Shah et al., Afr J Tradit Complement Altern Med., (2017) 14 (1): 251-262
doi:10.21010/ajtcam.v14i1.27

FABRICATION AND EVALUATION OF SMART NANOCRYSTALS OF ARTEMISININ FOR ANTIMALARIAL AND ANTIBACTERIAL EFFICACY.

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Abstract

Background: Nanocrystals have the potential to substantially increase dissolution rate, solubility with subsequent enhanced bioavailability *via* the oral route of a range of poor water soluble drugs. Regardless of other issues, scale up of the batch size is the main issue associated with bottom up approach.
Material and Methods: Smart nanocrystals of artemisinin (ARM) was produced relatively at large batch sizes (100, 200, 300 and 400ml) compared to our previously reported study by (Shah, et al., 2016). ARM nanosuspensions/nanocrystals were characterised using zeta sizer, SEM, TEM, DSC, PXRD and RP-HPLC. The nanosuspensions were finally subjected to *in vitro* antimalarial and antimicrobial activity.
Results: The average particle size (PS) for 400 ml batches was 126.5 ±1.02 nm, and the polydispersity index (PI) was 0.194 ± 0.04. The saturation solubility of the ARM nanocrystals was substantially increased to (725.4± 2.0 µg/ml) compared to the raw ARM in water 177.4± 1.3 µg/ml and stabilizer solution (385.3± 2.0 µg/ml). The IC_{50} value of ARM nanosuspension against *P.* vivax was 65 and 21 folds lower than micronized 19.5 ng/mL and unprocessed drug (6.4 ng/mL) respectively. The ARM nanosuspension was found highly effective compared to unprocessed drug against all the tested microorganism except *E. coli*, *Shigella* and *C. albican.*
Conclusion: The simple precipitation-ultrasonication approach was efficiently employed for fabrication of ARM nanosuspension to scale up the batch size. Similarly, the solubility, antimalarial potential and antimicrobial efficacy of ARM in the form of nanosuspension were significantly enhanced**.** Findings from this study can persuade research interest for further comprehensive studies using animals model.

Keywords: Artemisinin, Nanocrystals, Antimalarial, Antimicrobial activity.

Introduction

Malaria was one of the most challenging diseases until middle of the 20th century in Western medicine. In different parts of Europe and North America malaria was endemic at that time (Meshnick and Dobson, 2001; Tarning, 2016). About 10,000 malaria cases are reported annually in Europe and 1300 to 1500 in the United States (Taylor, et al., 2012). Pyremethamine, choloroquine, mefloquine and halofantrine are well-established drugs used for the treatment of this disease. Malarial parasites offered strong resistance to these drugs. Despite the fast development of new research approach in science and technology, there seem to be little or no promising and effective malarial vaccines available (Joshi, et al., 2008). From traditional Chinese medicine a new class of antimalarial drug (artemisinin) was derived. In China for at least 2000 years the use of *Artemisia annua* has been reported for the treatment of haemorrhoids and fever (Klayman, 1985). Artemesinin in addition to antimalarial effects is also found to have optimum antifungal and antibacterial effects and is been tested on various bacterial cultures of *Staphylococcus aureus*, *Pseudomonas aerueginosa*, *Mycobacterium intracellulare* and Escherichia coli (Sack, 1975).

In literature more than 40% of active pharmaceutical ingredients (APIs) coming from high thorough screening have the tendency of suffering from poor aqueous solubility with subsequent low rate of dissolution, bio-availability and hence erratic efficacy (Shegokar and Müller, 2010). To address the problem of poor solubility, multiple approaches are being employed and among which nanoparticles are becoming a fast platform solution. The stability of suspensions relies on the method of generating particle and associated formulation components (Khan, et al., 2013). To control particle sizes and particle size distribution currently static mixer and impinging reactors have been reported (David, 2001). In nanoprecipitation, nucleation is very key to be controlled for tailoring particle sizes and their distribution (Hu, et al., 2011). A numbers of factors are responsible for controlling nucleation including supersaturation level, mixing time, micro-mixing and mass transfer etc (Lonare and Patel). Irradiation has been known to be effective for mixing, to accelerate molecular diffusion and have the immense intensity for mass transfer (Lonare and Patel).

Artemisinin is a poorly water soluble drug which causes the erratic absorption and poor bioavailability with consequent less antimalarial effect. According to WHO If the *M. falciparum* develops resistance to the artemisinin and its derivatives, there will be no alternative effective compounds to treat malaria over the next ten years (Organization,

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2006). The aims of the current study was to scale up the batch size of artemisinin nanosuspension, investigation of physico-chemical stability, saturation solubility of the produced nanocrystals compared to its unprocessed and micronized drug and finally to assess whether the artemisinin nanocrystals possess an enhance antimalarial and antimicrobial efficacy compared its unprocessed drug (Raw artemisinin/ Standard Drug).

