FORMULATION OF ORAL PHARMACEUTICAL DOSAGE FORMS CONTAINING CRUDE EXTRACTS OF *ARTEMISIA ANNUA*

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ABSTRACT

Artemisia annua is an asiatic plant with curative properties against malaria, containing artemisinin. This active compound, and its derivatives are the last few antimalarial drugs that remain effective against multidrug resistant strains of *Plasmodium falciparum*. But they are very expensive and not accessible to poor African countries.

The aim of this study is to obtain effective pharmaceutical dosage forms against malaria, starting from extracts of *Artemisia annua* cultivated in Benin.

Several extraction methods were compared based on the extraction yield of artemisinin and antiplasmodial activity tests. Artemisinin was quantified in each extract by an HPLC method with derivatization of artemisinin in Q260. The extraction yield of artemisinin was the highest by using Soxhlet extraction with petroleum ether 40-60 (0,262%). The infusion method led to the weakest extraction yield (0,194%). Intermediate results were obtained for maceration in oil ether (40-60) process (0,199%) and in the mixture water/ ethanol (50/50) (0,227%). The antiplasmodial tests *in vitro* were carried out by the method of Delhaes et al and revealed that maceration in water/ethanol would be most effective with a IC50 equal to 2,849 and very near to the results of the artemisinin as a reference (IC50 = 2,731). The hydro-alcoholic extract was evaporated in order to obtain a dry powder for the preparation of two oral drug dosage forms (hard gelatin capsules and oral suspension). These two formulations were tested for content uniformity, stability, and microbiological properties and all the tests were found to be conform to the Pharmacopeias recommendations.

The use of crude extracts of the plant can give cheaper and efficient medicines containing artemisinin and also other compounds of the plant with antipyretic properties and which can potentiate artemisinin antimalarial activity.

Keywords: Herbal medicinal products, Pharmaceutical dosage forms, drugs made with plants.

INTRODUCTION

Malaria is a tropical infectious disease which touches approximately 500 million people in the world each year. Between 2 and 3 million of those people dies and the majority are children of less than 5 years old (WHO, 2003).

Research in order to lead to a vaccine against this frightening parasitic disease was not crowned yet success, so the scientists were especially interested in research of drugs. Quinine, chloroquine, antifolinic and others antimalaric was thus used. But the problems of tolerance and resistance generated by these products took along the scientists to be turned to other plants which played a significant role in old Chinese medicine. They discovered a 2000 years old receipt prepared from *Artemisia annua* which is known to have curative property of malaria.

The species *Artemisia annua L* belongs to the family of Asteraceae. Its old Chinese name is Qing Hao, which means literally "green grass". It is pratically the only species which contains artemisinin. Artemisinin content obtained from cultivated plants vary from 0.01% to up to 1.4% dry weight according to various factors such as the plant's origin, its stage of development and the cultivation conditions (Ferreira et al, 1997).

Let us mention that this substance was found only in negligible quantities in two other species: *Artemisia opiacea* and *Artemisia lancea* (Tan et al, 1998).

Artemisinin is very effective against malaria even in its resistant forms.

All the drugs based on artemisinin found on the pharmaceutical market for the moment are developed starting from pure artemisinin and are very expensive.

It is absolutely non sense for the poor African countries, to obtain pure artemisinin, which is very expensive, rather than use rough extracts of the plant. In fact, extracts

can be obtained by less complicated operations and they contain others compounds which can be interesting in malaria treatment.

It had been showed that 5g of *A. annua*'s tea in 1L of water/day within 7 days given to patients made it possible to have 70 to 100% of cure (Anamed, 2004) against 72 to 90% of cure with the tablets of artesunate (Karbwang, 1994).

The use of crude extracts of the plant can give cheaper medicines containing artemisinin and also other compounds of the plant with antipyretic properties and which can potentiate artemisinin antimalarial activity.

The aim of this study is to obtain simple galenic formulations starting from rough extracts of *Artemisia annua* cultivated in Benin.

MATERIAL AND METHODS

Plant material

The seedlings *of Artemisia annua* used in this study were provided by the Laboratory of Ecology Applied (LEA) of the Agronomic Faculty of Sciences of Abomey Calavi University in Benin. In fact, this Asian plant had been acclimatized and put in culture in Benin and the seeds used were from Anamed in Germany.

