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Full Length Article

Comparative Evaluation of Biological Activities of Native and Nanosuspension of *Terminalia arjuna*

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Abstract

Present study was aimed to enhance the biological activities of *Terminalia arjuna*, the renowned beneficial medicinal plant of Pakistan for the treatment of infectious diseases including fungal and bacterial. To enhance the biological activities of bioactive components of *T. arjuna* bark extract nanosuspension technology was successfully employed. To obtain efficacious nanosuspension, important formulative parameters were optimized by employing statistical experimental design of response surface methodology. Smallest particle size and polydispersity index values of the formulated nanosuspensions were the selection criteria and were determined by dynamic light scattering technique using Malvern zetasizer. Optimized nanosuspension (nanosuspension with smallest particle size and PDI value) was characterized by zeta potential, scanning electron microscopy, atomic force microscopy and Fourier transform infrared spectroscopy. Efficacy of optimized nanosuspension was determined by measuring its antimicrobial and antioxidative potential using native suspension as a reference. Optimized nanoformulation showed mean particle size of 79.1 nm with PDI and zeta potential values of 0.244 and -22.1 mV respectively. AFM analysis confirmed the nanosizing of *T. arjuna* plant extract with uniform particles having average size of 70nm. SEM photographs illustrated that particles were little bit flower type shape with uniform size. FTIR studies demonstrated the presence of H-bonding between plant extract and stabilizer. Significantly enhanced antioxidant and antifungal potential of nanosuspension of *T. arjuna* as compared to its native suspension demonstrated that nanosuspension technology can be used as an effective approach to enhance its biological activities.

Keywords: Nanosuspension; Zeta potential; Atomic force microscopy, Antifungal activity; *T. arjuna*

Introduction

Nature has bestowed Pakistan with wide range of flora which is a rich source of novel biologically active compounds with diverse biological activities. These biological activities of medicinal plants are due to the presence of plants secondary metabolites such as phenolic and flavonoid contents which can lower the risk of numerous degenerative diseases including cancer and cardiovascular disorders (Chen and Zuo, 2007; Wang *et al*., 2012). These phytoconstituents have attracted the attention of public and scientists owing their free radical scavenging potential (Zhang and Zuo, 2004). The medicinal plants have also gained fascinated interest in the field of plant diseases control, particularly plant extracts with antimicrobial properties contain a spectrum of secondary metabolites (Sales *et al*., 2016). Although human and animal fungal infections causes serious medical and veterinary issues, but fungal infections of plants epitomize substantial losses of agricultural products. Up to now, more than 100,000 fungal species are considered as natural contaminants of agricultural and food industry. Fungi, particularly the *Aspergillus* species are among the major genera that causes mycotoxins during storage (Gautam *et al*., 2009; Rizwana, 2018). The produced mycotoxins lower the quality of food products as well as decrease the remedial properties of medicinal plants (Avasthi *et al*., 2010). The plant extracts with enhanced biological activities can play an imperative role in the preservation of food staffs against fungi and to control plant diseases owing to their antifungal activities (Sati and Joshi, 2011).

Terminalia arjuna (*T. arjuna*) commonly known as arjun, is a versatile traditional medicinal plant belong to the family Combretaceae. It is usually found growing on river banks and dry river beds (Haq *et al*., 2012) and is commonly planted as a road side tree all over the Pakistan. It is one of the most important plant that is extensively used for the treatment of cardiovascular diseases by the CAM practitioners of Pakistan, particularly its bark possesses numerous beneficial phytoconstituents (Asha and Taju, 2012). Along with its remarkable cardioprotective potential *T. arjuna* possesses significant antioxidant, anti-mutagenic,

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anti-diabetic and antimicrobial properties (Jahan *et al*., 2011a, b). The beneficial pharmaceutical characteristics of *T. arjuna* are due to the presence of secondary metabolites such as flavonoids, phenolics, condensed and hydrolysable tannins that are key bioactive compounds accountable for the management of infectious diseases (Shanbhag and Khandagale, 2011). Various bioactive polyphenols are identified in the bark extract of *Terminalia arjuna* such as catechin, gallocatechin, 3-*O*-methyl-ellagic acid 4-*O*-β-D-xylopyranoside, quercetin, myricetin, kaempferol, ellagic acid, 3-*O*-methyl ellagic acid 3-*O*-rhamnoside and proanthocyanidins (Saha *et al.*, 2012).

