Comparative study of the processes for obtaining secondary metabolites from leaves and stems of *Artemisia annua* (*Asteraceae*)

Etude comparative des procédés d'obtention de métabolites secondaires des feuilles et tiges de *Artemisia annua* (Asteraceae)

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Abstract

Artemisia annua is a plant widely used to treat diseases including malaria. Manipulation conditions andtreatment of plant influence the physico-chemical characteristics and the composition. In order to identify the best process for obtaining the extract with optimal pharmacotechnical characteristics and phytochemical properties for drugs formulations, four processes were performed.From the raw powder of stems and leaves of *Artemisia annua*, 4 types of extracts were obtained: a dry infused, a dry hydro-ethanolic macerate, a freeze-dried infused, freeze dried hydro-ethanolic macerate. Physico and phyto-chemical, microbiological characterization was implemented to compare them. The freeze-dried hydro-ethanolic macerate was uncharacterizable. Freeze-dried infused, dry hydro-alcoholicmacerate and dry infused exhibited respectively an extraction yield of 11.7 ± 0.3%; 8.4 ± 0.4% and 6.8 ± 0.3%. Powderswith diameter 0-2.75 µm and D_{50}<125 µm were fine. Freeze-dried infused had a porphyritic morphology while both dry macerate and infused had respectively a sticky and crystalline appearance. All powders were microbiologically clean and hydro-ethanolic macerate was qualitatively the richest extract in phytochemicals. Freeze-dried infused had the highest concentration of artemisinin (3322.5 +/- 0.6 µg/mL) compared to dry infused (1308.9 +/- 0.1 µg/mL), dry macerate (1296.2 +/- 0.3 µg/mL) and raw powder (2190.8 +/- 0.5 µg/mL). In contrast, phytochemical constituents such as flavonoids were more abundant in dry macerate than dry infused respectively at 28.7 +/- 0.2 mg EQ/g and 11.4 +/- 0.2 mg EQ/g when they were not quantifiable in lyophilizate. Infusion followed by Freeze-drying was the best process and should be recommended for *Artemisia annua* metabolites extraction.

Keywords: *Artemisia annua*, freeze-drying, Hight Performance Liquid Chromatography, infusion, maceration.

Résumé

Artemisia annua est une plante largement utilisée pour soigner des maladies dont le paludisme. Les conditions de manipulation et de traitement de la plante influencent les caractéristiques physico-chimiques et la composition. Afin d'identifier le meilleur procédé pour obtenir l'extrait avec des caractéristiques pharmacotechniques et des propriétés phytochimiques optimales pour les formulations de médicaments, quatre procédés ont été réalisés. A partir de la poudre brute de feuilles et tiges de *Artemisia annua*, 4 types d'extraits ont été obtenus : un infusé sec, un macérât hydro-éthanolique sec, un infusé lyophilisé, un macérât hydro-éthanolique lyophilisé. Des analyses physico et phytochimique, une caractérisation microbiologique ont été mises en place pour les comparer. Le macérât hydro-éthanolique lyophilisé était non caractérisable. L'infusé lyophilisé, le macérât hydroalcoolique sec et l'infusé sec ont présenté respectivement un rendement d'extraction de 11,7 ± 0,3 % ; 8,4 ± 0,4 % et 6,8 ± 0,3 %. Les poudres de diamètre 0-2,75 µm et D50<125 µm étaient fines. L'infusé lyophilisé avait une morphologie porphyrique tandis que le macérât sec et l'infusé avaient respectivement un aspect collant et cristallin. Toutes les poudres étaient microbiologiquement propres et le macérât hydro-éthanolique était qualitativement l'extraitle plus riche en composés phytochimiques. L'infusé lyophilisé avait la plus forte concentration en artémisinine (3322,5 +/- 0,6 µg/mL) par rapport à l'infusé sec (1308,9 +/- 0,1 µg/mL), le macérât sec (1296,2 +/- 0,3 µg/mL) et la poudre brute (2190,8 +/- 0,5 µg/mL). En revanche, les constituants phytochimiques tels que les flavonoïdes étaient plus abondants dans macérât sec que dans l'infusé secrespectivement à 28,7 ± 0,2 mg EQ/g et à 11,4 ± 0,2 mg EQ/g lorsqu'ils n'étaient pas quantifiables dans l'infusé lyophilisé. L'infusion suivie d'une lyophilisation était le meilleur procédé et devrait être recommandé pour l'extraction des métabolites d'Artemisia annua.

