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Formation of *Amomum subulatum* Nano-Suspension and Evaluation of Angiotensin Converting Enzyme (ACE) Inhibition Potential

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Abstract. *Amomum subulatumis* act as a excellent antihypertensive and its bioactives exhibited detox properties. Low bioavailability and solubility of bioactive compounds as a result of complex structure is the main problem of less therapeutic efficiency of medicinal plants. The main objective of this research was formulation of *Amomum subulatum* nanosuspension to enhance ACE inhibition potential. *A. subulatum* nanosuspension was prepared by anti-solvent precipitation method by using sodium lauryl sulphate stabilizer and optimized by Response Surface Methodology. The prepared nanosuspensions were characterized by zeta sizer and scanning electron microscopy. Nanosuspension with 136 nm particle size and 0.215 PDI value was optimized and selected for further *in vitro* study. The scanning electron microscopy showed spherical shaped nanoparticles with smooth topology which confirmed formulation of nanosuspension. Optimized nanosuspension showed better ACE inhibition potential of 80% as compared to crude extract (36%). Results of antimicrobial activity showed greater zone of inhibition for nanosuspension against *E. coli* (8.5±2.061 mm) and *B. subtilus* strains (8.25±2.031 mm) as compared to crude extract (4.5±1.5 mm) and (4±2 mm) respectively at 150 mg/mL. Optimized nanosuspension showed greater percentage antioxidant potential as compared to the crude extract.

Keywords: *Amomum subulatum*, nanosuspension, ACE inhibition potential

Introduction

Nanotechnology as a most emerging technique in this era includes the manipulation of physical and bio-chemical properties at nano-scale having 200-600 nm dimensions. Nanotechnology revolutionized the pharmaceutical industry by enhancing bioavailability, solubility, therapeutic efficacy and dissolution rate of drugs or bio-actives (He *et al*., 2015). Medicinal plants are extensively used for treatment and management of aliments all over the world especially in developing countries (Bisht *et al*., 2011). The therapeutic effect of herbal plants is mainly due to specific primary and secondary bioactive compounds (Nazish *et al*., 2016). High blood pressure is a silent killer, causing many serious diseases such as heart stroke, kidney and heart failure. Since the proportion of hypertension among people has increased rapidly, so new advancements in the treatment and management of hypertension are necessary. ACE inhibitors are medicines that are used mainly in the treatment of hypertension and heart related diseases. Also used as a remedy by some patients with diabetes, kidney disease and heart protective after a heart attack through relaxation of blood vessels during hypertension. Synthetic ACE inhibitors have a number of side effects such as skin rash, persistent dry cough and headache etc. Nanosuspensions formulation of medicinal plants increased its ACE inhibition potential with less side effects as well as less draw backs.

Amomum subulatum belongs to *Zingeberaceae* family commonly known as Bari Ilaichi, Black cardamom, Nepal cardamom, Great Indian cardamom. Its bio-active spossess excellent therapeutic properties: increase of appetite, acts as antidote against poison, stimulant, causing the dermal cells contraction and cardio protective (Bimala *et al*., 2017). Plant fruit is essential for various types of disease including *i.e*. hypertension, problem in digestion, vomiting and other abdominal issues. Similarly, it can also be used as flavouring agent due to its fragrance and taste. The main bioactives of large cardamom are 1,8-cineole (65-80%) and limonene

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10.3% (Bisht *et al*., 2011). Among all of them monoterpene hydrocarbons (5-17%), terpinenes, limonene, sabeinene and pinenes are the most important compounds (Johannes *et al*., 2017).

These bioactive compounds of medicinal plants are not able to cross the cell membrane of biological cell due their large and complex structures decrease solubility, dissolution velocity, absorption and bioavailability (Karakucuk *et al*., 2016; Ajazuddin and Saraf, 2010). To deal with the problems of low solubility and erratic bioavailability nanosuspension is very effective technique to solve such issues and make them more beneficial (He *et al*., 2015).

Some conventional methods were used to enhance the solubility and bioavailability of bio-actives including solid dispersion, solubilisation using co-solvent and precipitation (Cristescu *et al*., 2016). All of these techniques have some limitations. Nanosuspension is the technique to overcome the problems related to the delivery of lipid soluble and water soluble drugs and it is distinctive because of its benefits and applicability to all drugs (Danhier *et al*., 2014). Nanosuspension is colloidal dispersion of bioactives with the size smaller than one millionth of a meter and stabilized by surfactant for up to 6 months. Nanosuspension prepared by different approaches that reduce diameter of particles(Patel *et al*., 2016).

The main objective of this research was formulation of *Amomum subulatum* nanosuspension to increase its ACE inhibition potential and bioavailability. Decrease in particle size results in increased surface area ratio, high dissolution rate and bioavailability of bioactive. Particle size reduction to nano level prevents the crystal growth and maintains stability of nanosuspension for long time (Frank and Georg, 2016; Chiang-Po, 2011). The main objective of this research was formulation of *Amomum subulatum* nanosuspension to increase its ACE inhibition potential and bioavailability.

Materials and Methods

Preparation of *Amomum subulatum* extract. *Amomum subulatum* (great cardamom) was selected for the formulation of nanosuspension on the basis of its medicinal importance and low bioavailability. Soxhlet apparatus was used for extraction and 20 g of plant powder was filled in cellulose extraction thimble, *n*-hexane was added to the flask as defatting solvent that removed all oily components. Then this defatted powder was treated with 300 mL methanol for extraction (Gao *et al*., 2011).