Regardless of diverse biological activities of plants secondary metabolites their lower bioavailability causes a problem to effectively use them to treat infectious diseases. Recently nanosuspension technology has successfully applied to improve the biological activities of drugs having solubility issues (Raj *et al*., 2016). Nanosuspensions are extremely fine, dispersed solid drug particles in an aqueous phase stabilized by surfactant, polymer or a mixture of both (Amudha and Komala, 2014; Steffi and Srinivasan, 2014). The size of nano-formulated drug particles is usually smaller than 1 micrometer with an average size ranged from 200 nm to 600 nm (Thadkala *et al*., 2015; Pawar *et al*., 2017). These nano-formulations have low processing cost, high dug loading and diminutive side effect by excipients (Chandiran and nandakirouchenane, 2014). Owing to the improved surface to volume ratio of nanosuspensions, drug particles exhibit greater saturation solubility and faster dissolution rate which ultimately improves the bioavailability (Priyanka *et al.*, 2013) and allows the treatment dosage to be reduced (Pawar *et al*., 2017).

In the present research nanosuspension of *T. arjuna* plant extract was formulated to enhance the biological potential of its secondary metabolites (specifically flavonoids). To best of our knowledge it was the first time that an attempt was made to enhance the antimicrobial and antioxidant potential of *T. arjuna* plant extract by formulating its nanosuspension.

Materials and Methods

Plant Collection and Extract Preparation

T. arjuna (bark) was collected form University of Agriculture, Faisalabad, identified from plant taxonomist (Dr. Mansoor Hameed) at Department of Botany and issued voucher number (228-1-2016) was noted. Collected plant material was washed with distilled water, dried under shade and grounded to fine powder. Plant powder was de-fatted with n-Hexane using Soxhlet apparatus. The defatted plant material was extracted with ethanol for about 6-8 h and filtered extract was concentrated by using rotary evaporator (BUCHI Rotavapor II) and residue obtained was used for the formulation of nanosuspension.

Formulation of Nanosuspension

Nanoprecipitation method was employed for the formulation of nanosuspensions by following the method of Hong *et al.* (2014) with some modifications. For the formulation of nanosuspension plant extract was dissolved in ethanol and filtered. The resulting organic phase was slowly injected (1 mL/min) into aqueous phase containing stabilizer (Polysorbate-80) with continuous mechanical stirring at 6000 rpm for 6 h at room temperature.

Optimization of Formulation Parameters

For the formulation of stable nanosuspension of *T. arjuna*, important formulative parameters such as amount of plant extract (A) concentration of stabilizer (B) and anti-solvent to solvent (AS/S) ratio (C) were optimized by using central composite design (CCD) of response surface methodology (RSM) (Pandya *et al*., 2011). Average particle size (Z-average; nm) and polydispersity index (PDI) were selected as response parameters. Experimental design used for the optimization study is given in Table 1.

Lyophilization of Nanosuspension

Optimized nanosuspension (nanosuspension with minimum particle size and PDI) was frozen and lyophilized for 72 h at -40ºC. Freeze-dried sample was used for solid state characterization (Yadav *et al*., 2012).

Characterization of Nanosuspension

Particle size, polydispersity index and zeta potential: The mean particle size (Z-average; nm), PDI and zeta potential of the prepared nanosuspensions were measured by dynamic light scattering (DLS) technique using Malvern Zetasizer (Nano ZS) (Thakkar *et al.*, 2011).

Atomic Force Microscopy (AFM): Three-dimensional (3D) characterization of optimized nanosuspension was done by atomic force microscopy (Shimadzu WET-SPM 9600, Tyoto Japan). For AFM analysis lyophilized nanosuspension was redispersed in small volume of deionized water. Silicon nitride AFM probe (model OMCL-TR800PSA-1) having microcantilever with 100 μm thickness and force constant of 0.57/m were used to obtain scans. SPM Manager Software provided by the AFM system supplier was used to analyze data (Hameed *et al*., 2017).