Mots clés : *Artemisia annua*, lyophilisation, Chromatographie Liquide Haute Performance, infusion, macération.

Introduction

The resurgence of drug resistance, accentuation of side effects, high costs associated with certain conventional drugs and their unavailability are pushing people to resort more and more to medicinal plants **(1).** Rational use of these medicinal plants requires the standardization of their treatments, such as the extraction and drying processes in order to lead to standardized, reproducible preparations presenting physico-chemical properties and stable phytochemical components allowing the formulation of quality drugs **(2–4)**. The choice focused on the study of *Artemisia annua* plant, an asteraceae: for its indication in the treatment of malaria. Malaria causing many deaths especially in children; for one of its active ingredients, artemisinin, used in the manufacture of ACTs, which is recommended as a first-line drug by the National Malaria Control Program (PNLP) in malaria-endemic countries, even if unfortunately they know resistances; for its use for more than 2000 years by the Chinese to treat malaria without any resistance being observed.

In the leaves and stems of *Artemisia annua*, sesquiterpene lactones including artemisinin and many other major and minor secondary metabolites have been found to act synergistically against malaria parasites **(5–7).** Many plant constituents, including artemisinin, are found at low concentrations in herbal tea and undergo degradation after 24 hour due to their instability in solvents **(8–10),** hence the interest of this work, which aims to compare four processes of obtaning extracts, in order to identify the best extract with pharmaco-technical, phyto-chemical, physico-chemical and microbiological levels to produce galenic formulations.

1. Material and methods

1.1. Plan material

The plant material were the leaves and stems of cultivated plants of Artemisia annua (Asteraceae) in plantations of APESE, an association in Bangangté, in west Cameroon. The harvest was made as soon as first flower buds appeared. After blowing leaves and stems at 40°C, the drying was carried out on racks in a clean and ventilated room for 3 to 5

days. Dried fresh plant loss its 60% water content. When their weight no longer varied, these parts were ground using a RESTCH brand at 4000 rpm for 3 minutes to obtain a coarse powder. A branch of a dried plant containing leaves, stems and flower bud has been identified at the National Herbarium of Cameroon on Voucher number 65647/HNC.

1.2. Chemical drug

Standard artemisinin and standard quercetin were from Sigma-Aldrich Co. (USA).

1.3. Preparation of extracts

Two extraction methods were used, namely extraction by aqueous infusion and hydro-ethanolic maceration on the basis of its frequent use in traditional medicine as a method of extraction About drying, two methods have also been carried out, namely freeze-drying and evaporation under vacuum followed by drying in an oven considered as modern method **(11).**

The combination of these extraction and drying powder methods allowed us to develop four processes to obtain secondary metabolites in the different types of extracts of *Artemisia annua*:

1.3.1. Aqueous infusion, concentrated by evaporation under vacuum and dried in an oven (process 1)

Extract obtained by 3 successive infusions required 520 g of coarsely ground leaves and stems which were introduced into 3 liters of boiling water. After 15 minutes of infusion, a simple filtration with a sieve and then with Wattman No. 1 filter paper was carried out. The infusion was taken over the marc simultaneously twice; and filtrate was concentrated on a rotary evaporator at 45°C under vacuum then dried in an oven at 45°C for 3 days.

1.3.2. Aqueous infusion dried by freeze-drying (process 2)

Extract was obtained by 3 successive infusions as described above and filtrate was frozen in flasks then freeze-dried for 24 to 48 hours.

1.3.3. Hydro-alcoholic maceration followed by vacuum evaporation and oven drying (process 3)

520 g of coarsely ground leaves and stems were introduced into 3 liters of an Ethanol/Water mixture in proportions 70/30 (v/v) and left to macerate with stirring for 48 h. A simple filtration was carried out and macerate been concentrated on a rotary evaporator at 45°C under vacuum and dried in an oven at 45°C for 3 days.

1.3.4. Hydro-alcoholic maceration followed by freeze-drying (process 4)

520 g of coarsely ground leaves and stems were introduced into 3 liters of an Ethanol/Water mixture in proportions 70/30 (v/v) and left to macerate with stirring for 48 h. A simple filtration was carried out and filtrate was frozen in flasks then freeze-dried for 24 to 48 hours.