Preparation of nanosuspension. *Amomum subulatum* nanosuspension was prepared by antisolvent precipitation method. Plant extract (0.25 g) was dissolved into organic solvent such as ethanol. Another solution of (0.25 g) stabilizer in 100 mL water was prepared. Stabilizer solution was mixed with second solution by applying constant agitation with mechanical stirrer. Nanosuspensions were prepared with different stabilizers including PEG (poly ethylene glycol) 400, SLS (sodium lauryl sulphate), PVA (polyvinyl alcohol), HPMC (hydroxyl propyl methyl cellulose), PVP (polyvinyl pyrrolidone) and Tween 80.

Optimization of nanosuspension. The effect of different independent variables on synthesis of *Amomum subulatum* nanosuspension was optimized by response surface methodology. An optimization method, a statistical tool central composite design (CCD) of response surface methodology (RSM), was used to study the effects of independent variables on dependent variables. These included the concentration of stabilizer and amount of anti-solvent. The response variables were particle size (nm) and polydispersity index. All the *Amomum subulatum* nanosuspensions were prepared according to the experimental design and the effect of independent variables was evaluated. Thus, these two variables with three levels were evaluated and experimental trials were performed at all 13 possible combinations. The responses obtained after the characterization of nanosuspensions were filled in the design.

Characterization of nanosuspension. Particle size of the nanosuspension was determined by zeta size in National Textile University Faisalabad. Poly disparity index and zeta potential gave information about the stability of nanosuspension. By varying the amount of anti-solvent and stabilizer, nanosuspension with maximum stability and particle size in nano range was selected (Bhadoriya *et al*., 2012).

Scanning electron microscopy (SEM). Particle morphology was observed using scanning electron microscopy (SEM) Shape of the drug particles and the value of average particle size can be visualized by SEM.

Physical stability. Physical stability deals with time for which the nanosuspension remained stable apparently. Some of the stabilizers showed agglomeration in suspension just the day after formation. Time required

by nanosuspension to remain stable was noted. Physical stability includes apparent changes such as colour change, crystal growth and agglomeration.

Anti-microbial assay. Antimicrobial activity of *Amomum subulatum* nanosuspensions was assessed by well diffusion method. Different steps were involved in this method as follow, gram-positive (*Bacillus subtilus*) and gram-negative strains (*Escherichia coli*) of micro-organisms were selected for determination of anti-bacterial activity. Microorganisms were collected from Department of Microbiology, University of Agriculture Faisalabad, Pakistan. Nutrient agar was dissolved in distilled water, trypton 7 g, NaCl 2.5 g and yeast extract 7.5 g in 500 mL distilled water and pH 7 of the media was adjusted by addition of acid or base. After properly plugged with cotton, this flask containing media was kept in autoclave for sterilization at 121 °C for 15 min. After sterilization, this media was cooled at room temperature.

Sterilized medium (15 mL) was speeded into petri plates uniformly in the form of thin film of gel of 2-3 mm thickness. Different concentrations of *Amomum subulatum* crude extract its nanosuspension and ciprofloxacin (positive std.) were applied to agar by well diffusion method. At the end of incubation, the zone of inhibition growth (mm) were measured by zone reader (Ayesha *et al*., 2013).

Antioxidant assays. The antioxidant activity of *Amomum subulatum* nanosuspension was evaluated by following antioxidant assays:

***DPPH radical scavenging assay*.** The assay of DPPH radical quenching activity was carried out by the following method: Freshly prepared solution of DPPH in methanol (1 mL, 0.1 mM) was mixed with five different concentrations (20-100 µg/mL) of *Amomum subulatum*, nanosuspension and ascorbic acid mixture incubated for half an hour. Appearance of blue colour was the indication of reduction reactions. Absorbance of the incubated sample was noted at 517 nm (Bharathi *et al*., 2016). DPPH percentage inhibition was calculated by this formula:

$$\% \text{ inhibition of DPPH radical} = \frac{A_o - A_s}{A_o} \times 100$$

where:

A_0 = absorbance of control; As = absorbance of sample

***Reducing power determination*.** Reducing power was measured according to the following method: Various concentrations (2-10 mg/mL water) *Amomum subulatum*, nanosuspension and ascorbic acid (positive Std.) were prepared. Equal volumes (2.5 mL) of phosphate buffer (2.5 mL, 0.2M, pH 6.6) and potassium ferricyanide (1%) was mixed with each sample (2.5 mL) in a test tube. The control contained all the reaction reagents except sample. The mixture was kept for 20 min at 50 °C in oven. The reaction was stopped by the addition of TCA (2.5 mL 1% w/v) and centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) of centrifuged mixture was diluted with water (2.5 mL). Absorbance of this solution was noted at 700 nm after the addition of 0.5 mL ferric chloride solution (0.1% w/v) (Cristescu *et al*., 2016).

***Lipid peroxidation inhibition assay*.** The reaction mixture contained samples (500 µg), emulsion of linoleic acid (2.5 mL) and buffer (2 mL, 0.04 M, pH 7). It was kept at 37 °C for 72 h for fast oxidation process. After 24 h, 2 mL of the incubated sample was removed and 0.5 mL of $FeCI_2$ (0.02 M) and 0.5 mL of 30% (w/v) ammonium thiocyanate was added. Amount of peroxide was evaluated by measuring the absorbance at 500 nm. Samples (*Amomum subulatum* nanosuspension and ascorbic acid) were analysed at every 24 h. The ascorbic acid was used as standard. All the tests were performed in triplicate (Nazish *et al*., 2011).

$$\text{Lipid peroxidation inhibition percentage} = 100\text{-}[As/Ac \times 100]$$

where:

As = absorbance of sample; Ac = absorbance of control

***Super oxide scavenging assay*.** In super oxide scavenging assay sodium carbonate (10%, 1 mL), NBT (150 µM, 0.4 mL), EDTA (1%, 0.2 mL) were mixed with 1 mL different concentrations (20-100 µg/mL) of *Amomum subulatum* crude extract, nanosuspension and ascorbic acid. First absorbance was noted immediately after mixing all solutions at 560 nm wavelength. After few minutes 0.4 mL hydroxyl amine chloride was added into the solution and recorded the absorbance after 5 min incubation time (Fatima *et al*., 2014).