Materials and Methods

Artemisinin (ARM) was kindly given by Institute of Material Medica, China, Batch No: (110916), polyvinyl alcohol (M. Wt 30,000-70,000) was purchased from Sigma Aldrich, (USA) batch No: (MKBR5960V), dimethylformamide (DMF) (Batch No 1373199), were purchased from Sigma Aldrich, (USA).

Preparation and scale up of ARM nanosuspension

ARM nanosuspension was fabricated and scaled up from 20ml batch size to 100, 200, 300 and 400ml using the optimized approach as reported in our previous work by shah et al., with modification in power and length of time for ultrasonication (Shah, et al., 2016). Briefly, 10mg/ml artemisinin was dissolved in dimethylformamide (DMF) with the final volume of 10ml, 20ml, 30ml and 40ml, respectively. Also 0.15% PVA (anti-solvent (aqueous) solutions having final volume of 90, 180, 260 and 240 ml were prepared. The organic (Solvent) and aqueous (Anti-solvent) solutions were filtered through 0.4 µm filter to ensure maximum clarity. Afterwards 10ml, 20ml, 30ml and 40 ml of the solvent containing drug were injected in to (pre-cooled at 4°C) 90, 180, 260 and 240 ml of anti-solvent solution containing (PVA) respectively, with simple stirring at 1500 rpm. The resulted precipitated artemisinin suspension was followed by ultra- sonication at different ultrasonic input 200, 250 and 300 Watt for different length of time 15, 20, 25 and 30 min. The time of ultra-sonication burst was set to 3sec with a pause of 5sec between two ultra-sonication burst. During fabrication of the temperature of the vial was controlled with ice cold water. After ultra-sonication at above mentioned parameters, the particle size measurements were carried out using Zetasizer Nano-ZS instrument, and the nanocrystals were subjected to characterization and *in vitro* antimalarial and antimicrobial activity.

Characterization of ARM Nanocrystals
Particle size Measurement

Malvern Zetasizer Nano-ZS dynamic light scattering (DLS) (Malvern Instruments, UK) was used to determine the particle size of ARM nanocrystals in the form of nanosuspensions.

Zeta Potential Measurements

Malvern Zetasizer Nano-ZS dynamic light scattering (DLS) (Malvern Instruments, UK) was used to determine the zeta potential of the produced ARM nanosuspensions. For zeta potential measurements samples were prepared according to the method reported by S. Plakkot et al., (Plakkot, et al., 2011). The original samples of the produced nanosuspensions were further diluted with the dispersion medium. One millilitre samples of nanosuspensions were further diluted with 2 ml of the dispersion medium. All the samples were analysed in triplicate.

Scanning Electron Microscopy (SEM)

Scanning electron microscope (SEM) (Quanta 400 SEM, FEI Company, Cambridge U.K) was used to evaluate the morphology of unprocessed (Raw) ARM. Photomicrographs of ARM were taken at different ranges of magnifications. However, images of ARM obtained at 220X were more prominent and descriptive. The particles of ARM were coated with gold prior to morphological studies.

Transmission Electron Microscopy (TEM)

To confirm the particle size of ARM nanocrystals transmission electron microscopy (TEM) (TEM-1200Ex, Japan Electron optics laboratory corporation Japan) was used at 120KV. Nanosuspension of ARM was deposited onto a grid of 200 mesh capper grid coated with formvar/carbon (code No; S162) followed by drying the samples at ambient temperature. The samples were stained negatively by using 2% magnesium uranyl acetate solution due to low conductivity.

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) module thermal analysis instruments (Q2000 series, TA instrument West U.K) was used to determine the melting point (M.P) and heat fusion of unprocessed and processed ARM (400 ml batch size). The instrument was calibrated with 99% indium having melting point 156.6°C and zinc with melting point

doi:10.21010/ajtcam.v14i1.27
419.5°C. Then under the stream of nitrogen gas the unprocessed and processed sample of artemether was then scanned at a flow rate of 50 ml/minute with temperature ranging 25°C -200°C.

Powder X-Ray Diffraction Studies (PXRD)

The crystallinity of raw and processed ARM previously was recovered from nanosuspension by centrifugation at 16000 rpm for 60 minutes and drying at ambient temperature was determine by using powder X-ray diffraction (Bruker D-8 powder diffractometer, Brukes, Kahsruhi, Germany). Samples were scanned over the range 5-500 2Ɵ at rate of 10 2Ɵ /min, at wave length 1.542Å with I mm Slit by using copper Kα as a radiation source in silicon-well sample holder. The PXRD was calibrated with corundum standard.