Scanning Electron Microscopy (SEM): Surface morphology of optimized nanosuspension was evaluated by using SEM. A scanning electron microscope (JEOL, JSM-6400, Japan) equipped with secondary electron detector was employed to get digital image at an accelerating voltage of 15 kv (Sohail *et al*., 2014).

Fourier Transforms Infrared Spectroscopy (FTIR): The optimized nanosuspension was subjected to FTIR

spectroscopy (Perkin Elmer Spectrum, version 10.4.3) to study the drug excipient interactions. The spectra were recorded for crude herbal extract, optimized nanosuspension and stabilizer (polysorbate-80). For analyzing samples, small amount of sample was placed on the lens by using spatula and pressure was applied through screw up to the specified mark. The scanning range was between 4,000-450 cm^{-1} (Sohail *et al*., 2014).

Determination of Antimicrobial Activity

Antimicrobial potential of optimized nanosuspension and native plant suspension was determined by disc diffusion method following the method of Zia-ud-Den and Shahid (2017) using two bacterial strains (*Escherichia coli* and *Bacillus subtilis*) and one fungal strain (*Aspergillus niger*). For determining the antibacterial activity nutrient agar (28.08 g/L) was added in sterilized petri plates and inoculated with the bacterial cultures. Very small filter paper discs impregnated with native plant suspension and nanosuspension (30 μL, 20 mg/mL) were placed flatly on the growth media and petri dishes were incubated for 24 h at 37ºC. Methanol and rifampicin were used as negative and positive controls respectively. Sample possessing antibacterial potential inhibited the growth of bacteria and resulted in the form of clear zone. The zones of inhibition were measured in millimeters using zone reader.

For the evaluation of antifungal activity, potato dextrose agar (39.06 g/L) was added in sterilized petri dishes and inoculated with the fungal specie. Appropriately cut discs of filter paper impregnated with native plant suspension and nanosuspension (30 μL, 20 mg/mL) were placed flatly on growth media. The plates were incubated for 48 h at 2ºC and the antifungal activity was determined by measuring the zones of inhibition using zone reader. Fluconazole (5 μL, 15 mg/250 μL) was used as a positive control to determine the antifungal activity.

Determination of Antioxidant Activity

Antioxidant activity of native plant suspension and optimized nanosuspension was evaluated by DPPH assay following the method of Zafar *et al*. (2016). Five varied concentrations of native plant suspension and nanosuspension ranged from 0.02-0.1 mg/mL were prepared. Aliquot (3 mL) of these concentrations was taken and freshly prepared DPPH solution (0.1 m*M*, 1.0 mL) was added to it. These solutions were allowed to stand at room temperature for 30 min. The absorbance of resultant solutions was noted at 517 nm by means of UV-vis spectrophotometer (Shimadzu, Japan). Decrease in absorbance with increase in concentrations showed high free radical scavenging activity. Ascorbic acid was used as standard compound to compare results. A blank solution was also run in similar manner. All the experiments were repeated thrice, and average results were used. The formula employed for the calculation of percentage inhibition of DPPH radical is given as follows:

$$\text{Percentage inhibition of DPPH} = [1-A_1/A_0] \times 100$$

Where

A_1 = Absorbance of samples
A_0 = Absorbance of control

Statistical Analysis

The CCD of RSM was used for the optimization of formulation parameters (A, B and C). Design Expert Software (version 7.1, Stat-Ease, Inc. USA) was used for generation and evaluation of statistical experimental design (Jin *et al*., 2011). Antioxidant activity was expressed in terms of IC_{50} values and results of antimicrobial activity were presented as mean ± SD (n = 3).

Results

Formulation and Optimization Study

In the present study, nanosuspensions were formulated by employing nanoprecipitation method using polysorbate 80 as a stabilizer. All the nanosuspensions formulated according to the CCD of RSM were found stable with particle size ranging from 79.67 nm to 1007.22 nm. The PDI of the formulated nanosuspensions was in the range of 0.13 to 0.87. Based on statistical results quadratic model was selected to explain the relationship between independent and response variables.