***Nitric oxide scavenging assay*.** In nitric oxide scavenging assay different concentration (20-100 µg/mL) of plant extract in water, *Amomum subulatum* nanosuspensions and ascorbic acid as standard were prepared. Sodium nitroprusside solution (2 mL) was prepared in

phosphate buffer saline and 2 mL of plant extract, nanosuspension and ascorbic acid was added into the mixture separately and kept it at ambient temperature for 30 min. Control was also run without adding the sample solution with same procedure. Now 1.5 mL of greiss reagent and incubated sample solution were mixed and again left for 30 min. The absorbance of the coloured solution was noted after half an hour with spectrophotometer at wavelength 546 nm (Ayesha *et al*., 2013). Nitric oxide inhibition was measured by formula:

$$\% \text{ of nitric oxide inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

where:

A_c = absorbance of control; A_s = absorbance of sample

Haemolytic assay. Haemolytic activity of *Ammomum subulatum* extract and its nanosuspension was assessed through comparatively simple method. Human blood (3 mL) was added to sterilized 15 mL falcon tube. The blood cells were washed by centrifuging the blood with 5 mL of PBS for 5 min until two layers separated. Then 180 µL of blood cells were added to 20 µL plant extract and nanosuspensions solutions in eppendrofs. Centrifuged the eppendrof tubes for 5 min and 100 µL of upper layer was separated and added to sterilized and chilled PBS solution. PBS was taken as negative control and 0.1% Triton was considered as positive control. In the end the absorbance of sample solution was noted at 576 nm on ELISA plate reader.

$$\% \text{ hemolysis} = \frac{\text{absorbance of sample} - \text{absorbance of negative control}}{\text{absorbance of positive control}} \times 100$$

Angiotensin converting enzyme (ACE) inhibitory activity assay. *Lung acetone powder preparation*. Rabbits were collected from market and their lungs were extracted and washed with 0.8% saline solution. Lungs were grind and centrifuged for 10 min at 4000 rpm with phosphate buffer saline solution. After the removal of upper layer the residues were passed through acetone along with continuous stirring for dehydration with magnetic stirrer. Excessive acetone drained off and remaining was dried overnight. In the next step it was converted to fine powder and preserved at 4 °C.

Enzyme extraction. The powder of lungs acetone was mixed with 10 mL of 100 mM borate buffer solution of pH 8.3. This mixture was stirred on magnetic stirrer overnight and centrifuged for 45 min at 4000 rpm. The upper layer obtained through centrifugation was passed through dialyzing membrane for 2 days to get concentrated solution. In the end the extra water was removed from solution by lyophilisation.

ACE inhibition activity test. ACE activity was assessed as 50 µL of ACE enzyme solution was incubated with 50 µL of borate buffer for 10 min at 37 °C. A 150 µL solution of substrate having Hip-His-Leu (8.3 mM) in borate buffer, was combined with incubated reaction mixture for 1 h and 20 min at 37 °C. The reaction mixture was inhibited by adding 250 µl HCl to it. 1500 µL ethyl acetate was mixed with the reaction mixture and centrifuged at 400 rpm for 15 min. The 750 µL sample from the upper layer was extracted and added to test tube and dried it at 7 °C under air flow. For every sample its blank and reaction was performed separately. But in reaction blank HCl was added before addition of substrate to stop the reaction.In this way inhibition potential of crude, nanosuspension and captopril as standard were determined with same procedure by replacing buffer with (1 mg/mL) sample solution. ACE inhibition was noted according to this equation:

$$\% \text{ IACE} = \frac{[(A - B) - (C - D)]}{A - B} \times 100$$

where:

A = absorbance of reaction in ACE; B = absorbance of reaction blank with ACE; C = absorbance of sample reaction; D = absorbance of reaction blank

Results and Discussion

Screening of stabilizers. *A. subulatum* nanosuspension was prepared to enhance the ACE inhibition potential, solubility and bioavailability of *A. subulatum* bioactives. Nanosuspension formulations involves decrease particle size, increase surface area, adhesiveness with cells and enhance dissolution rate. Large surface area increases the Gibb's free energy of particles and thermodynamically unstable. Nanoparticles tend to minimize total energy by agglomeration. Stabilizeract as wetting agent, used to reduce the interfacial tension between the particles and dispersion medium. Different stabilizers including sodium lauryl sulphate (SLS), polyethylene glycol (PEG 400), polyvinyl alcohol (PVA), hydroxypropyl methyl cellulose (HPMC), polysorbate 80 (Tween 80), polyvinyl pyrrolidone (PVP) were screened for the formulation of *A. subulatum* nanosuspension. Further-

more, the type of stabilizer may have distinctive influence on size of nanoparticles (Yancai *et al*., 2013).

Physical appearance. Physical analysis of all nanosuspension formulations was done on the basis of colour change and agglomeration over different days. Sedimentation of particles in nanosuspensions formulated by PEG 400, Tween 80 and PVP occurred within 3 days. On the other hand, nanosuspension was prepared with SLS, HPMC and PVA remained stable and did not sediment at the time of visual observation study.

