Examining The Extraction Of Artemisinin From Artemisia Annua Using Ultrasound

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Abstract.

Artemisinin suppresses the life-cycle of the plasmodium parasite which causes malaria. It is found naturally occurring within the trichome glands of the *Artemisia annua* plant.

Traditional methods for extracting artemisinin are time-consuming and have high environmental impact due to the temperatures and organic solvents which must be employed. Ultrasound decreases these through acoustic streaming and micro-jets. But to fully utilise this technology parameters, such as frequency, temperature and the properties of leaf and solvent, must be explored.

As with the extraction process there is also no set analysis method for identification of artemisinin. Therefore several methods of analysing these extracts are employed. Initial results indicate that sonication is able to enhance levels of artemisinin extracted when compared to the conventional/traditional extraction process. In addition Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) have been shown to have a high level of reproducible calibration.

Keywords: Ultrasound, Sonochemistry, Food, Extraction, Malaria.
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INTRODUCTION

The statistics of how malaria impacts the world's population are widely reported and it is known to be a disease that can disturb up to 500 million and devastate 5 million lives a year. A huge burden is therefore placed upon over 90 countries in tropical climates where malaria is rife. Over the last 50 years the World Health Organisation (WHO) has strived to rid these 90 countries from the ravages of malaria through the use of antimalarial medicaments, such as: chloroquine, quinine, mefloquine (Larium), primaquine, halofantrine and anti- folates such as sulfadoxine-pyrimethamine, which were initially extremely effective. However in the 1970s resistance of the parasites to these drugs began to be a problem. As a result of the increased resistance of the parasites to these compounds alternative sources of treatment was sought.

A potential source of anti-malarial medicines are those known as Artemisinin derivatives which are often extracted from the Artemisia annua plant [1]. These have been established from a Chinese herbal remedy denoted qinghao or Artemisia and comprise of Artemisinin, Artesunate, Artemether and Artether. Clear knowledge on the specifics of the manner in which Artemisinins act does not exist but many theories have been offered and a widely held speculation is that they act differently from previously used treatments [2,3]. One theory is that the peroxide bridge linkage found on the artemisinin ring results in destructive oxygen radical species being released due to a reaction with ferrous iron from the heme released by parasite-infected red blood cells [4,1].

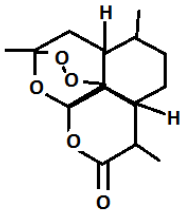

FIGURE 1. Structure of artemisinin

Artemisinin itself is contained within the leaf surfaces, flowers and buds of the plant *Artemisia annua. T*he content of artemisinin found in *A. annua* falls within the range of 0.01%-1.4% and is explained by the fact that discrepancies in yield can occur due to different plant origins, stage in growth and how they have been cultivated. However this % yield of active ingredient is extremely low therefore it is of importance that the extracted yield of artemisinin is maximized. Currently traditional extraction processes involve steeping or stirring the leaves of the A*rtemisia annua* plant in a solvent for several hours. There are discrepancies between solvents employed (hexane, toluene, petroleum ether, ethanol), times of extraction, with times quoted varying between 10 and 48 hours, and also extraction temperatures employed (40 °C to reflux) [5,1].

Ultrasound offers some important benefits for extraction. These include the disruption of cell walls to aid the release of contents, greater penetration of solvents, and improved mass transfer [6]. Ultrasound aided extraction is used for the extraction of bioactive products, oils, aromatics and proteins from plants. The use of ultrasound in extraction can show benefits such as increased product yield, reduced extraction time required, reduced temperature and energy required and less reliance on the specific solvent used and there are many such examples in current literature. A study in 2004 by Albu et al [7] found that in the extraction of antioxidants from *Rosmarinus officinalis*, ultrasound techniques are more efficient than traditional solvent extractions, extracting most material in under 15 minutes whereas a thermal process could take up to 180 minutes to extract a comparable amount of carnosic acid. This study also found that the efficiency of ethanol as an extraction solvent is greatly improved when in combination with ultrasound. Another study conducted by Dong et al [8] found that the extraction of salvianolic acid B from *Salvia miltiorrhiza* root could be significantly improved from the reflux extraction with the use of ultrasound. They found that under the conditions of a 25 minute extraction at 30°C, the use of 45 kHz ultrasound increased the salvianolic acid B yield from 28.76 mg/g (using reflux extraction) to 33.93 mg/g. Londono et al [9] investigated the recovery of antioxidant flavonoids from citrus peel using an optimized aqueous ultrasound assisted extraction method with high yield being obtained (40.25 ± 12.09 mg of flavonoid fraction/g peel) compared to conventional extraction. Whilst Caresa et al [10] investigated the ultrasonically assisted extraction of bioactive principles from Quillaja Saponaria Molina. They determined, by using HPLC, that there was no influence of ultrasound on the natural components extracted however extraction time could be reduced when using sonication. Many such reports point towards the benefits of employing ultrasound to aid extraction of natural products.

