteristics of sesquiterpene synthases, such as a broad pH optimum around 6.5-7.0, a molecular mass of 56 kDa, and a  $K_m$  of 0.6  $\mu\text{M}$ . The structure and configuration of amorpho-4,11-diene, its low content in *A. annua* and the high activity of amorpho-4,11-diene synthase all support that amorpho-4,11-diene is the likely olefinic sesquiterpene intermediate in the biosynthesis of artemisinin.

150. Brown, Geoffrey D., Sy, Lai-King. In vivo transformations of dihydroartemisinic acid in *Artemisia annua* plants. *Tetrahedron*. 60(5). 26 January, 2004. 1139-1159.

Database: Agricola

[15- $^{13}\text{C}^2\text{H}_3$ ]-dihydroartemisinic acid (2a) and [15- $\text{C}^2\text{H}_3$ ]-dihydroartemisinic acid (2b) have been fed via the root to intact *Artemisia annua* plants and their transformations studied in vivo by one-dimensional  $^2\text{H}$  NMR spectroscopy and two-dimensional  $^{13}\text{C}$ - $^2\text{H}$  correlation NMR spectroscopy ( $^{13}\text{C}$ - $^2\text{H}$  COSY). Labelled dihydroartemisinic acid was transformed into 16 12-carboxy-amorphane and cadinane sesquiterpenes within a few days in the aerial parts of *A. annua*, although transformations in the root were much slower and more limited. Fifteen of these 16 metabolites have been reported previously as natural products from *A. annua*. Evidence is presented that the first step in the transformation of dihydroartemisinic acid in vivo is the formation of allylic hydroperoxides by the reaction of molecular oxygen with the  $\Delta^{4,5}$ -double bond in this compound. The origin of all 16 secondary metabolites might then be explained by the known further reactions of such hydroperoxides. The qualitative pattern for the transformations of dihydroartemisinic acid in vivo was essentially unaltered when a comparison was made between plants, which had been kept alive and plants which were allowed to die after feeding of the labelled precursor. This, coupled with the observation that the pattern of transformations of 2 in vivo demonstrated very close parallels with the spontaneous autoxidation chemistry for 2, which we have recently demonstrated in vitro, has led us to conclude that the main 'metabolic route' for dihydroartemisinic acid in *A. annua* involves its spontaneous autoxidation and the subsequent spontaneous reactions of allylic hydroperoxides which are derived from 2. There may be no need to invoke the participation of enzymes in any of the later biogenetic steps leading to all 16 of the labelled 11,13-dihydro-amorphane sesquiterpenes which are found in *A. annua* as



natural products.

151. Cai Y., J.W. Jia, J. Crock, Z.X. Lin, X.Y. Chen, R. Croteau. A cDNA clone for beta-caryophyllene synthase from *Artemisia annua*. *Phytochemistry*, 2002, Volume 61, Issue 5, Pages 523-9. Database: Pubmed.

An homology-based cloning strategy yielded a full-length cDNA from *Artemisia annua* that encoded a protein of 60.3 kDa which resembled a sesquiterpene synthase in sequence. Heterologous expression of the gene in *Escherichia coli* provided a soluble recombinant enzyme capable of catalyzing the divalent metal ion-dependent conversion of farnesyl diphosphate to beta-caryophyllene, a sesquiterpene olefin found in the essential oil of *A. annua*. In reaction parameters and kinetic properties, beta-caryophyllene synthase resembles other sesquiterpene synthases of angiosperms. The beta-caryophyllene synthase gene is expressed in most plant tissues during early development, and is induced in mature tissue in response to fungal elicitor thus suggesting a role for beta-caryophyllene in plant defense.

152. Chang YJ, Song SH, Park SH, Kim SU. Amorpha-4,11-diene synthase of *Artemisia annua*: cDNA isolation and bacterial expression of a terpene synthase involved in artemisinin biosynthesis. *Archives of Biochemistry and Biophysics*, 2000, Volume 383, Issue 2, Pages 178-84. Database: pubmed.

*Artemisia annua*, an indigenous plant to Korea, contains an antimalarial sesquiterpene, artemisinin. The first committed step of artemisinin biosynthesis is the cyclization of farnesyl diphosphate by a sesquiterpene synthase to produce an amorphane-type ring system. The aims of this research were to molecularly clone and express amorpha-4,11-diene synthase for metabolic engineering. PCR amplification of genomic DNA with a pair of primers, designed from the conserved regions of sesquiterpene synthases of several plants, produced a 184-bp DNA fragment. This fragment was used in Northern blot analysis as a probe, showing approximately 2.2 kb of a single band. Its sequence information was used to produce 2106 bp of a full-length cDNA sequence including 1641 bp of open reading frame for 546 amino acids (kcs12) through a rapid amplification of cDNA ends (RACE). The deduced amino acid sequence displayed 36% identity with 5-epi-aristolochene synthase of *Nicotiana tabacum*. A soluble fraction of *Escherichia coli* harboring kcs12 catalyzed the cyclization of farnesyl diphosphate to produce a sesquiterpene, which was identified through GC-MS analysis as amorpha-4,11-diene.

153. Chen D, Ye H, Li G. Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. *Plant Science* 2000, Volume 155, Issue 2, Pages 179-185. Source: Pubmed.

An *Agrobacterium tumefaciens*-mediated transformation system was developed for *Artemisia annua* L. Using this system a cDNA encoding farnesyl diphosphate synthase (FDS placed under a CaMV 35S promoter) was transferred into *A. annua* via *A.*

tumefaciens strain LB4404. Leaf or leaf discs were used as explants to be infected with *A. tumefaciens* and an optimal concentration of 20 mg/l kanamycin was applied to select kanamycin resistant shoots. Forty-five lines of resistance kanamycin shoots transformed with FDS were established. Analysis of PCR showed that at least 20 shoots transformed with the FDS gene were PCR positive. Southern blot analysis suggested the foreign FDS gene had been integrated into the *A. annua* genome, and Northern blot analysis revealed that the foreign FDS gene expressed at the transcriptional level in five shoot lines (F-1, F-4, F-61, F-62 and F-73 shoot lines). Analysis of artemisinin demonstrated that about 8 approximately 10 mg/g DW of artemisinin were then detected in transgenic plants regenerated from five shoot lines, this is about 2-3 times higher than that in the control.

154. D'Andrea, S., Caramiello, R., Ghignone, S., Siniscalco, C. Systematic studies on some species of the genus *Artemisia*: Biomolecular analysis. *Plant Biosystems*. 137(2). 2003. 121-130. Database: Biosis.

The internal transcribed spacers (ITS) of the ribosomal DNA gene of 11 taxa of the genus *Artemisia* were sequenced and compared with other 14 species taken from GenBank. The aims of this study are to clarify phylogenetic relationships for 25 taxa within the genus *Artemisia*, and to highlight the phylogenetic position of some species of geobotanical interest from the Alps or from other European areas. The results support the monophyly of the genus *Artemisia*, and the presence of the five main clades, corresponding to the morphologically based sections, *Absinthium*, *Artemisia*, *Seriphidium*, *Dracunculus* and *Tridentatae*. Only *A. annua* and *A. genipi* are not classified in the section in which they were traditionally included: *A. annua* is assigned to *Seriphidium* and not *Artemisia*, and *A. genipi* to *Absinthium* and not *Artemisia*. The basal structure of the tree differed in the 45 equally parsimonious MP trees, and thus appeared as a polytomy in the consensus tree. This does not allow us to completely solve the relationships among the clades. The molecular data are complementary with the morphological and biogeographical information and all are essential to draw valid conclusions on relative closeness of the various taxa.

155. Dhingra V, Narasu ML. Purification and characterization of an enzyme involved in biochemical transformation of arteannuin B to artemisinin from *Artemisia annua*. *Biochemical and Biophysical Research Communication*, 2001, Volume 281, Issue 2, Pages 558-61. Database: Pubmed

The protein involved in the conversion of arteannuin B to artemisinin has been purified from the leaves of *Artemisia annua*. The pure protein found to be homogenous on Native gel electrophoresis showed two major bands of 21 and 11 kDa on 12% SDS-PAGE. Molecular weight estimation of native protein indicated an apparent molecular mass of 66,000 Daltons. This protein is able to achieve 58% conversion. It has a  $K_m$  of 0.5 mM for arteannuin B and a pH optima between 7.0-7.2. It is maximally active at 30 degrees C.

156. Greenhagen, Bryan T., Rising-Manna, Schoenbeck, Mark A., Chappell, Joe Characterization of sesquiterpene cyclase activity in the antimalarial herb *Artemisia annua*. *Plant Biology (New York)*. 1999 1999. 80-81. Database: Biosis.

157. Hua L, Matsuda SP. The molecular cloning of 8-epicedrol synthase from *Artemisia annua*. *Archives of Biochemistry and Biophysics* 1999, volume 369, Issue 2, Pages 208-12. Database: Pubmed.

A cDNA library was prepared from *Artemisia annua*, and a 129-bp fragment was amplified from this library using primers corresponding to sequences conserved in known dicot sesquiterpene synthases. A 1641-bp open reading frame that encoded a predicted protein 35-38% identical to dicot sesquiterpene synthases was cloned using this fragment as a hybridization probe. The gene product expressed in *Escherichia coli* cyclized farnesyl diphosphate to a 96:4 mixture of (-)-8-epicedrol and cedrol. Neither cedrol epimer was detected by GC-MS in an *A. annua* extract prepared from the same specimen as the cDNA.

158. Iskra, Timothy, Weathers, Pamela J., Wobbe, Kristin K. Evidence for the involvement of ascorbate peroxidase in the degradation of artemisinin. *Plant Biology (New York)*. 2000 2000. 125-126. Database: Biosis.

159. Iskra, Timothy, Weathers, Pamela J., Wobbe, Kristin K. Enigmatic role of peroxidases in *Artemisia annua*: Destruction or synthesis of artemisinin? *Plant Biology (New York)*. 1999 . 91. Database: Biosis

160. Jia JW, Crock J, Lu S, Croteau R, Chen XY. (3R)-Linalool synthase from *Artemisia annua* L.: cDNA isolation, characterization, and wound induction. *Archives of Biochemistry and Biophysics*, 1999, Volume 372, Issue 1, Pages 143-9. Database: Biosis.

*Artemisia annua* is an annual herb used in traditional Chinese medicine. A cDNA library was constructed from leaves of *A. annua* seedlings and target sequences were amplified by PCR using degenerate primers derived from a consensus sequence of angiosperm terpene synthases. Two clones, QH1 and QH5, with high sequence similarity to plant monoterpene synthases were ultimately obtained and expressed in *Escherichia coli*. These cDNAs encode peptides of 567 aa (65.7 kDa) and 583 aa (67.4 kDa), respectively, and display 88% identity with each other and 42% identity with *Mentha spicata* limonene synthase. The two recombinant enzymes yielded no detectable activity with isopentenyl diphosphate, dimethylallyl diphosphate, chrysanthemyl diphosphate, farnesyl diphosphate, (+)-copalyl diphosphate, or geranylgeranyl diphosphate, but were active with geranyl diphosphate in yielding (3R)-linalool as the sole product in the presence of divalent metal cation cofactors. QH1-linalool synthase displays a  $K_m$  value of 64  $\mu\text{M}$  for geranyl diphosphate, which is considerably higher than other known monoterpene synthases, and a  $K_m$  value of 4.6 mM for  $\text{Mg}^{+2}$ . Transcripts of QH1

and QH5 could be detected by RT-PCR in the leaves and inflorescence of *A. annua*, but not in the stem stele or roots; transcripts of QH5 could also be detected in stem epidermis. Linalool could not be detected by GC-MS in the essential oil of *A. annua*, nor in acid or base hydrolysates of aqueous extracts of leaves. RT-PCR demonstrated a wound-inducible increase in QH1 and QH5 transcript abundance in both leaves and stems over a 3-day time course.

161. Liu Yan, Ye He-Chun, Wang Hong, Li Guo-Feng. Molecular cloning, *Escherichia coli* expression and genomic organization of squalene synthase gene from *Artemisia annua*. *Acta Botanica Sinica*. 45(5). May 2003. 608-613. Database: Biosis.

A 1 539 bp squalene synthase (AaSQS) cDNA was cloned from a high-yield *Artemisia annua* L. strain 001 by reverse transcription-polymerase chain reaction (RT-PCR). The amino acid sequence of AaSQS is 70%, 77%, 44% and 39% identical to that of squalene synthases from *Arabidopsis thaliana*, tobacco, human and yeast, respectively. The AaSQS genomic DNA has a complex organization containing 14 exons and 13 introns. Full-length or C-terminal truncated cDNA was subcloned into prokaryotic expression vector pET30a and the constructed plasmid was introduced to *Escherichia coli* strain BL21 (DE3) for induced overexpression. No squalene synthase protein with expected molecular mass was observed in *E. coli* containing the putative full-length squalene synthase cDNA, however, overexpression in *E. coli* was achieved by truncating 30 amino acids of hydrophobic region at the carboxy terminus.

162. Liu Yan, Ye He-Chun, Li Guo-Feng. Cloning, *E. coli* expression and molecular analysis of a novel sesquiterpene synthase gene from *Artemisia annua*. *Acta Botanica Sinica*. 44(12). December 2002. 1450-1455. Database: Scifinder scholar.

A 1 886 bp full-length sesquiterpene synthase (AaSES) cDNA was cloned from a high-yield *Artemisia annua* L. strain 001 by a rapid amplification of cDNA and (RACE) strategy. AaSES is 59% identical to *Artemisia* cyclase cDNA clone cASC125, 50% identical to epi-cedrol synthase from *A. annua*, 48% identical to amorpho-4, 11-diene synthase from *A. annua*, 39% identical to the 5-epi-aristolechene synthase from tobacco, 38% identical to vetispiradiene synthase from *H. muticus*, 41% identical to the delta-cadinene synthase from cotton. The coding region of the cDNA was inserted into a prokaryotic expression vector pET-30a and overexpressed in *E. coli* BL21 (DE3). The cyclase proteins extracted from bacterial culture were found largely in an insoluble protein fraction. AaSES expresses in leaves, stems and flowers, not in roots as indicated by Northern blotting analysis.

163. Lu S, Xu R, Jia JW, Pang J, Matsuda SP, Chen XY. Cloning and functional characterization of a beta-pinene synthase from *Artemisia annua* that shows a circadian pattern of expression. *Plant Physiology* 2002, Volume 130, Issue 1, Pages 477-86.

Database: Pubmed.

*Artemisia annua* plants produce a broad range of volatile compounds, including monoterpenes, which contribute to the characteristic fragrance of this medicinal species. A cDNA clone, QH6, contained an open reading frame encoding a 582-amino acid protein that showed high sequence identity to plant monoterpene synthases. The prokaryotically expressed QH6 fusion protein converted geranyl diphosphate to (-)-beta-pinene and (-)-alpha-pinene in a 94:6 ratio. QH6 was predominantly expressed in juvenile leaves 2 weeks postsprouting. QH6 transcript levels were transiently reduced following mechanical wounding or fungal elicitor treatment, suggesting that this gene is not directly involved in defense reaction induced by either of these treatments. Under a photoperiod of 12 h/12 h (light/dark), the abundance of QH6 transcripts fluctuated in a diurnal pattern that ebbed around 3 h before daybreak (9th h in the dark phase) and peaked after 9 h in light (9th h in the light phase). The contents of (-)-beta-pinene in juvenile leaves and in emitted volatiles also varied in a diurnal rhythm, correlating strongly with mRNA accumulation. When *A. annua* was entrained by constant light or constant dark conditions, QH6 transcript accumulation continued to fluctuate with circadian rhythms. Under constant light, advanced cycles of fluctuation of QH6 transcript levels were observed, and under constant dark, the cycle was delayed. However, the original diurnal pattern could be regained when the plants were returned to the normal light/dark (12 h/12 h) photoperiod. This is the first report that monoterpene biosynthesis is transcriptionally regulated in a circadian pattern.

164. Magazova, N.S., Kunaeva, R.M., Baltabaeva, G.R., Ermekbaeva, L.A., Lopatina, N.P. Some properties of beta-galactosidase from *Artemisia annua* L. Stems, leaves, inflorescences. *Biochemistry. Nov 1983 (pub. 1984). v. 48 (11,pt.1) p. 1541-1545*  
Database: Agricola

165. Matsushita Y, Kang W, Charlwood BV. Cloning and analysis of a cDNA encoding farnesyl diphosphate synthase from *Artemisia annua*. *Gene. 1996, Volume 172, Issue 2, Pages 207-9.* Database: Pubmed.

A cDNA encoding farnesyl diphosphate (FPP) synthase (FPPS) has been cloned from a cDNA library of *Artemisia annua*. The sequence analysis showed that the cDNA encoded a protein of 343 amino acid (aa) residues with a calculated molecular weight of 39420 kDa. The deduced aa sequence of the cDNA was highly similar to FPPS from other plants, yeast and mammals, and contained the two conserved domains found in polyprenyl synthases including FPPS, geranylgeranyl diphosphate synthases and hexaprenyl diphosphate synthases. The expression of the cDNA in *Escherichia coli* showed enzyme activity for FPPS in vitro.

166. McCoy, Mark C., Smith, Tara, Weathers, Pamela J. The effects of phytohormone interaction and media alteration on growth and secondary metabolite production using

transformed roots of *A. annua* as a plant model. *Plant Biology (New York)*. 2001 2001. 82.  
Database: Biosis

167. Mercke P, Bengtsson M, Bouwmeester HJ, Posthumus MA, Brodelius PE. Molecular cloning, expression, and characterization of amorpha-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L. *Archives of Biochemistry and Biophysics*, 2000, Volume 381, Issue 2, Pages 173-80.  
Source: Pubmed.

In plants, sesquiterpenes of different structural types are biosynthesized from the isoprenoid intermediate farnesyl diphosphate. The initial reaction of the biosynthesis is catalyzed by sesquiterpene cyclases (synthases). In *Artemisia annua* L. (annual wormwood), a number of such sesquiterpene cyclases are active. We have isolated a cDNA clone encoding one of these, amorpha-4,11-diene synthase, a putative key enzyme of artemisinin biosynthesis. This clone contains a 1641-bp open reading frame coding for 546 amino acids (63.9 kDa), a 12-bp 5'-untranslated end, and a 427-bp 3'-untranslated sequence. The deduced amino acid sequence is 32 to 51% identical with the sequence of other known sesquiterpene cyclases from angiosperms. When expressed in *Escherichia coli*, the recombinant enzyme catalyzed the formation of both olefinic (97.5%) and oxygenated (2.5%) sesquiterpenes from farnesyl diphosphate. GC-MS analysis identified the olefins as (E)-beta-farnesene (0.8%), amorpha-4,11diene (91.2%), amorpha-4,7(11)-diene (3.7%), gamma-humulene (1.0%), beta-sesquiphellandrene (0.5%), and an unknown olefin (0.2%) and the oxygenated sesquiterpenes as amorpha-4-en-11-ol (0.2%) (tentatively), amorpha-4-en-7-ol (2.1%), and alpha-bisabolol (0.3%) (tentatively). Using geranyl diphosphate as substrate, amorpha-4,11-diene synthase did not produce any monoterpenes. The recombinant enzyme has a broad pH optimum between 7.5 and 9.0 and the  $K_m$  values for farnesyl diphosphate,  $Mg^{2+}$ , and  $Mn^{2+}$  are 0.9, 70, and 13 microM, respectively, at pH 7.5. A putative reaction mechanism for amorpha-4,11-diene synthase is suggested.

168. Mercke P, Crock J, Croteau R, Brodelius PE. Cloning, expression, and characterization of epi-cedrol synthase, a sesquiterpene cyclase from *Artemisia annua* L. *Archives of Biochemistry and Biophysics* 1999, Volume 369, Issue 2, Pages 213-22.  
Database: Biosis.

Sesquiterpene cyclases (synthases) catalyze the conversion of the isoprenoid intermediate farnesyl diphosphate to various sesquiterpene structural types. In plants, many sesquiterpenes are produced as defensive chemicals (phytoalexins) or mediators of chemical communication (i.e., pollinator attractants). A number of sesquiterpene synthases are present in *Artemisia annua* L. (annual wormwood). We have isolated a cDNA clone encoding one of these, epi-cedrol synthase. This clone contains a 1641-bp open reading frame coding for 547 amino acids (63.5 kDa), a 38-bp 5'-untranslated end, and a 272-bp 3'-untranslated sequence. The deduced amino acid sequence was 32 to 43% identical with the sequences of other known sesqui-terpene cyclases from angiosperms.

When expressed in *Escherichia coli*, the recombinant enzyme catalyzed the formation of both olefinic (3%) and oxygenated (97%) sesquiterpenes from farnesyl diphosphate. GC-MS analysis identified the olefins as alpha-cedrene (57% of the olefins), beta-cedrene (13%), (E)-beta-farnesene (5%), alpha-acoradiene (1%), (E)-alpha-bisabolene (8%), and three unknown olefins (16%) and the oxygenated sesquiterpenes (97% of total sesquiterpene generated, exclusive of farnesol and nerolidol) as cedrol (4%) and epi-cedrol (96%). epi-Cedrol synthase was not active with geranylgeranyl diphosphate as substrate, whereas geranyl diphosphate was converted to monoterpenes by the recombinant enzyme at a rate of about 15% of that observed with farnesyl diphosphate as substrate. The monoterpene olefin products are limonene (45%), terpinolene (42%), gamma-terpinene (8%), myrcene (5%), and alpha-terpinene (2%); a small amount of the monoterpene alcohol terpinen-4-ol is also produced. The pH optimum for the recombinant enzyme is 8.5-9.0 (with farnesyl diphosphate as substrate) and the K(m) values for farnesyl diphosphate are 0.4 and 1.3 microM at pH 7.0 and 9.0, respectively. The K(m) for Mg(2+) is 80 microM at pH 7.0 and 9.0.

169. Geng Sa; Ma Mi ; Ye He-chun ; Liu Ben-ye ; Li Guo-feng ; Chong Kang  
Effects of ipt gene expression on the physiological and chemical characteristics of *Artemisia annua* L. *Plant Science (Shannon)*. 160(4). March, 2001. 691-698.  
Database: Biosis.

An isopentenyl transferase gene (ipt) from T-DNA was transferred into *Artemisia annua* L. via *Agrobacterium tumefaciens*. The ipt gene was placed in a binary vector under the control of the CaMV 35S promoter. Leaf explants were infected with *A. tumefaciens* LBA4404 containing pBIipt to induce the buds. Nineteen shoot lines were selected, which were resistant to kanamycin. Polymerase chain reactions and Southern blotting confirmed that at least five shoot lines contained the foreign gene. The results of RT-PCR and Northern blotting analyses suggested that the foreign ipt gene of the transgenic shoot was expressed. Cytokinins, chlorophyll and artemisinin contents were found increased at different degree. Content of cytokinins (iPA and iP) was elevated 2- to 3-fold, chlorophyll increased 20-60% and artemisinin increased 30-70% compared with the control plants, respectively. A direct correlation was found between the contents of cytokinins, chlorophyll and artemisinin. This may be the first report on the relationship between endogenous cytokinin content and the production of secondary metabolites in plants.

170. Sangwan, N.S., R.S. Sangwan, S. Kumar. Isolation of Genomic DNA from the Antimalarial Plant *Artemisia annua*. *Plant Molecular Biology Reporter*, 1998, Vol. 16 Issue 4, p365. Database: Scifinder Scholar.

171. Souret, Frederic F., Weathers, Pamela J., Wobbe, Kristin K. The mevalonate-independent pathway is expressed in transformed roots of *Artemisia annua* and regulated

by light culture age. *In Vitro Cellular & Developmental Biology-Plant*. 38(6). 2002. 581-588. Database: Biosis

*Artemisia annua* produces a large number of unique terpenoids, making it of particular interest as a source of phytochemicals and a useful model plant for studying terpenoid metabolism. The ability to engineer fast-growing in vitro cultures to produce terpenoids in high yield would be a dramatic step towards commercial use. Two distinct pathways have been characterized in higher plants leading to the biosynthesis of isopentenyl diphosphate, the common precursor to all terpenes: the cytosolic mevalonate pathway and the plastid-localized mevalonate-independent pathway. While transformed roots of *A. annua* have been demonstrated to be superior to whole plants in terms of yield of the sesquiterpene artemisinin, they appear to lack functional chloroplasts, bringing into question the presence of a functional mevalonate-independent pathway. Using a cDNA library made from these roots, we isolated two clones encoding deoxy-D-xylulose-5-phosphate synthase (DXPS) and deoxy-D-xylulose-5-phosphate reductoisomerase (DXPR). The biochemical function of both enzymes was confirmed by complementing *E. coli* dxps- and dxpr-mutants. Northern blot analysis showed that the transformed root cultures expressed these genes at different levels during the culture cycle. In addition, cultures grown in continuous light showed substantial increases in DXPS transcript levels compared to dark-grown cultures. These results represent an important step towards demonstrating the presence of the plastid-localized terpenoid biosynthetic pathway in these easily engineered in vitro cultures.