Solubility Studies

Solubility study of ARM nanocrystals was carried out by using the reported centrifugation method (Liu, et al., 2011; Thakkar, et al., 2011; Van Eerdenbrugh, et al., 2010). For the nanosuspension, approximately 1.5ml of the nanosuspension was filled in 1.5ml centrifugation tube and kept for 24 hrs followed by centrifugation at 1600 rpm for 1 hr using a sigma centrifuge (Scientific Lab supplier, Model: Sigma 0II5982IIII). To make sure complete solubility of the produced nanocrystals the supernatant layer was taken and filtered through 0.02 µm filter (Syringe Filter: 20 nm, Whatmananotop, Germany). The filtrate was analysed for the ARM contents by RP-HPLC, Waters 2695, system connected to a UV detector and Ultra II TM C18 5µm 250×4.6mm column. The samples were analysed using modified method as reported in reference (Kakran, et al., 2010). Solubility study of unprocessed ARM in pure water and stabilizer solution 0.15% w/w PVA was also carried out to evaluate the effect of nanocrystals on saturation solubility of ARM. Sufficient quantity of ARM in pure water and stabilizer solution was put in vials and sonicated for two hours followed by the same procedure mentioned for nanocrystals.

Stability Studies

Physico-chemical stability of nanosuspensions been reported to be a major issues (Khan, et al., 2013). Owing to increased surface free energy of the produced nanocrystals which can potentially lead to their altered physico-chemical attributes. It is therefore important to carry out physico-chemical stability studies on the produced ARM nanosuspensions. Physical stability of the produced ARM nanosuspensions was monitored by measurements of the particles sizes and zeta potential values. However, the chemical stability was assessed by determination of the active contents of ARM for seven days. The produced nanosuspensions were evaluated for their respective active contents using the reported modified method of Kakran et al., (Kakran, et al., 2010). In addition, the ARM nanosuspension (batch size 100 ml) was subjected to long term 90-day stability studies and stored at refrigerated conditions 2-8°C, room 25°C and 40°C. The aims of this study were to determine the extent of particle growth through aggregation and Ostwald ripening. The particles size measurements of the ARM stored samples were carried out for 3 months at regular intervals 0, 10, 15, 30, 45, 60, 75 and 90 days by using DLS.

***In vitro* antimalarial activity**

In vitro antimalarial activity of artemisinin (ARM) nanosuspension (processed), its unprocessed (Raw) and micronized drug were performed against *P. vivax*. The specie was previously identified by trained microbiologist of Teaching Hospital (Hayat Abad Medical Complex, Peshawar, Pakistan) using simple microscopic techniques. The plasmodium culture was maintained as described by Trager and Jensen 1976 (Trager and Jensen, 1976). Stock solutions of the ARM were prepared in solvent as described by Wahajuddine et. al (Trager and Jensen, 1976). The concentration of ARM nanosuspension and its unprocessed and micronized drug were in range of 1 to 70 ng/mL. The culture (*P. vivax*2-3% and hematocrit 2-3%) was exposed to various concentrations of the processed, unprocessed and micronized ART for 72 hrs (37 °C, 5 % CO2). To each well Lytic buffer comprised on (20 mM Tris pH 7.5, 5 mM EDTA, 0.008% saponin, and 0.08% triton X-100) and sybergreen-I was added followed by incubation for 3 hrs in dark at room temperature. Parasites in the thin films was counted against 250 erythrocytes each. The experiment was performed in triplicate and the mean parasitemia was calculated. The mean parasitemia in the drug-free control wells was served as the parameter of optimum and relative growth inhibition in the drug wells and was calculated on the basis of formula as suggested by Fidock et al (2004) (Fidock, et al., 2004). All the plates were read under fluorescence and IC50 was determined.

Preparation of Bacterial and Fungal Cultures

Strains of gram positive *(Staphylococcus aureus* and *Bacillus subtilis)*, gram negative bacteria *(Escherichia coli, Salmonella sp) and Candida albicans* were cultured respectively on Nutrient agar and Sabouraud Dextrose Agar (SDA). The produced cultures were then tested for antimicrobial effects keeping the standard environmental conditions.

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Antimicrobial Disk Diffusion Assay

The culture media of Nutrient agar and Sabouraud Dextrose Agar (SDA) were prepared and microbes were cultivated by spreading technique using the method reported by Suganthi et al., (Appalasamy, et al., 2014). For negative and positive controls filter paper discs impregnated with 1 μL of polyvinyl alcohol (PVA) solution in water and streptomycin were used, respectively. Artemisinin nanosuspensions 400 ml batch size and unprocessed drug were impregnated on the filter paper discs accordingly. The produced cultures were then incubated keeping the standard environmental conditions. During the incubation period of 48h, the diameters of the zones of inhibition were measured every six hours. All the tests were performed in triplicate.