EXPERIMENTAL

Conventional Extraction

5g of dried and ground *Artemisia annua* leaf was added to 250 ml of hexane, in a sealed conical flask. The flask was heated to temperatures of 25°C, 35°C, 45°C and 65°C as appropriate. Extraction times of 15, 30, 60, 90, 120 minutes were employed. Each experiment was performed in triplicate (n=3).

Ultrasonic Extraction

20 kHz Frequency

A 20 kHz probe system was used (Sonics Vibra-cell (Model: VCX600) with a model CV26 horn: Power= 14.08 W; Intensity=12.45 W/cm^3). The probe tip was submerged into the flask containing 5g of dried *Artemisia annua* leaves and 250 ml hexane. Foil was wrapped around the top of the flask to seal the vessel. Ultrasound was applied at temperatures 25°C, 35°C, 45°C and 65°C was maintained using a water bath as appropriate. Extraction times of 15, 30, 60, 90, 120 minutes were employed. Each experiment was performed in triplicate (n=3).

40 kHz Frequency

A 40 kHz ultrasonic bath system was used (Rieber 375TT (Model: S0375T SONOMATIC): Power= 10.54 W; Intensity=0.60 W/cm^3). The sealed flask containing 5g of dried *Artemisia annua* leaves and 250 ml hexane was submerged into the ultrasonic bath. Ultrasound was applied at temperatures of 25°C, 35°C, 45°C and 65°C and was maintained using a water bath as appropriate. Extraction times of 15, 30, 60, 90, 120 minutes were employed. Each experiment was performed in triplicate (n=3).

ANALYSIS

Thin Layer Chromatography (TLC)

This is a semi-quantitative method of analysis. 1µl spots of standards or samples are micropipetted onto a TLC plate (Silica gel 5x10cm) 7 mm apart and 1cm from the bottom. The plates are developed in an elution solvent of ethyl

acetate:hexane (1:4), for 5 minutes, dried then immersed into a visualisation dip containing 12 g vanillin, 3 ml concentrated sulphuric acid, 190 ml absolute ethanol and 20 ml water. Artemisinin is visualized by the formation of blue spots at an R.F value of approximately 0.25. The colour intensity of the spots is determined using the trace method on the Sigma Scan computer program. A linear calibration curve was constructed using 100, 200, 400, 600 and 800ppm of pure artemisinin standards (supplied by Sigma Aldrich) and the concentration of artemisinin present in the samples was calculated using the equation of the slope and the intensity of colour values.

High Performance Liquid Chromatography (HPLC)

A Shimadzu HPLC machine was used with a Supelcosil LC-18 column (25cm x 4mm x 5µm) with a 1:1 Acetonitrile:Water solvent at a flow rate of 1ml/min. The oven was set at 30°C and runs were isocratic for 20 minutes with an injection volume for each sample of 10 µL. The retention time of Artemisinin was observed as around 10 minutes. A linear calibration plot was constructed using 100, 250, 500 and 750 ppm standards in ethanol.

RESULTS

Analytical Protocols

An added complexity to improving the extraction of artemisinin from *Artemisia. annua* is a lack of standard analysis protocols. The fundamental problems with ascertaining the concentration of artemisinin is its instability and the fact that the whole molecule has staining problems. Thin Layer Chromatography is a 'semi-quantitative' technique and is fairly undemanding. However as artemisinin doesn't stain very well it may result in an under estimation of the concentration of actual artemisinin present. HPLC with ultra-violet detection has been observed to be the most suitable analysis technique however its principle limitation is the lack of any subgroup within the artemisinin molecule which will absorb UV light effectively. As a result both these methods were all employed in an effort to identify the most reproducible and reliable analytical procedure.

Figure 2 below shows the results of the extraction of tablets, containing known amounts of artemisinin active ingredient, using ethanol solvent and analysed by the methods described above.