ANOVA for Response Surface Quadratic Models for the Formulation of *T. arjuna* Nanosuspensions

Very small probability values (p<0.0001) reflected the significance of selected quadratic models for both response parameters, particle size (R1) and PDI (R2), with model F-value of 1269.71 and 55.67 respectively (Table 2 and 3). Level of significance was set to 0.05 and model terms with p-values less than 0.05 were considered as significant, whereas, terms with p-values greater than 0.05 were categorized as non-significant. Analysis of variance (ANOVA) study for first response parameter (R1) indicated the significant impact of amount of *T. arjuna* plant extract (A), concentration of stabilizer (B), AS/S ratio (C), interactive effect of amount of plant extract with stabilizer concentaration (AB), interactive effect of amount of plant extract with AS/S ratio (AC) as well as quadratic effect of stabilizer concentration (B^2) on particle size reduction of *T. arjuna* nanosuspension (Table 2).

ANOVA for second response parameter illustrated the significance of linear (A, B, C) and quadratic model terms (A^2, B^2, C^2) in reducing PDI. Moreover, interactive effect of plant extract with stabilizer (AB) as well as stabilizer with AS/S ratio (BC) were also found statistically significant.

Table 1: Experimental design for the optimization of formulative parameters

S. No	Amount of plant extract (g)	Concentration of stabilizer (%)	Antisolvent to solvent ratio
1	0.25	0.25	10.00
2	1.00	0.25	10.00
3	0.25	2.00	10.00
4	1.00	2.00	10.00
5	0.25	0.25	20.00
6	1.00	0.25	20.00
7	0.25	2.00	20.00
8	1.00	2.00	20.00
9	0.00	1.13	15.00
10	1.26	1.13	15.00
11	0.63	0.00	15.00
12	0.63	2.60	15.00
13	0.63	1.13	6.59
14	0.63	1.13	23.41
15	0.63	1.13	15.00
16	0.63	1.13	15.00
17	0.63	1.13	15.00
18	0.63	1.13	15.00
19	0.63	1.13	15.00
20	0.63	1.13	15.00

Table 2: ANOVA for response surface quadratic model for particle size of *T. arjuna* nanosuspensions

Source	Sum of Squares	df	Mean Square	F-value	p-value Prob> F	
Model	1.30E+06	9	1.45E+05	1269.71	< 0.0001	Significant
A-Plant	2.73E+05	1	2.73E+05	2401.2	< 0.0001	
B-Stabilizer	6.29E+05	1	6.29E+05	5522.8	< 0.0001	
C-ratio	9151.02	1	9151.02	80.39	< 0.0001	
AB	3.48E+05	1	3.48E+05	3052.74	< 0.0001	
AC	1956.04	1	1956.04	17.18	0.002	
BC	434.34	1	434.34	3.82	0.0793	
A^2	232	1	232	2.04	0.1839	
B^2	39722	1	39722	348.95	< 0.0001	
C^2	232	1	232	2.04	0.1839	
Residual	1138.34	10	113.83			
Lack of Fit	45.92	5	9.18	0.042	0.9983	non significant
Pure Error	1092.42	5	218.48			
Cor Total	1.30E+06	19				
R^2	0.9991			Adj R^2	0.9983	
Pred R^2	0.9985			CV %	2.85	
Adeq Precision	123.138					

Pred R^2 = Predicted R^2, Adeq Precision= Adequate Precision, Adj R^2 = Adjusted R^2, CV = Coefficient of Variation

The only non significant term in reducing PDI was the interaction of plant extract with AS/S ratio (AC) (Table 3).

The non significant lack-of-fit F-value of R1 (0.04) and R2 (4.52) showed good predictability of the model. The R^2 value of 0.9991 and 0.9804 for R1 and R2 indicated that 99.91% (R1) and 98.04% (R2) variability in the responses was explained by the statistical model. The values of predicted R^2, adjusted R^2, adequate precision and coefficient of variation (CV) of both response variables confirmed the suitability of quadratic model in explaining the relationship between independent and response variables (Table 2 and 3).

Effect of Independent Variables on Particle Size and PDI Reduction of *T. arjuna* Nanosuspensions

Combined effect of all the independent variables (A, B and C) on response parameters (R1 and R2) for the formulation of *T. arjuna* nanosuspensions was evaluated by three-dimensional (3D) response surface plots. In each plot the combined effect of two variables was determined simultaneously, whereas third factor was kept at its middle value.