Zeta size and particle size distribution analysis. Stabilizers for nanosuspension preparation with minimum particle size (200-600 nm) and PDI (less than 0.5) was selected for further study. Nanosuspension formulated with SLS stabilizer possessed zeta size of 290 nm. Particle size ranged from 274 nm for PVA to 280 nm for HPMC, which seemed to be affected by relative viscosity of polymeric dispersion in the presence of stabilizer. PVA based formulation gave smaller nanoparticles as compared to HPMC containing nanosuspension, which was a bit bigger molecule. PDI value of nanosuspension prepared with SLS (0.323), PVA (0.374) and HPMC (0.306) showed narrow distribution of particles (Fig. 1a & b). Particle size analysis was performed by zeta sizer that gave particle size and charge distribution values.

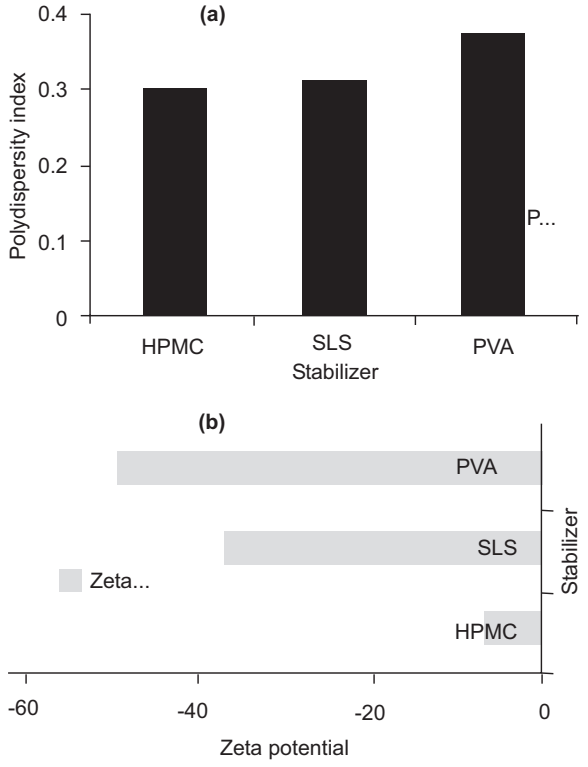

Fig. 1a & b. Particle size, PDI and Zeta potential of *Amomum subulatum* nanosuspension formulated with HPMC, SLS and PVA stabilizer.

Zeta potential of nanosuspension. Zeta potential of nanosuspension formulated with SLS was -36 mV. The lowest zeta potential of nanosuspension prepared with HPMC was -6 mV that might induce fast aggregation of particles. PVA containing nanosuspension showed highest value of zeta potential of -48.6 mV. PVA is the steric surfactant and provide combined electrostatic and steric stabilization to nanosuspension and minimum zeta potential of ±20 mV is desirable (He *et al*., 2015). This results may be attributed to the zeta potential and molecular weight of stabilize itself (Fig. 1a & b).

Optimization of *A. subulatum* nanosuspension. Response surface methodology (RSM) was applied to optimize the effect of independent variables on response variables. Some important formulation factors effected the stability of nanosuspension are anti-solvent, stirring time, drug and stabilizer amount ratio. So, independent variables selected for optimization were following: concentration of stabilizer (g) and volume of anti-solvent (mL) (Table 1). Two response variables zeta size (nm) and polydispersity index PDI were selected (Naresh and Geeth, 2014).

Analysis of variance. Analysis of variance (ANOVA) used to identify the significant terms in quadratic model.

Table 1. Responses of experiment designed by central composite design CCD

Runs	Stabilizer (g)	Anti- solvent (mL)	Particle size (nm)	PDI
1	1.00	200.00	1496	1
2	0.63	79.29	162.9	0.421
3	0.63	150.00	236	0.285
4	0.09	150.00	136.8	0.215
5	0.63	150.00	236	0.285
6	0.63	150.00	236	0.285
7	0.25	100.00	290.3	0.315
8	0.25	200.00	291	0.361
9	0.63	220.71	1453	1
10	0.63	100.00	275	0.331
11	1.00	100.00	226.7	0.367
12	0.63	150.00	236	0.285
13	1.16	150.00	244.7	0.305

The analysis of variance (ANOVA) for response surface quadratic model has been showed significant effect of independent variables on responses particle size and PDI. The P values were used as a tool for the significance of each coefficient. Model terms having P>0.05 were considered significant, where as P>0.001 was categorized as highly significant. Model terms with P>0.10 were tested as non-significant. In the present study F value 47.29 for particle size and 24.95 for PDI. And P values showed that quadratic model was highly significant for both responses.

Effect of independent variables on particle size. The response surface graph revealed the effect of two independent variables, conc. of stabilizer and volume of anti-solvent on particle size (Z-average-nm), while dealing with particle size of nanosuspension, it was observed that minimum particle size was obtained at smallest value of stabilizer (0.09 g) and 150 mL of anti-solvent. Hence amount of stabilizer played an important role in particle size of nanosuspension.

Out of the prepared nanosuspensions two nanosuspensions S_2 and S_4 showed good results of minimum particle size of *A. subulatum* nanosuspension. Moreover, by using 79.9 mL anti-solvent and 0.63 g stabilizer concentration, the particle size of 162.9 nm was obtained that confirmed the formulation of nanosuspension. S_2 and S_4 nanosuspension (with 1:0.36 drugs to stabilizer ratio) showed better results of zeta size 162.9 and 136 nm, respectively.