The seedlings were harvested just before flowering. This period is recommended to obtain a maximum extraction yield of artemisinin in the plant. The leaves and small stems of the plant were dried in the shade, at ambient temperature not exceeding 40°C for 72 hours. They were then crushed into powder with a mixer.

Preparation of crude plant extracts

Various extractions were carried out:

- Soxhlet extraction by petroleum ether

5 g of *Artemisia annua* powder are extracted with the soxhlet by 250 ml of petroleum ether 40 – 60° during 4 hours. The extraction is repeated twice time on the residue. The three extracts are separately evaporated.

- Maceration with petroleum ether

5 g of *Artemisia annua* powder are extracted by maceration in 250 ml of petroleum ether 40 – 60°. Maceration was done for 1h30 under agitation is then filtered. The residue is rinsed with 30 ml of solvent. The extraction is repeated twice time on the residue. The three extracts are separately evaporated.

- Infusion in water

5 g of *Artemisia annua* powder are extracted by mixed with 1 liter of ebullient water, then left in infusion (under agitation) during 10 minutes. The mixture then is filtered then evaporated.

- And maceration in the mixture water/ethanol (50:50)

5 g of *Artemisia annua* powder are extracted by maceration with 1L of the mixture water/ethanol (50:50) during 1h30 under agitation. After filtration, the residue is rinsed with 30 ml of solvent.

The extraction is repeated twice time on the residue. The three extracts are separately evaporated.

All the solvent-free extracts were stored frozen until quantification.

Artemisinin quantification

Artemisinin quantification in the various extracts was done by HPLC according to the method of Zhao and Zeng (1985).

Artemisinin which absorbs slightly in UV undergoes a prédérivatisation. This allows the UV detection of the resulting product at 260 nm (Figure 1).

FIGURE 1

Separations were performed on a reversed-phase column Lichrospher RP-18 (250 x 4.6 mm). The mobile phase was isocratic and constituted of phosphate buffer 0.01M pH 7.9 and methanol (55:45).

The flow during the analysis was 1ml/min with a stop time of 15 minutes.

All the dry extracts obtained are solubilised into 25 ml of ethanol before HPLC quantification.

For this quantification, prederivatization is carried out by taking into a 10 ml measuring flask, 1 ml of the solution obtained and addying 4 ml of NaOH 0.2% (m/v).

The mixture is carried to 50°C during 30 minutes. After that, we let cool during 10 minutes, and then 1ml of ethanol was added. Finally 0.2 N acetic acid solution was filled up to the mark.

The calibration line was obtained starting from standards with respective concentrations in Q260 of 10, 20, 40 and 60 µg/ml prepared thanks to a stock solution of pure artemisinin in ethanol (1 mg/ml).

We respectively took 0.1 ml, 0.2 ml, 0.4 ml and 0.6 ml of stock solution into 10 ml measuring flask and we carried out prederivatization as described above.

Artemisinin rate were calculated in relation with the weight of plant used initially.

Antiplasmodial activity of the extracts

The antiplasmodial activity of the extracts was carried out into microplaques with 96 sterile wells.

The method used is based on Delhaes and al (1999), called "*microculture tetrazolium assay» (*MTA). This method gives us the different IC_{50}.

Stability studies:

Preparations of ethanolic and hydro alcoholic solutions of pure artemisinin were made and were stored at ambient temperature (25 ± 2°C). Then we realized periodic prelevements over 3 months for HPLC quantification.

Hydro alcoholic extracts were resolubilized in ethanol and solutions were stored at 7°C (fridge) and 40°C (drying oven). Periodic prelevements were done during 2 months for HPLC quantification.

Galenic formulations and quality control

We have realized with the extract a powder which will be reconstitute just before administration. Reconstitution will be done by adding 150 ml of water and shaking.

Patients must drink 10 ml of this suspension, three times a day during 5 days to be safe of malaria.

The powder obtained by addying Aerosil was also used to realized capsules which were tested after.

We studied the pharmaceutical quality of this suspension after reconstitution by:

- Identification and quantification of the active compound (artemisinin)

- Physicochemical controls *(rheological properties – pH – density)*

- Evaluation of the microbiological quality according to the European Pharmacopeia recommendations: (enumeration of total aerobic germs - enumeration of enterobacteria - *Escherichia Coli* – Salmonella/shigella).