The yield of the extractions was calculated according to the following formula:

$$\%R = \frac{\text{Dried extract Mass}}{\text{Dried raw plant material}} X\ 100$$

1.4. Raw Powder and extracts Tests

1.4.1. Determination of residual moisture

We used thermogravimetric method to carry out raw powder and extracts **(12).**

1.4.2. Macroscopic and sensory examinations

The raw powder and extracts were taken to sense organs to determine taste, color and odor

1.4.3. Particle size analysis

According to decision tree, due to the particle size more than 50 µm the sieve method was used to analyse raw powder **(12).** Analysis was validated when sum of the oversize from each sieve did not exceed more than 2% **(12)**.

Regarding the extract, the sieve method was not indicated because of small particle size (less than 50 µm) thus the evaluation of particle size distribution was done by microscopy.

Particles of various extracts were observed and length of them (Martin's diameter) was measured using OPTIKA microscope connected to a computer. The shapes of particles were described and particle size analysis was carried out.

Range was determined by formula $Span = (D_{90} - D_{10})/D_{50}$. Modal diameter was determined by average diameter of most frequented class. D_{90}, D_{10} and D_{50} representing 90%, 10% and 50% respectively of cumulative particle distribution were determined.

1.4.4. Solubility test

Extract solubility test was carried out according to OECD 105 method **(14,15)** for these different solvents conventionally used in the manufacture of drugs (water, glycerin, sorbitol, propylene glycol, 70% alcohol, 95% alcohol, petroleum jelly) and on 1 g extract sample test at room temperature.

Determination of pH:

pH of extracts was determined with a pH meter after realize dilution to 1/10

1.4.5. Phytochemical screening

It was performed qualitatively on raw powder and extracts samples after extraction with appropriate solvents using a standard method based on staining and precipitation reactions as described by Jean Bruneton **(12).**

1.4.6. Identification and concentration of markers in powder

These markers were artemisinin and total flavonoids expressed as quercetin equivalent per gram of dry matter (DM). The assay methods were validated by checking linearity, repeatability, detection and quantification limits.

a) Identification and concentration of artemisinin

Identification of artemisinin in plant powder was made by thin layer chromatography using 100 mg of *Artemisia* leaves and stems powdered and mixed in a mortar with 5 ml of toluene. After centrifugation, 20 µL of extract (filtrate) and 10 µL of artemisinin standard dosed at 250 ng/µL were deposited on a TLC plate. After migration, the plate was revealed under a UV lamp at 280 nm with 10% dilute sulfuric acid. The frontal ratios (Rf) of different tasks were calculated.

Artemisinin dosing assay was performed by HPLC according to method described by Zhao and Zeng **(16).** The maceration of 250g of powder with 200 mL of dichloromethane were conducted for 5 hours and 3 times successively. Evaporated filtrates were then treated with a mixture of water and acetone (8 volumes/10 volumes). A white precipitate (artemisinin) obtained was washed with an excess of water, dried and purified with hexane, then dissolved in 10 mL of acetonitrile and analyzed by HPLC on a C18 column in reverse phase, with standard artemisinin as internal standard. A linear gradient (80:20) of acetonitrile and 10 mM phosphate buffer was applied as mobile phase at a flow rate of 0.6 mL/min. An iodine strip detector was used and reading was taken at a wavelength of 260 nm.

b) Concentration of flavonoids

Flavonoids dosing assay was performed using a Waters Alliance 2998 PDA HPLC and a waters e2695 separation module with temperature oven and EMPOWER3 operating software. 20 µL of each extract obtained by dissolving 1 mg of sample in 1 mL of solvent, were injected onto a C18 reverse phase type column, of dimensions equal to 125×4.6 mm. The mobile phase consists of three eluents: distilled water, methanol, acetic acid (50:47:2.5) (V/V/V). The applied elution gradient was spread over 7 min. The flow rate is 1 mL/min **(17)**. The detection was carried out by a UV-Vis detector at a wavelength equal to 254 nm and an iodine plate detector.