It was observed that particle size of the nanosuspension was lower when the concentration of stabilizer and anti-solvent volume was also decrease. The particle size of *A. subulatum* nanosuspension was higher when concentration of stabilizer was increased from 0.25 mg to1mg. The zeta size of *A. subulatum* nanosuspension was also increased with increase in volume of anti-solvent from 100 mL to 200 mL. The maximum particle size value of nanosuspension 1496 nm was obtained at maximum concentration of stabilizer (1 g) and anti-solvent (200 mL). The least value of zeta size was 136 nm when anti-solvent volume was 150 mL and 0.09 g of stabilizer concentration, while 162.9 nm zeta size of nanosuspension was also obtained in nano range with 79.29 mL anti-solvent and 0.63 g concentration of sodium lauryl sulphate. The nanosuspension with minimum particle size 136 nm and 0.215 PDI value was selected for further activities (Fig. 2a & b).

Effect of independent variable on PDI. The response surface graph obtained by using design expert software showed effect of independent parameters on poly-dispersity index of nanosuspensions. The concentration of stabilizer (sodium lauryl sulphate) and volume of anti-solvent (water) were two independent parameters selected to show the response (PDI) of the nanosus-

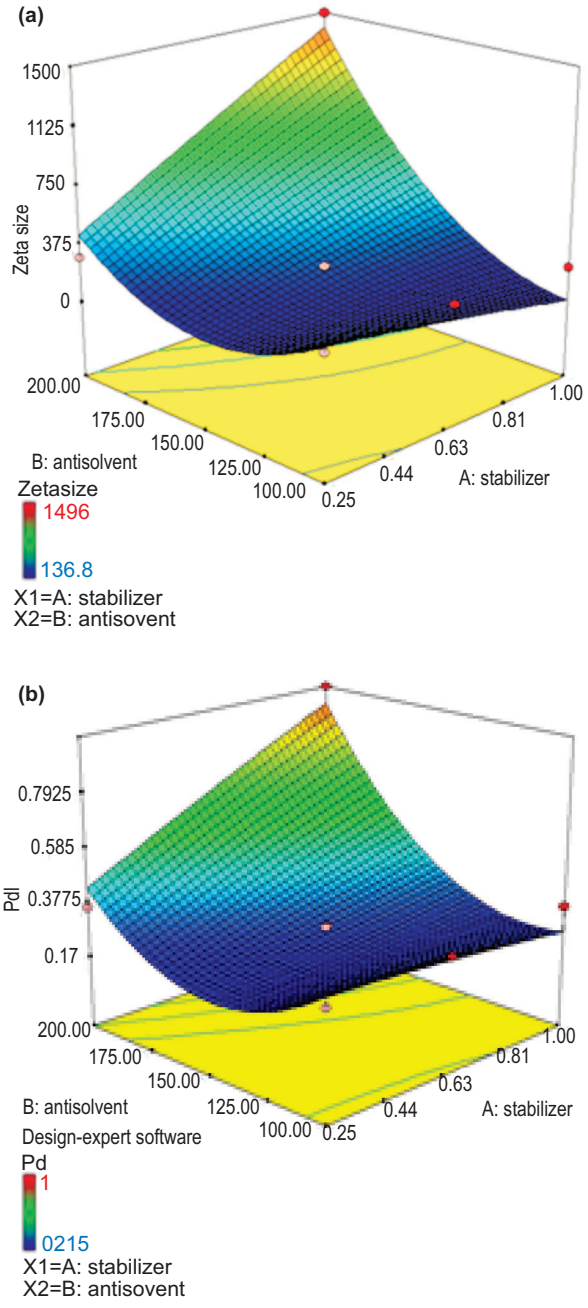

Fig. 2a & b. Graphical representation for effect of stabilizer concentration and antisolvent volume on particle size and PDI of *A. subulatum* nanosuspension.

pensions. Statistical significance test for quadratic model showed that concentration of stabilizer and volume of water as antisolvent were significant (P<0.05). Both of these variables have significant effect on polydispersity index of the *A. subulatum* nanosuspension.

From the values it was observed that least value of PDI 0.215 was obtained when concentration of stabilizer sodium lauryl sulphate (SLS) was lowest (0.09 g). By increasing the concentration of stabilizer PDI value also exceed upto 0.421 (Fig. 3a & b). Even when the concentration of stabilizer was 1 g then maximum value of PDI was observed (Fig. 2a & b). By further increasing the concentration of stabilizer the small decrease in