RESULTS AND DISCUSSION

Artemisinin contents of the extracts and the seedlings of *Artemisia annua*

According to our study, the content of artemisinin of the seedlings of *Artemisia annua* cultivated in Benin borders 0.262% of the weight of dry leaves (Table 1). This value is low compared to the contents obtained by cultivating this plant in other countries. Indeed, ILLO (2002) had found content between 0.53% and 0.60% for the sample cultivated in RDC. Gaudin and Simmonet (2002) found a content of 0.54% after analysis of a sample cultivated in Switzerland. This Asian plant is also cultivated in several countries of under area, particularly in Burkina Faso, Madagascar, Cameroun and the contents obtained were also higher.

This difference would be explained by the fact that it is the first harvest of this plant, after experimentation's starting in Benin. New plantations have been implemented, in order to optimize the output of the seedlings and their artemisinin content.

TABLE 1

The comparison of the different extracts (Table 1) has shown that extracts by petroleum ether are the richest in artemisinin (p= 0,000). Such results had been establish by Klayman et al (1984); they affirmed that petroleum ether was the most selective solvent for artemisinin extraction and that it was the best solvent.

However, with the use of these extracts we would have the problem of residual petroleum ether which must be eliminating; and this is a supplementary phase.

Extract for the galenic formulation and studies of stability

Antiplasmodial activity *in vitro*

Analyse of the *in vitro* antiplasmodial activity (Table 1) has shown that the therapeutic effectiveness is not inevitably in relation to the rate of artemisinin because the extract with soxhlet, richer in artemisinin gave an IC50 equal to 3.69 µg/ml.

This report confirms the assumption of other substances (like flavonoids) contained in the plant which would potentiate the antipaludic action of artemisinin (Bhakuni et al, 2002; Elford et al, 1987; Mueller et al, 2000).

The hydro alcoholic extract of *Artemisia annua* appears interesting (extraction rate of artemisinin equal to 87% and *in vitro* activity which is the most significant and very near to that of the artemisinin of reference : **2.849 µg/ml against 2.731 µg/ml)** .

Moreover, with this extract we have no problem of the residual solvent to eliminate since ethanol is not toxic.

So, we selected this extract for the therapeutic use.

Stability studies

Pure artemisinin is stable in ethanolic and hydro alcoholic solutions for the 3 months period of this study (Figure 2).

FIGURE 2

The artemisinin concentration is stable over two months period when the solution of extract is stored in fridge but degradation appears when the solution is stored in the drying oven with 40°C. (Figure 3)

FIGURE 3

The stability of artemisinin thus differs according to whether it is in pure solution or in the hydro alcoholic extract.

A permanent liquid form is thus not adequate for galenic formulation with this extract as not stable for more than a few days. So we decided to realize two different formulation: powder to reconstitute oral suspension and capsules.

Galenic form obtained and pharmaceutical quality control

Formulation of a dry powder for oral suspension to reconstitute was done with the selected extract and the composition is below:

*Artemisia annua's e*xtracts	15g
Aerosil 200	4.5g which is hier an adsorbant
Xanthan gum	1.5g to viscosify the suspension
Saccharose	15g to edulcorate the suspension.
Menthol spirit	3 drops
Distillate water	qsp 150 ml

Pf an oral suspension

After formulation, we obtain a homogeneous and brown powder.

The aspect and the taste of the suspension after reconstitution were acceptable.

The capsules obtained had a good aspect and mass uniformity and content uniformity tests were done with success (results not shown in this paper).

Quality control of powder to reconstitute oral suspension:

The powder was easy to put in suspension and the suspension was stable.

The identification and the study of stability of the active ingredient are in conformity.

The measured pH is 5.3. This value is adequate and we observe that the pH is also stable during the 15 days of reconstitution.

The determination of the density gave 1.12 and the analyses of rheological properties carried out during 15 days of reconstitution were in adequation with international recommendations.

The viscosity of the reconstituted suspension remains constant with superposition of the curves of day 1, 7 and 14 (Figure 4).

FIGURE 4

The results obtained for the microbiological quality of the suspension (Table 2) show that it is in conformity with Pharmacopoeias recommendations.

TABLE 2

The complete pharmaceutical quality control of the suspension (physico-chemical and microbiological controls) was thus carried out and it shows that the formulation meets the standards of the European Pharmacopeia.