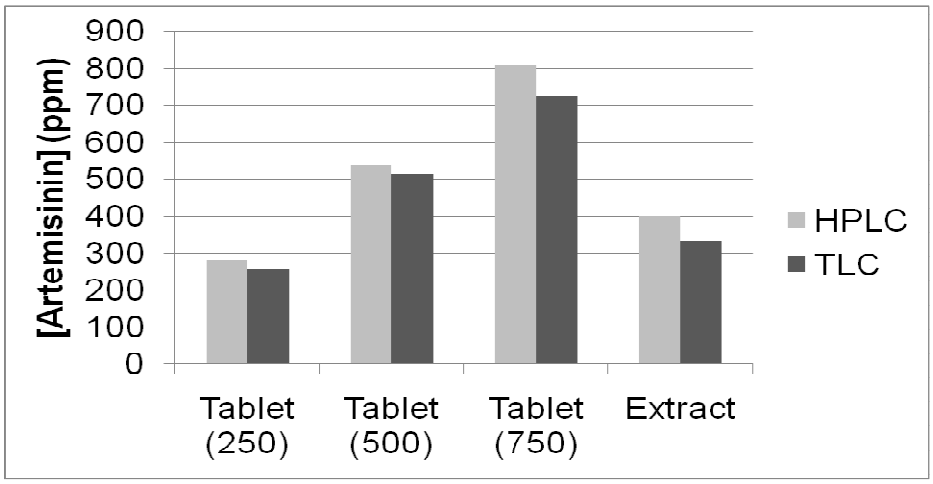

FIGURE 2. Comparison of Analysis Techniques against standard solutions of tablets containing 250, 500 and 750ppm of artemisinin and a conventional extraction using dried artemisinin leaves (n=3)

As can be seen both methods show good reproducibility with regard to the tablet samples. The TLC however indicates an underestimation of artemisinin content when compared to the HPLC technique. What is of interest however is the extraction of the actual dried leaf sample. 10% w/v *Artemisia annua* leaves in ethanol solvent were extracted by 20 kHz ultrasound for 20 minutes and analysed using both techniques. The HPLC results and TLC follow the same pattern as the tablet samples with the TLC slightly overestimating the HPLC values again by

approximately 5%. As a result the TLC technique appears to be a quick and viable analytical technique for determining artemisinin content within solvent extracts.

Ultrasonic extraction

20 kHz Frequency

Samples of dried leaf were extracted using ultrasound at 20 kHz frequency. Extraction temperatures ranged between 25 °C and 65 °C and extraction times between 15 and 120 minutes. The results obtained can be seen in Figure 3 below.

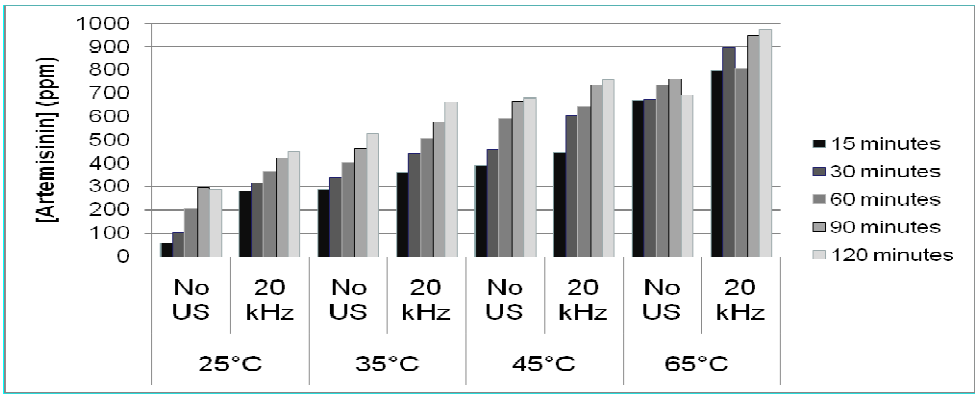

FIGURE 3. Comparison of ultrasonic extraction (20 kHz) in hexane with conventional extraction at varying temperatures and extraction times (n=3)

As can be observed longer extraction times increase the yield of artemisinin. At higher temperatures however the major part of the artemisinin within the dried leaves appears to be extracted within 15 minutes. Subsequent extraction times do increase the amount of artemisnin extracted but not to as great an extent. For example at 25°C conventional extraction yields 50 ppm of artemisinin after 15 minutes which increases to 300 ppm after 90 minutes a gain of 250 ppm. Whilst at 65 °C 15 minutes achieves 690 ppm increasing to 760 ppm after 90 minutes a gain of only 70ppm. Temperature clearly enhances the solubility of the artemisinin in the solvent for both conventional and ultrasonic methods.