172. Souret, Frederic F., Weathers, Pamela J., Wobbe, Kristin K. Dual analysis of the mevalonate and mevalonate-independent pathways involved in terpenoid biosynthesis using transformed roots of *Artemisia annua* as a model system. *Plant Biology (New York)*. 2001 2001. 148. Database: Biosis

173. Van Geldre E, De Pauw I, Inze D, Van Montagu M, Van den Eeckhout E. Cloning and molecular analysis of two new sesquiterpene cyclases from *Artemisia annua* L. *Plant Science*, 2000, Volume 158, Issue 1-2, Pages 163-171. Database: Pubmed

*Artemisia annua* L. is the only source of artemisinin, a new promising antimalarial drug (Qinghaosu Antimalarial Coordinating Research Group, *Chin. Med. J.* 92 (1979) 811). Our efforts are focused on the overproduction of this valuable medicine by genetic engineered *A. annua* plants. Therefore, we decided to isolate the gene(s) encoding sesquiterpene cyclase(s) in *A. annua* as a first step in improving artemisinin yield. Four partial genomic clones, gASC21, gASC22, gASC23 and gASC24, were isolated through polymerase chain reaction (PCR) with degenerated primers based on homologous boxes present in sesquiterpene cyclases from divergent sources. Intron-exon organisation of those partial genomic clones was analysed and it was shown that *A. annua* contains a gene family for sesquiterpene cyclases. Based on gASC21, gASC22, gASC23 and gASC24 sequences, the full-length cDNA clones cASC34 and cASC125 were subsequently isolated by rapid amplification of cDNA ends PCR. The derived amino acid sequences of both full-length clones show high homology with sesquiterpene cyclases

from plants. Reverse transcription-PCR analysis revealed transient and tissue specific expression patterns for cASC34 and cASC125, in contrast to the constitutively expressed 8-epicedrol synthase, a previously reported sesquiterpene cyclase from *A. annua*. Both cASC34 and cASC125 could only be detected in flowering plants when artemisinin concentration is at highest.

174. Vepari, Charu P., Weathers, Pamela J. Localization of peroxidases in hairy roots of *Artemisia annua*. *Plant Biology (New York)*. 1999 1999. 91. Database: Biosis

175. Wallaart TE, Bouwmeester HJ, Hille J, Poppinga L, Maijers NC. Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. *Planta*. 2001, Volume 212, Issue 3, Pages 460-5. Source: Pubmed

The sesquiterpenoid artemisinin, isolated these from the plant *Artemisia annua* L., and its semi-synthetic derivatives are a new and very effective group of antimalarial drugs. A branch point in the biosynthesis of this compound is the cyclisation of the ubiquitous precursor farnesyl diphosphate into the first specific precursor of artemisinin, namely amorpha-4,11-diene. Here we describe the isolation of a cDNA clone encoding amorpha-4,11-diene synthase. The deduced amino acid sequence exhibits the highest identity (50%) with a putative sesquiterpene cyclase of *A. annua*. When expressed in *Escherichia coli*, the recombinant enzyme catalyses the formation of amorpha-4,11-diene from farnesyl diphosphate. Introduction of the gene into tobacco (*Nicotiana tabacum* L.) resulted in the expression of an active enzyme and the accumulation of amorpha-4,11-diene ranging from 0.2 to 1.7 ng per g fresh weight.

176. Wallaart ET, Pras N, Quax WJ. Seasonal variations of Artemisinin and its biosynthetic precursors in tetraploid *Artemisia annua* plants compared with the diploid wild-type. *Planta Medica* 1999, Volume 65, Issue 8, Pages 723-8. Source: Pubmed.

Using colchicine we induced tetraploidy in *Artemisia annua* L. plants. During a vegetation period we monitored the time course of the levels of artemisinin, its direct precursors, the biosynthetically related sesquiterpenes and the essential oil content in the diploid (wild-type) and tetraploid *A. annua* plants. The averaged artemisinin level in tetraploids was 38% higher than that of the wild-type as measured over the whole vegetation period. In contrast, the averaged essential oil content of the tetraploids over this period was 32% lower. This might suggest a reciprocal correlation between artemisinin (sesquiterpenes) and the essential oil content (monoterpenes). The averaged biomass of the leaves of the tetraploid plants was lower compared to the wild-type plants. Therefore, the artemisinin yield per m<sup>2</sup> tetraploids was decreased by 25%. Although the tetraploid plants were smaller than the wild-type plants, certain individual organs like the leaves were considerably larger, and seeds obtained by cross pollination between tetraploid *A. annua* plants had a spectacular size. In principle, tetraploid *A. annua* can be

a useful starting material for a breeding program in order to obtain larger and faster growing plants, which produce higher levels of artemisinin.

### **Pharmacology Literature on *Artemisia annua*:**

177. Amos, S.; Chindo, B.A.; Abbah, J.; Vongtau, H.O.; Edmond, I.; Binda, L.; Akah, P.A.; Wambebe, C.; Gamaniel, K.S. Postsynaptic dopamine (D<sub>2</sub>)-mediated behavioural effects of high acute doses of artemisinin in rodents. *Brain Research Bulletin*, 2003, *Volume 62, Issue 3, Pages 255-260*. Database: Academic Search Premier

Artemisinin or qinghaosu is the active principle of qinghao (*Artemisia annua* L.) developed from Chinese traditional medicine, which is now widely used around the world against falciparum malaria. Behavioural effects of high acute doses of artemisinin were studied on spontaneous motor activity (SMA), exploratory behavior, apomorphine-induced stereotype behavior and pentobarbital sleeping time in mice and rats in order to provide additional evidence on its safety profile on the central nervous system (CNS). Effects of the drug on bromocriptine-induced hyperactivity in short term reserpinised mice were also evaluated. Intraperitoneal (i.p.) injection of artemisinin at doses of 50 and 100 mg/kg, significantly ( $P < 0.05$ ) reduced the SMA in mice, prolonged the pentobarbital sleeping time in rats, and attenuated the apomorphine-induced stereotypy in mice. Mice pretreated with reserpine, showed a significant decrease in locomotor activity compared to the saline-treated group. Bromocriptine, a D<sub>2</sub> receptor agonist, induced locomotor activity in mice pretreated with reserpine which was attenuated by artemisinin. The results suggest that artemisinin possesses sedative property, which may be mediated via postsynaptic dopamine (D<sub>2</sub>) receptor in the CNS.

178. Avery MA, Alvim-Gaston M, Rodrigues CR, Barreiro EJ, Cohen FE, Sabnis YA, Woolfrey JR. Structure-activity relationships of the antimalarial agent artemisinin. 6. The development of predictive in vitro potency models using CoMFA and HQSAR methodologies. *Journal of Medicinal Chemistry*, 2002, *Volume 45, Issue 2, Pages 292-303*. Database: Pubmed

Artemisinin (1) is a unique sesquiterpene peroxide occurring as a constituent of *Artemisia annua* L. Because of the effectiveness of Artemisinin in the treatment of drug-resistant *Plasmodium falciparum* and its rapid clearance of cerebral malaria, development of clinically useful semisynthetic drugs for severe and complicated malaria (artemether, artesunate) was prompt. However, recent reports of fatal neurotoxicity in animals with dihydroartemisinin derivatives such as artemether have spawned a renewed effort to develop nontoxic analogues of artemisinin. In our effort to develop more potent, less neurotoxic agents for the oral treatment of drug-resistant malaria, we utilized comparative molecular field analysis (CoMFA) and hologram QSAR (HQSAR), beginning with a series of 211 artemisinin analogues with known in vitro antimalarial activity. CoMFA models were based on two conformational hypotheses: (a) that the X-ray structure of artemisinin represents the bioactive shape of the molecule or (b) that the

hemin-docked conformation is the bioactive form of the drug. In addition, we examined the effect of inclusion or exclusion of racemates in the partial least squares (pls) analysis. Databases derived from the original 211 were split into chiral (n = 157), achiral (n = 34), and mixed databases (n = 191) after leaving out a test set of 20 compounds. HQSAR and CoMFA models were compared in terms of their potential to generate robust QSAR models. The  $r(2)$  and  $q(2)$  (cross-validated  $r(2)$ ) were used to assess the statistical quality of our models. Another statistical parameter, the ratio of the standard error to the activity range (s/AR), was also generated. CoMFA and HQSAR models were developed having statistically excellent properties, which also possessed good predictive ability for test set compounds. The best model was obtained when racemates were excluded from QSAR analysis. Thus, CoMFA of the n = 157 database gave excellent predictions with outstanding statistical properties. HQSAR did an outstanding job in statistical analysis and also handled predictions well.

179. Bailey, N., Yulan Wang, Julia Sampson, Wendy Davis, Ian Whitcombe, Peter J. Hylands, Simon L. Croft and Elaine Holmes. Prediction of anti-plasmodial activity of *Artemisia annua* extracts: application of  $^1\text{H}$  NMR spectroscopy and chemometrics *Journal of Pharmaceutical and Biomedical Analysis*, 2004, Volume 35, Issue 1, Pages 117-126. Database: Academic Search Premier.

We describe the application of  $^1\text{H}$  NMR spectroscopy and chemometrics to the analysis of extracts of *Artemisia annua*. This approach allowed the discrimination of samples from different sources, and to classify them according to anti-plasmodial activity without prior knowledge of this activity. The use of partial least squares analysis allowed the prediction of actual values for anti-plasmodial activities for independent samples not used in producing the models. The models were constructed using approximately 70% of the samples, with 30% used as a validation set for which predictions were made. Models generally explained >90% of the variance,  $R^2$  in the model, and had a predictive ability,  $Q^2$  of >0.8. This approach was also able to correlate  $^1\text{H}$  NMR spectra with cytotoxicity ( $R^2=0.9$ ,  $Q^2=0.8$ ).

This work demonstrates the potential of NMR spectroscopy and chemometrics for the development of predictive models of anti-plasmodial activity.

180. Beekman, A.C., Wierenga, P., Woerdenbag, H., Uden, W. van., Pras, N., Konings, A., El-Feraly, F.S., Galal, A.M., Wikstrom, H.V. TI - Artemisinin-derived sesquiterpene lactones as potential antitumour compounds: cytotoxic action against bone marrow and tumour cells. *Planta Medica* 1998, Volume 64, Pages 615-619. Database: Agricola.

181. Beekman, A., Woerdenbag, H., Kampinga, H., Konings, A. Cytotoxicity of artemisinin, a dimer of dihydroartemisinin, artemisitene and eupatoriopicrin as evaluated by the MTT and clonogenic assay. *Phytotherapy Research*, 1996, Volume 10, Issue 2, Pages 141-144. Database: Agricola.

Artemisinin and its derivatives possess an endoperoxide bridge, which is thought to lead to the production of free-radical species. The cytotoxicity of some of these agents to a murine Ehrlich ascites (EN19) and a human HeLa S3 cancer cell line was determined using the MTT and the clonogenic assay. The MTT assay cannot distinguish between growth inhibition and cell killing, while the clonogenic assay detects actual cell death. The use of both assays to test a certain drug may give information on the mode of its cytotoxicity (i.e. growth inhibition versus cell killing). The endoperoxides artemisinin and the dimer of dihydroartemisinin showed much higher cytotoxicity in the MTT assay compared with the clonogenic assay. Thus these drugs mainly induced growth inhibition. For artemisitene and eupatoriopicrin, which possess an exocyclic methylene with alkylating properties, both tests yielded comparable results. For these compounds the MTT assay merely determined cell killing. For the reference drugs cisplatin and doxorubicin the MTT assay showed lower cytotoxicity than the clonogenic assay. This may be explained by the metabolic activity of cells that were clonogenically dead. Moreover, our experiments have shown that the MTT assay may lead to misinterpretations concerning the mode of action of certain drugs, when it is used as a substitute for the clonogenic assay.

182. Benakis A, Paris M, Loutan L, Plessas CT, Plessas ST. Pharmacokinetics of artemisinin and artesunate after oral administration in healthy volunteers. *American Journal of Tropical and Medical Hygiene*, 1997, Volume 56, Issue 1, Pages 17-23. Database: Pubmed.

This study was designed to determine the pharmacokinetic parameters of a new pharmaceutical form of artemisinin (a natural substance extracted from the *Artemisia annua* L. plant) and of one of its derivatives, artesunate, a semisuccinate of 12-hydroxy-artemisinin. These two compounds are widely used in the treatment of malaria. The new oral forms of these two compounds, in 250-mg tablets, were used in two parallel pharmacokinetic studies. For artemisinin, the mean pharmacokinetic parameters were maximum drug concentration ( $C_{max}$ ) = 0.36 microgram/ml; peak time ( $t_{max}$ ) = 100 min; appearance half-life ( $t_{1/2 \text{ max}}$ ) = 0.62 hr; distribution half-life ( $t_{1/2 \text{ alpha}}$ ) = 2.61 hr; decline half-life ( $t_{1/2 \text{ beta}}$ ) = 4.34 hr; and total area under the concentration-time curve (AUC) = 1.19 micrograms.hr/ml. For artesunate, its main metabolite, dihydroartemisinin, was measurable in the plasma. The mean pharmacokinetic parameters for dihydroartemisinin were appearance rate constant ( $K_a$ ) = 2.11 hr<sup>-1</sup>; elimination rate constant ( $K_e$ ) = 1.18 hr<sup>-1</sup>; biotransformation half-life = 0.33 hr; elimination half-life = 0.65 hr; and AUC = 0.74 microgram.hr/ml. Both pharmaceutical forms were well-tolerated and no undesirable side effects were observed in any of the subjects.

183. Bharate, Sandip B. Medicinal plants with anti-HIV potential. *Journal of Medicinal & Aromatic Plant Sciences*. 25(2). June 2003, 427-440. Database: Agricola.

Since ancient times, plants have been an exemplary source of medicine. Ayurveda and other literature mention the use of plants in treatment of various human ailments. India

has about 45,000 plant species and among them, several thousands have been claimed to possess medicinal properties. The discovery of medicinal agents capable of specifically inhibiting human immunodeficiency virus (HIV) is urgently needed due to its globally widespread infection. More than 50% of world's marketed drugs have their natural origin. Natural products, of which structural diversity is so broad, are good sources for the effective discovery of anti-HIV agents with decreased toxicity. Over the past decade, substantial progress has been made in research on the natural products for the treatment of AIDS. Several plants have shown anti-HIV activity. The present paper reviews 52 such medicinal plants and their products (active natural principles and crude extracts) that have shown anti-HIV activity. Medicinal plants which are most effective and the most commonly studied in relation to AIDS and its complications are: *Acer okomotoanum*, *Aesculus chinensis*, *Andrographis paniculata*, *Annona glabra*, *Ardisia japonica*, *Ancistrocladus korupensis*, *Arctium lappa*, *Artemisia annua*, *Buchenavia capitata*, *Callophyllum* spp, *Castanospermum australe*, *Celastrus hindsii*, *Chassalia parvifolia*, *Conospermum incurvum*, *Crataegus pinatifida*, *Croton tiglium*, *Curcuma longa*, *Evodia roxburghiana*, *Ferula sumbul*, *Ganoderma lucidium*, *Garcinia multiflora*, *Gelonium multiflorum*, *Glycyrrhiza lepidota*, *Homalanthus nutans*, *Hopea malibato*, *Houttuynia cordata*, *Hypericum perforatum*, *Kadsura lansilimba*, *Leitneria floridana*, *Lepidobotrys staudtii*, *Litsea verticillata*, *Maclura tinctoria*, *Maprounea africana*, *Marila laxiflora*, *Momordica charantia*, *Myrianthus holstii*, *Palicourea condensate*, *Phyllanthus myrtifolius*, *Polyalthia suberosa*, *thomorphe peltata*, *Schisandra sphaerandra*, *Stephania cepharantha*, *Symphonia globulifera*, *Symplocos setchuensis*, *Syzygium claviflorum*, *Terminalia bellerica*, *Terminalia chebula*, *Toddalia asiatica*, *Trichosanthes kirilowii*, *Tripterygium wilfordii*, *Wikstroemia indica*, *Xanthoceras sorbifolia*. All plants have shown varying degree of anti-HIV activity.

184. Bilia AR, Lazari D, Messori L, Taglioli V, Temperini C, Vincieri FF. Simple and rapid physico-chemical methods to examine action of antimalarial drugs with hemin: its application to *Artemisia annua* constituents. *Life Sci.* 2002, Volume 70, Issue 7, Pages 769-78. Database: Pubmed.

Malaria is a major health problem in many countries and according to an estimate of the WHO, more than 500 million infections occur per year. Artemisinin, a sesquiterpene from *Artemisia annua* L., has received considerable attention as a promising and potent antimalarial drug for its stage specificity, its rather low toxicity, effectiveness against drug-resistant Plasmodium species and activity against cerebral malaria. From recent studies it seems that hemin is primarily involved in the antimalarial activity of the constituents of *Artemisia annua* L. Thus, the interaction of a compound with hemin may represent a crucial screening test to define its efficacy. In this study the interaction between artemisinin and hemin was investigated by UltraViolet/Visible (UV/Vis) spectrophotometry and High Performance Liquid Chromatography/Diode Array Detector/Mass Spectrometry (HPLC/DAD/MS). In addition, some flavonols isolated from *Artemisia annua* L. were also tested to investigate their possible role in the interaction between artemisinin and hemin. These two simple physico-chemical methods can be useful as rapid and widespread screening methods for the search of other

alkylating antimalarial constituents from natural sources or for the evaluation of the activity of semisynthetic analogues of artemisinin.

185. Brown, G. D.  $^{13}\text{C}$ - $^2\text{H}$  correlation NMR spectroscopy studies of the in vivo transformations of natural products from *Artemisia annua*. *Phytochemistry Reviews*. 2(1-2). 2003. 45-59. Database: Agricola.

186. Chan KL, Yuen KH, Jinadasa S, Peh KK, Toh WT. A high-performance liquid chromatography analysis of plasma artemisinin using a glassy carbon electrode for reductive electrochemical detection. *Planta Med*. 1997, Volume 63, Issue 1, Pages 66-9. Database: Pubmed.

A high-performance liquid chromatography assay equipped with a glassy carbon electrode for electrochemical detection (HPLC-ECD) was developed at reductive mode for the analysis of artemisinin, the antimalarial drug from *Artemisia annua* (Asteraceae) in human plasma. This method was selective, sensitive, and produced satisfactory recovery, precision, and accuracy. Analysis of plasma samples from 8 male volunteers given 10 mg kg<sup>-1</sup> of artemisinin orally as an aqueous suspension showed a mean peak plasma concentration (C<sub>max</sub>) of 580.89 ng ml<sup>-1</sup> +/- 88.64 SD at 2.5 h +/- 0.5 SD after dosing, and the mean area under the plasma concentration-time curve (AUC<sub>0-infinity</sub>) was 2227.57 ng h ml<sup>-1</sup> +/- 677.22 SD. In addition, the elimination rate constant (K<sub>e</sub>), elimination half-life (t<sub>1/2</sub>), and apparent volume of distribution (V<sub>d</sub>) were calculated to be 0.2971 h<sup>-1</sup> +/- 0.0644 SD, 2.42 h +/- 0.46 SD, and 16.26 l kg<sup>-1</sup> +/- 3.44 SD, respectively.

187. Chawira AN, Warhurst DC, Robinson BL, Peters W. The effect of combinations of qinghaosu (artemisinin) with standard antimalarial drugs in the suppressive treatment of malaria in mice. *Trans R Soc Trop Med Hyg*. 1987;81(4):554-8. Database: Pubmed.

Artemisinin is a novel antimalarial drug isolated in China from the wormwood plant *Artemisia annua* L. Studies with rodent malaria were carried out to detect antagonism and synergism with a variety of antimalarial drugs. Isobolograms of drug interaction were plotted at the ED<sub>90</sub> level. With a normally susceptible strain of *Plasmodium berghei*, marked potentiative synergism was found with mefloquine, tetracycline and spiramycin. There was some synergism also with primaquine. Combinations of artemisinin with dapson, sulfadiazine, sulfadoxine, pyrimethamine, pyrimethamine/sulfadoxine and cycloguanil showed antagonism. A high degree of potentiation was shown between artemisinin and primaquine with a primaquine-resistant strain, whilst the combination with mefloquine showed enhanced potentiation with a mefloquine-resistant strain. Combinations of artemisinin with mefloquine, primaquine, tetracycline or clindamycin showed marked potentiation with an artemisinin-resistant strain. The mechanisms underlying the drug interactions observed are discussed.

188. Chen Huan-Huan, H.J. Zhou G.D. Wu X.E. Lou. Inhibitory Effects of Artesunate on Angiogenesis and on Expressions of Vascular Endothelial Growth Factor and VEGF Receptor KDR/flk-1 *Pharmacology 2004, Volume 71, Pages 1–9*. Database: Academic Search Premier.

Artesunate (ART) is a semi-synthetic derivative of artemisinin extracted from the plant *Artemisia annua* is a safe and effective antimalarial drug. In the present investigation, ART was found also to inhibit angiogenesis in vivo and in vitro. The anti-angiogenic effect in vivo was evaluated in nude mice by means of human ovarian cancer HO-8910 implantation and immunohistochemical stainings for microvessel (CD31), vascular endothelial growth factor (VEGF) and VEGF receptor KDR/flk-1. Tumor growth was decreased and microvessel density was reduced following drug treatment with no apparent toxicity to the animals. ART also remarkably lowered VEGF expression on tumor cells and KDR/flk-1 expression on endothelial cells as well as tumor cells. The in vitro effect of ART was tested on models of angiogenesis, namely, proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVEC). The results showed that ART significantly inhibited angiogenesis in a dose-dependent form in the range of 0.5F50 Ìmol/l. Additionally, the inhibitory effect of ART on HVUEC proliferation was stronger than that on Hela, JAR, HO-8910 cancer cells, NIH-3T3 fibroblast cells and human endometrial cells, indicating that ART was selectively against HUVEC. These findings and the known low toxicity of ART are clues that ART may be a promising angiogenesis inhibitor.

189. Cheng, F., Jianhua Shen, Xiaomin Luo, Weiliang Zhu, Jiande Gu, Ruyun Ji, Hualiang Jiang and Kaixian Chen. Molecular docking and 3-D-QSAR studies on the possible antimalarial mechanism of artemisinin analogues. *Bioorganic & Medicinal Chemistry, vol. 10, issue 9, Pages 2883*. Database: Academic Search Premier.

Artemisinin (Qinghaosu) is a natural constituent found in *Artemisia annua* L, which is an effective drug against chloroquine-resistant *Plasmodium falciparum* strains and cerebral malaria. The antimalarial activities of artemisinin and its analogues appear to be mediated by the interactions of the drugs with hemin. In order to understand the antimalarial mechanism and the relationship between the physicochemical properties and the antimalarial activities of artemisinin analogues, we performed molecular docking simulations to probe the interactions of these analogues with hemin, and then performed three-dimensional quantitative structure–activity relationship (3-D-QSAR) studies on the basis of the docking models employing comparative molecular force fields analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA). Molecular docking simulations generated probable ‘bioactive’ conformations of artemisinin analogues and provided a new insight into the antimalarial mechanism. The subsequent partial least squares (PLS) analysis indicates that the calculate binding energies correlate well with the experimental activity values. The CoMFA and CoMSIA models based on the bioactive conformations proved to have good predictive ability and in turn match well with the docking result, which further testified the reliability of the docking model.

Combining these results, that is molecular docking and 3-D-QSAR, together, the binding model and activity of new synthesized artemisinin derivatives were well explained.

190. Classen W, Altmann B, Gretener P, Souppart C, Skelton-Stroud P, Krinke G. Differential effects of orally versus parenterally administered qinghaosu derivative artemether in dogs. *Experimental Toxicology and Pathology*, 1999, Volume 51, Issue 6, Pages 507-16. Database: Pubmed.

Artemether (AM) is an antimalarial drug derived from artemisinin (Qinghaosu), an extract of the herb *Artemisia annua* L., sweet wormwood. Its antiparasitic effect is that of a schizontocide and is explained by rapid uptake by parasitized erythrocytes and interaction with a component of hemoglobin degradation resulting in formation of free radicals. It has been shown to exhibit a high clinical cure rate. Previous animal safety studies with Qinghaosu derivatives revealed dose-dependent neurotoxicity with movement disturbances and neuropathic changes in the hindbrain of intramuscularly treated dogs, rats and monkeys. Such effects have not been seen in man. The objective of our present studies was to compare the effects of high levels of AM administered to dogs p.o. versus i.m. In a pilot study 20 mg/kg/day of AM was given i.m. to groups of 3 male Beagle dogs for 5 and 30 days, respectively. Clinical signs of neurotoxicity were noted in some individual dogs from test day 23 on. One dog had to be sacrificed pre-term. Hematologic findings indicated a hypochromic, microcytic anemia. Microscopic examination demonstrated neuropathic changes only at 30 days, but not at 5 days. The animals had neuronal and secondary axonal damage, most prominent in the cerebellar roof, pontine and vestibular nuclei, and in the raphe/paralemniscal region. The affected neurons showed loss of Nissl substance, cytoplasmic eosinophilia, shrinkage of the nucleus and in advanced stages scavenging by microglia. In a subsequent experiment, AM was administered to groups of 4 male and 4 female dogs, respectively, at 8 daily doses of 0, 20, 40 and 80 mg/kg i.m., or 0, 50, 150 and 600 mg/kg p.o. Neurologic signs were seen at high i.m. doses only. In most animals they were inconspicuous and consisted of reduced activity with convulsions seen in single dogs shortly before death. Neuronal damage occurred in all animals at 40 and 80 mg/kg following i.m. treatment. At 20 mg/kg minimal effects occurred in 5/8 dogs only, indicating that this level was close to tolerated exposure. No comparable lesions were observed after oral administration. Both i.m. and p.o. exposure at high dose levels was associated with a prolongation of mean QT interval of ECG, suggesting slowing of repolarization of the myocardium. Individual data indicated that in 1 of 4 females at 80 mg/kg i.m. this prolongation was above the 25% level considered as threshold for concern. After intramuscular administration pharmacokinetics indicated peak plasma levels of AM at 2 to 4 hours post-dose, slow elimination and a tendency to accumulate after repeated administration. Only low levels of the major metabolite, dihydroartemisinin (DHA), were found. AM levels in the cerebrospinal fluid (CSF) were < 10% of plasma levels. After oral administration AM concentrations were considerably lower than after i.m. administration. The concentration of DHA was high on day 1 but almost nil on day 7 indicating its fast inactivation in dogs. Two hours after the 8th oral administration neither AM nor DHA was detected in CSF which may explain the absence of neurotoxicity in dogs after oral administration of AM.