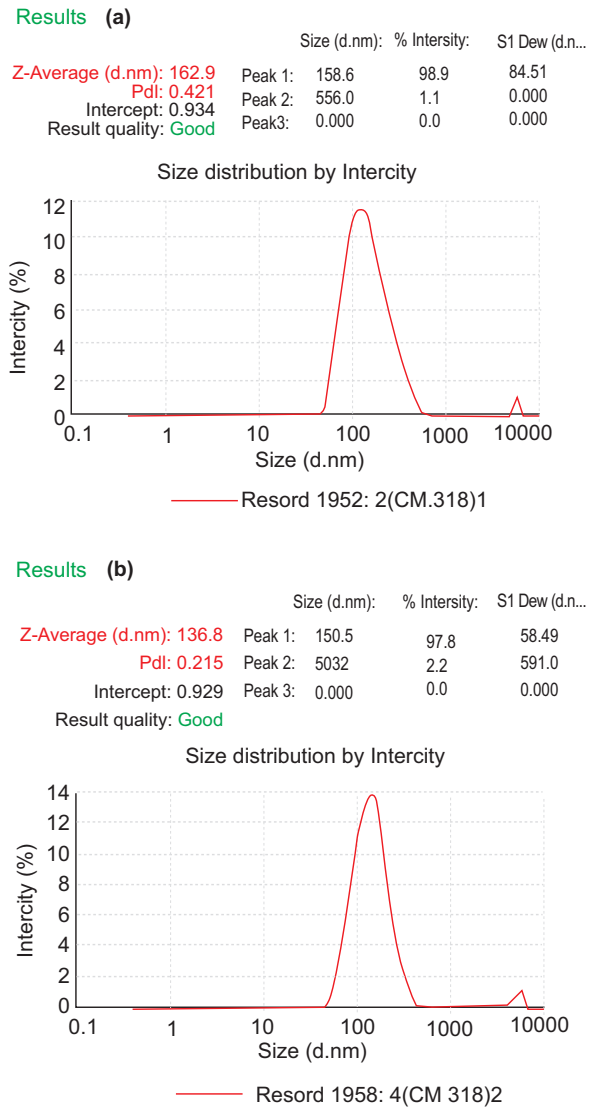

Fig. 3a & b. Particle size of optimized nanosuspensions.

polydispersity index value was observed. This trend in the PDI value clearly showed that *A. subulatum* nanosuspension depends on another factor.

SEM analysis. Scanning electron microscopy (SEM) analysis was done was done to study the size and morphology of optimized nanosuspension. A scanning electron microscope (SEM) with a secondary electron detector was used to obtain digital images of the samples at an accelerating voltage of 15 kV (Peng-Fei *et al.*, 2014). Scanning electron images of surface morphology of prepared nanosuspension revealed their smooth texture. Their pictures showed that most of the particles were having spherical shape and smooth topology (Fig. 4a, b & c).

Angiotensin converting enzyme (ACE) inhibition evaluation. In case of S_2 and S_4 nanosuspensions drug to stabilizer ratio was kept 1:2.5 and 1: 0.36, respectively. ACE inhibition percentage, with 1 mg/mL concentration of *A. subulatum* crude extract, S_2 and S_4 nanosuspensions, were 16.1, 27 and 0.69%, respectively. At higher concentration (2.5 mg/mL), the greater ACE inhibition potential of *A. subulatum* crude extract (25%), S_2 nanosuspension (50%) and S_4 nanosuspension (13.4%). ACE inhibition potential of S_4 nanosuspension was less than S_2 nanosuspension may be due to the greater amount of sodium lauryl sulphate in S_4 nanosuspension (0.63 g) as compared to the S_2 nanosuspension (0.09). ACE inhibition potential of *A. Subulatum* crude extract 36% S_2 nanosuspension 70% and S_4 nanosuspension 60% was attained at 5 mg/mL. Captopril was taken as standard (Fig. 5).

Compounds that have ability to make hydrogen bonds with the active sites of ACE enzyme inhibit the activity of enzyme. This hydrogen bonding inhibits the production of angiotensin II that release the pressure on blood vessels. In previous studies, it was reported that the C-3 hydroxyl group does not play a significant role in ACE inhibition. The common characteristics for all the flavonoid glycosides examined were the presence of heterocyclic oxygen and C-7, C-5 and C-30 hydroxyl groups. The presence of hydroxyl groups at the C-7 and C-8 positions was found to be effective in ACE inhibition rather than the hydroxyls attached to other positions of the flavonoid structure. Qualitative analysis of *A. subulatum* extract divulge that tannins, carbohydrates, amino acids, quercetin, triterpenoids, steroids, glycosides (Kare *et al.*, 2012), flavanone, anthocyanins are present in great cardamom (Robin *et al.*, 2013). It has been also reported that anthocyanins and plant

extracts rich in anthocyanins can inhibit ACE in *in vitro* systems and cell culture due to the presence of hydroxyl groups. Several studies reported that quercetin can reduce plasma extravasation by inhibiting ACE and endopeptidases.

The sodium lauryl sulphate stabilizer have active sulphate group that produce hydrogen bonding with the ACE enzyme and block its active sites. The carboxylic group present in the protocatechuic acid and protocatechuldehyde produce hydrogen bonding with the active sites of ACE enzyme.