The formula applied to find concentrations of artemisinin and quercetin was as follows:

$$\text{Concentration} = \frac{SE}{ST} \times \frac{Pt}{VtT} \times t\,\% \times \frac{VE}{PE}$$

SE: test area, ST: control area, Pt: control mass, Vt: control volume, t%: standard titer /100, VE: test volume, PE: test mass

1.4.7. Microbiological control

The objective of this control was to evaluate microbial load on raw powder and extracts by counting germs on a solid medium. Two techniques were used, the first consisted of depth or mass inoculation for enumeration of Aerobic Mesophilic Germs (MAG) and thermotolerant coliforms while the second was carried out on surface for detection of *Escherichia coli*, *Staphylococcus aureus*, yeasts, molds and *Pseudomonas aeruginosa*. A series of two dilutions was carried out for each preparation **(18)**.

1.4.8. Statistical analysis

Kruskal-wallis test was used to compare Artemisin concentration in differents powders, extraction yield

2. Results

2.1. Extraction yield, residual moisture of raw powder and extract test results

The results of the various tests, the yield of the extraction, the loss on drying of raw powder and extracts were recorded in the following table 1.

Freeze-dried hydro-ethanolic macerate was abandoned due to the difficulties in recovering the lyophilizate which had stuck strongly to the walls of the freeze-dryer balloons. Thus the analyzes only focused on the three other processes

Table 1: Extraction yield and residual moisture content of different powders

Powder or Extract Type	Yield (%)	Residual moisture
Raw powder	**Not applicable**	9.1± 0.3%
Infused dry	6.8 ±0.3%	11, 1 ± 0.2%
Dry hydro ethanolic macerate	8.4 ±0.4% ** p = 0.0023 compare to infused dry yield	12.1±0.2%
Infused freeze-dried	11.7±0.3% *** p = 0.0001 compare to infused dry yield	9.8±0.2% ** p = 0.0015 compare to infused dry of residual moisture

2.2. Macroscopic characteristic of raw powder and extracts

The macroscopic organoleptic characters of different powders were showed is table 2.

Table 2: Macroscopic and sensory results of different powders

Types of powder	Organoleptic's characters				
	Odour	Flavour	color	touch	picture
Raw powder	Pleasant Flavored Camphor smell	Bitter and astringent	Greyish-green	Crunchy and grainy	
Infused dry	Pleasant, camphor aroma	bitter	Green-dark	crystalline powder	
Dry hydro ethanolic macerate	strong and pleasant smell	bitter	marbled color with yellow and orange reflections	Soft extract, very sticky, shiny rubber aspect	
Infused freeze-dried	Very flavored and camphorated	bitter	Green-Brown	Porphyritic appearance, spongy, very hygroscopic	

2.3. Particle size analysis of raw powder and 3 extracts

Particle size distribution in raw powder sieve method revealed that 10% of particles size were less than 50 µm (D_{10} < 50 µm), with median particle size around 360 µm (D_{50} = 360 µm), D_{90} = 2000 µm and Span = 0.05% **(Figure 1)**.

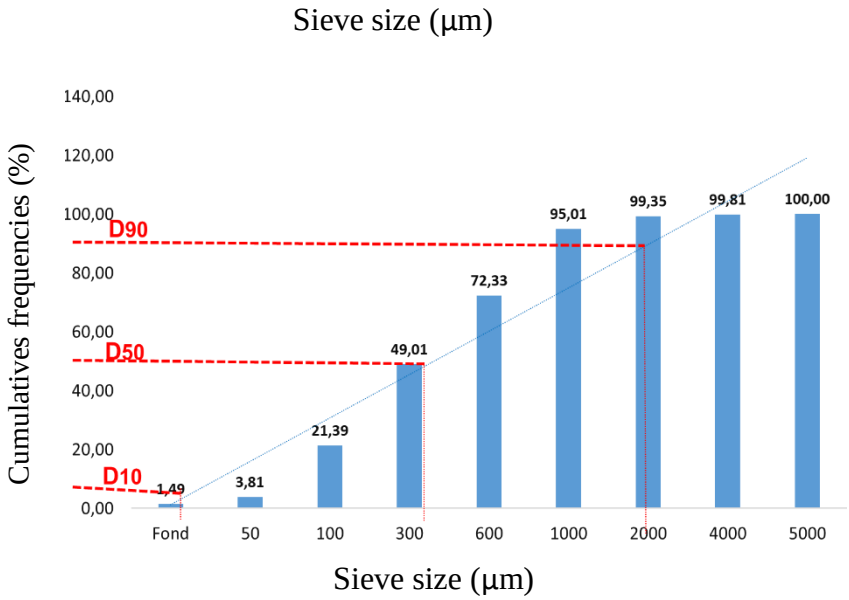

Figure 1: **Particle size distribution of *A. annua raw powder***

About extracts, particle size analysis by microscopic method showed the D_{10}, D_{50}, D_{90} and the span of different extracts **(Table 3)**