191. Dias PC, Foglio MA, Possenti A, Nogueira DC, de Carvalho JE. Antiulcerogenic activity of crude ethanol extract and some fractions obtained from aerial parts of *Artemisia annua* L. *Phytotherapy Research* 2001, Volume 15, Issue 8, Pages 670-5. Database: Pubmed.

The resulting enriched sesquiterpene lactone fraction and the crude ethanol extract of *Artemisia annua* L. aerial parts, showed antiulcerogenic activity when administered orally, on the indomethacin induced ulcer in rats. The sesquiterpene lactone fraction yielded three different polarity fractions on column chromatography as follows: non-polar, medium polarity and polar fraction. When submitted to the same indomethacin-induced ulcer in rats they resulted in different levels of inhibition of the ulcerative lesion index. The participation of nitric oxide was evaluated on an ethanol-induced ulcer model which had a previous administration of L-NAME, a NO-synthase inhibitor. Under these conditions, the medium polarity fraction maintained the antiulcerogenic activity, suggesting that nitric oxide could not be involved in the anti-ulcerogenic activity. When the animal groups were treated with N-ethylmaleimide, an alkylator of sulphhydryl groups, using the same experimental model, the medium polarity fraction maintained its antiulcerogenic activity, suggesting that the pharmacological mechanism is not related to non-protein sulphhydryl compounds. On the ethanol-induced ulcer with previous indomethacin treatment, the medium polarity fraction lost its antiulcerogenic activity indicating that the active compounds of *Artemisia annua* L. increase the prostaglandin levels in the gastric mucosa. This hypothesis was reinforced by an increase of adherent mucus production by the gastric mucosa, produced by the medium polarity fraction on the hyperthermic restraint stress induced ulcer model.

192. Eckstein-Ludwig U, R.J. Webb, I.D. Van Goethem, J.M. East, A.G. Lee, M. Kimura, P.M. O'Neill, P.G. Bray, S.A. Ward, S. Krishna. Artemisinin target the SERCA of *Plasmodium falciparum*. *Nature* 2003, Volume 424, Issue 6951, Pages 957-961. Database: Pubmed.

Artemisinins are extracted from sweet wormwood (*Artemisia annua*) and are the most potent antimalarials available, rapidly killing all asexual stages of *Plasmodium falciparum*. Artemisinins are sesquiterpene lactones widely used to treat multidrug-resistant malaria, a disease that annually claims 1 million lives. Despite extensive clinical and laboratory experience their molecular target is not yet identified. Activated artemisinins form adducts with a variety of biological macromolecules, including haem, translationally controlled tumour protein (TCTP) and other higher-molecular-weight proteins. Here we show that artemisinins, but not quinine or chloroquine, inhibit the SERCA orthologue (PfATP6) of *Plasmodium falciparum* in *Xenopus* oocytes with similar potency to thapsigargin (another sesquiterpene lactone and highly specific SERCA inhibitor). As predicted, thapsigargin also antagonizes the parasitocidal activity of artemisinin. Esoxyartemisinin lacks an endoperoxide bridge and is ineffective both as an inhibitor of PfATP6 and as an antimalarial. Chelation of iron by desferrioxamine abrogates the antiparasitic activity of artemisinins and correspondingly attenuates inhibition of PfATP6. Imaging of parasites with BODIPY-thapsigargin labels the

cytosolic compartment and is competed by artemisinin. Fluorescent artemisinin labels parasites similarly and irreversibly in an Fe<sup>2+</sup>-dependent manner. These data provide compelling evidence that artemisinins act by inhibiting PfATP6 outside the food vacuole after activation by iron.

193. Efferth T, Dunstan H, Sauerbrey A, Miyachi H, Chitambar CR. The anti-malarial artesunate is also active against cancer. *International Journal of Oncology*, 2001, Volume 18, Issue 4, pages 767-73. Database: Pubmed.

Artesunate (ART) is a semi-synthetic derivative of artemisinin, the active principle of the Chinese herb *Artemisia annua*. ART reveals remarkable activity against otherwise multidrug-resistant *Plasmodium falciparum* and *P. vivax* malaria. ART has now been analyzed for its anti-cancer activity against 55 cell lines of the Developmental Therapeutics Program of the National Cancer Institute, USA. ART was most active against leukemia and colon cancer cell lines (mean GI50 values: 1.11±0.56 µM and 2.13±0.74 µM, respectively). Non-small cell lung cancer cell lines showed the highest mean GI50 value (25.62±14.95 µM) indicating the lowest sensitivity towards ART in this test panel. Intermediate GI50 values were obtained for melanomas, breast, ovarian, prostate, CNS, and renal cancer cell lines. Importantly, a comparison of ART's cytotoxicity with those of other standard cytostatic drugs showed that ART was active in molar ranges comparable to those of established anti-tumor drugs. Furthermore, we tested CEM leukemia sub-lines resistant to either doxorubicin, vincristine, methotrexate, or hydroxyurea which do not belong to the N.C.I. screening panel. None of these drug-resistant cell lines showed cross resistance to ART. To gain insight into the molecular mechanisms of ART's cytotoxicity, we used a panel of isogenic *Saccharomyces cerevisiae* strains with defined genetic mutations in DNA repair, DNA checkpoint and cell proliferation genes. A yeast strain with a defective mitosis regulating BUB3 gene showed increased ART sensitivity and another strain with a defective proliferation-regulating CLN2 gene showed increased ART resistance over the wild-type strain, wt644. None of the other DNA repair or DNA check-point deficient isogenic strains were different from the wild-type. These results and the known low toxicity of ART are clues that ART may be a promising novel candidate for cancer chemotherapy.

194. Etkin, Nina L. The co-evolution of people, plants, and parasites: Biological and cultural adaptations to malaria. *Proceedings of the Nutrition Society*. 62(2). May 2003. 311-317. Database: Biosis.

The urgency generated by drug-resistant strains of malaria has accelerated anti-malarial drug research over the last two decades. While synthetic pharmaceutical agents continue to dominate research, attention increasingly has been directed to natural products. The present paper explores the larger context in which plant use occurs and considers how the selection of medicinal plants has evolved over millennia as part of the larger human effort to mediate illness. First attention is directed to indigenous medicinal plants whose anti-malarial activity is based on an oxidant mode of action, by which intracellular constituents lose electrons (become more electropositive). Next, parallels are drawn

between these plant substances and a suite of malaria-protective genetic traits: glucose-6-phosphate dehydrogenase deficiency; haemoglobins S, C and E; alpha-and beta-thalasseмии. These erythrocyte anomalies are classic examples of Darwinian evolution, occurring in high frequency in populations who have experienced considerable selective pressure from malaria. Characterized by discrete loci and pathophysiologicals, they are united through the phenomenon of increased erythrocyte oxidation. In this model, then, oxidant anti-malarial plants are culturally constructed analogues, and molecular mimics, of these genetic adaptations. To further reinforce the scheme, it is noted that the anti-malarial action of pharmaceutical agents such as chloroquine and mefloquine duplicates both the genetic anomalies and the folk therapeutic models based in oxidant plants. This discussion coheres around a theoretical foundation that relates plant secondary metabolites (oxidants) to plasmodial biochemistry and human biological and cultural adaptations to malaria. Co-evolution provides a theoretical link that illuminates how medical cultures manage the relationships among humans, plants, herbivores and their respective pathogens.

195. Foglio MA, Dias PC, Antonio MA, Possenti A, Rodrigues RA, da Silva EF, Rehder VL, de Carvalho JE. Antiulcerogenic activity of some sesquiterpene lactones isolated from *Artemisia annua*. *Planta Medica* 2002, Volume 68, Issue 6, Pages 515-8. Database: Pubmed.

Artemisinin 1, dihydro-epideoxyarteannuin B 2 and deoxyartemisinin 3 were isolated from the sesquiterpene lactone-enriched fraction obtained from the crude ethanolic extract of *Artemisia annua* L. These compounds were tested on ethanol and indomethacin-induced ulcer models. Compound 1 did not afford cytoprotection under the experimental models tested. Only compounds 2 and 3 decreased the ulcerative lesion index produced by ethanol and indomethacin in rats. These compounds did not demonstrate antiulcerogenic activity when tested on the ethanol-induced ulcer model, with previous administration of indomethacin, suggesting that the antiulcerogenic activity is a consequence of prostaglandin synthesis increase.

196. Fuchs, E., Albers, K., Kopan, R. Terminal differentiation in cultured human epidermal cells. *Advances in Cell Culture*. 1988. v. 6 p. 1-33. Database: Agricola.

197. Funk, Jens Oliver; Rauch,; Weber, H. Oliver; Efferth. The anti-malarial artesunate acts also as a potent anti-cancer drug via both p53-dependent and -independent pathways *Archives of Dermatological Research*. 293(1-2). February, 2001. 66. Database: Biosis.

198. Jiang, J.B., Jacobs, G., Liang, D.S., Aikawa, M. Qinghaosu-induced changes in the morphology of *Plasmodium inui*. *American Journal of Tropical Medicine & Hygiene*. 1985. v. 34 (3) p. 424-428. Database: Agricola.

199. Kar, K., Nath, A., Bajpai, R., Dutta, G.P., Vishwakarma, R.A. Pharmacology of alpha/beta arteether--a potential antimalarial drug. *Journal of Ethno-Pharmacology*. 1989. v. 27 (3) p. 297-305. Database: Agricola.

Pharmacological studies on  $\alpha/\beta$  arteether (a 30:70 mixture of isomers), a potential drug for the treatment of cerebral malaria, were carried out in experimental animals by giving the drug in arachis oil suspension. The compound appears to be devoid of significant pharmacological activity on the central nervous, cardiovascular and urinary systems. It lacks an anti-inflammatory response in rats up to 50 mg/kg intramuscularly but at 50–200 mg/kg it has some antianaphylactic potential.

200. Malik, Heetika, Puri, Sunil K., Singh, Chandan. Amino functionalized 1,2,4-trioxanes: Synthesis and in vivo antimalarial activity. *Medicinal Chemistry Research*. 12(6-7). 2003. 287-288. Database: Biosis.

201. Meshnick, Steven R. Artemisinin: Mechanisms of action, resistance and toxicity. *International Journal for Parasitology*. 32(13). 4 December 2002. 1655-1660. Database: Biosis.

Artemisinin and its derivatives are widely used throughout the world. The mechanism of action of these compounds appears to involve the heme-mediated decomposition of the endoperoxide bridge to produce carbon-centred free radicals. The involvement of heme explains why the drugs are selectively toxic to malaria parasites. The resulting carbon-centred free radicals alkylate heme and proteins, one of which is the translationally controlled tumour protein. Clinically relevant artemisinin resistance has not been demonstrated, but it is likely to occur since artemisinin resistance has been obtained in laboratory models. At high doses, artemisinin can be neurotoxic but toxicity has not been found in clinical studies. The mechanism of neurotoxicity may be similar to the mechanism of action.

202. Mueller, S., Schmitt, M., Dekant, W., Stopper, H., Schlatter, J., Schreier, P., Lutz, W.K. Occurrence of emodin, chrysophanol and physcion in vegetables, herbs and liquors. Genotoxicity and anti-genotoxicity of the anthraquinones and of the whole plants. *Food & Chemical Toxicology*, 1999, Volume 37, Issue 5, Pages 481-491. Database: Agricola.

203. Niu XY, Ho LY, Ren ZH, Song ZY. Metabolic fate of Qinghaosu in rats; a new TLC densitometric method for its determination in biological material. *Eur J Drug Metab Pharmacokinet*. 1985 Jan-Mar;10(1):55-9. Database: Pubmed.

Since the sixties, the emergence of malarial parasites resistant to the most potent anti-malarials has posed a serious problem to the therapy of malaria. Qinghaosu, a new sesquiterpene isolated from a Chinese medicinal herb Qing-hao (*Artemisia annua* Linn) is being used for the treatment of malaria in China with good results even in cases resistant to common anti-malarial agents. In this paper, a sensitive method of high specificity using TLC for the determination of Qinghaosu in biological specimens and in the study of the metabolism of the drug in rats is described. Qinghaosu was shown to be completely and rapidly absorbed after oral administration. However, a very low plasma level was

obtained even after a dose of 300 mg/kg. Liver was found to be the chief site of its inactivation. When Qinghaisu was given intramuscularly, significant and more persistent plasma levels were detected. Qinghaisu was shown to pass the blood-brain and blood-placenta barriers after i.v. injection. Very little unchanged Qinghaisu was found in the urine and feces in 48 hours regardless of administration route (i.v., i.m. or p.o.).

204. Qiao Guo-Fen, Yang Bao-Feng, Li Wen-Han, Li Bai-Yan. Effects of artemisinin on action potentials from C-type nodose ganglion neurons. *Acta Pharmacologica Sinica*. 24(9). September 2003. 937-942. Database: Biosis.

To investigate the effects of artemisinin (Art) on the action potentials (AP) recorded from identified C-type nodose neurons and study its anti-arrhythmic and anesthetic mechanisms.

Neonatal and adult rats were selected for the preparation of isolated nodose ganglia neurons (NGN) and nodose ganglion-vagus slice preparation. Somatic AP were recorded from both isolated and slice NGN using whole-cell patch technique. Conduction velocity (CV) was measured using slice preparation. The effects of Art on AP were evaluated with the reference to ketamine.

Effects of Art on AP were that: (1) AP depolarizing profiles were inhibited without changing resting membrane potential (RMP). The peak of AP (APpeak) and upstroke velocity (UVAPD50 and UVmax) decreased markedly ( $P < 0.01$ ). (2) The duration of AP at the point of half repolarization (APD50) was obviously prolonged ( $P < 0.01$ ). (3) Art also slowed down AP repolarization profiles (downstroke velocity, DVAPD50, and DVmax) and the peak of after-hyperpolarization (AHPpeak) was less negative. (4) Total inward and outward currents over the course of AP were significantly reduced in the presence of Art. (5) CV did not changed by Art. (6) The effects of Art on AP were concentration-dependent and resembled with those of ketamine except for CV.

Art inhibited both depolarization and repolarization of AP, suggesting that the effects of Art were probably, due to the blockade of Na<sup>+</sup> and K<sup>+</sup> ion channels.

205. Perazzo, F.F.; Carvalho, J.C.T.; Carvalho, J.E.; Rehder, V.L.G. Central properties of the essential oil and the crude ethanol extract from aerial parts of *Artemisia annua* L. *Pharmacological Research*, 2003, Volume 48, Issue 5, Pages 497-502  
Database: Academic Search Premier.

The present study evaluated the central activity of the essential oil and the ethanolic extract from *Artemisia annua* L. in animals as a part of a psychopharmacological screening of this plant. The extract was prepared with fresh leaves in ethanol (AEE) and the essential oil (AEO) was obtained by hidrodestillation. The ED<sub>50</sub> and the LD<sub>50</sub> obtained for the essential oil were 470 mg/kg (correlation coefficient  $r=0.97333$  and linear regression  $y=-26.52x+0.158$ ) and 790 mg/kg, and for the extract, 450 mg/kg (correlation coefficient  $r=0.99266$  and linear regression  $y=-27.34+0.156$ ) and more than 2 g/kg, respectively. The doses increased the latency time to convulsions induced by picrotoxin and pilocarpine but prevented the onset of pentylenetetrazol and strychnine induced

seizures. In addition to, the products have caused marked inhibition in the Rota-rod assay. According to the results, the AEO has a high acute toxicity and a possible cholinergic action, and the AEE showed a possible central activity as dopaminergic and cholinergic agents, and did not present a significant acute toxicity. These differences should be due to chemical substances present in each product. These products had no significant effect as an anticonvulsant, while exhibited a strong depressant activity on the CNS.

206. Rajanikanth, M., Madhusudanan, K. P., Gupta, R. C. Liquid chromatographic-mass spectrometric method for the determination of alpha-,beta-arteether in rat serum. *Journal of Chromatography B*. 783(2). 15 January, 2003. 391-399. Database: Biosis.

This study reports the development and validation of a sensitive and selective assay method for the determination of alpha-,beta-arteether in rat serum by liquid chromatography-mass spectrometry. The mobile phase was composed of methanol-0.1 mM sodium acetate (pH 5) (80:20%) at a flow-rate of 1 ml min<sup>-1</sup> and chromatographic separations were achieved on a Ultracarb, 5 ODS 20, Phenomenex column (5 µm, 30 mmX4.6 mm I.D.). The total effluent from the column was split so that one-tenth was injected into the electrospray LC-MS interface. ESI-MS analysis was carried out using a Micromass Quattro II Triple Quadrupole Mass Spectrometer equipped with an electrospray source. The MS analysis was carried out at a cone voltage of 52 V with a scan range of 100-400 Da. The analytes were quantified from the (M+Na)<sup>+</sup> ion chromatograms of alpha-,beta-arteether at m/z 335 and artemisinin at m/z 305. A simple liquid-liquid extraction with 2X2 ml n-hexane was used to isolate alpha-,beta-arteether from rat serum. The method was validated in terms of recovery, linearity, accuracy and precision (within- and between-assay variation). The recovery from spiked control samples ranged from 88.41 to 96.17% with a maximum CV of 10.8% for alpha-arteether and 69.83-79.69% with a maximum CV of 17.06% for beta-arteether. Linearity in serum was observed over the range 20-320 ng ml<sup>-1</sup>. Percent bias (accuracy) was well within the acceptable range. Within- and between-assay precision were less than 15%. The assay method described here is being applied to study the pharmacokinetics of CDRI developed intramuscular formulation Emal (alpha-/beta-arteether in the ratio of 30:70) in rats. The method is sensitive enough to monitor alpha-,beta-arteether up to 24 h after a single 30 mg kg<sup>-1</sup> i.m. dose.

207. Severina, Irina S., Pyatakova, Natalya V., Bussygina, Olga G., Mikhailitsyn, Felix S. Khropov, Yuri V. Inhibition of nitric oxide-dependent activation of soluble guanylyl cyclase by the antimalarial drug, artemisinin *European Journal of Pharmacology*. 438(1-2). 1 March, 2002. 69-73. Database: Biosis.

The influence of artemisinin on the activity of human platelet soluble guanylyl cyclase was investigated. Artemisinin (0.1-100 µM) had no effect on the basal activity of the enzyme. Artemisinin inhibited in a concentration-dependent manner the sodium nitroprusside-induced activation of human platelet guanylyl cyclase with an IC<sub>50</sub> value 5.6 µM. Artemisinin (10 µM) also inhibited (by 71±4.0%) the activation of the

enzyme by the thiol-dependent nitric oxide (NO) donor, the derivative of furoxan, 3,4-dicyano-1,2,5-oxadiazole 2-oxide (10  $\mu$ M), but did not influence the stimulation of soluble guanylyl cyclase by protoporphyrin IX. Inhibition of guanylyl cyclase activation by NO donors but not by protoporphyrin IX represents a possible additional mechanism of the pharmacological action of this drug.

208. Stermitz, Frank R., Scriven, Lacey N., Tegos, George, Lewis. Two flavonols from *Artemisa annua* which potentiate the activity of berberine and norfloxacin against a resistant strain of *Staphylococcus aureus*. *Planta Medica*. 68(12). December 2002. 1140-1141. Database: Biosis.

Bioassay-guided fractionation of an extract of *Artemisia annua* L. (Asteraceae) was conducted in order to assess the possible presence in the plant material of inhibitors of bacterial multidrug resistance pumps. Fractions were tested for *Staphylococcus aureus* growth inhibition in the presence of a subinhibitory dose of the weak antibacterial alkaloid berberine. Active fractions yielded the flavones chrysosplenol-D and chrysosplenetin, which themselves had very weak growth inhibitory action, but which made a potent combination with berberine. In comparison with work on other flavonols, it is likely that potentiation is due to the inhibition of an *S. aureus* multidrug resistance (MDR) pump. These same two flavonols were earlier reported to potentiate the activity of artemisinin against *Plasmodium falciparum*.

209. Raeth K, K. Taxis, G. Walz, C.H. Gleiter, S.M. Li, L. Heide. Pharmacokinetic study of artemisinin after oral intake of a traditional preparation of *Artemisia annua* L. (annual wormwood). *American Journal of Tropical Medicine and Hygeine*, 2004, Volume 70, Issue 2, Pages 128-32. Database: Pubmed

*Artemisia annua* L. (annual wormwood) contains the antimalarial artemisinin. Aqueous preparations of the dried herb are included in the pharmacopoeia of the People's Republic of China for treatment of fever and malaria. Fourteen healthy male volunteers received one liter of tea prepared from nine grams of *Artemisia annua* leaves. Blood samples were taken and artemisinin was detected by reversed phase high-performance liquid chromatography. The mean  $\pm$  SD maximum plasma concentration of artemisinin was 240  $\pm$  75 ng/mL and the mean  $\pm$  SD area under the plasma concentration-time curve was 336  $\pm$  71 ng/mL x hr. Artemisinin was absorbed faster from herbal tea preparations than from oral solid dosage forms, but bioavailability was similar. One liter of an aqueous preparation of nine grams of *Artemisia annua* contained 94.5 milligrams of artemisinin (approximately 19% of the usually recommended daily dose). Artemisinin plasma concentrations after intake of this herbal tea are sufficient for clinical effects, but insufficient to recommend such preparations as equivalent substitutes for modern artemisinin drugs in malaria therapy.

210. Schuster BG. Demonstrating the validity of natural products as anti-infective drugs.

*J Alternative and Complementary Medicine, 2001, Volume 7 Suppl 1, Pages S73-82.*  
Database: pubmed.

This presentation reviews the synthetic or classical development pathway of drug development and contrasts it with developing natural products as drugs. Also presented is an example of a traditional medicine that has been developed from a natural product and has become a "new/old" antiparasitic drug used in the treatment of malaria. The classic paradigm of synthetic drug development breaks down into drug discovery, drug design, preclinical studies, and clinical studies. This paradigm, constructed to weed out failures, results in a drug-development process that is high risk, time consuming, and expensive. The process requires screening an average of 10,000 active compounds to find a single compound that successfully makes its way through validation to drug approval and the marketplace. Following this paradigm, researchers progress from identifying a chemical lead to testing the compound in humans. The World Health Organization (WHO) Guidelines for the Assessment of Herbal Medicines are based on the classical guidelines and follow the classical approach to validating quality, safety, and efficacy--with one major difference. The starting point is to look at the natural product in humans. By taking into account the traditional experience with the product, the validation standard for safety and efficacy of natural products allows for the prolonged and apparently uneventful use of a substance to offer testimony of its safety. The reliance, then, is on experience--or what Western regulatory agencies would call "anecdotal information." Since most phytomedicines are a combination of several active ingredients, the WHO guidelines cover two kinds of combination products: Combinations that are already used in traditional medicine are considered "old" combination products. "New" combination products are well-known substances that are now being used in combination. *Artemisia annua*, a pervasive weed, has been referred to in Chinese medicine for thousands of years as a treatment for fever. In 1971, an extraction of *artemisia* yielded activity against *Plasmodium berghei*, a mouse model for malaria. The isolated compound, artemisinin, is an example of a traditional medicine that started out in humans, but which then provided a lead structure for a standard drug-development paradigm. Today, artemisinin derivatives are being used widely in combination therapy, especially in areas of the world where there is multidrug-resistant malaria.

211. Shen M, Ge HL, He YX, Song QL, Zhang HZ. Immunosuppressive action of Qinghaosu. *Sci Sin [B]*. 1984 Apr;27(4):398-406. Database: Pubmed.

Qinghaosu, isolated and purified from the Chinese herb, *Artemisia annua* Linn, and identified as a sesquiterpene with a peroxide bridge and lactone structure, is a highly potent and non-toxic new antimalaria drug. This paper reports the immunosuppressive action of its water soluble derivative (hemisuccinate NA, QHS). The remarkable suppression by QHS of the *in vitro* 3HTdR incorporation by mitogen-stimulated mouse spleen cells and human peripheral lymphocytes, as well as the spontaneous incorporation by mouse thymocytes and blood cells from some leukemia patients is presented and its

characteristics are described. The in vivo effect as shown by quantitative PFC is studied and the difference between the present in vitro and in vivo effects is investigated. The possible mechanism of inhibition and discrepancy in effects are discussed.

212. Tan Y, Zhao Y, Lin Q, Xie G, Yang P, Yin X. Experimental study on antiendotoxin effect of extracts from *Artemisia annua* L. [Article in Chinese]. Not reviewed by team. *Zhongguo Zhong Yao Za Zhi*. 1999, Volume 24, Issue 3, Pages 166-71, 192. Database: Pubmed.