Three-dimensional response surface plot between amount of plant extract and concentration of stabilizer showed a sharp increase in particle size by increasing the amount of plant extract from 0.25 to 1 g. However, no significant influence was observed by increasing the concentration of stabilizer. Minimum particle size was achieved at smaller amount of plant extract and stabilizer concentration (0.25 g plant and 0.25% stabilizer) (Fig. 1A). 3D response surface, plotted between amount of plant extract and AS/S ratio also illustrated greater influence of amount of plant extract in reducing particle size of *T. arjuna* nanosuspensions as compared to AS/S ratio (Fig. 1B). A steady decrease in particle size was observed by increasing the amount of stabilizer (Fig. 1C) and minimum particle size was noted at highest concentration of stabilizer (2%). However, particle size remained unaffected by varying the AS/S ratio. 3D response surface plots for second response parameter (PDI) also illustrated the remarkable influence of all the three formulative variables on PDI reduction however, the impact of amount of plant extract and concentration of stabilizer was more pronounced as compared to AS/S ratio (Fig. 2A, B and C).

Regression Analysis

Second order polynomial equations showing the effect of independent variables on response variables in terms of coded factor are given below (Equation 1 and 2). The positive coefficients of equations demonstrated the synergistic effect of independent variables on responses, whereas, negative sign showed contradictory effect.

Polynomial Equation in Terms of Coded Factors

***T. arjuna* (Size-nm) (R1)** = +332.73 + 141.47A - 214.56B - 25.89C - 208.42AB -15.64AC +7.37BC+ 4.01A2+ 52.50B2+ 4.01C2 **(Equation 1)**

***T. arjuna* (PDI) (R2)** = +0.33 - 0.15A - 0.059B + 0.035C - 0.17AB -0.019AC +0.044BC+ 0.045A2+ 0.064B2+ 0.095C2 **(Equation 2)**

Desirability and overlay plots created by using design expert software indicated that minimum particle size (79.57 nm) and PDI (0.209) was obtained when nanosuspension was formulated by using 1 g plant

Table 3: ANOVA for response surface quadratic model for PDI value of *T. arjuna* nanosuspensions

Source	Sum of Squares	df	Mean Square	F-value	p-value Prob> F	
Model	0.82	9	0.091	55.67	< 0.0001	Significant
A-Plant	0.31	1	0.31	191.12	< 0.0001	
B-Stabilizer	0.047	1	0.047	28.87	0.0003	
C-ratio	0.017	1	0.017	10.23	0.0095	
AB	0.24	1	0.24	145.74	< 0.0001	
AC	2.78E-03	1	2.78E-03	1.69	0.2226	
BC	0.015	1	0.015	9.39	0.012	
A^2	0.029	1	0.029	17.75	0.0018	
B^2	0.058	1	0.058	35.63	0.0001	
C^2	0.13	1	0.13	79.53	< 0.0001	
Residual	0.016	10	1.64E-03			
Lack of Fit	0.013	5	2.69E-03	4.52	0.0617	non-significant
Pure Error	2.97E-03	5	5.94E-04			
Cor Total	0.84	19				
R^2	0.9804			Adj R^2	0.9628	
Pred R^2	0.8732			CV %	8.69	
Adeq Precision	28.165					

Pred R^2 = Predicted R^2, Adeq Precision= Adequate Precision, Adj R^2 = Adjusted R^2, CV = Coefficient of Variation

extract, 2% stabilizer and AS/S ratio of 19.99 (Fig. 3A and B). These outcomes were in conformity with the experimental results where same experimental conditions provided comparable results (Fig. 4A).

Characterization of Optimized Nanosuspension

Zeta potential: Zeta potential value (-22 mV) of optimized nanosuspension confirmed the stability of optimized nanosuspension (Fig. 4B).

AFM analysis of *T. arjuna* nanosuspension: AFM image of *T. arjuna* nanosuspension revealed that particles were little bit porous in nature with non-uniform distribution. Average size of particles was in the range of 70 nm (Fig. 5).

SEM analysis of *T. arjuna* nanosuspension: SEM photograph of *T. arjuna* nanosuspension is given in Fig. 6 which illustrated that particles were little bit flower type shape with uniform size at certain places.