What is of most interest however is the yield gained through the use of ultrasound as an extraction technique. What can be clearly observed when employing ultrasound is that at all the temperatures examined ultrasound greatly enhances the extraction of artemisinin with the most significant enhancements being observed at lower temperatures. 15 minutes conventional extraction at 25° C yielded 50 ppm artemisinin whilst the comparable ultrasonic extraction yielded 280 ppm a gain of 230 ppm in the same time period. This is almost the same amount as a 90 minute conventional extraction at the same temperature. In addition an actual 90 minute and 120 minute ultrasonic extraction at 25 °C resulted in 120 and 150 ppm greater yields than the corresponding conventional extractions respectively.

Higher temperatures for extraction also exhibit an ultrasonic benefit. A 15 minute ultrasonic extraction at 65°C provides 800 ppm artemisin which is greater than the 760 ppm obtained after a 90 minute comparable conventional extract at the same temperature. The greatest yields are observed at 65 °C with a 120 minute extraction providing 980 ppm of artemisinin far greater than any yield observed for a conventional procedure under similar temperaures and times.

Ultrasound is clearly very efficient at extracting artemisinin from the dried leaf. The effective outcome of sonication is the formation of cavitation bubbles which when formed within a heterogeneous system can collapse assymetrically resulting in the formation of microjects of solvent directed towards the leaf surface. The consequence of this is enhanced cell disruption which is particularly beneficial for artemisinin which is sited in trichome glands at

the leaf surfaces. In addition to this greater penetration of solvent into the leaf material occurs resulting in improved mass transfer of components thus an increase in artemisinin yield is observed.

40 kHz Frequency

The trends observed with the 20 kHz ultrasonic extraction frequency are mirrored with the results obtained using a 40 kHz extraction frequency seen below in Figure 4. Again ultrasound is seen to enhance extraction at all temperatures above the comparable conventional method with the greatest effect observed at lower temperatures.

Here 15 minute conventional extraction provides 50 ppm compared to 180 ppm when using sonication a gain of 130ppm. A 120 minute conventional extraction achieves levels of 300 ppm artemisinin and 40 kHz sonication again improves the extract amount with 420 ppm obtained.

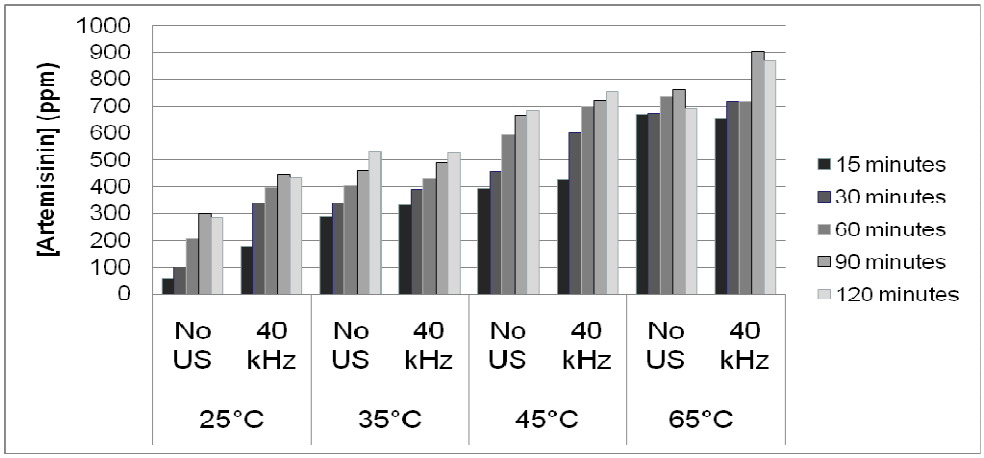

FIGURE 4. Comparison of ultrasonic extraction (40 kHz) in hexane with conventional extraction at varying temperatures and extraction times (n=3)

The fact that 40 kHz ultrasound shows benefits to extraction is promising as this frequency offers real scale up possibilities from the use of simple large scale ultrasonic baths and/or submersible transducers without the need for bespoke equipment.

CONCLUSIONS

Ultrasound has been shown to clearly benefit extraction at all temperatures and times examined with both 20 kHz and 40 kHz frequencies showing much enhanced yields when compared to the comparable conventional extraction process. This is very promising as it offers the genuine possibility low temperature extraction thus reducing operational costs of any process and in addition reducing economic impact. It may also offer the possibility of a low temperature extract which may be 'cleaner' thus avoiding co-extraction of unwanted components and hence reducing the need for further purification stages.

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