**OBJECTIVE:** To explore the antiendotoxin effect of extracts from *Artemisia annua* (AA) and qinghaosu (QHS). **METHOD:** LPO and SOD in chondriosome, ACP in lysosomes, tumor necrosis factor-alpha (TNF-alpha) and endotoxin in plasma, and P450 concentration in hepatic microsomes of rats were determined. Mortality of endotoxemic mice and histomorphology were observed.

**RESULT:** LPO, ACP, endotoxin, TNF-alpha and P450 content were decreased with AA and QHS. SOD activity was increased with AA and QHS. At the same time, mortality was decreased. Histomorphology of lysosomes and chondriosome of rats were protected from endotoxin. **CONCLUSION:** AA and QHS possess an antiendotoxin effect.

213. Tawfik AF, Bishop SJ, Ayalp A, el-Ferally FS. Effects of artemisinin, dihydroartemisinin and arteether on immune responses of normal mice. *International Journal of Immunopharmacology*, 1990, Volume 12, Issue 4, Pages 385-9. Database: Pubmed.

Artemisinin (Qinghaosu) is a potent antimalarial sesquiterpene lactone isolated from the Chinese herb *Artemisia annua*. Arteether, a potent semisynthetic analogue of dihydroartemisinin is being developed by the World Health Organization as the artemisinin derivative of choice for the treatment of malaria. All three agents in doses of 400 and 600 mg/kg body weight were found to exhibit marked suppression of humoral responses, as measured by the hemolytic plaque assay, with arteether being the most potent. These agents did not alter the delayed-type hypersensitivity response to sheep erythrocytes at the same dose levels. In addition, all three agents were found not to possess any anti-inflammatory activity when tested on carrageenan-induced oedema. These results indicated that these agents have a selective immunosuppressive activity. They did not exhibit immunostimulating activity in contrast to what has been reported for sodium artesunate.

214. Titulaer, H., Vink-Blijleven, N. Assay of artelinic acid in serum by high-performance liquid chromatography. *Journal of Chromatography. A.*, 1993. v. 612 (2) p. 331-335. Database: Agricola. Not reviewed by team.

215. Wang Q, L.M. Wu, A.Y. Li, Y. Zhao, N.P. Wang. Experimental studies of antitumor effect of artesunate on liver cancer. *Zhongguo Zhong Yao Za Zhi*, 2001, Volume 26,

Issue 10, Pages 707-8, 720. Article in Chinese. Not Reviewed by team. Database: Pubmed.

**OBJECTIVE:** To observe the inhibiting effect of Artesunate on liver cancer in vitro and in vivo. **METHOD:** The mice bearing H22 solid and ascitic liver tumor were applied in vivo experiments. Microculture tetrazolium assay and colony-forming unit assay were applied to test the cytotoxicity to human hepatocarcinoma SMMC-7721 cell line in vitro. **RESULT:** The growth of solid tumor were obviously inhibited by Artesunate at the dose of 300 mg.kg-1.d-1 ig for 7 days. The tumor inhibiting rates of Artesunate were 49.1%, 48.7%, 46.6% in 3 experiments respectively. After administration of Artesunate, the survival rate of the mice bearing H22 ascitic liver tumor were increased to 45%. Compared with the control groups, the difference was statistically significant ( $P < 0.01$ ). In additional, Artesunate can synergize the antitumor activity of 5-fluorouracil. Artesunate showed evident cytotoxicity to human hepatocarcinoma SMMC-7721 cells, the IC50 of Artesunate being 2.07 micrograms.ml-1 in MTT experiment and 2.48 micrograms/ml in colony-forming unit experiment. **CONCLUSION:** Artesunate has marked antitumor activity in vitro and in vivo.

216. Webb, G. Immunomodulating compounds from traditional Chinese herbs. *Herbalgram. Fall 1997, Issue 41, Pages 19*. Database: Agricola.

217. Zhang, Junfeng; Tan, Jian; Pu, Qiang; Liu, Yinghua; He, Kaize. Study of antiviral activity against HSV-2 of the extract from *Artemisia annua* L. *Tianran Chanwu Yanjiu Yu Kaifa (2003), 15(2), 104-108*. Database: Scifinder Scholar.

The anti-herpes simplex virus type 2 activity of the crude water ext. of *Artemisia annua* L. was proven by cytopathogenic effect (CPE) method. By re-extg. with different solvents, the partially sepd. components maintaining antiviral activity were obtained. The 50% cytotoxicity concn. (CC50), 50% inhibition concn. (IC50) and therapy index (TI) of the crude water ext. and the partially sepd. components were 4.94 mg/mL, 0.128 mg/mL, 38.6 and 5.29 mg/mL, 1.45 mg/mL, 3.65, resp. measured by MTT method with 0.5 mg/mL of ACV as the pos. control, showing that the partially sepd. components had remarkable anti-HSV-2 activity in vitro.

218. Zhao KC, Song ZY. The pharmacokinetics of dihydroqinghasu given orally to rabbits and dogs. Article in Chinese. Not reviewed by team. *Yao Xue Xue Bao. 1990;25(2):147-9*. Database: Pubmed.

Qinghaosu (QHS), also known as artemisinin and arteannuin, is isolated from the Chinese herb *Artemisia annua* L. It is highly active against both chloroquine-sensitive and chloroquine-resistant strains of *P. berghei* and has been approved by the Ministry of Health for the treatment of malaria. When QHS is treated with sodium borohydride, dihydroqinghaosu (DH QHS) is resulted with the antimalarial activity enhanced several

fold. This paper reports the pharmacokinetics of DHQHS studied with the radioimmunoassay method. When the drug was given orally in tablet form to rabbits at doses of 10, 20 and 30 mg/kg, peak serum levels of 0.03, 0.05 and 0.13 micrograms/ml, respectively, were obtained in 1 to 2 h. The corresponding T<sub>1/2</sub> of the drug were found to be 1.19, 1.00 and 1.10 h and the MRTs were 1.73, 1.36 and 1.53 h. No significant difference between dosages used was observed. When dogs were given DHQHS tablets at the dose of 20 mg/kg, a peak serum concentration of 0.13 micrograms/ml was reached in about 2 h with a T<sub>1/2</sub> of 2.10 h and an MRT of 3.04 h. However, when dogs were given QHS tablets at the dose of 70 mg/kg, no drug was detected in the serum. It would appear that the bioavailability of DHQHS tablets is much higher than that of QHS when given orally to the dog.

219. Zhao KC, Song ZY. Pharmacokinetics of dihydroqinghaosu in human volunteers and comparison with qinghaosu. Article in Chinese. Not reviewed by team. *Yao Xue Xue Bao*. 1993;28(5):342-6. Database: Pubmed.

Qinghaosu (QHS), also known as artemisinin and arteannuin, is a novel type of sesquiterpene with a peroxide linkage isolated from the Chinese herb *Artemisia annua* L. Since its discovery as an antimalarial with low toxicity, hundreds of derivatives have been synthesized among them artesunate (ATS), artemether (ATM) and dihydroqinghaosu (DHQHS) were found to be more active than QHS itself. A suppository of QHS, a dual-pack dosage form of ATS (artesunic acid to be dissolved in sodium bicarbonate solution just before iv injection) and an oil solution of ATM for im injection had been approved by our Ministry of Health for clinical use. However, a preparation for oral administration is still not available. We have reported that when dogs were given QHS tablets orally at the dose of 70 mg/kg, no drug was detected in the serum using the RIA method, whereas appreciable serum concentration was found by the same method when dogs were given DHQHS tablets at a dose as low as 10 mg/kg. This paper reports the pharmacokinetics of DHQHS in man studied with the RIA method and compared with QHS. When DHQHS in tablet form was given to human volunteers at doses of 1.1-2.2 mg/kg, peak serum levels of 0.13-0.71 micrograms/ml were obtained in 1.33 h with MRT of 2.26-2.36 h. When QHS tablets were given at the dose as high as 15 mg/kg, however, the peak serum level found in 1.5 h was only 0.09 microgram/ml with MRT of 1.33 h. Therefore, the bioavailability of QHS tablets is only 1.62-10.08% that of DHQHS.

220. Zheng GQ. Cytotoxic terpenoids and flavonoids from *Artemisia annua*. *Planta Medica* 1994, Volume 60, Issue 1, Pages 54-7. Database: Pubmed.

The cytotoxic activity of nine terpenoids and flavonoids isolated from *Artemisia annua* was tested in vitro on several human tumor cell lines. These compounds are artemisinin, deoxyartemisinin, artemisinic acid, arteannuin-B, stigmaterol, friedelin, friedelan-3 beta-ol, artemetin, and quercetagenin 6,7,3',4'-tetramethyl ether. Friedelane-type triterpenoids were isolated for the first time from this plant. Artemisinin and

quercetagenin 6,7,3',4'-tetramethyl ether showed significant cytotoxicity against P-388, A-549, HT-29, MCF-7, and KB tumor cells.

221. Ziffer, H., Highet, R., Klayman, D.L. Artemisinin: an endoperoxidic antimalarial from *Artemisia annua* L. *Fortschritte der Chemie Organischer Naturstoffe*.1997. *Volume72 Pages 121-214*. Database: Agricola.

Antimalarial drug from China. *Science*, 1985, Volume 227, Issue 4697, Pages 1049  
Database: Academic Search Premier.

The herb *Artemisia annua* has been used for many centuries in Chinese traditional medicine as a treatment for fever in malaria. In 1971, Chinese chemists isolated from the leafy portions of the plant the substance responsible for its reputed medicinal action. This compound, called qinghaosu, has been used successfully in several thousand malaria patients in China.

222. Balint GA. Artemisinin and its derivatives: an important new class of antimalarial agents. *Pharmacology and Therapeutics*, 2001, *Volume 90, Issue 2-3, Pages 261-5*.  
Database: Pubmed.

Artemisinin and its derivatives are a potent new class of antimalarials, originated from *Artemisia annua*, L. The clinical efficacy of these drugs is characterized by an almost immediate onset and rapid reduction of parasitaemia. Their efficacy is high in such areas as well where multidrug-resistance is rampant, but in these areas, their combination with other (effective) antimalarials (e.g., mefloquine) is highly recommended. In this short review, the chemical structures, pharmacological properties, and clinical uses of artemisinin drugs are discussed.

223. Bodeker, Gerard. Searching for Antimalarials in Plants  
*Journal of Alternative & Complementary Medicine*, 2000, Vol. 6 Issue 2, Pages 127-129.  
Database: Academic Search Premier.

Focuses on experiments conducted by pharmacists on herbal medicines for the treatment of malaria. Number of people infected by malaria worldwide; Therapeutic use of the plant *Artemisia annua*; Resistance of the malaria parasite to artemisinin

224. Bodrug, M.V. Sweet wormwood [*Artemisia annua*], a promising essential oil plant. *Izvestiia Akademii Nauk Moldavskoi Ssr. [Kishinev, "Shtiintsa"]* 1980. (4) p. 79-80. In Russian. Not reviewed by team. Database: Agricola.

225. Bruce-Chwatt, L.J. Qinghaosu: A new antimalarial. *British Medical Journal*. Mar 13, 1982. v. 284 (6318) p. 767-768. Database: Agricola.

Qinghaosu and its synthesized derivatives, by-product of plant *Artemisia annua*, possible new antimalarial, preliminary reports show it may be superior to quinine for cases of cerebral malaria resistant to chloroquine, further study needed.

226. Chaudhary, S., Puri, S., Singh, C. Orally active artemisinin derivatives. *Medicinal Chemistry Research*. 12(6-7). 2003. 362. Database: Agricola.

227. Chen Y, Zhu SM, Chen HY. Progress in studies on the artemisinin (qinghaosu)-type antimalarials. Article in Chinese. Not Reviewed by team. *Yao Xue Xue Bao* 1998, Volume 33, Issue 3, Pages 234-9. Database: Pubmed.

228. Coghlan, Andy. New Scientist, 2003, Vol. 179 Issue 2409, Page 16  
Database: Academic Search Premier.

A hitherto unknown but vital weakness in the malaria parasite has been exposed by studying extracts from ancient Chinese anti-fever remedies. The discovery opens a new front in the fight against the parasite, which has become resistant in most parts of the world to the most common anti-malarial drug, chloroquine. Derived from the Chinese herb qinghao, or sweet wormwood (*Artemisia annua*), the extracts have already helped millions of patients in south-east Asia who would otherwise have suffered or died when conventional drugs failed (New Scientist, 13 July 1996). Now researchers have discovered how the drugs, called artemisinins, actually work, revealing a chink in the *Plasmodium falciparum* parasite's armour. The chink is one of the two enzymes that enable the parasite to pump the correct amount of calcium into its cell membranes. "Artemisinin hits one of those pumps directly," says Sanjeev Krishna of St George's Hospital Medical School in London, the head of the research team. Once the calcium pump has been disabled, the parasite dies within hours, although Krishna doesn't yet know the precise mechanism. The discovery of the enzyme, called *Plasmodium falciparum* ATP6, or PfATP6, provides a juicy new target for drug makers and for researchers like Krishna who want to improve the killing power of artemisinins. What is more, the gene that encodes the pump can now be monitored in parasites worldwide to see if it mutates to make the parasite resistant to artemisinins. "We could look for and anticipate resistance, instead of responding when it happens," says Krishna. The discovery that artemisinins hit the enzyme came as a surprise, because the assumption till now has been that the extracts damage chambers where the parasite digests blood meals. To prove the enzyme was the key, Krishna's team isolated it by injecting the messenger RNA that codes for PfATP6 into eggs of the frog *Xenopus laevis*. By comparing the effects of artemisinins with a chemical known to block the enzyme's action, as well as with drugs such as chloroquine, they were able to show that artemisinins block PfATP6 both in the eggs and in intact malarial parasites (Nature, vol 424, p 957).

Manufactured in China and Vietnam, artemisinins are already having a huge impact in areas of south-east Asia where resistance to other drugs is rife. Krishna says that the

drugs are now beginning to prove their worth in Africa too, and combinations of artemisinin and other drugs are proving most effective. Together with Peter Kremsner of the University of Tübingen in Germany, Krishna's team is testing a combination of artemisinins and amodiaquine on children in Gabon. Robert Ridley, coordinator of product development for tropical diseases at the World Health Organization in Geneva, says that the discovery should allow new artemisinins to be developed that work in 3 to 4 days, rather than the week that current formulations take. This would make it easier for patients to stick to a drug regime, he says. And the discovery of the vulnerable enzyme should encourage the search for new drugs, which are desperately needed as resistance to older drugs escalates. "The extracts have already helped millions of patients in south-east Asia who would otherwise have suffered or died when conventional drugs failed"

229. Delfosse M. *Artemisia annua* for the treatment of malaria. Article in French. *J Pharm Belg*, 1998, Volume 53, Issue 4, Pages 276-7. Database: Pubmed, Scifinder Scholar.

230. Dhingra V, Vishweshwar Rao K, Lakshmi Narasu M. Current status of artemisinin and its derivatives as antimalarial drugs. *Life Sciences*, 2000, Volume 66, Issue 4, Pages 279-300. Database: Pubmed.

Artemisinin is a promising and a potent antimalarial drug, which meets the dual challenge posed by drug-resistant parasites and rapid progression of malarial illness. This review article focuses on the progress achieved during the last years in the production of artemisinin from *Artemisia annua*. The structure, biosynthesis and analysis of artemisinin and its mode of action are described. The review also focuses on clinical studies, toxicity studies, pharmacokinetics and activity of artemisinin related compounds. The production strategies including organic synthesis, extraction from plants, in vitro cultures and alternative strategies for enhancing the yields are also discussed.

231. Dobson MJ. Bitter-sweet solutions for malaria: exploring natural remedies from the past. *Parassitologia* 1998, Volume 40, Issue 1-2, Pages 69-81. Database: Pubmed.

This paper explores "a wonderful cure" for malaria used successfully by Robert Talbor, an apothecary's apprentice in the English marshes, to treat Essex smugglers and European Royalty in the seventeenth century. The basis of this cure is identified as "quinquina" from the bark of the South American *Cinchona* tree. The story of Robert Talbor and his secret remedy for malaria opens up a set of intriguing questions about the early history of "quinquina", the subsequent development of quinine, the use of higher plants for antimalarial drugs, including the Chinese plant *Artemisia annua* L., and the value of unlocking the secrets of the past in our search for strategies to control malaria.

232. Gerriets, M. Wormwood: a new crop to combat malaria. *Agricultural Research* 1992. v. 40 (6) p. 24-25. Database: Agricola.

233. Huang L, Liu JF, Liu LX, Li DF, Zhang Y, Nui HZ, Song HY, Zhang CY. Antipyretic and anti-inflammatory effects of *Artemisia annua* L. Article in Chinese. Not reviewed by team. *Zhongguo Zhong Yao Za Zhi*. 1993 Jan;18(1):44-8, 63-4. Database: Pubmed.

The antipyretic, heat-resisting anti-inflammatory analgesic and bacteriostasis effects of water extracts ethyl-acetate and n-butyl alcohol extracts of *Artemisia annua* are reported. Animal experiment has demonstrated that qinghao acid is one of the actively bacteriostatic constituents. Scopoletin is one of the anti-inflammatory constituents of *Artemisia annua*.

234. Jung, Mankil, K.Lee,H. Kim, M. Park. Recent Advances in Artemisinin and Its Derivatives as Antimalarial and Antitumor Agents. *Current Medicinal Chemistry*, 2004, Volume 11 Issue 10, Pages 1265-1284. Database: Academic Search Premier.

Artemisinin, the first and last naturally occurring 1, 2, 4-trioxane originated from *Artemisia annua*, L. and its derivatives are a potent class of antimalarial drugs. The clinical efficacy of these drugs is characterized by an almost immediate onset and rapid reduction of parasitemia, and it is high in such areas as well where multidrug-resistance is rampant. Furthermore, artemisinin and many of its analog possess not only antiparasitic effect against *Plasmodium falciparum*, *Schistosoma japonicum* and *Clonorchis sinensi* but also immuno-modulation effects, and antitumor activities. This review covers the chemistry of artemisinin including synthesis of acetal-, non acetaltype C-12 analogs, C-11- and C-13 derivatives from artemisitene, ring-contracted derivatives, dimers, and trimers. Modes of biological action of artemisinin - derived analogs are also reviewed. The main objective of this article is to review the literatures of recent progress taken place in chemistry, mode of biological actions of artemisinin, and its derivatives as antimalarial and antitumor agents during the last three years (1999-2001).

235. Klayman, D.L. *Artemisia annua*: from weed to respectable antimalarial plant. *Acs Symposium Series*. 1993. (534) p. 242-255. Database: Agricola.

In the early 1970's, a potent and essentially non-toxic antimalarial agent was isolated from qing hao (*Artemisia annua*), a plant used in Chinese folk medicine for some 20 centuries. The active compound, artemisinin (qinghaosu), is a sesquiterpene lactone bearing an unusual endoperoxide group that is essential for its activity. Artemisinin is effective against drug-sensitive and -resistant *Plasmodium falciparum* and *P. vivax*. Oil-soluble derivatives of artemisinin, such as artemether and arteether, and water-soluble derivatives, such as sodium artesunate and sodium artelinate, have increased potency and allow fewer recrudescences. The latter two have special application for the treatment of potentially fatal cerebral malaria. Recent animal studies suggest that the artemisinin class of compounds may be effective when administered transdermally.

236. Klayman DL. Qinghaosu (artemisinin): an antimalarial drug from China. *Science*. 1985 May 31;228(4703):1049-55. Database: Pubmed.

The herb *Artemisia annua* has been used for many centuries in Chinese traditional medicine as a treatment for fever and malaria. In 1971, Chinese chemists isolated from the leafy portions of the plant the substance responsible for its reputed medicinal action. This compound, called qinghaosu (QHS, artemisinin), is a sesquiterpene lactone that bears a peroxide grouping and, unlike most other antimalarials, lacks a nitrogen-containing heterocyclic ring system. The compound has been used successfully in several thousand malaria patients in China, including those with both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. Derivatives of QHS, such as dihydroqinghaosu, artemether, and the water-soluble sodium artesunate, appear to be more potent than QHS itself. Sodium artesunate acts rapidly in restoring to consciousness comatose patients with cerebral malaria. Thus QHS and its derivatives offer promise as a totally new class of antimalarials.

237. Lee MR. Plants against malaria, part 2: *Artemisia annua* (Qinghaosu or the sweet wormwood). *J R Coll Physicians Edinb*. 2002, Volume 32, Issue 4, Pages 300-5 Database: Pubmed.

238. Li Y, Wu YL. An over four millennium story behind qinghaosu (artemisinin): A fantastic antimalarial drug from a traditional Chinese herb. *Current Medicinal Chemistry*. 10(21). November 2003. 2197-2230. Database: Biosis.

239. Li Y, Wu YL. How Chinese scientists discovered qinghaosu (artemisinin) and developed its derivatives? What are the future perspectives? *Med Trop (Mars)* 1998, Volume 58 (3 Suppl), Pages 9-12. Database: Pubmed.

Since the middle of this century and especially since the 1960s and 1970s. Chinese scientists have put considerable effort and resources into the search for new antimalarial compounds extracted from Chinese traditional herbs. Archaeological findings indicate that qinghao (*Artemisia annua* L.) has been used as a traditional remedy in China for over two thousand years. Its antimalarial principle was finally isolated in 1971 and named artemisinin or qinghaosu (meaning the principle of qinghao in Chinese). Its rapid action, low toxicity and powerful effect against *falciparum* malaria made it a favored subject for research. In 1976, the unique structure of the molecule, characterized by an endoperoxide and an alternative O-C-O-C segment, was identified. The specific lactone reduction discovered during the determination of the structure opened the way for the synthesis of qinghaosu derivatives, and thereafter a series of more active and more oil- or water-soluble derivatives was developed. Subsequent studies of the structure/activity relationship led to the discovery of dihydroartemisinin, artemether and artesunate. Now qinghaosu and these three derivatives are being used around the world as effective new

antimalarial drugs in the fight against falciparum malaria, including multi-drug-resistant *Plasmodium falciparum*. At the present time new qinghaosu analogues or derivatives are being developed and studies of their structure/activity relationships, their antimalarial mechanisms, their interaction with ferrous ions and the DNA damage associated with these processes are being actively pursued. In addition, recent studies also indicate that some qinghaosu derivatives have other bioactivities, including antiparasitic (against *Schistosoma japonicum*, *Toxoplasma gondii* and so on) and anticancer activities. Research into qinghaosu and its derivatives has already produced and will no doubt continue to produce results of the utmost importance in the fight against malaria and other diseases.

240. Lwin M, Maun C, Aye KH. Trial of antimalarial potential of extracts of *Artemisia annua* grown in Myanmar. *Trans R Soc Trop Med Hyg.* 1991 Jul-Aug; 85(4):449. Database: Pubmed.

241. Mercereau-Puijalon, Odile, Fandeur, Thierry. Antimalarial activity of artemisinin: Identification of a novel target? *Lancet (North American Edition).* 362(9401). 2003 2035-2036. Database: Agricola.

242. Moneton P, Ducret JP. Positioning, labelling and control of medical information: artesunate strategy and Arsumax development story. *Med Trop (Mars).* 1998, Volume 58, Issue 3 Suppl, Pages 70-2. Database: Pubmed.

Sanofi decided, some years ago, to help developing a molecule of a new type called artesunate, a semisynthetic derivative from Qinghaosu, or artemisinin, a sesquiterpenic lactone extracted from the leaves of a world wide spread plant: *Artemisia annua*. After having signed a secrecy agreement with a local pharmaceutical company in China manufacturing artesunate, Sanofi started, in 1993, the assessment of all the data transmitted as well as the plant involved in the artesunate tablet manufacturing. As conclusions of the whole audit performed on this project were judged satisfactory, Sanofi signed a cooperation agreement with this Chinese pharmaceutical firm in order to be allowed to start further development work required to guarantee quality and safety of artesunate 50 mg tablets called Arsumax, and to complete a registration dossier acceptable for Health Authorities in French-speaking Africa. Pharmaceutical, pharmacotoxicological and clinical documentation were widely completed, and entirely reformed according to European guidelines. The registration dossier was submitted in all French speaking African countries. The approval has been obtained in 13 countries. Due to its fast efficacy, its absence of undesirable effects, its presentation in tablets, its quite simple dosage (one box for a treatment), Arsumax positioned itself as an accurate second line antimalaric treatment.

243. Mueller MS, N. Runyambo N, I. Wagner, S. Borrmann, K. Dietz, L. Heide. Randomized controlled trial of a traditional preparation of *Artemisia annua* L.

(Annual Wormwood) in the treatment of malaria. *Trans R Soc Trop Med Hyg.* 2004, Volume 98, Issue 5, Pages 318-21. Database: Pubmed.

The Chinese medicinal plant *Artemisia annua* L. (Annual Wormwood) contains the antimalarial compound artemisinin. The locally grown herb may offer an additional tool for the control of malaria, especially in poor countries where modern antimalarial drugs are often unavailable. In an open, randomized, controlled pilot trial, we investigated the efficacy and safety of traditional tea preparations of *Artemisia annua* in the treatment of uncomplicated malaria. Treatment resulted in a quick resolution of parasitaemia and of clinical symptoms. After 7 d of medication, cure rates were on average 74% for the *Artemisia* preparations compared with 91% for quinine. However, recrudescence rates were high in the *Artemisia* groups. Therefore, monotherapy with *Artemisia annua* L. cannot be recommended as alternative to modern antimalarials, but may deserve further investigation.