FTIR analysis of *T. arjuna* native extract and nanosuspension: FTIR spectrum of *T. arjuna* nanosuspension was analogous with plant extract rather than stabilizer (Polysorbate 80) (Fig. 7A, B and C). However, the broad peak of hydroxyl group at 3363.24 cm^{-1} become minimized at 3445.42 cm^{-1}. The peaks at 2918.32 cm^{-1} and 2850.62 cm^{-1} in the spectrum of plant extract were similar to peaks of nanosuspension (2916.69 cm^{-1} and 2849.61 cm^{-1}) respectively. In comparing the remaining peaks there was no significant change in the peak positions apart from the peak intensity.

Comparative Antimicrobial Potential of *T. arjuna* Nanosuspension and Native Suspension

Results of antimicrobial activity of native plant suspension and nanosuspension are given in Table 4. Comparative

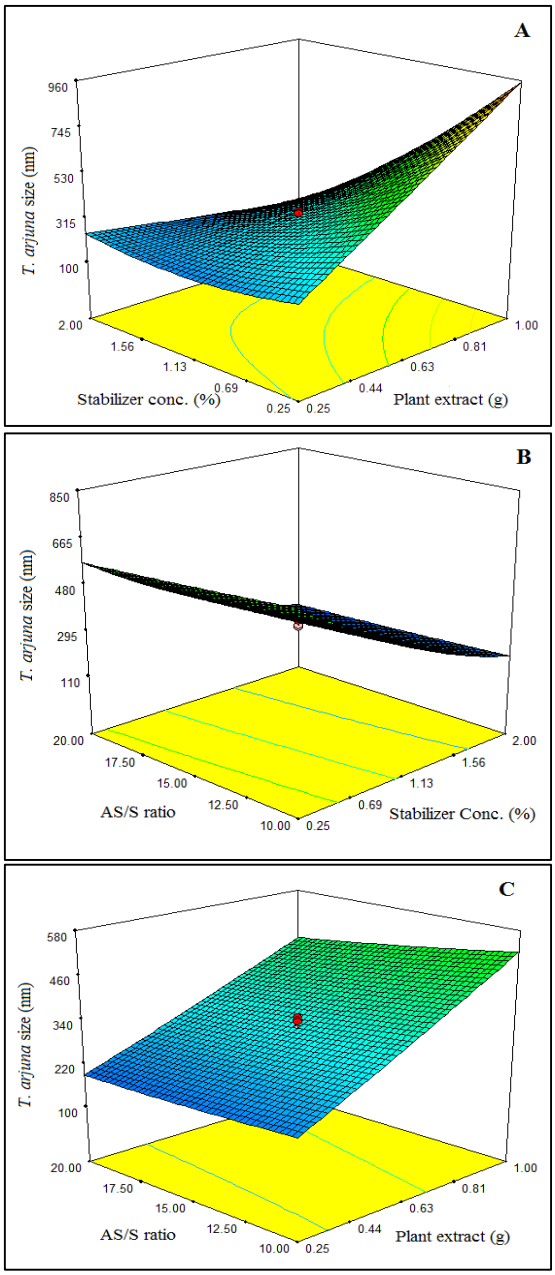

Fig. 1: 3D response surface graphs illustrating the combined effect of **(A)** amount of plant extract and concentration of stabilizer **(B)** concentration of stabilizer and AS/S ratio and **(C)** amount of plant extract and AS/S ratio on particle size reduction of *T. arjuna* nanosuspensions

evaluation of native plant suspension and nanosuspension revealed significantly higher (p<0.05) antifungal activity for nanosuspension. However, significantly (p<0.05) greater antifungal activity was observed for fluconazole (positive control) as compared to both treatments. Regarding antibacterial activity, remarkably greater (p<0.05) inhibition zones were observed for *T. arjuna* native suspension than its nanosuspension