Minimum Inhibition Concentration (MIC) Measurement

Minimum inhibition concentration (MIC) was determined for each microbe based on the least concentrations of artemisinin nanosuspensions (processed) and unprocessed (Raw). A serial dilution of artemisinin nanosuspensions and it unprocessed drug was done using the sample in concentration 0.1mg/ml to 3mg/ml. On each plate of tested microbes six disks of all the six concentrations were impregnated. All the test was done in triplicates. The Minimum bactericidal concentration (MBC) value was determined by sub culturing the test dilution (which showed no visible turbidity) on to freshly prepared nutrient agar media. The plates were incubated further for 18-42 h at 37 °C. The highest dilution that yielded no single bacterial colony on the nutrient agar plates was taken as MBC.

Statistical Analysis

All the data were run in triplicate and results were subjected to statistical analysis using statistics 8.1. Mean values were compared using ANOVA followed by Tukey's *post hoc* test. The difference was considered as significant at $P < 0.05$.

Results and Discussions

Preparation of Artemesinin nanosuspensions.

The batch size of ARM nanosuspensions was successfully increased to 400 ml. The initial particle size of ARM unprocessed drug was confirmed by using SEM, which was found to be about 200 µm (Figure 1 A). The particle size (PS) and polydispersity index (PI) values of the produced ARM nanosuspensions using simple stirring and combined approach (Simple stirring) using PVA solution (Anti-solvent) 0.15% pre-cold at 4°C, are shown in Table 1. Simple stirring at 1500 rpm was sufficient to produce the PS below 500 nm for all the samples. The minimum PS 126.5 ±1.02 nm and PDI 0.197±0.09 for 400 ml batch was achieved at ultrasonication input of 300 Watt within 30 minutes, The PS of nanocrystals was also confirmed using TEM (Figure 1B). Similarly, by further increase in the power inputs and time for fabrication no substantial decreased was observed in the PS and PI. However, for effective micromixing the stirring speed, ultrasonication power input and time for ultrasonication was slightly increased (Table 1). The results demonstrated that by increasing the batch sizes no substantial increased was observed in the PS and PI from the previously reported particle size which was 98.77 ±1.05nm having PI 0.186±0.01 (Shah, et al., 2016). Therefore, this technology was effectively applied to scale up from 20 ml to 400 ml batch size for fabrication of ARM nanosuspension. During the physical stability studies for 90 days it was found the nanosuspensions stored at 2-8°C (Figure 2A) and 25°C (Figure 2B) was most stable compared to sample stored at 40°C (Figure 2C). Also, the percent recovery of active contents was maximum 97.02± 2.30, which shows both efficiency of the technology and stability of the drug as well (Table 2). Furthermore, by controlling the key parameters like concentration of stabilizer, ultrasonic inputs, length for fabrication it could be possible to scale up this novel antimalarial drug for commercial use in the form of nanosuspension.

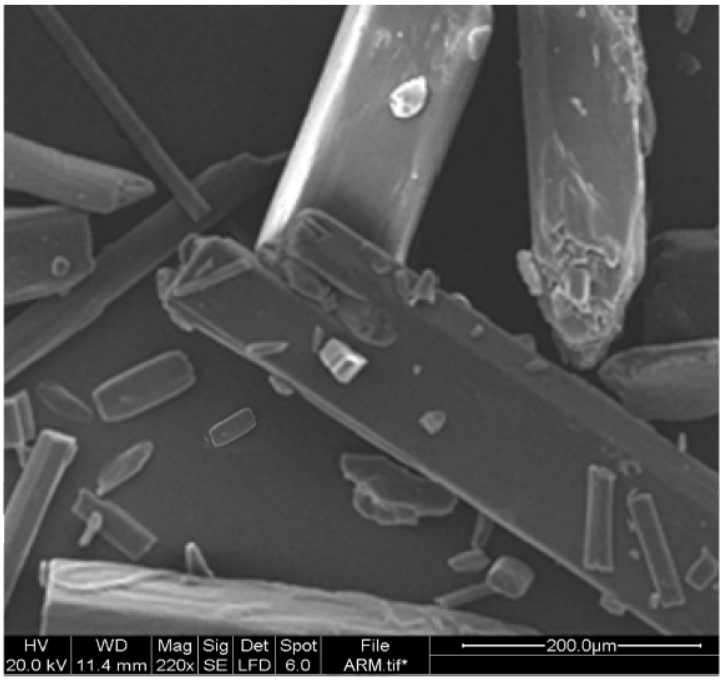
Zeta Potential

The values of zeta potential of ARM nanosuspensions were found to be within the reported range ±20 mV as presented and no significant difference was observed between tested formulation. The zeta potential results of 100 ml and 400 ml batch was found to be ±24.6 mV and ±25.1 mV respectively. The results demonstrated that PVA alone is sufficient to stabilize nanosuspensions (Figure 3). This polymers has been reported previously for stabilization of drug nanocrystals using bottom up approach (Xia, et al., 2010). Measurements of zeta potential have also been reported to predict physical stability of formulated nanosuspensions. The zeta potential measurements depend both on surface of the drug particles and composition of the stabiliser medium. A range of zeta potential values have previously been reported to give stable nanosuspensions, which include ±30 mV and ±20 mV for electrostatically and sterically stabilised systems, respectively (Jacobs and Müller, 2002; Yang, et al., 2008). In addition a minimum zeta potential value of ±20 mV has been proposed for stability of nanosuspensions where both ionic and non-ionic polymers and surfactants have been used as stabilizers (Ali, et al., 2009).