Table 3: Particles distribution of 3 extracts according to Ferêt diameter

Settings	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	Span(%)	Powder type
Dry infused (I)	0.125	0.375	0.625	0.01	**Very fine**
Freeze-dried infused (L)	0.125	0.50	0.750	0.01	**Very fine**
Dry hydro-alcoholic macerate(M)	0.125	0.225	0.700	0.02	**Very fine**

The D_{50} of all extracts is less than 125 µm (D_{50} <125 µm) which allows them to be classified as very fine powders. Each of curves admits a plateau, testifying to homogeneity of the extracts.

2.4. Extract solubilization and pH tests

Solubility

Among 7 solvents (water, sorbitol, glycerol, propylene glycol, alcohol at 70°C v/v, alcohol at 95% v/v and petroleum jelly), water and sorbitol presented best solvation for infused extracts with values of solubility between 33 and 50g/L. Concerning dry hydroethanolic macerate, solubility was higher in 70°C v/v alcohol with a solubility value of 95g/L. All the extracts were insoluble in 95° ethanol and in petroleum jelly.

pH of powder and extracts

Dry infused, freeze-dried infused, macerate and raw powder had pH values below 8 **(Table 4)**

Table 4: pH of powder and different extracts

Powder Type	pH	Temperature (°C)
Raw powder	7.65± 0.2	24.6
Infused dry	6.02 ± 0.5	23.4
Freeze-dried	5.89 ± 0.4	23.4
Hydroalcoholic dry macerate	6.9 ± 0.5	23.4

2.5. Phytochemical screening of powder and extracts

The chemical constituents characterized in the raw powder and in the extracts are presented in Table 5.

Table 5: Qualitative phytochemical screening of powder and extracts

Secondary metabolites	Raw powder	Infused dry	Infused freeze-dried	Hydroalcoholic dry macerate
Alkaloids	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	-	+
Phenols	+	+	-	+
Quinone	+	+	+	+
Sterols	+	-	-	++
Terpenes	+	+	+	-
Saponosides	+	+	+	-
Coumarins	+	+	+	+
Anthocyanins	-	-	-	-

presence: +: Absence: -

2.6. Identification and assay of markers (artemisinin and quercetin) in raw powder and extracts

TLC shows the brick red spot confirming the presence of artemisinin at frontal ratio 0.35 **(Figure 2)**

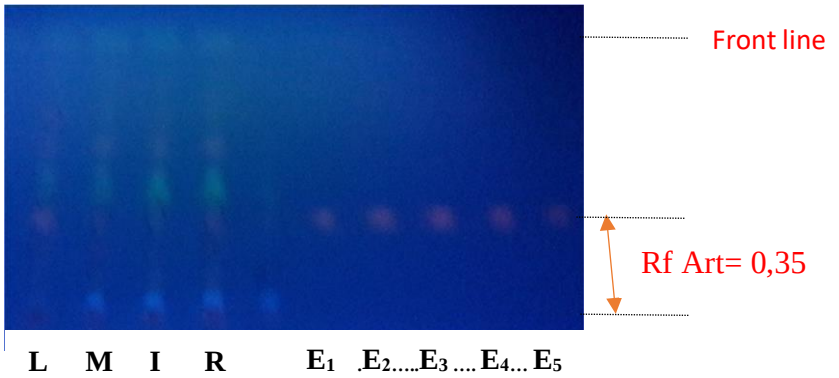

Figure 2 : artemisinin identification chromatograph in raw powder and extracts

L: Freezed dry infused; M: Hydroalcoolic dry maceration; I: Dry infused; R: Raw powder; E: Artemisinin standard; $E_1E_2E_3E_4E_5$: Increasing concentrations