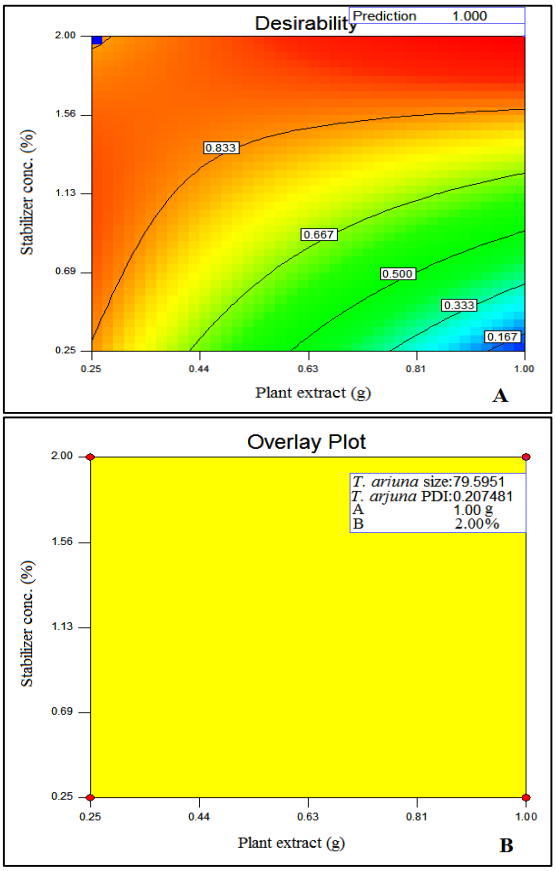

Fig. 3: Desirability and overlay plots showing the interactive effect of formulative variable A and B on particle size and PDI values of *T. arjuna* nanosuspensions

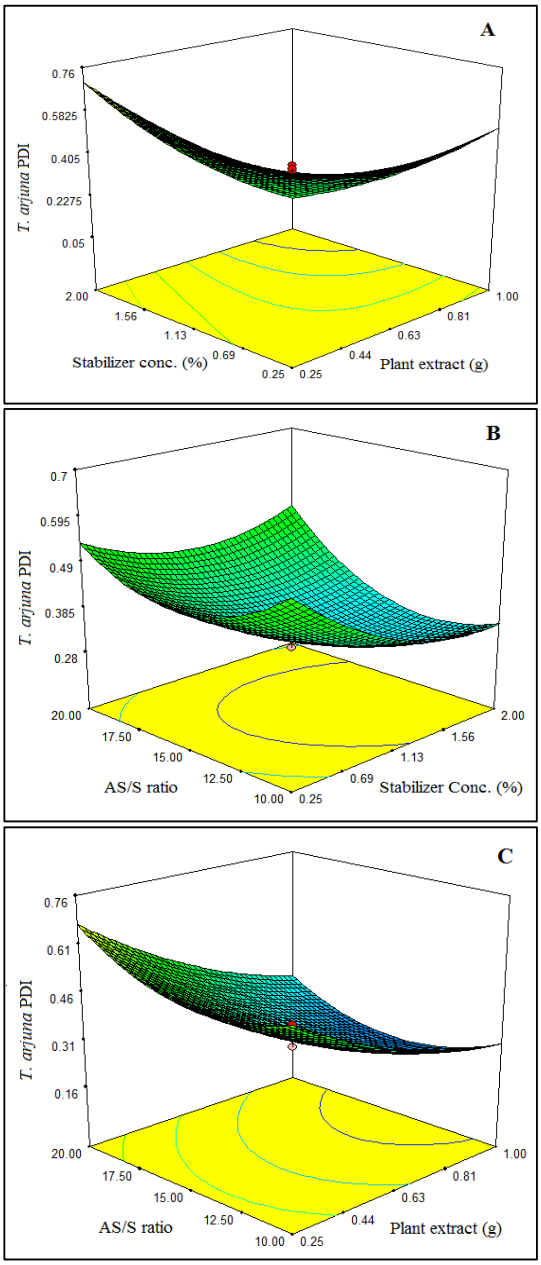

Fig. 2: 3D response surface graphs illustrating the combined effect of **(A)** amount of plant extract and concentration of stabilizer **(B)** concentration of stabilizer and AS/S ratio and **(C)** amount of plant and AS/S ratio on PDI reduction of *T. arjuna* nanosuspensions

against both bacterial strains (*E. coli* and *B. subtilis*). However, rifampicin (positive control) illustrated significantly (p<0.05) enhanced antibacterial activity as compared to both suspensions (native as well as nanosuspension).