CONCLUSION

At the term of these studies, the hydro alcoholic extract of *Artemisia annua*, showing a good *in vitro* antiplasmodial activity, was chosen in order to carry out antipaludic galenic formulations.

Stability studies were realized and then we prepared a dry powder for oral suspension. The pharmaceutical quality of this formulation obtained from the hydro alcoholic extracts of *Artemisia annua* has been studied and it was found to be conform to the Pharmacopoeias recommendations.

Simple galenic forms, obtained easily with minimum equipment can thus be prepared with hydro alcoholic extracts of *Artemisia annua* and can be used by populations in order to treat simple malaria at low cost.

This study also showed us that it is possible to cultivate *Artemisia annua* in Benin, even if studies are still in hand in order to improve the rates of artemisinin contained in the seedlings.

The use of rough extracts of *Artemisia annua* constitutes a salutary way for treatment of malaria for the countries in the process of development like Benin.

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Table 1: Artemisinin contents (expressed compared to the mass of dry sheets) and antiplasmodial activity *in vitro* of various extracts of *Artemisia annua L.*

Extracts	Artemisinin content (% dry weight of leaves) (n = 3)	**IC50 (µg/ml) (n = 3)**
Soxhlet extract with petroleum ether	0.262 ± 0.003	3.690 ± 1.315
Macerate in petroleum ether	0.199 ± 0.003	3.278 ± 2.001
Macerate in water/EtOH 50:50	0.227 ± 0.022	2.849 ± 1.215
Infusion in water	0.194 ± 0.001	3.394 ± 0.390

Table 2: Microbiological quality control of the suspension after reconstitution

Microbiologic Tests	Results obtained	Normal values according to pH Eur [7]	Conclusion
Total aerobic microbial count	Bacteria: 8000 / ml Fungi: 6000 / ml	Bacteria:maximum 10^5 per ml Fungi: maximum 10^4 per ml	Conform
Enterobacteria count	10^2 - 10^3 per 1ml	under 10^3 bacteria per ml	Conform
E. Coli	No colonies	No colonies	Conform
Salmonella	No colonies	No colonies	Conform