Artemisinin concentrations in raw material and extracts are shown in Figure 3

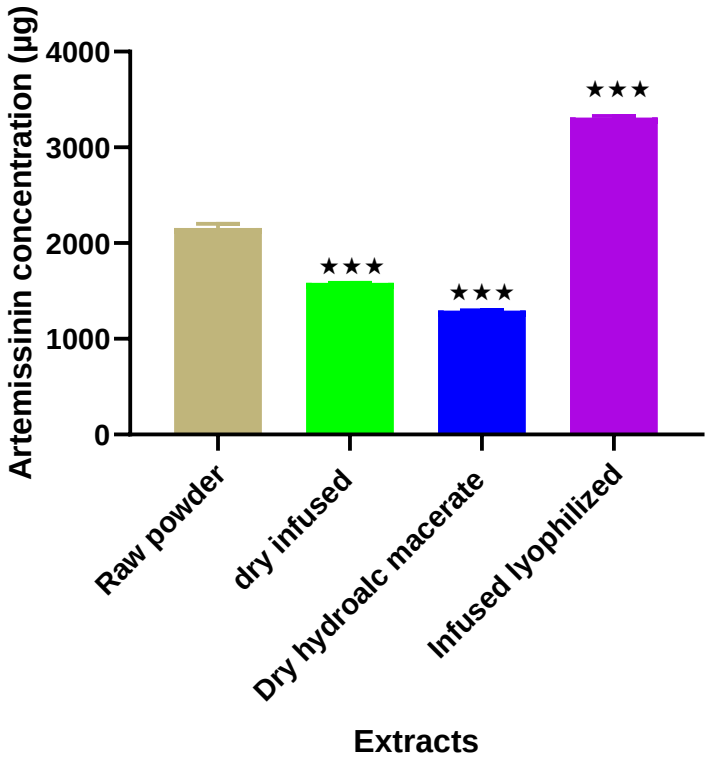

Figure 3: Evolution of artemisinin concentration in extracts according to method of extraction

Kruskal-wallis test: Values expressed mean standard deviation; risk α=5%;

Significant difference artemisinin concentration compared to raw powder: Dry infused (p = 0.0001); Dry hydroalc macerate (p = 0.0001); Infused lyophilized **(p = 0.0001)

On Figure 4 were presented all total flavonoids of different powders

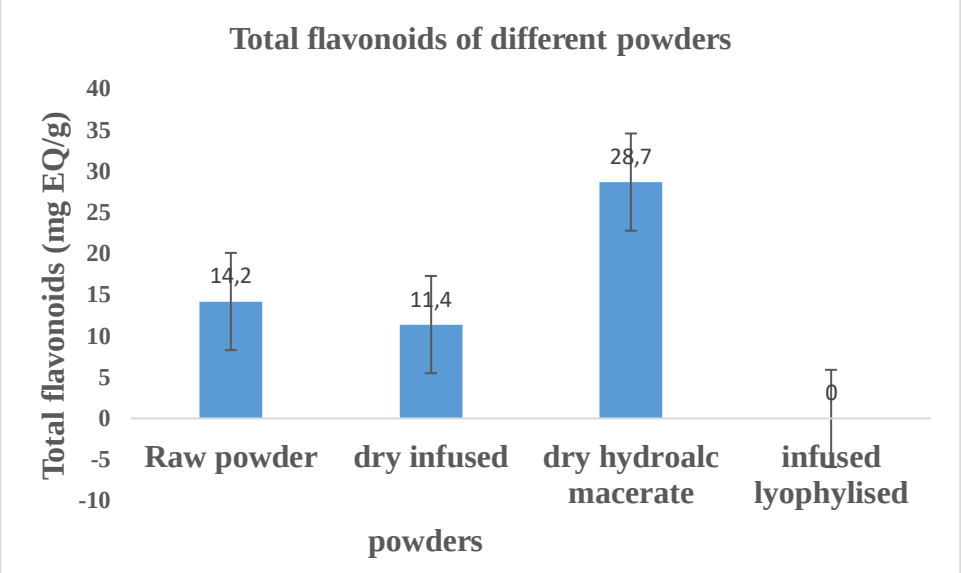

Figure 4: Evolution of total flavonoids of different powders according to method of extraction

Dry hydroalcolic extract have the highest total flavonoids

2.7. Microbiological results of powders and extracts:

An acceptable amount of germs was only identified in the raw powder **(Table 6)**.