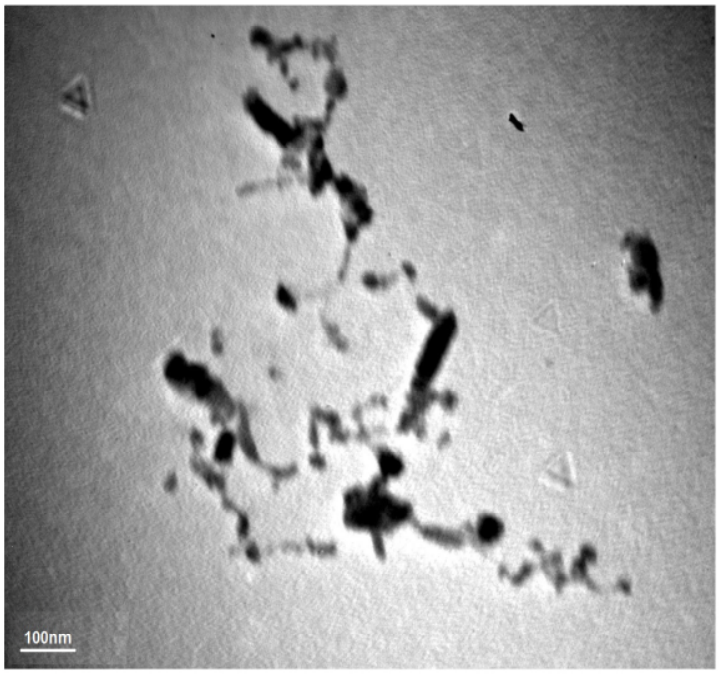
Solubility Studies

Solubility profile of artemesinin (ARM) nanocrystals and unprocessed ARM in pure water and stabilizer (polyvinyl alcohol) solution has been shown in (Figure 4) The solubility of ARM nanocrystals was found to be 752.4 ± 2 µg/mL, an almost 3.0-fold and 6-fold increase compared to the solubility of unprocessed ART in stabilizer solution 358.3± 2.0 µg/mL and pure water 171.4± 1.3 µg/mL. Owing to increased surface area of the produced nanocrystals, a significant difference

doi:10.21010/ajtcam.v14i1.27
(*P<0.001*) was observed in solubility of the processed ARM compared to the solubility in water and stabilizer solution. There has been previously reported that water soluble polymers and surfactants (HPMC, PVP and SLS) can increase solubility of drug compounds (Aziz, et al., 2012; Leuner and Dressman, 2000). We also observed an increase in solubility of ARM in stabilizer solution (PVA). The nanocrystals have however substantially increased the saturation solubility indicted that solubility of ARM has been purely increased because of small particle size of the particles not due to the polymers.



(A) SEM Image of Unprocessed ARM



(B) TEM Image of ARM Nanocrystals

Figure 1: (A) Scanning electron micrographs of unprocessed artemisinin, (B) transmission electron micrographs of artemisinin nanocrystals