244. Peters W, Robinson BL, Rossier JC, Jefford CW. The chemotherapy of rodent malaria. XLVIII. The activities of some synthetic 1,2,4-trioxanes against chloroquine-sensitive and chloroquine-resistant parasites. Part 1: Studies leading to the development of novel cis-fused cyclopenteno derivatives. *Ann Trop Med Parasitol.* 1993, Volume 87, Issue 1, Pages 1-7. Database: Pubmed.

The new Chinese antimalarial blood schizontocide, artemisinin, derived from the plant *Artemisia annua*, displays a high level of activity against polyresistant *Plasmodium falciparum*. Several synthetic 1,2,4-trioxanes were examined in a search for compounds that exhibit a similar type of action against drug-resistant parasites. This paper, the first of a series, describes the examination of these trioxanes against drug-sensitive and drug-resistant malaria parasites in a rodent model, using artemisinin and arteether as comparison standards. Cis-fused cyclohexeno-1,2,4-trioxanes (10-17) substituted with various side-chains revealed for the most part variable but weak antimalarial activity. On the other hand, cis-fused cyclopenteno-1,2,4-trioxanes (18-19) showed greater activity, 19 showing about 1/30th of the activity of arteether against drug-sensitive *Plasmodium berghei* in vivo, thereby providing a clue to the structure-activity relationship.

245. Phan VT. Artemisinine and artesunate in the treatment of malaria in Vietnam (1984-1999). Article in French. *Bull Soc Pathol Exot.* 2002, Volume 95, Issue 2, Pages 86-8. Database: Pubmed.

The long history of the use of *Artemisia annua* L. to treat malaria (called Qinghao in China and Thanh hao in Vietnam) has led Vietnamese scientists to manufacture locally preparations of artemisinine and artesunate, to test their tolerance for human beings as well as their efficiency in treating *P. falciparum* and *P. vivax* infections. Associating these drugs with antibiotics (such as tetracycline or doxycycline) could be an interesting topic for future research. Under the auspices of the National Program against Malaria, specialists will try to prevent the occurrence of drug resistance in *Plasmodium* and to propose new associations of drugs.

246. Phillipson JD, Wright CW. Can ethnopharmacology contribute to the development of antimalarial agents? *J Ethnopharmacol.* 1991, Volume 32, Issue 1-3, Pages 155-65. Source: Pubmed.

The resistance of *Plasmodium falciparum*, the cause of tertian malaria, to synthetic antimalarials, together with the resistance of the vector mosquitoes to insecticides, has resulted in a resurgence in the use of quinine and a search for new antimalarial agents. In recent years, artemisinin, isolated from *Artemisia annua* which is used in Chinese traditional medicine for the treatment of malaria, has proved to be effective in the treatment of cerebral malaria due to chloroquine-resistant strains of *P. falciparum*. The development of in vitro tests utilising *P. falciparum* obtained from malaria patients means that it is possible to use bioassay guided fractionation of active extracts in order to isolate active principles. A number of laboratories throughout the world are currently investigating plants used in traditional medicine for their active constituents. Some of their results will be described and in particular two aspects of our investigations with species of Simaroubaceae and Menispermaceae will be discussed. There is every possibility that such approaches which use leads from Ethnopharmacology will result in the development of new antimalarial agents. It is vitally important to those populations relying on traditional medicines for the treatment of malaria that the safety and efficacy of such medicines be established, their active principles determined and that reproducible dosage forms be prepared and made available for use.

247. Tagboto S, Townson S. Antiparasitic properties of medicinal plants and other naturally occurring products. *Advances in Parasitology* 2001, Volume 50, Pages 199-295. Database: Pubmed.

Parasitic diseases remain a major public health problem affecting hundreds of millions of people, particularly in tropical developing countries. The limited availability and affordability of pharmaceutical medicines means that the majority of the world's population depends on traditional medical remedies, and it is estimated that some 20,000 species of higher plant are used medicinally throughout the world. Many well-known drugs listed in the modern pharmacopoeia have their origins in nature, including, for example, quinine from the bark of the *Cinchona* tree for the treatment of malaria, which has been followed by the subsequent development of the synthetic derivatives chloroquine, amodiaquine, primaquine and mefloquine. More recently, the wider recognition of the antimalarial activity of artemisinin from the herb *Artemisia annua* has led current research to focus on the development of a large number of synthetic and semisynthetic compounds, which are more active than artemisinin. There is an increasing awareness of the potential of natural products, which may lead to the development of much-needed new antiparasitic drugs. In this chapter, we have drawn together a comprehensive list of medicinal plants and other natural products that have been shown to have activity against human and, to a lesser

extent, animal parasites. In addition, some of the opportunities and difficulties in working with natural products have been reviewed and discussed, including the problems involved with evaluating complex mixtures of compounds which may occur in extracts, problems associated with differentiating between general cytotoxicity and genuine antiparasitic activity, and the hope that new technologies will rapidly accelerate new drug discovery and development in this field. Nevertheless, the way forward for natural product medicines, including the conservation of recognized natural products and protection of general biodiversity, the discovery and development process, and the promotion and usage of existing remedies, presents some difficult challenges. Following an initiative by the World Health Organization in August 2000, there is now the opportunity to evaluate scientifically many more traditional medicines and other natural products in validated antiparasite and toxicity screens, which will help establish which substances have potential for new pharmaceutical products. The use of 'untested' traditional medicines will no doubt continue, and there is an urgent need to distinguish between the efficacious and safe products and the ineffective and/or unsafe products, particularly since many remedies are being more widely promoted in developing countries.

248. van Agtmael MA, Eggelte TA, van Boxtel CJ. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. *Trends Pharmacol Sci.* 1999, Volume 20, Issue 5, Pages 199-205. Database: Pubmed.

Registration in Europe of several artemisinin drugs for the treatment of malaria can soon be expected. Artemisinin is isolated from the herb *Artemisia annua*, in use in China more than 2000 years as a herbal tea against fever. Artemisinin drugs are being used extensively in South-East Asia and increasingly in Africa. Active derivatives have been synthesized - artemether, arteether and artesunate - which are used for oral, intramuscular, rectal and intravenous administration. The origin, mechanism of action, efficacy and safety in patients, the pharmacokinetics and the position of this group of compounds among existing antimalarials are discussed in this review.

249. Wan YD, Zang QZ, Wang JS. Studies on the antimalarial action of gelatin capsule of *Artemisia annua*. Article in Chinese. Not reviewed by team. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* 1992;10(4):290-4. Database: Pubmed.

The pharmacological and clinical effects of gelatin capsule of *Artemisia annua* (GCAA) were investigated. The results revealed that the LD50 was 162.5 +/- 10.1g (crude drug)/kg and ED50 was 11.9 +/- 2.4g (crude drug) for clearance of parasitemia in mice infected with *Plasmodium berghei* therapeutic index being 13.6, which was 3.5 times more than that of artemisinin. The cure rate of COEA for *Plasmodium berghei* and *P. vivax* infections was 100%, as well as just like that of the extract tablets of *Artemisia annua* and chloroquine. This formulation was found to be better than chloroquine in fever subsidence and disappearance of malarial symptoms, while the recrudescence rate was

still high, the latter could be inhibited by increasing therapeutic course or daily dosing time or by combination with primaquine.

250. White NJ. Artemisinin: current status. *Trans R Soc Trop Med Hyg.* 1994, Issue 88, Suppl 1, Pages S3-4. Database: Pubmed.

The compounds derived from the Chinese medicinal plant qinghao (*Artemisia annua*) are the most rapidly acting of all antimalarial drugs. They are effective when given parenterally, orally or by suppository. No serious adverse effect has yet been reported in humans. The artemisinin derivatives already have an established role in the treatment of multi-drug resistant falciparum malaria, but their wider use will depend on the results of current mortality and toxicity studies.

251. Woerdenbag HJ, Pras N, van Uden W, Wallaart TE, Beekman AC, Lugt CB. Progress in the research of artemisinin-related antimalarials: an update. *Pharm World Sci.* 1994, Volume 16, Issue 4, Pages 169-80. Database: Pubmed.

Artemisinin, a sesquiterpene lactone endoperoxide isolated from *Artemisia annua* L., and a number of its semisynthetic derivatives have shown to possess antimalarial properties. They are all effective against *Plasmodium* parasites that are resistant to the newest and commonly used antimalarial drugs. This article gives a survey of the literature dealing with artemisinin-related antimalarial issues that have appeared from the end of 1989 up to the beginning of 1994. A broad range of medical and pharmaceutical disciplines is covered, including phytochemical aspects like the selection of high-producing plants, analytical procedures, and plant biotechnology. Furthermore, the organic synthesis of artemisinin derivatives is discussed, as well as their mechanism of action and antimalarial activity, metabolism and pharmacokinetics, clinical studies, side-effects and toxicology, and biological activities other than antimalarial activity.

252. Woerdenbag HJ, Lugt CB, Pras N. *Artemisia annua* L.: a source of novel antimalarial drugs. *Pharm Weekbl Sci.* 1990 Oct 19;12(5):169-81. Database: Pubmed.

*Artemisia annua* L. contains artemisinin, an endoperoxide sesquiterpene lactone, mainly in its leaves and inflorescences. This compound and a series of derivatives have attracted attention because of their potential value as antimalarial drugs. In this review a survey of the currently available literature data is given. It includes phytochemical aspects, such as constituents of *A. annua*, the artemisinin content during the development of the plant and its biosynthesis, isolation, analysis and stability. Total chemical synthesis of artemisinin is referred to, as well as structure-activity relationships of derivatives and simplified analogues. Pharmacological studies are summarized, including the mechanism of action, interaction of the antimalarial activity with other drugs, possible occurrence of resistance to artemisinin, clinical results, toxicological aspects, metabolism and pharmacokinetics. Finally, plant cell biotechnology is mentioned as a possible means to

obtain plants and cell cultures with higher artemisinin contents, allowing an industrial production of pharmaceuticals containing this novel drug.

253. Ziffer H, Highet RJ, Klayman DL. Artemisinin: an endoperoxidic antimalarial from *Artemisia annua* L. *Fortschr Chem Org Naturst.* 1997, Volume 72, Pages 121-214. Database: Pubmed.

#### **Antimicrobial and AntiProtozoal Literature on *Artemisia annua*:**

254. Juteau F, Masotti V, Bessiere JM, Dherbomez M, Viano J. Antibacterial and antioxidant activities of *Artemisia annua* essential oil. *Fitoterapia* 2002, Volume 73, Issue 6, Pages 532-5. Source: Pubmed.

The essential oil of *Artemisia annua* aerial parts, consisting of camphor (44%), germacrene D (16%), trans-pinocarveol (11%), beta-selinene (9%), beta-caryophyllene (9%) and artemisia ketone (3%), was screened for its antimicrobial activity. The essential oil remarkably inhibited the growth of tested Gram-positive bacteria *Enterococcus hirae* and both tested fungi. This oil has shown an antioxidant activity equivalent to 18% of the reference compound (alpha-tocopherol).

255. Liu CH, Zou WX, Lu H, Tan RX. Antifungal activity of *Artemisia annua* endophyte cultures against phytopathogenic fungi. *Journal of Biotechnology*, 2001, Volume 88, Issue 3, Pages 277-82. Source: Pubmed.

*Artemisia annua*, well recognized for its production of antimalarial drug artemisinin, is seldom attacked by any of phytopathogenic fungi, which could be partially associated with the presence of endophytes. Present investigation is aiming at disclosing whether the endophytes inside *A. annua* produce antifungal substances. A total of 39 endophytes were isolated and fermented, and the ferment broth was evaluated in vitro for the antifungal activity against crop-threatening fungi *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, *Helminthosporium sativum*, *Fusarium graminearum*, *Gerlachia nivalis* and *Phytophthora capsici*. These plant pathogens are still causing wheat take-all, sharp eyespot, common rot, scab, snow mould, and pepper phytophthora blight, respectively. Out of 39 endophytes investigated, 21 can produce in vitro substances that are inhibitory to all or a few of the tested phytopathogens whereas the rest yielded nothing active. Moreover, the most active broth of endophyte IV403 was extracted with EtOAc and n-butanol, and comparisons of the antifungal activity of the extracts indicated that the major active metabolites were EtOAc-extractable.

256. Stermitz F.R., L.N. Scriven, G. Tegos, K. Lewis. Two flavonols from *Artemisia annua* which potentiate the activity of berberine and norfloxacin against a resistant strain of *Staphylococcus aureus*. *Planta Medica* 2002, Volume 68, Issue 12, Pages 1140-1.

Source: Pubmed.

Bioassay-guided fractionation of an extract of *Artemisia annua* L. (Asteraceae) was conducted in order to assess the possible presence in the plant material of inhibitors of bacterial multidrug resistance pumps. Fractions were tested for *Staphylococcus aureus* growth inhibition in the presence of a subinhibitory dose of the weak antibacterial alkaloid berberine. Active fractions yielded the flavones chrysofenol-D and chrysofenetin, which themselves had very weak growth inhibitory action, but which made a potent combination with berberine. In comparison with work on other flavonols, it is likely that potentiation is due to the inhibition of an *S. aureus* multidrug resistance (MDR) pump. These same two flavonols were earlier reported to potentiate the activity of artemisinin against *Plasmodium falciparum*.

257. Bailey NJ, Y. Wang, J. Sampson, W. Davis, I. Whitcombe, P.J. Hylands, S.L. Croft, E. Holmes. Prediction of anti-plasmodial activity of *Artemisia annua* extracts: application of <sup>1</sup>H NMR spectroscopy and chemometrics. *Journal of Pharmaceutical and Biomedical Analysis*, 2004, Volume 35, Issue 1, Pages 117-126. Source: Pubmed.

We describe the application of <sup>1</sup>H NMR spectroscopy and chemometrics to the analysis of extracts of *Artemisia annua*. This approach allowed the discrimination of samples from different sources, and to classify them according to anti-plasmodial activity without prior knowledge of this activity. The use of partial least squares analysis allowed the prediction of actual values for anti-plasmodial activities for independent samples not used in producing the models. The models were constructed using approximately 70% of the samples, with 30% used as a validation set for which predictions were made. Models generally explained >90% of the variance, R(2) in the model, and had a predictive ability, Q(2) of >0.8. This approach was also able to correlate <sup>1</sup>H NMR spectra with cytotoxicity (R2=0.9, Q2=0.8). This work demonstrates the potential of NMR spectroscopy and chemometrics for the development of predictive models of anti-plasmodial activity.

258. Devi, C. Usha, Valecha, Neena, Atul, Pillai, C. R. Antiplasmodial effect of three medicinal plants: A preliminary study. *Current Science (Bangalore)*. 80(8). 25 April, 2001. 917-919. Database: Biosis.

259. Kim JT, Park JY, Seo HS, Oh HG, Noh JW, Kim JH, Kim DY, Youn HJ. In vitro antiprotozoal effects of artemisinin on *Neospora caninum*. *Vet. Parasitol.* 2002, Volume 103, Issue 1-2, Pages 53-63. Source: Pubmed.

*Neospora caninum* is an intracellular apicomplexan parasite that infects a wide range of mammals and has been associated with abortion in cattle worldwide. Artemisinin is an effective antimalarial compound derived from a traditional Chinese herbal remedy, qinghao or *Artemisia annua* L. In the study reported, the cultured host cells (vero cells or mouse peritoneal macrophages) infected with *N. caninum* tachyzoites were incubated

with alpha-MEM (minimal essential medium) 10%HS supplemented with various concentration or artemisinin (20, 10, 1, 0.1 and 0.01 microg/ml) to examine the efficacy of artemisinin against *N. caninum* tachyzoites intracellular multiplication. In long-term studies, at 20 or 10 microg/ml for 11 days, artemisinin reduced *N. caninum* and completely eliminated all microscopic foci of *N. caninum*. At 1 microg/ml for 14 days, artemisinin reduced *N. caninum* and completely achieved elimination of all microscopic foci of *N. caninum*. There was no apparent toxicity to host cells in long-term studies. In short-term studies, at  $>$  or  $=$  0.1 microg/ml, artemisinin reduced *N. caninum* tachyzoites intracellular multiplication, significantly ( $P < 0.05$ ) and appeared to depend on the artemisinin concentrations. Pretreatment of host cells or *N. caninum* tachyzoites with artemisinin had no effect on *N. caninum* tachyzoites intracellular multiplication. These results demonstrate that artemisinin inhibited *N. caninum* tachyzoites intracellular multiplication.

260. Olliaro, Piero L., Haynes, Richard K., Meunier, Bernard, Yuthavong, Yongyuth Possible modes of action of the artemisinin-type compounds. *Trends in Parasitology*. 17(3). March, 2001. 122-126. Database: Biosis.

Artemisinin-type compounds are used for the treatment of uncomplicated and severe forms of malaria. They reduce parasitaemia more rapidly than any other antimalarial compound known, and are effective against multidrug-resistant parasites. However, uncertainties remain as to how they act on the parasite and cause toxicity. In this review, we summarize current ideas.

261. Sharma P, Sharma JD. Plants showing antiplasmodial activity--from crude extracts to isolated compounds. *Indian Journal of Malariology*, 1998, Volume 35, Issue 2, Pages 57-110. Source: Pubmed.

The derivation of important antimalarial compounds started with the discovery of Cinchona bark powder with wine. Subsequently, post World War-I was a period of intensive work in maintaining such ethnobotanical records, in which the use of quinine has remained the drug of choice in malaria. After World War-II new chemical techniques were used to fractionate and isolate, and also for structure determinations, which led to an ever increasing number of potential antiplasmodial compounds. Recently experimental studies in animals and in clinical trials, showed the emergence of CQ-sensitive and CQ-resistant strains of Plasmodium. This paper is an attempt to update a historical list of antimalarial plants and their natural products as studied by pharmacognostic extraction methods of crude drug research of those times. Further an attempt has been undertaken to list the compounds as classified into three major groups, namely alkaloids, terpenes and quassinoids and aromatic and miscellaneous compounds. The most promising is a quassinoid, artemisinin derived from *Artemisia annua* which has caused a resurgence for the quest of newer antimalarial compounds.

262. Shuhua X, Chollet J, Weiss NA, Bergquist RN, Tanner M. Preventive effect of artemether in experimental animals infected with *Schistosoma mansoni*. Parasitol Int. 2000, Volume 49, Issue 1, Pages 19-24. Source: Pubmed.

The effect of artemether, an antimalarial drug developed from the plant *Artemisia annua*, has been tested against the larval stages of *Schistosoma mansoni* covering the time from skin penetration to the early adult liver-stage. The results show that the experimental animals used (hamster and mice) do not develop schistosomiasis *mansoni* if treated with artemether during the first month after infection. The parasite was found to be especially susceptible between the 3rd and 4th week after infection, resulting in worm reductions of 75.3-82.0% compared to non-treated controls. This level was boosted to 97.2-100% when the animals were subjected to various schedules of repeated treatment. Almost complete protection was also reached in parallel experiments with repeated infections carried out to mirror more closely the real situation of trickle infection.

***Artemisia annua* Patents of Interest to BIONNEX and Madagascar:**

263. Jain, Dharam Chand, Pant, Neerja, Gupta, Madan Mohan, Bhakuni, Rajendra Singh Verma, Ram Kishor, Tandon, Sudeep, Gupta, Shiv Kumar, Tewari, Amit, Kahol, Atul Prakash, Kumar, Sushil

Process for the isolation of compound scopoletin useful as nitric oxide synthesis inhibitor Official Gazette of the United States Patent & Trademark Office Patents. 1254(2). Jan. 8, 2002.

Database: Biosis

The invention relates to a process for the isolation of compound scopoletin which is used as nitric oxide synthesis inhibitor from ***Artemisia annua*** and other plant families, said process comprising extraction of dried powdered material of different plant parts with an aqueous acetone solvent in the ratio of 1:5 for 6 to 8 hrs., concentration of the extracted solvent upto 30% of its original extract under vacuum, partitioning the concentrated extract with halogenated solvent to transfer scopoletin in the non-polar halogenated solvent, drying halogenated solvent over anhydrous sodium sulphate and evaporating the solvent, crystallizing the residues in methanol and filtering the crystals, concentrating the filtrate and chromatographed on silica gel, eluting scopoletin in chloroform methanol mixture; and crystallization of the fractions containing the scopoletin to get the pure scopoletin compound.

264. Kumar, Sushil, Gupta, Shiv Kumar, Singh, Digvijay, Gupta, Madan Mohan, Jain, Dharam Chand, Kahol, Atul Prakash Khanuja, Suman Preet Singh., Ram, Govind

Process for isolating artemisinin from ***Artemisia annua***

Official Gazette of the United States Patent & Trademark Office Patents. 1279(1). Feb. 3, 2004.

The present invention relates to a process for the isolation of artemisinin, an antimalarial agent from the herb of the *Artemisia annua* plant, comprising of extracting the herb with

ethanol, partitioning of the extract between water and hexane, followed by evaporative crystallization of artemisinin from hexane phase to produce substantially pure artemisinin.

265. Kumar, Sushil, Gupta, Shiv Kumar, Gupta, Madan Mohan, Verma, Ram Kishor, Jain, Dharam Chand, Shasany, Ajit Kumar, Darokar, Mahendra Pandurang, Khanuja, Suman Preet Singh [Inventor]

Method for maximization of artemisinin production by the plant *Artemisia annua*  
Official Gazette of the United States Patent & Trademark Office Patents. 1258(4). May 28, 2002.

The invention provides a method for maximization of artemisinin yield of the plant *Artemisia annua*, said method comprising sowing seeds of *Artemisia annua* plant on raised bed nursery during second and third week of December and maintaining the moisture throughout; transplanting seedlings thus obtained bearing at least 5-15 leaves into the main field fertilized with fertilizer, preferably NPK @ 80,40,40 kg/ha to achieve a population density of 50,000 to 200,000 per ha followed by light irrigation in the second week of March and irrigation every fortnight thereafter; harvesting the crop four times by cutting the plant tops leaving 75-100 cm part of plant for further regeneration, the said harvests are performed in a manner that the first harvest is done in fourth week of May, second harvest in third week of July, third harvest in second week of September and fourth harvest in third week of October of each year; and at each harvesting time care is taken to care at least one green branch, and extracting artemisinin from the plant tissue immediately after each harvest.

266. Patra, Dharani Dhar, Kiran, Usha, Anwar, Mohammed, Chand, Sukhmal, Kumar, Sushil.

Formulation useful as a nitrification and urease inhibitor and a method of producing the same. Official Gazette of the United States Patent & Trademark Office Patents. 1252(2). Nov. 13, 2001.

The invention relates to a novel formulation useful as a nitrification and urease inhibitor, said formulation comprising an effective amount of nitrogenous fertilizer, castor oil and oil derived from *Artemisia annua*, in an amount sufficient to enhance the nitrification activity of the formulation, a method for producing the formulation and method for applying the same to soil.

267. Wheatley, Gary William, Chapman, Thomas Brian [Inventor]

Method for producing an extract containing artemisinin

Official Gazette of the United States Patent & Trademark Office Patents. 1242(5). Jan. 30, 2001.

Database: Biosis

A method of preparation of an artemisinin extract comprising the steps of extraction of **Artemisia annua** with liquid carbon dioxide and allowing the carbon dioxide to evaporate from the resultant mixture.

A quantity of roughly ground aerial parts of the herb *Artemisia annua* was packed into the pressure vessel and allowed to equilibrate at the desired pressure and temperature with the carbon dioxide. After this time, the flow rate was adjusted to allow an extraction ratio of 5:1 of carbon dioxide to substrate. Table summarises the results;

Experiment	Pressure/psi	Temperature /°C	Co-solvent	Yield /%	Artemisinin /%
1	1500	27	None	1.1	14
2	1500	27	10% Ethanol	3.2	3.3
3	4500	50	None	0.2	2.0
4	4000	50	10% Ethanol	3.7	1.0

268. Patent Referencing the extracting of *A. annua* leaves by hexane for the production of artemisinin. Full patent provided below, rather than abstract.

**United States Patent**

**4,952,603**

**ElFeryal, et al.**

**August 28, 1990**

---

Method for the isolation of *artemisinin* from *Artemisia annua*

**Abstract**

An improved method of producing artemisinin, an antimalarial agent, from the leaves of the plant *Artemisia annua* 1. comprising extracting the leaves of the plant with hexane, partitioning the hexane between hexane and acetonitrile following chromatographing the acetonitrile phase to produce substantially pure artemisinin.

---

Inventors: **ElFeryal; Farouk S.** (105 Virginia St., Oxford, MS 38655); **ElSohly; Hala N.** (41 Shelia Dr., Oxford, MS 38655)

Appl. No.: **208763**

Filed: **June 20, 1988**

**Current U.S. Class:** 514/450

**Intern'l Class:** A61K 031/335

**Field of Search:** 514/450

---

**References Cited**

## Other References

Acton et al., J. Chrom., 355 (1986), 448-450.  
Merck Index, 9th ed., p. 931, No. 6972, 1976.

*Primary Examiner:* Rollins; John W.  
*Attorney, Agent or Firm:* Stokes; William D.