Table 6: Microbiological characteristics of powder and extracts

Germs	**Raw powder (CFU/g)**	**Infused dry (CFU/g)**	**Freeze -dried (CFU/g)**	**Dry macerate (CFU/g)**
Thermophilic coliforms	-	-	-	-
Escherichia coli	-	-	-	-
Salmonella	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-
Staphylococcus aureus	-	-	-	-
yeasts and molds	$0.9x10^{2}$	-	-	-
Total Aerophilic Germs	$3x10^{1}$	-	-	-

2.8. Statistic results:

Kruskal-wallis test:

Showed the significant difference yield of infused lyophilized extract *** $p = 0.0001$ compare to infused dry yield followed by dry hydroethanolic macerate extract ** $p = 0.0023$ compare to infused dry yield:

Moisture content were lowest in infused lyophilized extract ** $p = 0.0015$ compare to infused dry of residual moisture. There were no significative difference between dry ethanolic macerate extract and dry infused extract.

There were also significant difference artemisinin concentration compared to raw powder: Dry infused ($p = 0.0001$); Dry hydroalc macerate ($p = 0.0001$); Infused lyophilized **($p = 0.0001$)

Discussion

The processes for obtaining secondary metabolites of a plant are numerous, varied and capital on industrial level in order to ensure optimum quality of the raw materials used in production. Talk about processes refers to the combination of both extraction and drying methods that lead to a quality raw material that can be ready to use in the formulation. Extraction and drying, are too critical operation on an industrial level that requires validation before. The aim of this work was to evaluate the influence of extraction and drying methods on extraction yield, physicochemical characteristics, phytochemical, microbiological and pharmacotechnical properties to produce galenic formulations.

Four processes were implemented to obtain secondary metabolites from the plant:

- Aqueous infusion followed by evaporation under vacuum and drying in an oven;
- Aqueous infusion followed by freeze-drying,
- Hydro-ethanolic maceration followed by evaporation under vacuum then drying in an oven.
- Hydro-ethanolic maceration followed by evaporation under vacuum then drying in an oven

The tests were performed in oder to identify the best processes that would produce an extract with an acceptable yield and optimal physicochemical properties,

➢ **Extraction yield and residual moisture and organoleptic characteristics of extracts and raw powder**

Infusion and maceration have been chosen to mimic extraction in traditional medicine.

The mode of extraction and drying method performed at different raw powders to obtain extracts influenced yield, appearance, smell, including colour of 3 extracts under study. Infusion for 48 hours followed by freeze-drying were the process which made possible to obtain highest yield of dry extract (11.7 ± 0.3%) compared to dry macerate (8.4 ± 0. 4%) and dry infused (6.8 ± 0.3%). Also this same process had well influence organoleptic characteristics giving a pleasant smell and touch as well as a beautiful colour. Guilbot et al**.** although having used characterized tinctures and extracts of glycerinated fluids which are other modes of extraction, come to the same conclusion as us, namely the influence of certain factors such as temperature, time, solid/solvent ratio used, contact surface with solvent are all elements that influenced extraction yields as well as organoleptic characteristics **(19)**.

The results of quality of lyophilizate obtained, conforms to standards of European Pharmacopoeia 10th edition **(20)** had confirmed once again that freeze-drying is the best drying method because this process considerably reduced humidity of extracts, thus limiting degradation risks while maintaining best organoleptic characteristics**.(21,22)**.

Hydro-ethanolic macerates and dry infused extracts gone under standards recommended by European Pharmacopoeia 10th edition, which makes those extracts obtained auspicious to micro-organisms development. However, some authors have shown that if drying plants quickly lowered water content of leaves below 12%, this was enough to stop biochemical reactions and avoid degradation of plant material during drying and storage **(23,24)**.

➢ **Particle size analysis of the raw powder by the sieve method.**

Raw powder was coarse as D50 was 360 µm. Percentage of rejection was 0.8% less than 2%, which validated particle size analysis. All these results are compliant with specifications of European Pharmacopoeia 10 Ed **(20).** All dry powdered extracts (dry infused, dry macerate and freeze-dried infused) were very fine with particle size analysis by microscopy gave similar results with spans between 0.01 and 0.02 showing a good distribution with D50 <125 µm.