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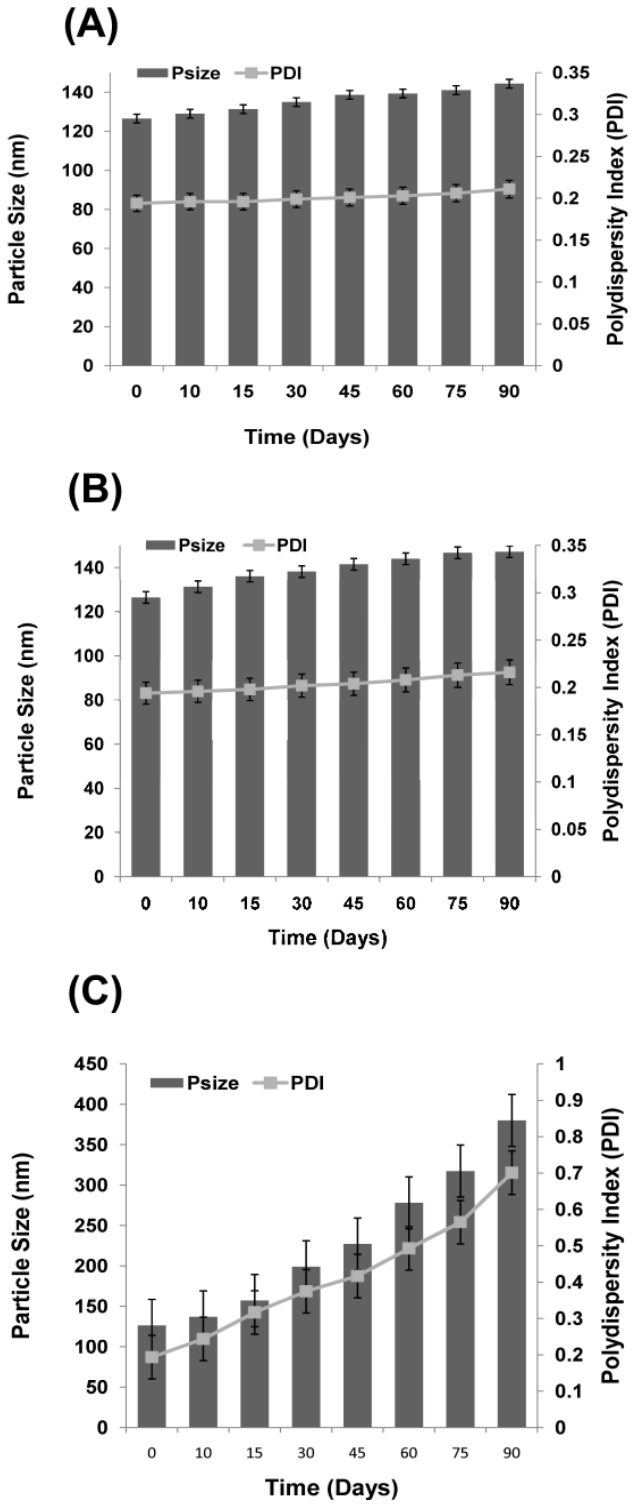

Figure 2: Stability of artemisinin nanocrystals in term of monitoring the particle size and polydispersity index at various time points on storage at, (A) 2-8°C, (B) 25°C, (C) 40°C.

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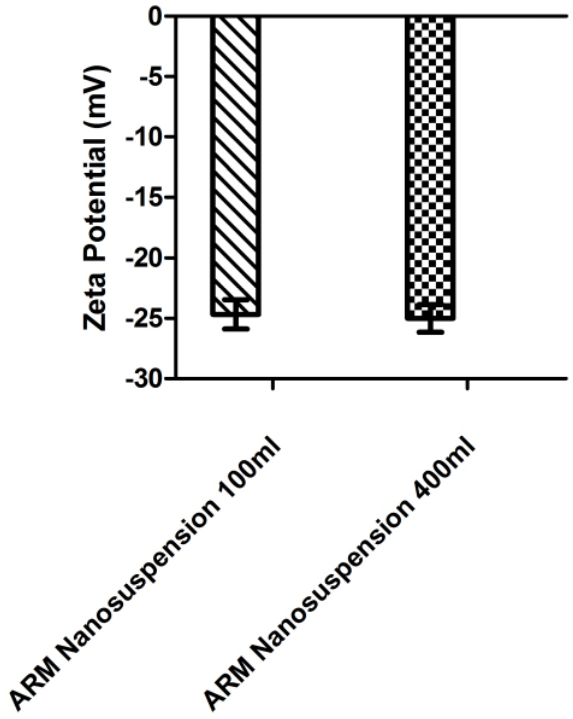

Figure 3: Zeta Potential of artemisinin nanosuspension**.**

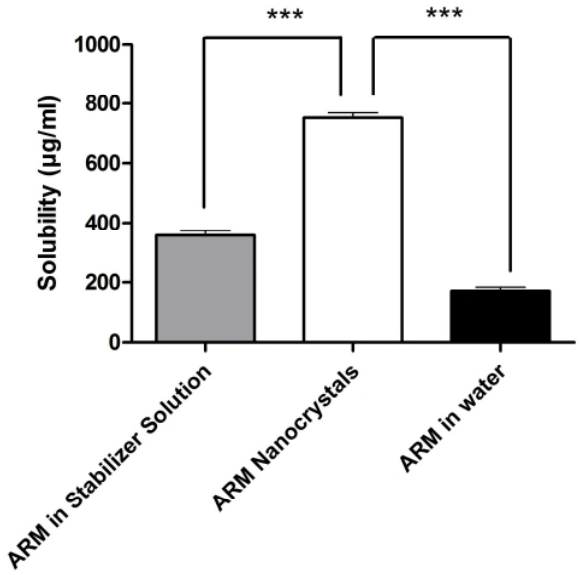

Figure 4: Solubility studies of artemisinin nanocrystals, unprocessed artemisinin in pure water and in stabilizer solution. Each bar represents mean ± SEM. One way ANOVA followed by *post hoc* Tukey's test. ***$P < 0.001$**.**