---

## Claims

---

We claim:

1. The process of producing artemisinin from the plant *Artemisia annua* comprising the steps of extracting the plant with hexane, partitioning the hexane extract between hexane and acetonitrile - water mixture, evaporation of the solvents to dryness, chromatographing the evaporated mixture on silica gel adsorbent with a solvent comprising ethyl acetate in hexane, and evaporating the acetonitrile phase followed by crystallization to produce substantially pure artemisinin.
2. The process of claim 1 wherein the dried leaves only of the plant is used.
3. The process of claim 1 wherein said hexane extract of the leaves of *Artemisia annua* is partitioned between presaturated hexane and aqueous acetonitrile.
4. The process of claim 3 wherein the 20% aqueous acetonitrile is used.
5. The process of claim 3 comprising the step of removing water from the partitioned extract prior to evaporation of the solvents to dryness.
6. The process of claim 3 comprising the step of saturating the partitioned mixture with sodium chloride followed by removing water from the mixture as brine.
7. The process of claim 3 comprising the step of crystallizing artemisinic acid out of the partitioned mixture prior to chromatographic step.
8. The process of claim 1 wherein the chromatographic step is carried out in columns having a solute to adsorbent ratio of about 1:10.
9. The process of claim 1 wherein chromatographic solvent comprises a mixture of about 10% to 20% ethyl acetate in hexane.
10. The process of claim 1 wherein the chromatographic step utilized one column volume of each of 10% ethyl acetate in hexane and 15% ethyl acetate in hexane, followed by one and one half column volume of 20% ethyl acetate in hexane.

11. The process of producing artemisinin from the plant *Artemisia annua* comprising the steps of extracting the dried leaves of the plant with hexane, Partitioning the hexane extract with about 20% aqueous acetonitrile in presaturated hexane thereby transferring artemisinin present into the acetonitrile phase, saturating the mixture with sodium chloride followed by removing the water from the mixture as brine, evaporating the acetonitrile phase to produce an oily residue, crystallizing artemisinic acid out using acetonitrile, evaporating the acetonitrile, chromatographing the residue over silica columns using a ratio of about 1:10 solute to adsorbent and a solvent system comprising a mixture of about 10% to 20% ethyl acetate in hexane to obtain an oily material, crystallizing substantially pure artemisinin from said oily material.

12. The method of claim 10 wherein the oily material obtained from the chromatographic step is subjected to treatment with an ether/hexane mixture to obtain crystalline artemisinin followed by recrystallization of said crystalline artemisinin with a mixture of hexane and methylene chloride to produce pure artemisinin.

---

### *Description*

---

## BACKGROUND OF THE INVENTION

Artemisinin (Qinghaosu) is a novel sesquiterpene lactone endoperoxide having potent antimalarial activity. Artemisinin is obtained from the leaves of *Artemisia annua* L., the well known traditional Chinese herbal remedy, Qinghao. The only reported method of extraction of artemisinin has been by ethyl ether. Moreover, literature has provided no details of any method of isolation of artemisinin. Investigators working at the Walter Reed Army Institute of Research located *Artemisia annua* growing in the Washington, D.C. area and reported two procedures for the isolation of artemisinin. One of those procedures depends upon the use of the Ito multi-layer separator extractor. This procedure is only suitable for small scale production. The second procedure, while being capable of producing relatively larger quantities of artemisinin, suffers from major disadvantages, among which is that the procedure depends upon chromatographing a relatively crude fraction on silica gel. This known technique necessitates the use of a large ratio of solute to adsorbent, for example, the order of 1:44. Another disadvantage is that the solvent system used in eluting the chromatographic column is 7.5% ethyl acetate in chloroform, accordingly, the bulk of the eluting system is chloroform which is dense, expensive and unstable. Moreover, the order of elution when using such solvent system is artemisinin (R<sub>f</sub> 0.83), arteannuin B (R<sub>f</sub> 0.72) and the artemisinic (qinghao) acid (R<sub>f</sub> 0.6). The acid being predominant, tends to elute with artemisinin, accordingly the fractions contain large amount of the acid that affects the purity of the desired artemisinin.

It is the principal objective of this invention to provide a simple, practical method for the isolation and recovery of artemisinin from plant material which yields artemisinin in quantities and purity heretofore unobtainable in the methods known in the art.

Still another objective of the invention is the provision of a method for the isolation and recovery of artemisinin which method allows the eluting columns to be reused resulting in enormous savings heretofore impossible using the know methods.

## SUMMARY OF THE INVENTION

The invention is a method for the production of artemisinin from the plant, *Artemisia annua* comprising the steps of extracting leaves of the plant with hexane, partitioning the hexane extract between hexane and acetonitrile, evaporation of the acetonitrile fraction followed by chromatographing the evaporated fraction on a silica gel using as an eluting solvent a mixture of ethyl acetate in hexane followed by evaporation and crystallization to yield pure artemisinin.

## DETAILED DESCRIPTION OF THE INVENTION

In accordance with the method of the invention, dried leaves of *Artemisia annua* are extracted with hexane. The twigs or stems of the plant, comprising about 80% of the plant are not extracted for the reason that it was found that the twigs or stems contained little or no artemisinin. In the preferred embodiment of the invention, the extract is then partitioned in order to concentrate the extract and remove as many impurities as possible from the concentrate. This intermediate step of partitioning greatly facilitates and improves the efficiency of the chromatographic separation step.

Partitioning of the hexane extract is carried out using presaturated hexane and aqueous acetonitrile. For each 1g of material to be partitioned, a ratio of 12ml hexane to 4ml of 20% aqueous acetonitrile gives excellent results. The partitioning step results in a substantially exclusive transfer of the artemisinin into the acetonitrile layer with concomitant reduction in the amount of material, i.e., on the order of 32% to 36% of the original hexane extract. It will be appreciated that concentration of the sample to this extent materially decreases the workload of the silica columns used in the chromatography step of the inventive method. Prior to the evaporation step, for simplicity as well as for technical reasons, it is preferred to remove any water present in the acetonitrile phase. The water present may be removed by such commonly known means as azeotropic techniques using benzene and absolute alcohol or by using anhydrous sodium sulfate; however, it was discovered that water present can be efficiently and inexpensively removed by saturating the mixture with sodium chloride followed by removing the water as brine.

Subsequent evaporation of the non-aqueous acetonitrile phase yielded an oily, yellowish-brown, residue containing artemisinin of about three times richer than the hexane extract. For the reason that the acetonitrile phase is also rich in artemisinic (qinghao) acid, partial crystallization of the acid from this fraction is possible through the use of acetonitrile. In this manner, about 10% of the weight of the acetonitrile phase may be simply and practically removed prior to the chromatography step.

In the chromatography step of the invention, a ratio of 1:10 (solute to absorbent) was discovered to yield excellent results. In the known chromatographic techniques, a ratio of substantially 1:44 has been required. In the solvent system of the invention, a mixture of ethyl acetate in the range of about 10% to 20% in hexane was found to be quite effective. Using the solvent system and technique of the invention, the elution of the acid is reversed when compared with the mechanics of a solvent system consisting of 5% to 7.5% ethyl acetate in chloroform. In other words, using the method of the invention, the acid elutes first followed by the artemisinin. Accordingly, substantially all of the acid present will be removed prior to elution of artemisinin thus enhancing the purity of the artemisinin finally recovered in the process. By way of example of the chromatographic step of the process elution of the artemisinic acid and artemisinin from the column required the use of one column volume of each of the eluting solvent systems consisting of 10% ethyl acetate/hexane and 15% ethyl acetate/hexane, followed by 20% ethyl acetate/hexane (one and one half column volume) at a filtration flow rate. Artemisinic acid was present in the fraction eluted with 10% ethyl acetate/hexane (one column volume) and 15% ethyl acetate/hexane (two third column volume). Artemisinin was obtained from the oily greenish-yellow fraction eluted with 15% ethyl acetate/hexane (one third column volume) and 20% ethyl acetate/hexane (one and one half column volume). Purification of artemisinin was carried by crystallization from ether/hexane (1:4) with a further purification by recrystallization from methylene chloride/ hexane (1:4).

As mentioned hereinbefore, one of the major advantages, particularly economic, of the process of the invention is that the packing material in the columns may be used in at least two runs. After each use, a simple washing, for example, using one and one-half column volumes of ethyl acetate will recondition the column. The polarity of the solvent system should be decreased in a succeeding run to compensate for the partial deactivation of the silica gel.

In accordance with the description hereinabove, the solvent composition used in a second run would be about 8% ethyl acetate in hexane (one column volume) 13% ethyl acetate in hexane (one column volume) and 18% ethyl acetate in hexane (one and one half column volume), thus compensating for the slight deactivation of the silica gel from the first run. The silica gel used as a packing material in the examples herein was Machery Nagel silica gel 60, Brinkmann, mesh size 70-270. The recovered ethyl acetate/hexane solvent mixture may be used over and over again after drying, for example, over anhydrous sodium sulfate (250g/3L) and adjusting its composition to the desired percentage level.

The following examples illustrate specific embodiments of the method of the invention.

#### EXAMPLE I

Dried unground leaves of *Artemisia annua* (250g) was extracted by continuous hot percolation over a period of 48 hours using n-hexane as a solvent. The solvent free extract (19.5g, 7.8%) was partitioned with n-hexane and 20% aqueous acetonitrile, presaturated with each other, using 12ml hexane per gram extract and one third of this amount (4 ml/g) of the 20% aqueous acetonitrile phase. Partitioning of the hexane phase

between 20% aqueous acetonitrile was repeated two additional times using the same solvent ratio. The combined 20% aqueous acetonitrile was back-washed using 10% of its volume with presaturated hexane (24ml). Sodium chloride (7g/100ml of 20% aqueous acetonitrile) was added to remove the water. Evaporation of the acetonitrile in vacuo provided 6.7g of an oily yellowish-brown residue. About 650 mg of artemisinic acid was crystallized from this acetonitrile phase and removed prior to chromatography. Column chromatography of the residue was conducted using Machery Nagel silica gel 60 (Brinkmann, mesh size 70-270) in the ratio of 1:10. The eluting system comprising 10% ethyl acetate/hexane (1:0 column volume), followed by 15% ethyl acetate/hexane (1.0 column volume) and 20% ethyl acetate/hexane (one and one-half column volume), at filtration flow rates, yielded artemisinin in the column fractions eluted with 15% ethyl acetate/hexane (last 1/3 of the column volume) and 20% ethyl acetate/hexane (1.5 column volume). Evaporation of the solvent produced 2.5g of a greenish-yellow oil that crystallized readily from ether/hexane (1:4) to yield 270mg of pure artemisinin.

The above procedure was repeated three times on the same scale and consistently provided the same yields of artemisinin.

#### EXAMPLE II

Unground leaves of *Artemisia annua* (400kg) were proportionally extracted, partitioned and chromatographed in accordance with the procedure of Example I. The weight of the soluble hexane extract was 29.2kg (7.3%) and that of the acetonitrile phase, 10.765kg (36.8% that of the hexane extract). Partial crystallization of artemisinic acid from the acetonitrile phase (using 680ml acetonitrile/kg) yielded 1.18kg. The total amount of isolated artemisinin from this 400kg batch was 485g.

#### EXAMPLE III

The extraction and partitioning of the hexane extract was carried out as described in Example I. A portion of the acetonitrile residue (466g) obtained from Example II was column chromatographed on fresh silica gel 60 (4.5kg, 18.times.41cm) yielding an artemisinin rich fraction (wt 72.6g) from which 23.45g of pure artemisinin was obtained (0.135%).

#### EXAMPLE IV

The extraction, partitioning and chromatography was carried out as described under Example III except that the silica gel 60 was reused. 452g of the acetonitrile phase was applied on a reused silica gel 60 column (4.5kg, 18.times.41cm) to yield an artemisinin rich fraction having a weight of 70.22g from which 22.15g of artemisinin was obtained (0.132%).

#### EXAMPLE V

The dried hexane extract (12.5g) from 160g of the dried unground leaves of *Artemisia*

annua was partitioned and chromatographed in accordance with the procedure of Example I. The acetonitrile phase, after evaporation to dryness, yielded a 6.3g residue. The residue, upon chromatography, provided an artemisinin containing fraction (2.24g) from which 170mg of pure artemisinin was obtained (0.106%).

#### EXAMPLE VI

The dried hexane extract (12.5g) from 160g of the dried unground leaves of Artemisia annua was partitioned using non-saturated phases of hexane and 20% aqueous acetonitrile, and then chromatographed as described in Example I. The acetonitrile phase, after evaporation to dryness, yielded a 4.05g residue. The residue upon being chromatographed, yielded an artemisinin containing fraction (0.75g) from which 137mg of pure artemisinin was obtained (0.085%).

#### EXAMPLE VII

The dried hexane extract (12.5g) from 160g of the dried unground leaves of Artemisia annua was partitioned using saturated phases of hexane and 20% aqueous acetonitrile. The 20% aqueous acetonitrile was evaporated directly without salting out the water with sodium chloride, then chromatographed in accordance with the procedure of Example I.

The acetonitrile phase, after evaporation, yielded a 4.5g residue. The residue, upon being chromatographed, yielded an artemisinin containing fraction (1.34g) from which 121mg of pure artemisinin was obtained (0.075%).

#### EXAMPLE VIII

Dried hexane extract (12.5g) from 160g of the unground dried leaves of Artemisia annua was partitioned using unsaturated phases of hexane and 20% aqueous acetonitrile. In this example, 20% aqueous acetonitrile was not back-washed with 10% of its volume with hexane and was directly evaporated without removing the water. This procedure yielded a 4.6g residue. The residue, upon being chromatographed in accordance with the procedure of Example I, yielded an artemisinin containing fraction (1.59g) from which 111mg of pure artemisinin was obtained (0.069%).

#### EXAMPLE IX

Dried hexane extract (obtained as under Example I), about 20g from 250g of Artemisia annua unground leaves, was partitioned between 240ml of hexane and 3.times.80ml of acetonitrile (both presaturated with each other). The combined acetonitrile fraction was back-washed with 24ml of hexane followed by evaporation in vacuo at 40.degree. producing a 9.7g residue. Artemisinin remained exclusively in the acetonitrile phase. Chromatography, in accordance with the procedure of Example I, yielded 250 mg of artemisinin (0.1% isolated yield).

The dry hexane extract of the plant leaves obtained as in Example I was partitioned

between 10% aqueous methanol and hexane, presaturated with each other. A thin layer chromatographic analysis of the methanolic and hexane phases on silica gel G revealed that artemisinin was distributed between the methanolic phase and the hexane phase, accordingly this procedure is not feasible. In another approach, dry hexane extract of the leaves was dissolved in methanol and an aqueous solution of lead subacetate added. A sticky residue deposited itself at the bottom of the decanter permitting easy decantation of the supernate. The residue was washed with methanol. The supernate and the methanol wash were combined, evaporated to dryness and the residue extracted with chloroform. Chromatography of the residue obtained from the chloroform fraction yielded artemisinin in such low yields as to represent no improvement over the art.

It will be appreciated from the examples that the method of the invention, as exemplified in Examples I through IX, provide pure artemisinin simply and practically in yields heretofore unobtainable in the methods known in the art and the other methods attempted by the inventors.

Full copy of published patent using solvent extraction provided on purpose as solvent extraction is recommended and care must be taken not to infringe inadvertently on a patent awarded.

We recommend obtaining legal counsel on the issues of using technology that is distinct from those for which patents have been awarded; or for licensing the use of any such patented technologies for which BIONNEX seeks to use (if any), and the law surrounding such issues of patents as it pertains to artemisinin extraction. We provide the background information based upon published information in the global scientific literature.

**Additional References Used- not directly including *Artemisia annua per se*:**

268. Luo and Shenm 1987. Meyer-Warnod, B, A. Camilli. 1984. Natural Essential Oils. Extraction Processes and Application to some major oils. Perfumer and Flavorist April/May: 93-101.

269. Mchugh, M.A. and V.J. Krukonis. 1994. Supercritical fluid extraction: Principles and Practice, Butterworth-Heinemann, Boston, 2nd ed.

270. Reverchon, E., G.D. Porta and F. Senatore. 1995. Supercritical CO<sub>2</sub> extraction and fractionation of lavender essential oil and waxes. J. Agric. Food Chem. 43: 1654-1658.

271. Rozzi, N.L., W. Phippen, J.E. Simon, and R.K. Singh. 2002. Supercritical fluid extraction of essential oil components from lemon-scented botanicals. Lebensm.-Wiss. U—Technol. 35:319-324.

272. Simandi, B. et al. 1998. Supercritical carbon dioxide extraction and fractionation of oregano oleoresin. Food Res. Int. 31:723-728.

273. Trivedi, R.K. 1997. Supercritical fluids technology for extraction and separation of components from plant materials. For the International Training Program in Aromatic and Medicinal Plants, Purdue University, West Lafayette, In.



## **DRAFT: Artemisia Date Collection Guide:**

### Objectives:

1. To assess the performance including yield (biomass and artemisinin content) for each line.
2. We define line here as an accession, landrace, selected line, or a commercial variety. Each line or entry into the germplasm study is to be considered separately and then all compared against each other.
3. The attached guide placed on EXCEL spreadsheet can be modified and adjusted upon your review and further discussion. For practical purposes, it lists the parameters that we generally record for *Artemisia annua* and will help answer the primary objective: Is this line adaptable for production in its current location and/or does it contain some of value that can be used to improve the current varieties that are available.
4. a major challenge is to examine each treatment, field site for its content of artemisinin over the growing season and in particular as it is time to harvest. To do this, once a plant is dried, and destemmed, and the dry leaves/plot are available, then a sub-sample of the dried leaves from each plot (as an aggregate mixed bulked random sample from dry leaves from the entire plot) should be packaged and sent for analysis. Rutgers is happy to do such analysis, and/or to help set-up a local lab to do such analysis using HPLC and/or GC. We are set-up for HPLC (using different detectors) and GC analysis. We have already provided protocols for analysis and analyzed over 100 Madagascan samples. Our own preference is for BIONNEX to purchase their own analytical equipment to run tests locally with the first time around we can confirm the results at Rutgers and share our and other published techniques. We have already given you hard copies of papers that provide detailed steps of the analytical protocols in the analysis, but it may be easier to re-send these and to provide them in step-wise protocol manner, should there be interest.
5. Final commercial harvest may take place just prior to and/or at first onset of flowering, but the optimum time of harvest is not the main question here as that has been well studied in many other papers. The key question is to obtain baseline data and to identify the best line(s). As such, the data templates were drafted with this purpose in mind.
6. Sampling will be a key issue. As such, we suggest the following:
  - 6.1. Take larger plant tissue samples. Take 10 plants and sample as your now doing; then after sampling each plant (leaves only) then harvest the entire plant, and dry it, de-stem main stem and then mix the entire mass of dried leaves and stems and lets run that analysis. This will allow us to better understand the relationship between a small leaf sample analyzed for artemisinin with the entire 'extractable content of artemisinin in the entire or whole plant'. This would better allow us to develop base-line data on the relationship between the 'tiny leaf sample which would provide the 'highest artemisinin content (% leaf dry wt)' versus the whole plant from which simulates more of the expected final commercial harvest.

- 6.2. Samples each 5 plants/field every week as plants get close to flowering. Test and see when artemisinin content is highest.
- 6.3. Retain 30 plants for continued testing just to confirm optimum harvest time relative to growth development. That is sample 5 plants each week just prior to flowering and then for 6 weeks (total of 30 plants) at one site. See 6.1. protocol as one could actually just do the protocol listed there as part of this overall sampling procedure and have the answers for next season.
- 6.4. See data template that follows.  
SAMPLING:
- 6.5. Document the stage of the plant (flowering, rel % flowering- early, mid, full post full bloom, post bloom).
- 6.6. Cut off 90% of the plants at the soil line.
- 6.7. Weigh the plant fresh;
- 6.8. dry the plant; and weigh fully dried plant;
- 6.9. separate all the branches with leaves and flowers from main stem;
- 6.10. weigh each one (we do this as the main stem has nothing of value in it, but on some varieties of Artemisia and spacing in the field dependent, meaning plants grown super close together will have thinner stems, etc. have very thick stems and thus a very high stem: leaf ration- so it could account for 10% of the plant weight!
- 6.11. Once the plant parts are dried and weighted, only the branches with leaves and flowers are then bagged and kept for our analysis. We can later go into sub-sampling.
- 6.12. For yield estimates, record the plant spacing between plants- that is if each plant is spaced 1 meter apart between plants within a row and if the rows are 1 meter apart, then each plant occupies 1 sq. meter. So we need to know the density in order to then calculate the area yield and the estimate larger hectare yields.
- 6.13. Record drying temperature.
- 6.14. Use oven method for % moisture determination.
- 6.15. Keep all data in notebooks.



## ***Artemisia annua* Crop Budget Records:**

### **Table: Production/Input costs:**

Seed Costs (you need to charge for your own seeds and calculate a seed production cost) :

Nursery Costs:

Watering:

Labor in nursery:

Transplanting Costs (labor and time involved):

Watering and Irrigation Costs in production:

Labor in weeding:

Land preparation, tillage/cultivation:

Land rental or land usage costs for field production

Labor for data collection:

Harvesting the field:

Drying and Handling:

De-stemming:

Transportation Costs:

Lime and soil ph adjustment

Fertilizers

Other Supplies:

Other production inputs

Testing artemisinin

Soil tests

Other analytical services  
(insects/diseases diagnoses/field surveys, etc.)

Harvested yields per unit area (fresh weight)

Total material going into dryer (fresh weight, but mark whether it the main stem was removed or not, etc.

Total weight of dried material coming out of dryer

Storage costs per unit area considering storage facility construction and maintenance costs

Suggestions for labor savings:

Suggestions for production:

Other comments:

Modify to include all other inputs such as land rental/land usage

This basic template will be useful to ensure your recording your unit production costs at each production site. This will later be compared with total yields, artemisinin extraction and recovery per site and the cost of production.



**Analysis of Artemisinin in *Artemisia annua* field-grown in Madagascar,  
A NUANPP Technical Report, 2005**

Submitted to BAMEX:

*Research conducted and submitted by:*

Qing-Li Wu, Chungheon Park, Diandian Shen, Rodolfo Juliani and James E. Simon

New Use Agriculture & Natural Plant Products Program

Department of Plant Biology and Pathology, Cook College, Rutgers University

*(December 2, 2005)*

*Summary:* The artemisinin contents in 28 *Artemisia annua* samples from Madagascar, were quantified by the LC/MS method developed by the NUAPP, Cook Collge/Rutgers. The artemisinin content in the 28 Madagascar samples varied from 0.379% to 1.038%.

## 1. Method

LC/MS Method developed by NUAPP, Cook Collge/Rutgers (see attached publication)

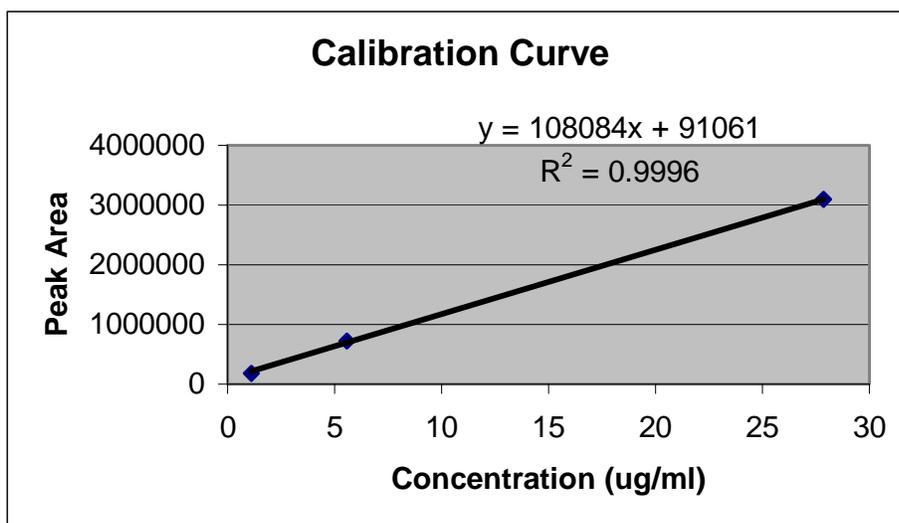
## 2. Material

The first set (7 samples) and the second set (21 samples) of *Artemisia annua* from Madagascar.

## 3. Experiment and Results

The contents of artemisinin in the 28 *Artemisia annua* samples from Madagascar were assayed by the developed method using selected-ion monitoring LC/MS and the results were listed in Table 1 and Table 2. Final artemisinin concentrations were determined based upon a calibration curve, as shown in Figure 1.

**Fig. 1. Calibration Curve for Determination of Artemisinin in *Artemisia annua* samples.**



**Table 1. Artemisinin content from the first seven *Artemisia annua* samples from Madagascar.**

<b>Sample name</b>	<b>Content (%)</b>
SABINAN Ech 1 19/10/05	0.472
SABINAN Ech 2 19/10/05	1.038
Echarhillan fugeain Dctc 15/10/05 By Beramarja last	0.674
Ben Ech Mat. 16/10/05	0.895
Antsirabe Ech 1 18/10/05	0.415
Antsirabe Ech 2 18/10/05	0.470
Antsirabe Ech 3 18/10/05	0.605

**Table 2. Artemisinin Content from the second set of *Artemisia annua* samples from Madagascar.**

<b>Sample name</b>	<b>Content (%)</b>
AB	04/11/05 0.467
E:1	22/10/05 0.533
E:1	31/10/05 0.585
E:1	04/11/05 0.754
E:2	22/10/05 0.499
E:2	31/10/05 0.730
E:2	04/11/05 0.543
E:3	22/10/05 0.539
E:3	31/10/05 0.503
E:3	04/11/05 0.655
E:4	22/10/05 0.672
E:4	31/10/05 0.659
E:4	04/11/05 0.617
Pat Bage	20/10/05 0.516
Pizicie Anksirabe terre noire	02/11/05 0.493
Saborsenamena (r.pr)	29/10/05 0.526
SB	04/11/05 0.519
Tanety Tony Terre noire	02/11/05 0.384

Tanety Jules Terre noire	02/11/05	0.379
TRM	05/11/05	0.400
TVM	05/11/05	0.630

---

#### 4. Reference

Wang, M.; Park, C.; Wu, Q. Analysis of Artemisinin in *Artemisia annua* L. by LC-MS with selected ion monitoring. *Journal of Agricultural and Food Chemistry* **2005**, 53, 7010-7013.