➢ **Physico-chemical characterizations**

✓ ***Solubility***

All extracts were soluble in water and sorbitol but macerate was more soluble in alcohol 70%. Samira et *al*., in 2018 showed that *Artemisia herba alba* extracts belonging to Asteraceae family as *Artemisa annua* were more soluble in polar solvents **(23)**

✓ ***pH***

Dry infused and freeze-dried infused had similar pH values, respectively around 5.69 and 6.2. Concerning macerate and raw powder, this pH was neutral and close to 6.9 and 7.65 respectively. Extraction solvent would have an influence on pH of extracts. Our results are in line with those of Youyou Tu, 2021 who found in his study on metabolic engineering of *Artemisia annua*, that e optimal pH values varied between 6.5 and 7 **(25)**.

➢ **Phytochemical screening**

By colour reactions revealed presence of alkaloids, flavonoids, polyphenols, sterols and polyterpenes in all extracts. However, saponosides and anthocyanins were absent in the infusion, macerate and lyophilizate. Flavonoids were concentrated in hydro-ethanolic extract, the best solvent for extracting them. Conventionally, phenolic compounds are extracted by maceration or reflux extraction with hydro-alcoholic mixture. Hydroalcoholic macerate was richer in the other metabolites; this is in agreement with Diawara et al studies **(26)**

➢ ***Dosage of markers (artemisinin and quercetin)***

✓ ***Artemisinin***

Infusion followed by freeze-drying made highest levels of artemisinin, while infusion and hydro-ethanolic maceration followed by vacuum evaporation and drying in an oven gave extracts containing artemisinin and quercetin with quite acceptable yields. This great variability in artemisinin concentration between different extracts could be due to temperature at which these operations were carried out. Indeed, the level and time of application of this heat for evaporation and drying (infused and macerate) would cause volatilization of compounds responsible for the low proportion of artemisinin with these processes. In contrast, cold desiccation carried out with freeze-drying would preserve thermolabile and denaturable substances.

Another factor which could influence these constituents in extracts is also extraction time. , the freeze-dried infused is richer in artemisinin than the dry infused this can be justified by the fact that artemisinin degrades in solution after 24 hours, the exercise thesis work by Chuipet demonstrated this when she evaluated the stability of the artemisinin contained in the herbal tea over time. She demonstrated that in solution, whatever the means of preservation, with the addition of a natural preservative (lemon) or in the refrigerator, artemisininin began to degrade after 24 hours and continued over time **(9)**. Extraction and/or purification process must therefore be rapid to reduce time extraction and limiting pre- and post-treatment steps **(27,28)**.

✓ **Flavonoïds**

Concerning Flavonoids, several studies have demonstrated the thermal stability of flavonoids during processes using positive temperatures **(29)**.

In the current state of our knowledge, there are very few works that have shown the stability of flavonoids at negative temperatures (freezing) since freeze-drying is a process that involves freezing before drying, since the reaction of Shibata showed the presence of flavonoids while the HPLC could not quantify quercetin, it would be good to investigate the influence of freezing on the stability of quercetin. Qiao et al demonstrated that quercetinin was degradedat low temperature and while an improvement was noted with increasing temperature this is corroborated with the

results found during the HPLC quantification of the flavonoid marker, quercetin that we used for the evaluation. These results could be different if we had used a flavonoid marker other than quercetin (**30)**.

➢ **Evaluation of microbiological quality**

Environmental cultivation places contamination, harvesting conditions and all manipulations up to final conditioning of plants, are more steps which can explain contamination of medicinal plants.

Number of molds and yeasts ($3x10^1$ CFU/g) was lower than maximum bacteria tolerate (10^4 CFU/g) in dry matter. Our results are different from those of Dony et *al* who found that bacterial load in their samples was greater than 10^3 CFU/g **(31,32)**. The low-level contamination of our plant material would not only be due to dry process as observed by Tharreau et al., but also to part of plant used, place of harvest and the climatic conditions during harvest and associated manipulations **(33).**

In total, infusion extraction process followed by freeze-drying gave us best results in terms of yield, moisture and artemisinin content. However major disadvantage of this type of extraction is financial cost which will have negative impact on cost of large-scale production of extracts and therefore on the cost of the drug.

Conclusion

Infusion followed Freeze-dried was the process that produce an extract whose powder was the richest in artemisinin (major component responsible for the antimalarial activity), in addition this process gave good extraction yield, low humidity rate and pulverulent appearance (powder) in accordance with e specifications of 10th pharmacopoeia Edition. Regardless cost of acquiring this equipment, this process should be retained for herbal galenic formulation. As an alternative, maceration can be used because it leads to obtain powdered extracts with best phytochemical qualities.

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