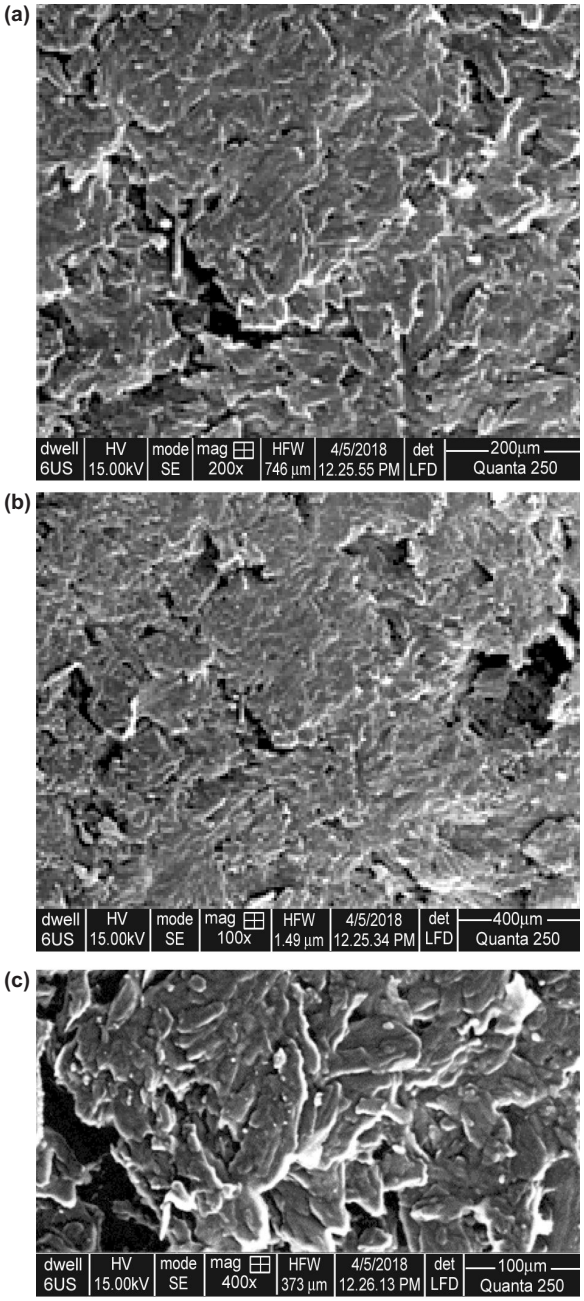

Fig. 4a, b & c. SEM images for *Amomum subulatum* nanosuspension.

Antioxidant activities. In great cardamom limonene and 1,8-cineole are very prominent components (Robin *et al*., 2013). Due to the presence of such active ingredients *A. subulatum* gives good scavenging activity of free radicals that are fatal for biological system. *A. subulatum* extract showed less radical quenching activity as compared to nanosuspension at 100 mg/mL concentration. The radical scavenging activity(85%) was observed for ascorbic acid at 100 mg/mL Fig. 6(a). The results were revealed that *A. subulatum* nanosuspension have the strongest antioxidant potential as compared to *A. subulatum* nanosuspension in this study. The DPPH assay was important for assessment of free radical scavenging potential of flavono lignans due to its ease and simplicity Fig. 6(d). (Cristescu *et al*., 2016; Nazish *et al*., 2011).

A. subulatum nanosuspension polar heads of active compounds flavanone, anthocyanins and tannins enhanced the radical scavenge activity of crude extract. It also possess antioxidant activity due to protocatechualdehyde and protocatechuic acid. They increased the ability to donate hydrogen of *A. subulatum* stabilized more easily by enhancing resonance with polar groups (Nazish *et al*., 2016). The direct reduction of $Fe[(CN)^6]^3$ to $Fe[(CN)^6]^2$ is helpful to measure the reducing capacity of reductants. And the increase in absorbance mixture would indicate the increase in reducing power due to complex formation Fig. 6(b,c,e).

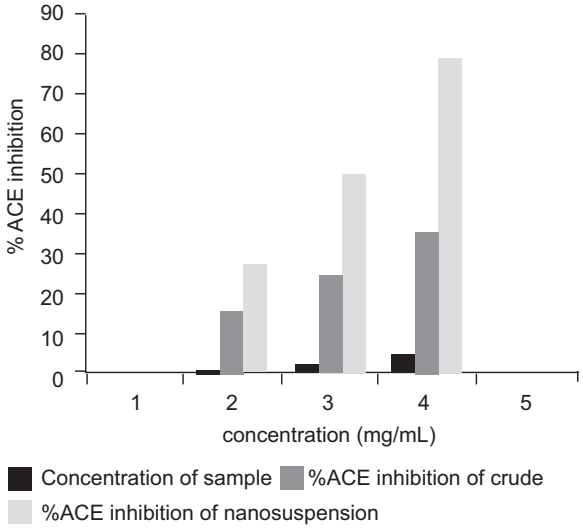

Fig. 5. % ACE inhibition assay.