**ANALYSIS OF ARTEMISININ FROM FIELD-GROWN ARTEMISA  
ANNUA PLANTS, MADAGASCAR, 2005**

**A Technical Report from Rutgers in Support of Our BAMEX Project**

*Submitted by:*

Qing-Li Wu, Chungheon Park, Diandian Shen, Rodolfo Juliani and James E. Simon

New Use Agriculture & Natural Plant Products Program

Department of Plant Biology and Pathology, Cook College, Rutgers University

*(January 13, 2006)*

**Subject: Quantitation of Artemisinin in *Artemisia annua* field grown in Madagascar Using LC/MS**

*Summary:* The artemisinin contents in 34 *Artemisia annua* samples from Madagascar, were quantified by the LC/MS method developed by NUAPP, Cook Collge, Rutgers University. The artemisinin contents in the 34 samples varied from 0.711% (M102 Tanety Jules) and 0.728 (M101 Tanety Tony) to 1.708%. (Sample M122 coded as C3).

### 1. Method

The LC/MS method of artemisinin analysis developed by NUAPP, Rutgers University (Wang et al. 2005) was used in this study.

### 2. Materials

All the 34 *Artemisia annua* samples were from the BIONNEX (Charles Gibrain's) project in Madagascar. Samples were first dried and shipped to MediPlant, Switzerland, and later in late December, MediPlant then shipped the samples to NUANPP.

### 3. Experimental Results

The contents of artemisinin in the 34 *Artemisia annua* samples from Madagascar were assayed by the developed method using selected-ion monitoring LC/MS and the results are listed in Table 1.

**Table 1. Content of artemisinin in the 34 *Artemisia annua* samples from Madagascar**

<b>Sample Code</b>	<b>Sample Name</b>	<b>Content (%)</b>
M100	Riziere Jules	0.922
M101	Tanety Tony	0.728
M102	Tanety Jules	0.711
M103	Tanety tony	0.817
M104	Riziere Jules	0.904
M105	Tanety Jules	0.741
M106	PB(sans B)	1.134
M107	C1	1.256
M108	NO4	1.087
M109	NO3	1.324
M110	NE1	1.322

M111	Parc a bois avec B	0.930
M112	NO2	1.020
M113	C3	1.173
M115	C2	1.323
M116	NE1	1.173
M117	NE3	0.993
M118	NO1	1.412
M119	C4	1.099
M120	NO3	1.561
M121	Parc a bois(sans B)	1.239
M122	C3	1.708
M123	NO4	1.249
M124	NO1	1.448
M125	NE3	1.082
M126	NE2	1.270
M127	P B(avec B)	1.032
M128	Mediplant	1.206
M129	NE4	1.079
M130	NE2	1.503
M131	NO2	1.439
M132	C4	1.087
M133	NE4	1.275
M134	C1	1.430

---

\* Sample M114 listed was not available

#### 4. Reference

2005. Wang, M., C.H. Park, Q. Wu, and J.E. Simon. Analysis of artemisinin in *Artemisia annua* L. by LC-MS with selected ion monitoring. **Journal Agric. Food Chem.** 53: 7010-7013.

#### Acknowledgements

This work is part of a larger project (Madagascar Business and Market Expansion (BAMEX) MOBIS No. GS-23F-9800H, Task Order #687-M-00-04-00212-00 of the USAID) and reflects only the chemical analysis of this particular set of *Artemisia annua* samples for which we were asked to quantitates the artemisinin content. We thank Charles Giblyn, BIONNEX, and also Jean Robert Estime, Chemonics International Inc. and USAID for their support of the BAMEX project.





**ANALYSIS OF ARTEMISININ FROM 77 SAMPLES of FIELD-GROWN ARTEMISA ANNUA PLANTS, MADAGASCAR, 2005**

**A Technical Report from Rutgers in Support of Our BAMEX Project**

*Submitted by:*

Qing-Li Wu, Chungheon Park, Diandian Shen, Rodolfo Juliani and James E. Simon

New Use Agriculture & Natural Plant Products Program

Department of Plant Biology and Pathology, Cook College, Rutgers University

*(February 8, 2006)*

**Subject: Quantitation of Artemisinin from 77 *Artemisia annua* field-grown samples in Madagascar Using LC/MS. Research conducted by Qing-Li Wu, Chungheon Park, Diandian Shen, Rodolfo Juliani and James E. Simon, NUANPP, Rutgers University, USA.**

*Summary:* The artemisinin contents in 77 *Artemisia annua* samples from Madagascar, were quantified by the LC/MS method developed by NUAPP, Cook Collge, Rutgers University. The artemisinin contents in the 77 samples varied from 0.450% (Sample M197, O3) to 1.189%. (Sample M170, SO4).

## 1. Method

The LC/MS method of artemisinin analysis developed by NUAPP, Rutgers University (Wang et al. 2005) was used in this study (Offprint of this publication in PDF is also attached with this report).

## 2. Materials

### *Plant Materials:*

All 77 *Artemisia annua* samples were shipped dried from BIONNEX (Charles Gibrain, CEO) project in Madagascar. This work is done in support of a collaborative project between Rutgers University and BAMEX, Chemonics Int. Inc. in which BIONNEX is a private sector partner and stakeholder.

### *Standard:*

Artemisinin (98%) was purchased from Sigma-Aldrich (Milwaukee, WI).

## 3. Experimental Results

The contents of artemisinin in the 77 *Artemisia annua* samples from Madagascar were assayed by the developed method using selected-ion monitoring LC/MS and the results are listed in Table 1.

**Table 1. Content of artemisinin in the 77 *Artemisia annua* samples from Madagascar**

<b>Sample Code</b>	<b>Sample Name</b>	<b>Content (%)</b>
M147	Tanety Tony	0.714
M148	Riziere Jules	0.666

<u>M149</u>	Terre Mouge Jules	0.630
<u>M150</u>	Tanety Jules	0.615
<u>M151</u>	SE 1	0.658
<u>M152</u>	SE 2	0.729
<u>M153</u>	SE 3	1.031
<u>M154</u>	SE 4	1.061
<u>M155</u>	NE 1	0.822
<u>M156</u>	NE 2	0.891
<u>M157</u>	NE 3	0.681
<u>M158</u>	NE 4	0.899
<u>M159</u>	NO 1	0.682
<u>M160</u>	NO 2	0.718
<u>M161</u>	NO 3	1.148
<u>M162</u>	NO 4	0.784
<u>M163</u>	S 1	1.017
<u>M164</u>	S 2	0.857
<u>M165</u>	S 3	1.128
<u>M166</u>	S 4	1.070
<u>M167</u>	SO 1	0.771
<u>M168</u>	SO 2	1.081
<u>M169</u>	SO 3	0.990
<u>M170</u>	SO 4	1.189
<u>M171</u>	O 1	0.881
<u>M172</u>	O 2	0.790
<u>M173</u>	O 3	0.680
<u>M174</u>	O 4	0.747
<u>M175</u>	C 1	0.877
<u>M176</u>	C 2	0.892
<u>M177</u>	C 3	1.089
<u>M178</u>	C 4	0.969
<u>M179</u>	Tanety Jules	0.545
<u>M180</u>	Tanety Tony	0.583
<u>M181</u>	Terre Mouge Jules	0.626
<u>M182</u>	Riziere Jules	0.572
<u>M183</u>	C 1	0.698

M184	C 2	0.728
M185	C 3	0.770
M186	C 4	0.526
M187	SO 1	0.582
M188	SO 2	0.691
M189	SO 3	0.720
M190	SO 4	0.738
M191	NO 1	0.786
M192	NO 2	0.846
M193	NO 3	0.758
M194	NO 4	0.730
M195	O 1	0.647
M196	O 2	0.768
M197	O 3	0.450
M198	O 4	0.628
M199	NE 1	0.530
M200	NE 2	0.680
M201	NE 3	0.585
M202	NE 4	0.644
M203	S 1	0.706
M204	S 2	0.932
M205	S 3	0.810
M206	S 4	0.824
M207	SE 1	0.519
M208	SE 2	0.800
M209	SE 3	0.650
M210	SE 4	0.881
M211	Terre Mouge Jules	0.678
M212	Riziere Jules	0.693
M213	Terre noire Jules	0.825
M214	Tanety Tony	0.790
M215	Tanety Jules	0.966
M216	Riziere Jules	0.833
M217	Tanety Tony	0.980
M218	Riziere Jules	0.950

M219	Tanety Jules	0.597
M220	Sabotsy Tony	0.799
M221	Tanety Tony	0.971
M222	Terre Mouge Jules	0.714
M223	S. MAT	0.985

#### 4. Reference

2005. Wang, M., C.H. Park, Q. Wu, and J.E. Simon. Analysis of artemisinin in *Artemisia annua* L. by LC-MS with selected ion monitoring. **Journal Agric. Food Chem.** 53: 7010-7013.

#### Acknowledgements

This work is part of a larger project (Madagascar Business and Market Expansion (BAMEX) MOBIS No. GS-23F-9800H, Task Order #687-M-00-04-00212-00 of the USAID) and reflects only the chemical analysis of this particular set of *Artemisia annua* samples for which we were asked to quantitate the artemisinin content. We thank Charles Giblain, BIONNEX, and also Jean Robert Estime, Chemonics International Inc. and USAID for their support of the BAMEX project.

## *Artemisia annua: A Primer for Production*

**James E. Simon, Chung Park and Rodolfo Juliani**

*Artemisia annua* L. is an annual aromatic herb of the Asteraceae family (formerly known as the Compositae) native to China and later naturalized to other regions of Southeast Asia, Eastern Europe and more recently North America. The plant, also known as Sweet Annie, annual wormwood or sweet wormwood, or Qinghai (Chinese name) is largely collected from the wild and cultivated in many countries as a source of natural anti-malarial compounds. Traditionally, *A. annua* as a Traditional Chinese Medicinal (TCM) was used against fevers and malaria. The plant is also used in the USA and Europe to a limited extent in the crafting of dried aromatic ornamental wreaths, dried flower arrangements and other herbal decorations. The plant, which can reach heights of 2m, is highly aromatic due to the volatile essential oils yet also contains a number of nonvolatile compounds and it is these nonvolatile sesquiterpenes, most notably artemisinin which are responsible for the anti-malarial activity. The essential oils of this plant have been used in the flavoring of beverages such as vermouth but to date; the essential oils from this species have not found a successful commercial niche. *A. annua* has generated much excitement in the scientific community around the world because this plant can provide a new generation of antimalarials effective against multi-drug resistant *Plasmodium* spp. due to the presence of Qinghaosu (artemisinin).

Artemisinin is found in the leaves and flowers and is a natural plant product identified as a sesquiterpene lactone endoperoxide (Klayman et al., 1984). While both the essential oils and sesquiterpenes such as artemisinin are associated with the secretory cells of the plant, the extraction of artemisinin is achieved by a solvent extraction (traditional and/or super critical fluid extraction such as CO<sub>2</sub>) rather than a water or steam distillation as the artemisinin and several other of these sesquiterpene lactones are not volatile, and therefore would not allow for the recovery of the artemisinin. One of the early reviews on the plants anti-malarial activity was published almost 20 years ago (Klayman, 1985).

The major sources of production and collection of this annual crop as a source of artemisinin are China and Vietnam. Cultivated on smaller scales have been reported in Brazil, South Africa, Switzerland, Tasmania and the United States. The plant can be easily introduced into new regions and has been found to be quite adaptable. Yet high yielding lines adaptable to specific regions/countries whose seed are both available in quantity and at a reasonable cost are now quite limited. The development of the specific commercial production practices that lead to an efficient and profitable opportunity for growers, both in minimizing risks, and maximizing performance remains a challenging task and requires new producers, or experienced producers introducing this species into a new region to first undergo local testing and screening. The objective of this production primer is to share our experiences in the production, harvesting, processing areas and to

provide specific initial recommendations in this plants' production that should later be tested and refined by region and local conditions and needs.

### **Plant Ecology**

One of the major issues in introducing *A. annua* into tropical areas is that this species is a determinate short-day plant. Non-juvenile plants are very responsive to photoperiodic stimulus and flower about two weeks after induction. This means that regardless of how long the plant has been growing in the field, or how tall the plant is, when the critical photoperiod reaches 13.5 hours, the plants will be induced to flower. While there may be a photoperiod x temperature interaction, the plant when grown in the northern temperate zones early in the growing season will typically flower in early September and produce a mature seed crop in October. If planted late, well into the growing season, the plants will begin to flower when very small in height and young in development resulting in a lower yield and limited seed set. As such, under those environmental conditions, early sowing and early field establishment increases yield significantly, while late sowing and/or late field establishment such as mid-way or later through the growing season results in plants that flower while the plants are very short and consequently results in very low yields. Unadapted *Artemisia annua* plants will therefore flower while the plants are still very young in the field in many tropical areas. To overcome this major limitation, non-photoperiodic plants must be selected and improved varieties introduced into those regions in order for the plants to grow to their biological potential. Such non-photoperiodic plants are available but quite limited in their seed availability and in the number of genetic lines available. In the northern zones, this is not a problem, as proper timing of sowing and planting will avoid this obstacle.



Close-up photo of field-grown *Artemisia annua*. Plant was approx. 1.5 meters in height.

### **Basics of Production**

Plants can be direct seeded or transplanted into the fields very early in the growing season. Seedlings appear to be tolerant of frost, a concern only in some regions. The plant is however, generally transplanted rather than sown into the field because the very small size of the seeds makes it difficult to obtain a good and uniform stand. Many researchers and small farmers first transplant seedlings but direct seeding will be required for larger-scale commercial production.

Seeds of this species are very small and difficult to handle and singulate for planting. Due to the very small seed size, growers use a few horticultural tricks to more easily handle the seed. We sometimes mix sand in with seeds and then sow the seeds with a 'flour sifter' into flats and place the flats into a mist house. The seeds are sown on the surface of the growth media as they need to be planted shallow and the watering of the seeds will physical contact of the tiny seeds onto the growth media surface result in most falling just

beneath the actual surface. The flats can be directly placed into a greenhouse without the use of a mist house, when we do this we often place a moist newspaper on top of the flat on purpose to retain moisture and also preclude direct water droplets contacting and potentially disrupting or washing away the seeds. Thus, at this initial stage the newly sown seeds need water but the water must be sprayed very gently onto the flats to keep the seeds in place. Seed viability is also an issue with this species in that the seeds can lose their viability relatively fast, and in particular if the seeds are not harvested at full maturity and stored under proper conditions. When the seedlings are 3 to 6 cm. tall, we replant each seedling into its own cell. Cell size may vary depending upon the size of the desired transplant, but we use flats with 52 cells as that provides to us a larger transplant, but a cell size that large is not required per se and sizes of 72 and smaller can be used. To minimize costs, some growers prefer not to use a greenhouse and incur the additional expenses of this approach but rather build an outdoor bed, sometimes a raised bed, into which *A. annua* seeds can be heavily sown. Then later and early in the growing season, the viable seeds germinate and emerge and thus form a bed of *A. annua*, in tune with the production cycle of the area. These beds may need to be watered and cared for but the level of management will depend on the actual site. In some cases, under northern temperate zone and where the plant is native or naturalized, there is little management provided to the beds under the presumption that natural rains will coincide with the beginning of the growing season. In these cases, the seeds are sown in late fall to mimic the natural seed sowing as the plants will under the appropriate growing conditions self-sow (mature seeds falling from the plant and onto the surrounding soil, only to naturally emerge the next growing season or under similar conditions conducive for germination. Seedlings can thus be dug and replanted into the field as needed either bare rooted or with some attached soil. This may be done when the seedlings reach a height of 3 to 6 cm. are then carefully removed ‘in mass’ and transplanted directly into the final field site

Plants are ready to transplant about 6 weeks following seeding. In many temperate regions, *A. annua* has been purposefully sown in the fall, emerged and survived the winter and later continued to grow the following spring. Plant populations that following spring have not been uniform, with stands uneven and too dense.



*Artemisia annua* seedlings growing in cell flats under greenhouse conditions



*Artemisia annua* plants that were transplanted into single pots from such cell trays shown in the prior picture. Note the upright growth, the single main stem, the beginning of the plant spread in width and the lush foliage. This plant is an exceedingly fast grower—once it reaches the seedling stage. The plants are larger and older than would be required for field transplanting. Plants grown here are being raised to collect seeds. (Rutgers University)

*Artemisia annua* is adaptable to a wide range of soil conditions. We have grown this plant in sandy, sandy-loam, loam, and clay loam soils each with success. Decreases in stand will result from excessive rains on heavier soils with poor drainage. There have been no studies indicating optimum soil pH nor fertility. We do, however, recommend a soil pH of 5.5 to 6.5. Our studies with nitrogen rates, although preliminary, indicate that plants only require about 67 kg N/ha (Simon et al., 1986; Simon et al., 1990). We have not yet observed any need for additional nutrients under the soil conditions used, but should this plant be introduced into soils of known mineral deficiencies and/or nutritional disorders, then we recommend keeping a close watch on the plant and taking the appropriate soil and tissue tests during the season. There is no guide established that links a range of recommended levels of nutrients in plant leaves to biomass and artemisinin yields from tissue testing. If your soil conditions are however known to be deficient in any macro- or minor nutrient (e.g. boron, copper, etc.) then we suggest using the same strategy of replenishing that nutrient back into the soil as you would when growing your traditional crops.

Optimum plant spacing is dependent upon the end-use of the plant. From a two year study evaluating different plant populations and levels of nitrogen, we found that the highest yields came from the most densely populated treatment of 111,111 plants/ha or an individual plant spacing of 30cm x 30cm (Simon et al., 1990). The biomass yields across all densities (27,778, 55,555, 111,111 plants/ha) and nitrogen levels (0, 67, 134kg N/ha) ranged from 21 to 35 metric tones/ha of fresh plant biomass. While this production primer is geared for the production of artemisinin, when the plant is grown for seed production greater spacing may be desirable between plants in order to maximize seed

yields, criteria that are distinct from artemisinin production. As the density increased, individual plant weight decreased, plant height increased, side-shoot and lateral growth decreased (Simon et al., 1990). The phenotypic characteristics are critical in the quality of the harvested and dried herb for processing. Plants that have high biomass yields but in which the main stem is large, the side shoots are numerous but the total leaf area per plant is low will result in lower area yields of the natural products as the products accumulate in the leaves and flowers. Rows 1 meter, with plants 0.6 to 1 meter apart will allow between row cultivation and a solid plant mass within the row. For seed production this and even lower populations would be used to achieve maximum seed production/plant. Final decisions on between row spacing should be guided by the cultivation system(s) used in the production area. As such, it appears more important to consider this plant as an industrial or agronomic crop, rather than a horticultural crop per se. Toward this approach, the costs in weed control may be among the highest input costs to a producer, so techniques employed to minimize weeding and to allow/foster mechanical cultivation and other weeding systems should factor into the final plant spacing decisions. Thus, we recommend adjusting the between row length to be compatible with the weed control strategies employed.

Plant height will be dependent upon the genetic line grown, cultural and management conditions, the season and very importantly, the time of planting. The goal should be for *A. annua* to reach heights of more than 2.5 meters. Be aware that the plant may only reach heights as low as 1 meter and in this case, a higher plant density would have been preferred a lower yields should be expected, presuming comparable levels of artemisinin. As the plant clearly has a main dominant growing point, it was of interest to growers to determine whether breaking that ‘apical dominance’ of that main stem would lead to a rapid proliferation of side stems and many leaves. That technique is used with many perennials and trees to shape and make the harvesting of the fruit or leaves (e.g. *Ginkgo biloba*) easier. In annual herbaceous plants, this is rarely employed and when we tried to ‘top’ the plant when it is young and or mature to encourage a copious growth of side laterals to achieve a small plant with more leaves on side branches, the resulting re-growth of lateral branches was poor (See table below). The plant does not easily nor rapidly grows back when ‘cut-back’ or pruned as it sometimes observed with tree crops. In one preliminary study to determine whether early topping or cutting the central stem would encourage greater branching and yield of shoots was evaluated in the results strongly show that this practice is not beneficial and should be avoided.

Treatment	Plant height (cm)	Plant Spread/Width (cm)	Plant Yield (fresh herbage Mt/ha)
No topping (control)	129	104	22
Topping	114	91	8*

\*showing that the topping of *Artemisia* in temperate zones where the growth season is limited, is not desirable due to the slow grow-back and final lower biomass yield.

## **Disease, Insect and Weed Control**

Diseases and Insects: No serious diseases or insects have been reported. From all our experiments and plantations for seed production and dried leaves, we have observed caterpillars on the leaves, but little visible feeding injury was observed and in most cases the caterpillars died, leading to the hypothesis that there may be an anti-insect compound in the leaves of the plant. We have recently been observing in our field trials, high aphid populations all concentrated at the growing tip of the plant. Continued monitoring for insects is highly recommended. Any insects found should be captured and properly identified.

Weed Control: The control of weeds is often most difficult to manage with new crops, and often represents one of the highest production costs and difficulties in crop management. Interestingly, artemisinin has been reported to be a potent plant inhibitor with potential as a natural herbicide (Duke et al., 1987). While it may control different weed species, weed control in the field, particularly in the early spring as the plants become established is necessary and weed pressure becomes less as the plant matures and its crop canopy develops. The production of this plant is for the pharmaceutical industry and as such to enter into that market, it is not necessary for the production system to be organic or non-organic. What is key is that the production is sustainable, profitable for the producer and processor and is done using Good Agricultural Practices and conforming to all pesticide and environmental laws, regulations of the producing country. A non-chemical pesticide (including herbicide) approach is highly desirable and preferred but will only work over the long- term if crop production system is profitable. We recommend local systems of weed control without the use of any pesticides, most notably for this discussion, herbicides, as the herbicide cost, the safety in handling, risk of exposure to workers and their families, proper storage, and disposal are issues that require training, interventions and monitoring. However, for large-scale production, herbicides have been used in this crop and as such, we present below some general observations.

We have conducted several herbicide studies to identify pre- and post-emergent herbicides (data not presented). In our research farms under experimental conditions, we use the herbicide, devrinol (2.2 kg/ha), as a pre-transplant incorporated herbicide with excellent results. However, as there are no legally registered chemicals for weed control and if a non-chemical approach is desired, alternative strategies need to be followed under commercial conditions. We typically work the fields twice prior to seeding and/or transplanting. The first time to prepare the soil for planting, and the second time after the initial spring weeds have emerged. This will reduce the early spring weed pressure. Transplanting healthy, vigorous plants as soon as the field has been worked the second time will help allow the plants to develop rapidly and begin to shade the soil prior to the development of new weeds below the plants' canopy. When introducing this plant into a new region, the most effective weed control strategies and management techniques employed with commercial crops of the region need to be considered.

When *A. annua* is directly sown into the field, the emergence will may take up to 3-6 weeks and by then in the early growing season, serious weed pressure will already be present. Direct sowing of the seeds manually is often done under small production conditions, but under larger-scale, a mechanical system of direct sowing is desirable yet the shape and the small seed size has not led to effective direct drilling even when using the small seed plates employed with the direct sowing of vegetable and agronomic seeds. Work toward seed coating, or the creation of a round consistent seed surrounded by inert material such as a clay, could in the future allow for efficient direct drilling of seeds. Seed costs of seed coating at present are unknown.

Proper site selection (areas of well drained soil, without noxious weed problems present) proper land preparation, removal of weeds at the initial stages of field sowing and transplanting are all key strategies toward the development of an effective weed control plan. Adjusting between row length to be narrow- but still allowing your tractor, animal or other weed control implementations between rows for easier tillage is desirable. Cultivating between the rows and possibly once through the fields with a crew to hand hoe should suffice. Once the plants start to shade the soil area below, few weeds are seen. The plant contains two compounds which actually inhibit the germination of other weed seeds. Once the plant is transplanted into the field, and is established, with adequate moisture and sunlight, the plant grows very fast, shading out potential weeds underneath.