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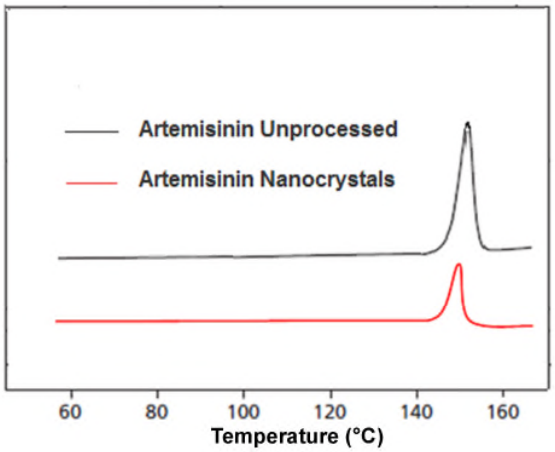

Figure 5: DSC profile of processed and unprocessed artemisinin particles.

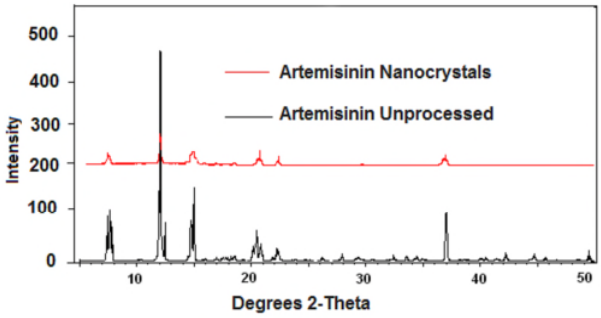

Figure 6: P-XRD patterns of processed and unprocessed artemisinin particles.

Table 1: Mean particle size (PS) of artemisinin in nanometre (nm) scale and particle size distribution (PDI), using simple precipitation and ultra-sonication techniques.

Method	Parameter	Batch Sizes			
		100 ml	200 ml	300 ml	400 ml
Simple Precipitation At 1500 rpm	PS (nm)	403.6 ± 1.03 nm	433.3 ±1.27 nm	441.6 ±2.0 nm	467.1 3 ±2.07
	PDI	0.290 ± 2.03 nm	0.337 ±1.01	0.381 ±1.75	0.301 ±2.24
Power (Watt)/ Time	Parameter	250 Watt/15 minutes	250 Watt/20 minutes	250 Watt/25 minutes	300 Watt/30 minutes
Precipitation Ultrasonication	PS (nm)	100.6 ±1.0 nm	111.1 ± 1.50 nm	119.7± 1.35	126.5 ±1.02 nm
	PDI	0.188 ±2.04	0.177± 1.0	0.191± 1.55	0.197± 1.09

(Values are expressed as mean ± SEM)

doi:10.21010/ajtcam.v14i1.27

Table 2: Chemical stability studies of Artemisinin nanosuspensions for seven days.

Stability Studies	Chemical Stability of Artemisinin Nanocrystals (% active contents± S.D)							
	Day-0	Day-1	Day-2	Day-3	Day-4	Day-5	Day-6	Day-7
Chemical Stability	97.02± 2.30	96.70± 3.20	96.35±0.01	96.03± 0.25	94.75± 0.13	94.23± 0.12	94.97 ±0.01	93.55 ± 0.21

Values are expressed as mean ± SD (Standard Deviation).

Table 3: Comparative antimicrobial activity of artemisinin nanosuspension (mg/ml) and Unprocessed drug using streptomycin (mg/ml) as positive control and PVA in water as negative control by disk diffusion assay. (a, b, c analysis of variance)

Microbes	Zone of Inhibition (mm)			
	Unprocessed Artemisinin	Nanoformulation Artemisinin	+Ve Control	6 ±0.00 -Ve Control
B. Subtilis	1.3 ± 0.91 ^{b}	2.13^{a} ± 0.76 ^{a}	1.4^{b}± 0.37 ^{b}	-
Streptococci	1.9 ± 0.52 ^{b}	3.86 ± 0.25 ^{a}	1.60 ± 0.42 ^{b}	-
Staphylococcus	2.33 ± 0.91 ^{b}	4.5 ± 0.71 ^{a}	2.11 ± 0.67 ^{b}	-
E. coli	-	-	3.5± 0.17	-
Salmonella	1.2 ± 0.15 ^{b}	2.1 ± 0.23 ^{a}	1.4 ± 0.33 ^{b}	
Shigella	-	-	-	
C. Albican	-	-	8.78± 0.81	
A. flavus	3.1 ± 0.15 ^{c}	5.6 ± 0.88 ^{b}	11.20± 1.11^{a}	
A. niger	2.6 ± 0.61 ^{c}	4.9 ± 0.20 ^{b}	9.12 ± 0.93 ^{a}	