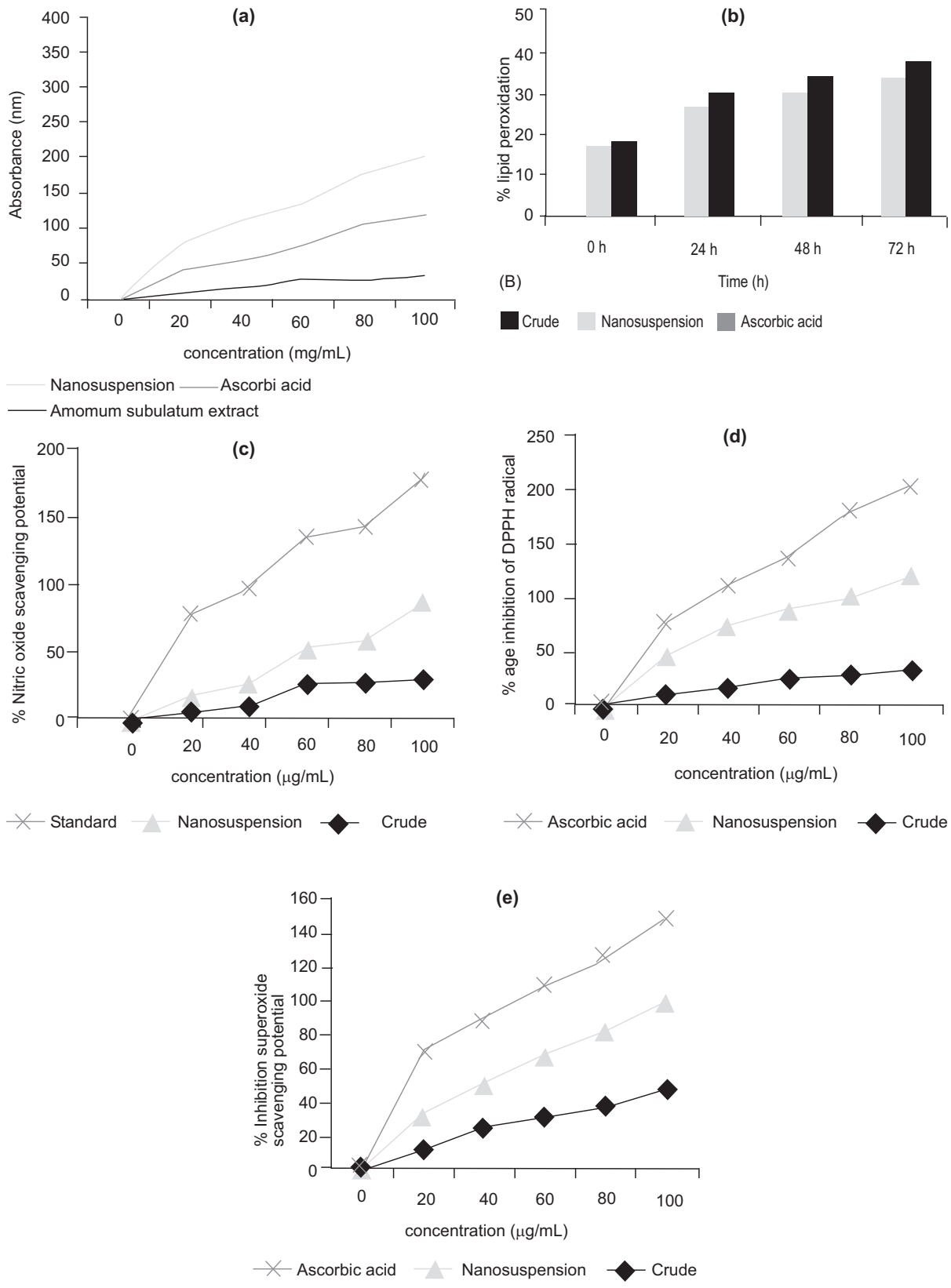

Fig. 6a-e. Antioxidant potential of *A. subulatum* crude and its nanosuspensions.

Antimicrobial activity. In this study, the antimicrobial activity of *A. subulatum* (great cardamom) extract and nanosuspensions has been quantitatively evaluated by well diffusion method. The antimicrobial activity of all samples were performed with gram negative (*E. coli*) and gram positive (*B. subtilis*) bacterial strains (Table 2 and 3). Both optimized nanosuspensions S_4 and S_2 showed better activity against microbes as compared to crude extract. S_2 nanosuspension produced greater zone of inhibition 8.25±2.031 mm against *B. subtilis* and 8.5±2.061 mm with *E.coli*. In S_4 nanosuspension the increased inhibition zone against *Bacillus subtitus* bacterial strains was 8.5±2.061 mm and with *E.coli* 8±2 mm at 150 mg/mL concentration. And during this antimicrobial assay *Ciprofloxacin* were taken as standard that showed inhibition zone 35±4.187 mm at 150 mg/mL.

The results of antimicrobial activity showed that nanosuspension exhibit better activity against microbes than crude extract. The hydroxyl groups present in flavonoids responsible for their antimicrobial activity. They depolarize the cell wall of bacterial cells and rupture the cell wall. *A. subulatum* nanosuspension contain active ingredients tannins, quercetin, triterpenoids, steroids, glycosides (Kare *et al*., 2012), flavanone, anthocyanins (Robin *et al*., 2013). Hence sodium lauryl sulphate have polar group's size reduction of polyphenols enhance the activity of *A. subulatum* hydroxyl groups.

Cytotoxicity activity. Cytotoxicity activity was assessed by haemolytic test deals with the red blood cells. *A. subulatum* crude extract showed percentage haemolysis 14.95% at 50 mg/mL. Nanosuspension showed 18.25 and 20.53% at the concentration of 20 mg/mL and 50 mg/mL respectively (Table 4). Increase in the percentage haemolysis of *A. subulatum* was due to the addition of sodium lauryl sulphate that increased polarity of crude extract. Sodium lauryl sulfate (SLS) is a sulfated surfactant that denatures membrane proteins of cells and pathogens (Dhananjay *et al*. 2010).

Table 2. Zone of inhibition with *B. subtilis* (mm)

Concentration (mg/mL)	Plant extract	Nanosuspension	Standard
50	3.5±1.32	7.5±1.936	25±3.5
100	3.75±1.69	8±2	30±2.87
150	4.5±1.5	8.5±2.061	35±4.187

Table 3. Zone of inhibition with *E. coli*

Concentration (mg/mL)	Plant extract	Nanosuspension	Standard
50	2.5±1.118	7±1.87	15.5±2.78
100	4±1.412	8±2	18±3
150	4.5±1.5	8.5±2.061	19.5±3.1224

Table 4. (%) Haemolysis of *A. subulatum* extract, nanosuspensions (S_2 , S_4) positive control and negative control

Samples	Concentration of sample	
	20 mg/mL	50 mg/mL
Amomum subulatum crude extract	12	14.95
Nanosuspension	18.25	20.53
Positive control	57.91	88.1
Negative control	0	0

Conclusion

The optimized *Amomum subulatum* nanosuspension showed higher ACE inhibition, antimicrobial and antioxidant potential as compared to crude pant extract. The *Amomum subulatum* nanosuspension being a potentent novel drug delivery system therefore may be used as a drug with higher bioavailability and therapeutic efficiency for hypertension and other hypertension related diseases.

Author's contribution. Munazza Rashid, M. Phil., performed the optimization, analysis and *in vitro* screening of *Amomum subulatum* nanosuspension. Zill-e-Huma and Talha Zulifqar contributed in writing, review and editing in manuscript. All the research work was planned under the kind supervision of Dr. Nazish Jahan and Prof. Dr. Khalil-ur-Rehhman. All the authors read and approved the manuscripts carefully.

Conflict of Interest. The authors declare no conflict of interest.

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