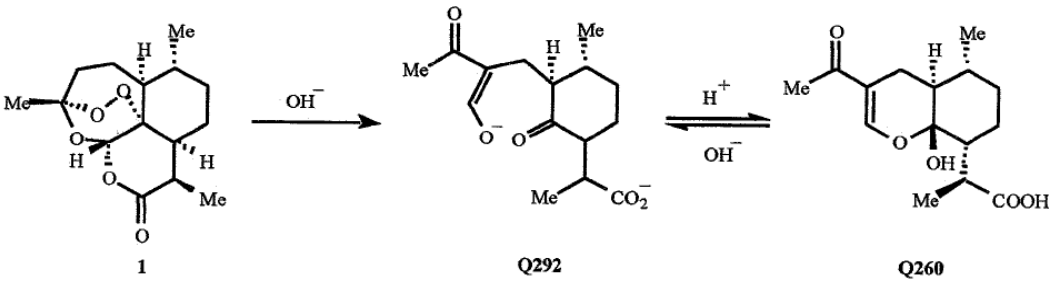

Figure 1: Derivatization of artemisinin in Q260

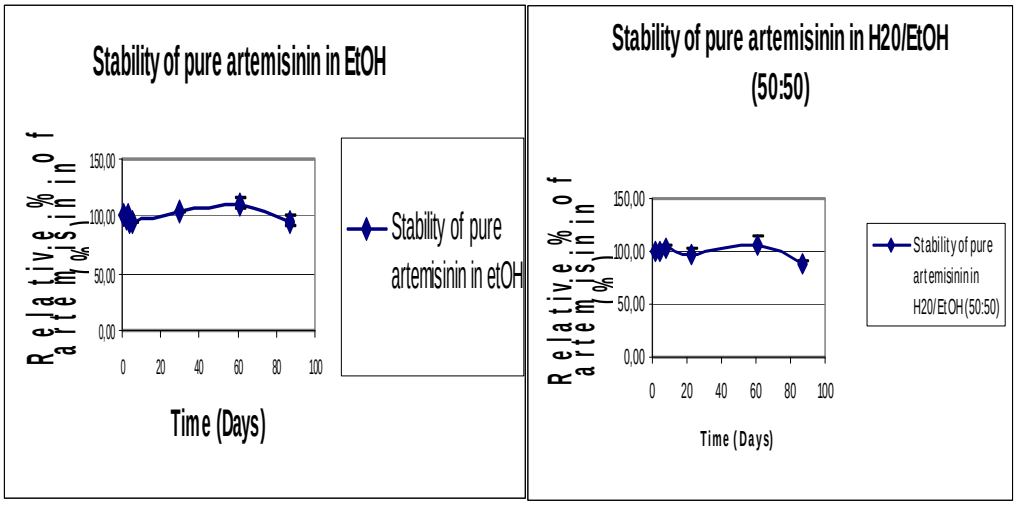

Figure 2: Stability of pure artemisinin in ethanolic solution 95% (left) and in hydroalcoholic solution 50:50 (right) stored at room temperature (25±2°C)

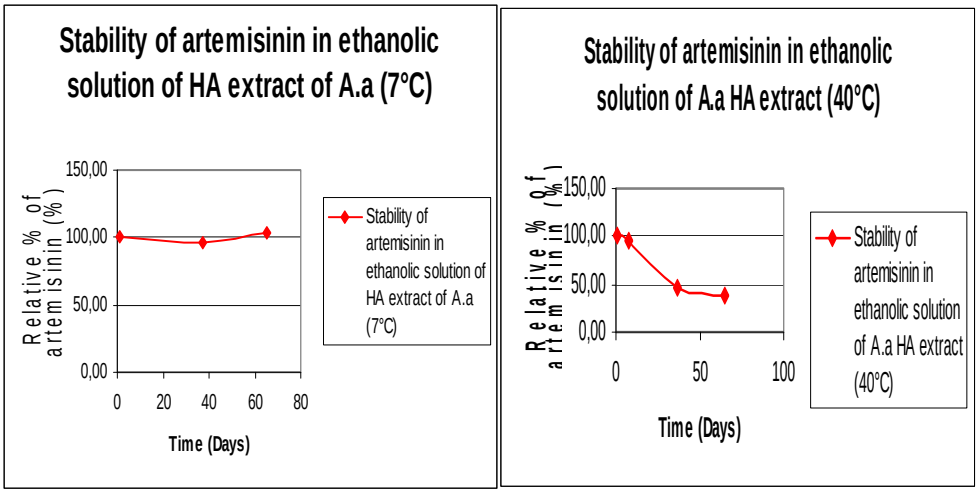

Figure 3: Stability of artemisinin in hydroalcoholic (HA) extract of *Artemisia annua* put in ethanolic solution and stored in fridge at 7°C (left) and in drying oven at 40°C (right)

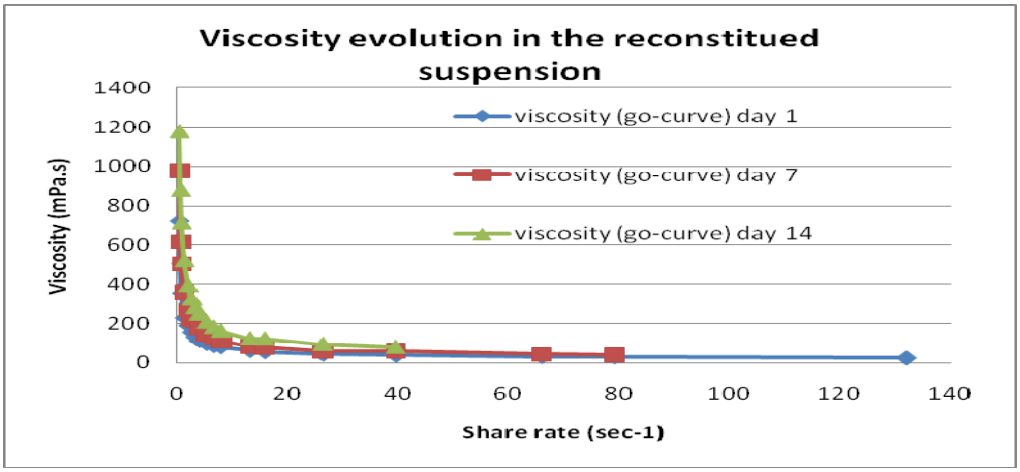

Figure 4: Determination of the rheological properties of the suspension during 15 days (Day 1, 7 and 14) using viscosimeter of Brookfield LVDV-e