Values in each column are significantly different (*P<0.05*)

Table 4: MIC and MBCs of artemisinin unprocessed drug and artemisinin nanosuspension**.**

Microbes	MIC and MBC (mg/ml)			
	MIC values of Artemisinin		MBC values of Artemisinin	
	Unprocessed	Nanosuspension	Unprocessed	Nanosuspension
B. Subtilis	0.87	0.39	2.1	0.55
Streptococci	0.71	1.53	2.21	2.67
Staphylococci	0.58	0.20	1.32	0.47
Salmonella	0.76	0.22	1.66	0.36
A. flavus	0.35	0.19	0.87	0.41
A. niger	0.71	0.33	1.19	0.73

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Dsc And P-Xrd Studies

Crystallinity of the processed artemisinin was assessed by using DCS and P-XRD, which were identified to be crystalline in nature. A single sharp endotherm was observed for both the nanocrystals and raw ARM having slight reduction in the melting onset temperature (T_{onset}) and in the melting peak maximum, of the nanocrystals compared to raw ARM. Also, dropped in heat of fusion for nanocrystals was observed (Figure 5). In P-XRD studies both nanocrystals and raw artemisinin produced sharp x-ray chromatograms which confirmed the crystalline nature of the produced nanocrystals (Figure 6). This decreased in heat of fusion is due to the adsorbed polymer/stabilizer(s) on the nanocrystals surface in trace amount (Valleri, et al., 2004). Also, this could be because of the low packing density in case of nanoparticles. In P-XRD studies there was observed that intensity of the produced nanoparticles reduced, broadened and disappearance of some peaks also occurred. Due to particle size broadening effect, the peak for ARM nanoparticles was slightly broadened, which is the key to attain low particle size (Khan, et al., 2014).

In vitro antimalarial activity

Artemisinin (ARM) showed significant (*P<0.05*) results against *P. vivax* at a very low concentration 0.3 ng/ml. The IC_{50} value of ARM nanosuspension was 65-fold lower than the IC_{50} value of micronized 19.5 ng/mL and 21 fold lower than the IC_{50} value of the unprocessed 6.4 ng/mL drug. This unique interaction of nanoparticles with biological system along with increased saturation solubility could be responsible for enhanced effectiveness.

Antimicrobial assay

Table 3 shows that ARM nanosuspension has strong effect against gram positive bacteria compared to the unprocessed ARM and positive control, whereas no significant difference was observed in the effects of unprocessed ARM and standard drug. However, both unprocessed and nanoformulation were found inactive against gram negative bacteria except the positive control. Both the unprocessed and nanoformulation showed no activity against *E-coli*, *Shigella* and *Candida albican*, while the positive control was found active against these microorganisms. These results clearly indicated that the processed nanoformulation was found highly effective and a significant difference was observed among the values of unprocessed ARM, nanoformulation of ARM and positive control against *Aspergillus flavus* and *Aspergillus niger*. Table 4 shows the values of MIC of unprocessed ARM which were found less 0.58 mg/ml in case of gram positive bacteria whereas 0.76mg/ml value was recorded against *Salmonella*. The produced ARM nanocrystals were found highly effective against gram positive bacteria, *Shigella* and both of the tested fungus. In case of MBC in general a significant high value was recorded as compared to MIC (Table 4). It was noted that ARM nanocrystals were highly effective against bacteria and fungus as compared to the unprocessed ARM in term of MBC. The inability of ARM nanosuspension to inhibit the *C. albican* was also reported by Galal et al., (Galal, et al., 2005). Different strains of bacteria have different cell permeability and which play a key role in te rate of large molecules penetrations (Lambert, 2002). Nanoparticles especially in submicron size (100 nm) have unique physicochemical properties including large surface to mass ratio, ultra-small size, high reactivity and unique interactions with biological systems (Zhang, et al., 2008).

Conclusion

This comprehensive study demonstrated that by controlling the process conditions using this technology, the batch size for production of stable nanocrystals can be successfully increased without any significant changes in the mean PS and PI. In addition, this technology can effectively produce large quantity of the ARM in the form of nanoformulations which could potentially be commercialised with a low dose while having maximum therapeutic potential. It will be a benefit to minimize the dose related toxicity and most importantly dose related resistance problems of this drug due to erratic bioavailability issues. Furthermore, production of stable ARM nanocrystals in large quantity can lead to established marketed products which in the very low dose will continue to improve the treatment of microbial infections, especially in life-threatening diseases like malaria.

Acknowledgments

I would like to acknowledge the financial support received from the International Research and Initiative Programme (IRSIP) of Higher Education Commission of Pakistan (HEC), Hayat Abad Medical Complex, Peshawar, Khyber Pakhtunkhwa and School of Pharmacy, Institute of Life Sciences Research, University of Bradford, West Yorkshire BD7 1DP, United Kingdom.

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