### **Plant Varieties and Plant Improvement**

This is one of the most important topics to cover in the production of Artemisia. Our early studies (Charles et al. 1990) showed that one can grow *A. annua*, and the natural products of interest may be in high, medium, or trace concentrations depending upon the source, or the genetics of the plant. To date, there are no physical or even morphological indicators one can use to predict final artemisinin concentration in the plant without the actual chemical screening for the compound. There are several rapid screening tools one may use to test high numbers of plants at a young growth stage and some that require only a small amount of leaves to be sampled, but at present the grower and the processor is faced with a major dilemma in that many commercially available lines of *Artemisia annua* are not uniform in growth or in artemisinin concentration, nor necessarily high in this targeted chemical. At present, due to the increased interest of this crop, there have been some lines sold as *A. annua* that may not even be the correct species. There are several commercial seed sources of *A. annua* on the market, but generally they have not been selected for artemisinin content per se or high biomass yields, and as a consequence some contain virtually no to small amounts of artemisinin. Rather, many seed companies procure their seed from the large wholesale market and some of this appears to come seed-stock from wild-grown plants. Selection and breeding programs in the USA, Brazil and Switzerland and other regions have shown that significant progress can be achieved by both selection and breeding. In our studies, we observed significant variability in the species and that many growth forms within a given population can be identified. We have isolated lines with a more upright tall growth pattern, another short and bushy, and others which remain vegetative for longer periods of time. Other breeding programs have

made excellent progress in identifying non-photoperiodic plants- most notably those in Switzerland and in Brazil. The hybrid Anamed, developed by MediPlant is reported to be an excellent plant, with a reported high artemisinin content and nonphotoperiodic character. Seed supply may be limited and the costs quite high, but this hybrid, if it grows well and provides the concentration of the artemisinin needed is an excellent option. However, limited seeds, high costs and the need to introduce more than one single line to an area are also highly desirable. The ability to first grow-out the commercially available lines, screen them for artemisinin content, evaluate them for their growth and biomass yield may also provide a clear roadmap as to the final choices of commercial seed lines acceptable within a given region to enter into larger-scale commercial production. As *A. annua* seeds do not maintain their viability for many years, and require proper storage (cold, dry conditions), developing and ensuring an adequate seed supply for commercial needs is also a major consideration, and suggests that the develop of acceptable open pollinated lines that can be actually grown for their seed in the country of commercial production may be a highly desirable option to protect and ensure one's own supply of high quality seed for the commercial industry in that same region. This approach is worthy to consider as it would lead to additional economic opportunities for those involved in seed production, and ensure a close connection between the producer and the seed supplier with lines that are adapted and suited for that locale. Development of such improved lines using an OP approach can also be done in concert with the in-country University and private sector (seed companies and others in the private sector) for local ownership of new and improved lines and varieties. For crops with more established histories and seed company involvement such an approach may not be as pertinent but when the commercial seed companies cannot guarantee a seed supply and seed supply of a consistent quality, this approach provides a safety net to a new production area. *Artemisia annua* produces copious amounts of seeds.

Ongoing selection and breeding programs are required to increase the artemisinin content and to develop high yielding plants adaptable to specific regions. Plants with both the desirable agronomical traits and chemical profile are needed. Most collections of *A. annua* derive from natural stands with highly variable artemisinin content, some as low of 0.01%. In one report, selections from Chinese *A. annua*, artemisinin varied from 0.05 to 0.21%, and since then, the upper end of artemisinin found was reported close to 1.00%. Swiss researcher N. Delabays reported a clonal selection derived from Chinese material which produces 1.1% artemisinin but is very late flowering; proprietary hybrids have been obtained with high content and earlier flowering, and newer hybrids now with high artemisinin and nonphotoperiodic are available. We predict that over the next several years, a range of improved *Artemisia* varieties (both hybrids and OP lines) will be available and suitable for either the tropical areas and more northern temperate zones and the goal is to make those improved lines available in the quantities needed and at affordable prices.

While the focus of this report is on artemisinin, it is important to recognize that this plant may serve also as a promising source for value-added additional products as well as a source of aromatic essential oils. In regard to the plant serving as a source of essential oil

which can be obtained by the water or steam distillation of the plant, the essential oil content of the plant is relatively high (approximately 0.05-0.5% or 0.4-1.2% fresh or dry tissue respectively, volume by weight basis). The main constituent in the oil is Artemisia ketone (Charles et al. 1991), which can account for more than half of the oil (on a percentage basis); yet different chemotypes can be developed to serve as rich sources of other aromas as well. Selection for high essential oil content or for higher individual chemical constituents can be accomplished by mass selection. Not surprisingly, there is significant variability in the aroma and chemical constituents between lines and plants.

### **Tissue Culture and BioReactor Potential for Production**

*A. annua* is easily propagated *in vitro* by standard protocols. *In vitro* propagation of shoot cultures has been reported by several groups who have published their methodology since the 1980's. Elite single plants can be clonally propagated via *in vitro* propagation and this technology offers an excellent vehicle for rapid single plant selection increases as well as a vehicle to study regulatory factors governing the biology of the plant.

Artemisinin is produced in shoots *in vitro* and despite the multitude of studies examining ways to produce the anti-malarials in culture, there is no evidence that *in vitro* production of artemisinin will be commercially feasible or competitive to field production. Studies employing hairy root cultures and other bioreacting systems to induce high accumulation of artemisinin *in vitro* have not been successful and to date no have been commercially employed.

### **Harvesting and Processing**

Unlike most other crops that have either multiple end use options and/or can be dried, stored and shipped, the linkage between the production and processing of *A. annua* is critical. Without processing facilities in place- regardless of the choice of processing technology (solvent extraction, super critical fluid extraction, others) there will no commercial outlet for this crop. The value of even high quality dried *A. annua* is not sufficiently high to warrant shipment to another country for processing. As such, the harvesting and post-harvest handlings are also key aspects in production and in developing a GAP system for Artemisia. Depending upon the final extraction system selected, certain final post-harvest handling practices may be different, but the goal is to maximize quality. Harvested plant yields of *Artemisia annua* need to be very high. In our cropping system studies, recorded yields of up to 35 metric tones of fresh plant material per hectare were obtained. In one study, we transplanted the *A. annua* each month for four months (April, May, June and July). Greatest plant yields were obtained from the May transplanted plants (Simon et al., 1990). The manner and timing of harvest depends upon the end-use. For artemisinin plants should be harvested at full bloom as that appears to be the time of maximum leaf content and maximum floral presence and content. As a source of other value-added products and essential oils, plants can also be harvested at the same stage of full flowering. With *A. annua*, the harvesting window is not exceptionally narrow in that the plant will have artemisinin present both prior to and

after the recommended time to recover the maximum amount on a dried wt. basis. This provides some degree of latitude for the grower and the processor in scheduling the harvest.

For artemisinin, plants can be mechanically cut and windrowed and then collected. Once the plants have been cut and harvested, plants are usually stacked horizontally on a flatbed truck and brought into a shaded area where further processing takes place. The main stem is not desired for processing as there is no artemisinin in the main stem, and thus this large bulk material needs to be discarded and the main stem removed. For solvent extraction, it may not be critical to remove the main stem, thus reducing post-harvest handling costs, but for other extraction technologies such as CO<sub>2</sub>, the main stems need to be removed and the plant further prepared for extraction. Plants also need to be dried yet sun-drying alone is not sufficient for certain extraction technologies as well. We recommend that the plants then be subjected to forced warm air that continually passes through the plant material to hasten the drying process and minimize both color loss, loss due to microbial spoilage and loss of natural products. The color loss is an example of a factor not relevant to the extraction of artemisinin but would be if dried for a medicinal tea or another final product. Temperatures within the stack of plants should not exceed 35°C. Artemisinin, the most biologically active sesquiterpene is unstable and the integrity of the peroxide bridge must be maintained for the chemical to retain its anti-malarial activity.

### **Extraction of Artemisinin**

The extraction of the artemisinin can be accomplished by a variety of methods. Each having their comparative strengths and weaknesses. Traditional solvent extraction is the lowest cost, now used around the world to extract artemisinin. The strengths include it is the lowest cost, relatively easy and introduces a moderate specialty technology. The weakness in the use of this technology is that the processing technology can be done without following GMP and thus the final product may not reach a certifiable standard. Other concerns however relate to the safety, handling and disposal of the traditional solvents now used in the extraction of artemisinin, and possible exposure to workers, their families and the facility and the potential for environmental contamination and difficulty in final storage and disposal unless handled properly. It is possible to use solvent extraction in a manner that overcomes the current concerns in the solvent extraction of artemisinin. Some of the solvents used include but are not limited to hexane, petroleum ether, and ethyl acetate.

Alternative processes can also be employed in the extraction of artemisinin and the one presented below involves the process of super critical fluid technology (SCFT) using CO<sub>2</sub> as the solvent. The advantage of this system is its high degree of control, its high efficiency, and that it lends itself to use of GMP. The system avoids the concerns listed above with petroleum solvent extraction, and the system is environmentally friendly and the products accepted as high quality. The disadvantage of this system is its initial high cost; despite that small custom tailored CO<sub>2</sub> units can be purchased and moved into a new location. The system would require a much higher level of trained management and

staff. This technology would be exciting to introduce into production regions as one can develop additional products from *A. annua* as well as use the same system for a wide range of other medicinal and food products. Thus, the recovery of additional value-added products from this plant and the high quality of the final product and the environmentally friendly processing system are among its major advantages.

### **Extraction of Essential Oil**

The volatile essential oils which are responsible for the intense aroma can be extracted by steam distillation in a manner similar to commercially produced and extracted aromatic plants (Simon et al., 1990). Essential oils can also be obtained as by products of other extraction techniques such as SCFT.

### **Toxicities and Other Concerns:**

There are few reports on any associated toxicity in handling the plant except when the plant has begun to flower. Those individuals that suffer from hay fever and are allergic to pollen can be adversely affected if handling this plant either in the field, or even after harvesting when pollen is present. Many other members of the Asteraceae family, to which *Artemisia* belongs, produce pollen that causes hay fever to those sensitive individuals.

### **Conclusion:**

*Artemisia annua* can be easily grown in most countries where it is introduced. The challenge is to ensure high yielding biomass lines rich in artemisinin. This is no easy task. The most important management problems will be to achieve uniform stands and weed control and to identify genetic lines that are adaptable and high yielding in the intended production areas. The plant is very vigorous should reach about 2m in height, and is essentially disease and pest free. When introduced into larger commercial areas, new diseases and insects may soon be identified and strategies developed. Studies have shown that the artemisinin concentration can range from trace amounts up to >1.0% dry weight of leaves, that is distinct from a 1.0% for the bulked harvested product, which includes the main stem, the side shoots etc. Till now, most of the limited information we have available is research oriented, and not area yields per se of the final extractable product. This is also because the efficiency in all the extraction technologies vary significantly that it makes assessing such yields far more difficult. Furthermore, research has shown that newer varieties and hybrids can exhibit artemisinin concentrations of greater than 1% dry wt and are non-photoperiodic. Improved genetic materials are being developed and we hope that a number of crop improvement programs and the commercial seed companies will over the next several years improve the productivity and artemisinin extractable yields under a wide range of environmental conditions and make such seed-stock available at affordable prices to those involved in the production.

**Acknowledgements:**

Wish to thank the following individuals for their suggestions, discussions and their own work toward the commercialization of this medicinal plant: Dr. Morales, Dr. Ferreira, Dr. Croom, Dr. Benakis, and Dr. Malghass.

**Selected References:**

Charles, D.J. and J.E. Simon, K.V. Wood and P. Heinstejn. 1990. Germplasm variation in artemisinin content of *Artemisia annua* using an alternative method of artemisinin analysis from crude plant extracts. *J. Natural Products*. 53(1):157-160.

Charles, D.J., E. Cebert and J.E. Simon. 1991. Characterization of the essential oil of *Artemisia annua* L. *J. Essential Oil Research* 3:33-39.

Charles, D.J., J.E. Simon, C.C. Shock, E.B.G. Feibert and R.M. Smith. 1993. Effect of water stress and drying on artemisinin content in the leaves of *Artemisia annua* L., pp. 640-643. In: J. Janick and J.E. Simon (eds). *New Crops: New Crops: Exploration, Research, Commercialization*. Proc. New Crops, Oct. 6-9, 1991, Indianapolis, IN. John Wiley & Sons, Inc., N. Y.

Delabays, N., A. Benakis, and G. Collet. 1993. Selections and breeding for high artemisinin (qinghaosu) yielding strains of *Artemisia annua*. *Acta Hort*. 330:203-207

Duke, S.O., K.C. Vaughn, E.M. Croom Jr. and H. N. Elsohly. 1987. Artemisinin, a constituent of annual wormwood (*Artemisia annua*), selective phytotoxin. *Weed Sci*. 35:499-505.

Ferreira, Jorge F.S., J.E. Simon, and J. Janick. 1997. *Artemisia annua*: Botany, horticulture, pharmacology. *Horticulture Reviews* 18:319-371.

Janick, J. 1985. Annual Wormwood. New Crop FactSHEET. Purdue New Crops <http://www.hort.purdue.edu/newcrop/cropfactsheets/artemisia>

Klayman, D.L. 1985. Qinghaosu (Artemisinin): an antimalarial drug from China. *Science* 228:1049-1055.

Klayman, D.L., A.J. Lin, N. Acton, J.P. Scovill, J.M. Hoch, W.K. Milhous and A.D. Theoharides. 1984. Isolation of artemisinin (Qinghaosu) from *Artemisia annua* growing in the United States. *J. Nat. Prod*. 47:715-717.

Laughlin, J. C. 1994. Agricultural production of artemisinin: A review. *Trans. Royal Soc. Trop. Med. Hyg.* 88 (Suppl.1):21-22.

Morales, M.R., Charles, D.J. and J.E. Simon. 1993. Seasonal accumulation of artemisinin in *Artemisia annua* L. International Symposium on Medicinal and Aromatic Plants, March 22-25, 1993, Tiberias, Israel. Acta Horticulturae 344: 416-420.

Simon, J.E. and E. Cebert. 1994. *Artemisia annua*: A Production Guide. Purdue University Fact Sheet.

Simon, J.E., D. Charles, E. Cebert, L. Grant, J. Janick and A. Whipkey. 1990. *Artemisia annua* L.: A promising aromatic and medicinal. In: Janick, J. and J.E. Simon (eds). Advances in New Crops. Timber Press, Portland, Oregon. (in press).

Simon, J.E., L. Grant and M.L. Overley. 1986. Effect of plant spacing and nitrogen on *Artemisia annua*. Presented at the 105<sup>th</sup> International Congress of Essential Oils, Fragrances and Flavors. Washington, D.C. November 19, 1986. Scientific Program and Abstract Book (Abstr. 93).

Woerdenbag, H.J., N. Pras, W. Van Uden. T.E. Wallaart, A.C. Beekman, and C.B. Lugt. 1994. Progress in the research of artemisinin-related antimalarials: An update. Pharm. World Sci. 16:169-180.

World Health Organization. 1981. Report of the Fourth Meeting of the Scientific Working Group on the Chemotherapy of Malaria. Beijing, People's Republic of China, October, October 6-10, 1981.

APPENXIX I

**Selected List of Our Published Works on *Artemisia annua*, provided to BAMEX and to BIONNEX, Madagascar by:**

**New Use Agriculture and Natural Plant Products Program (NUANPP)**

**Rutgers University, Cook College**

**59 Dudley Road, New Brunswick, New Jersey**

**Office Tel: 732-9711, ext.355; Fax: 732-932-9441**

**The Rutgers Artemisia Team includes:**

**Dr Qing-Li Wu (chemistry, extraction/processing)**

**Dr. Chung H. Park (plant improvement, tissue culture, production systems)**

**Dr. Rodolfo Juliani (essential oils, by-products, and post-harvest QC systems)**

**Dr. Jim Simon, Team leader ([jesimon@aesop.rutgers.edu](mailto:jesimon@aesop.rutgers.edu))**

**Research Papers on *Artemisia annua*:**

2005. Wang, M., C.H. Park, Q. Wu, and J.E. Simon. Analysis of artemisinin in *Artemisia annua* L. by LC-MS with selected ion monitoring. **Journal Agric. Food Chem.** 53: 7010-7013.

1997. Ferreira, J.F.S., J.E. Simon and J. Janick. *Artemisia annua* L.: Botany, Horticulture and Pharmacology. In: Janick, J. (ed). **Horticultural Reviews** 19:319-371.

1995a. Ferreira, J. J.E. Simon, and J. Janick. Developmental studies of *Artemisia annua* L.: Flowering and artemisinin production under greenhouse and field conditions. **Planta Medica**: 61:167-170.

1995b. Ferreira, J. J.E. Simon, and J. Janick. Relationship of artemisinin content of tissue-cultured, greenhouse-grown, and field-grown plants of *Artemisia annua* L. **Planta Medica**: 61:351-355.

1994. Ferreira, J.F.S., D.J. Charles, K. Wood, J. Janick and J.E. Simon. A comparison of gas chromatography and high performance liquid chromatography for artemisinin analyses. **Phytochemical Analysis** 5:116-120.

1993. Charles, D.J., J.E. Simon, C.C. Shock, E.B.G. Feibert and R.M. Smith. Effect of water stress and drying on artemisinin content in the leaves of *Artemisia annua* L., pp. 640-643. In: J. Janick and J.E. Simon (eds). *New Crops: New Crops: Exploration, Research, Commercialization*. Proc. New Crops, Oct. 6-9, 1991, Indianapolis, IN. **John Wiley & Sons, Inc., N. Y.**

1993. Morales, M.R., Charles, D.J. and J.E. Simon. Seasonal accumulation of artemisinin in *Artemisia annua* L. International Symposium on Medicinal and Aromatic Plants, March 22-25, 1993, Tiberias, Israel. **Acta Horticulturae** 344: 416-420.

1992. Whipkey, A., J.E. Simon, D.J. Charles and J. Janick. In vitro production of artemisinin from *Artemisia annua* L. **J. Herbs, Spices and Medicinal Plants** 1:15-25.

1991. Charles, D.J., E. Cebert and J.E. Simon. Characterization of the essential oil of *Artemisia annua* L. **J. Essential Oil Research** 3:33-39.

1990. Simon, J.E., D.J. Charles, E. Cebert, L. Grant, J. Janick and A. Whipkey. *Artemisia annua* L.: A promising aromatic and medicinal. *Advances in New Crops*, **Timber Press**, Portland, OR. pp. 522-526.

1990. Charles, D.J. and J.E. Simon, K.V. Wood and P. Heinstejn. Germplasm variation in artemisinin content of *Artemisia annua* using an alternative method of artemisinin analysis from crude plant extracts. **J. Natural Products**. 53(1):157-160.

*A Systems Approach for GAP on Artemisia annua:*

2003. WHO. WHO Guidelines on Good Agricultural and Collection Practices (GACP) for Medicinal Plants. **WHO**, Geneva, 2003. 72 pp. (Original Drafts and edits compiled by H. Fong, J.E. Simon and J. Regalado).

*A Recent Paper Using a Technology that could be Applicable for Artemisinin Quantitation:*

2006. Juliani H.R., J. Kapteyn, D. Jones, A. R. Koroch, O. Behra, L. Ranarivelo, A. Y. Mensah, C. Quansah, D. Acquaye, D. Charles, M. Wang, H. Moharram. and J.E. Simon. Applications Using Near Infrared Spectroscopy in Quality Control and Adulteration of African essential oils. **Phytochemical Analysis** **17**: 121-128.

Note: this paper is being sent as this technology is not that costly relative to other options, is robust, and can be used under industrial settings as found in Madagascar. However, we do not know its efficiency in the quantitation of artemisinin, and toward this end, we are now in discussion with the equipment manufacturer to see whether they would be interested in testing 20-30 of our samples. If it works, it could be better than LC and TLC systems now being considered to monitor field and processing aspects.

## APPENDIX II

A New Novel Technology Being Promoted by a Canadian Company for the Extraction of Artemisinin. This information comes directly from BIOEXX's information packet. **This is only provided as an illustrative example**, of how we have assisted linking BIONNEX to other companies that could provide unique services and/or technologies. We provided the contact information, assisted in forging a link and then each party can discuss and meet with each other. We assisted in making this type of information available to the private sector in Madagascar. This is not a recommendation to use BIOEXX but an example of our recommendation for BIONNEX to be engaged in discussions with any/all companies that are involved in this sector, thus enhancing their options and competitive decisions with whom they should partner and with which companies could they and others in Madagascar work. Thus, this represents just one of several private sector companies. Below is simply the private sector's companies background and technology information packet. Our role was to provide assistance in the identification of such private sector companies and organizations, provide contact information to BIONNEX and others in Madagascar and then contact the companies outside of Madagascar when applicable to get the company information to Madagascar.

---



## **BACKGROUND**

---

**Bio-Extraction Ltd.** (commonly referred to as “BioExx”), is a company formed in January 2003. BioExx controls patented technology that allows the user to extract organic materials (“active ingredients”) from biomass such as plant, fish and even animal materials. BioExx extracts this material for use as raw material for pharmaceutical and nutraceutical products. While these types of companies usually require FDA approval for their products, since BioExx supplies only the raw material, it does not require such approval for sales of its products.

In many cases, the patented technology results in a higher quality extract being prepared at a cost reduction of as much as 75% compared to conventional, and more hazardous, extraction methods. The patent was issued in the UK in late 2003.

In January 2003, BioExx used this technology to successfully establish its first application extracting *PACLITAXEL* from ground hemlock commonly referred to as yew trees. This development has resulted in the installation of a manufacturing facility in New Brunswick Canada that operates on a 24/7 basis. Paclitaxel is one of the leading ingredients used worldwide for the treatment of early stage cancer and experienced global sales approaching \$2 billion in 2003. Further applications of paclitaxel are being sought every year and growth in the use of the raw material remains very strong.

In addition to paclitaxel, BioExx has recently completed tests proving suitability for extracting from biomass sources including Artemisia, marigolds, crustacean shells, and approximately 50 other compounds.

BioExx has also formed an R&D partnership with the Food Technology Centre (“FTC”) in Prince Edward Island, Canada. FTC has obtained a \$4.9 million grant to add new research facilities to its current world leading base of extraction technology. While the new facility is centered on the BioExx technology, the facility also houses a complete super-critical CO<sub>2</sub> extraction facility and is capable of replicating hexane, methanol or other purely solvent based extractions. This wide array of equipment allows for complete side-by-side testing of all popular extraction methods so that the end user or customer can accurately predict which method will work best for a given biomass application. All advancements resulting from the R&D at FTC will belong to BioExx. The net result of this partnership is expected to be a continual flow of new products that are similar to paclitaxel as described above.

## **TECHNOLOGY**

---

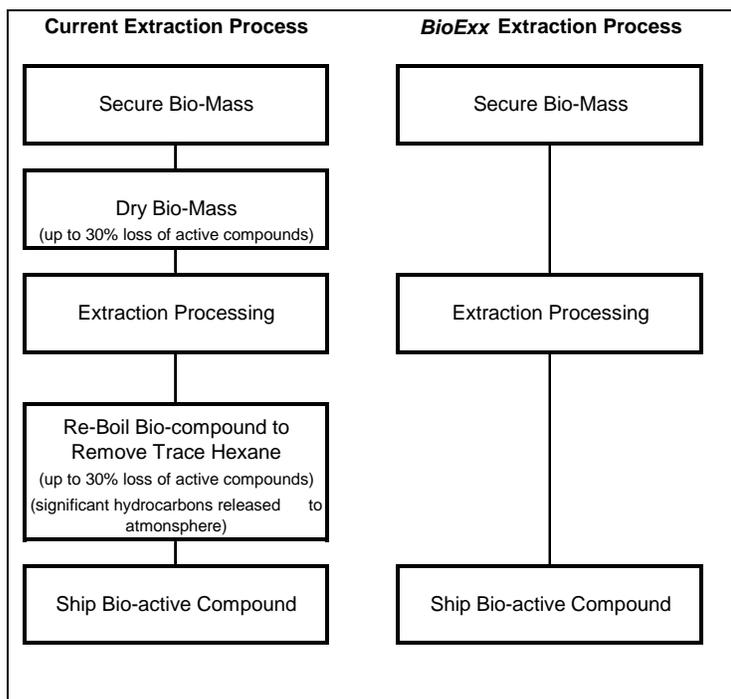
### *THE INDUSTRY Challenge*

Extracting vital compounds from plants and animal biomass is a process that has existed for centuries and is estimated to be a \$100+ billion industry annually on a global basis. Historically, the extractions use a range of environmentally dangerous, and in many cases, toxic compounds. The extracted end-products are used as the primary active ingredients for many drug products (pharmaceuticals), almost all vitamins (nutraceuticals), and many foods such as fish farm products and soy and canola based products

The most widely used existing technology involves a volatile solvent called hexane (a six octane gasoline) as the medium for extraction. The residual hexane is removed from the extracted compounds through re-heating the extracted compounds to high temperatures which, in turn, degrades the quality of the extracted material. Hexane processes also release significant amounts of hydro-carbon residues into the atmosphere. The result of this process is a high-cost, multi-step process that yields lower quality extracts than exists in the original medium being used in the extraction.

View the following websites for more detailed information regarding the concerns about Hexane: <http://www.healthfromthesun.com/HexaneFreePg.html> -  
<http://www.pnpi.com/Hexane.htm>

## THE *BIOEXX* SOLUTION



The BioExx extraction method uses an environmentally benign, non-petrochemical solvent with virtually no harmful emissions that leaves no harmful residues. The solvent works well with non-dried biomass where alternative solvents require the biomass to be pre-dried. This reduces the cost of the biomass by 60% or more.

As a result, there is no need to re-process the extracts at high temperatures to eliminate solvent residue. This eliminates two key costly steps in the extraction process and does not result in losses of the active ingredient during the drying or re-heating stage. This results in a substantially lower cost of production with better quality and yields of the desired compounds.

## APPENDIX I – AUTOMATED EXTRACTION FACILITY



Full time extraction facility is a closed-loop facility that operates below 300 psi. Solvents are neither carcinogenic nor flammable and are almost fully recovered during the extraction cycle.



Facility is 100% automated allowing for perfect repeatability during the extraction cycle. Programmable cycle parameters also allows for the facility to be easily converted for use with different types of biomass.