Comparative Antioxidant Potential of *T. arjuna* Nanosuspension and Native Suspension

Results of antioxidant activity of native plant suspension and nanosuspension were expressed in terms of amount required for 50% inhibition of DPPH radical (IC_{50}) and are given in Fig. 8. Ascorbic acid (the natural antioxidant) was used as a standard compound to compare the results and possessed IC_{50} value of 189.06 μg/mL. Comparative evaluation of *T. arjuna* nanosuspension and native suspension showed greater radical scavenging potential for nanosuspension with IC_{50} value of 192.9 μg/mL as compared to its native suspension (237.58 μg/mL).

Discussion

T. arjuna, an important medicinal plant, possess numerous biological activities owing to its diverse bioactive phytoconstituents. Among these constituents, phenolic and flavonoid contents are mainly responsible for its antimicrobial and antioxidant potential (Mandal *et al.*, 2013). However, low water solubility associated with flavonoid contents (kaempferol, quercetin and catechin) of *T. arjuna* limits its biological potential. Therefore, in the present study an attempt was made to enhance the biological

Table 4: The antimicrobial potential of *T. arjuna* nanosuspension and native suspension

Plant/Standard	Antifungal activity (diameter in mm)	Antibacterial activity (diameter in mm)	
Strain used	*Aspergillus niger*	*Escherichia coli*	*Bacillus subtilis*
T. arjuna Nat. sus	5.0 ± 0.02^{a}	18.75 ± 0.15^{a}	16.5 ± 0.14^{a}
T. arjuna Nano	18.5 ± 0.16^{b}	12 ± 0.06^{b}	13.5 ± 0.10^{b}
Fluconazole	43.5 ± 0.23^{c}		
Rifampicin	-	37.5 ± 0.13^{c}	27 ± 0.17^{c}
Methanol	-	00.0 ± 0.00^{d}	00.0 ± 0.00^{d}

Values are expressed as mean ±SD (n=3) indicating the diameter of zone of inhibition in mm

Values sharing similar letter within column are non-significantly different ($p<0.05$)

Nano=nanosuspension, *Nat. sus*=native suspension

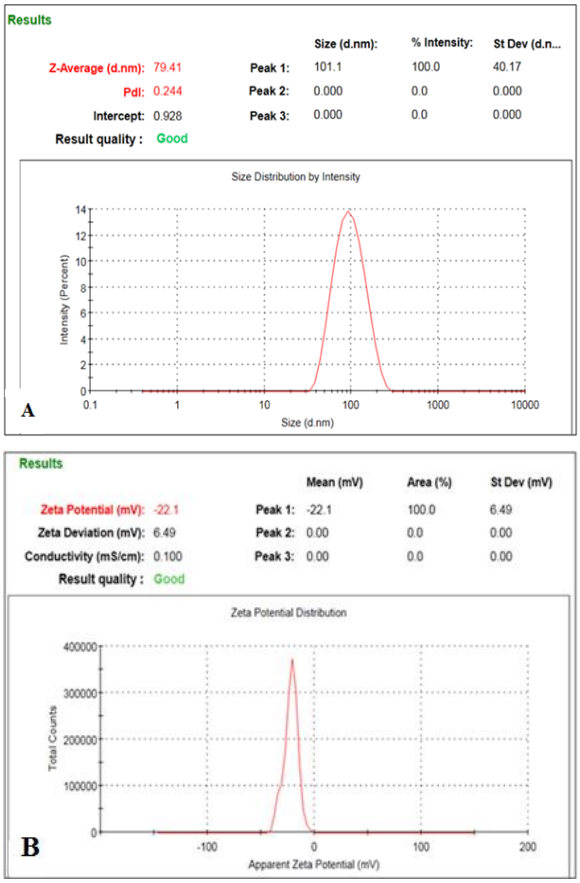

Fig. 4 (A): Zeta size, PDI and **(B)** zeta potential value of optimized nanosuspension of *T. arjuna*

activities of *T. arjuna* plant extract by formulating its nanosuspension. To formulate more efficacious nanosuspension, important formulation parameters (amount of plant extract, concentration of stabilizer and AS/S ratio) were optimized by using central composite design of RSM.

Based on CCD with three formulation parameters, a set of 20 experiments was performed and the obtained results were inserted in the software. The design identified the optimum conditions for the formulation of *T. arjuna*

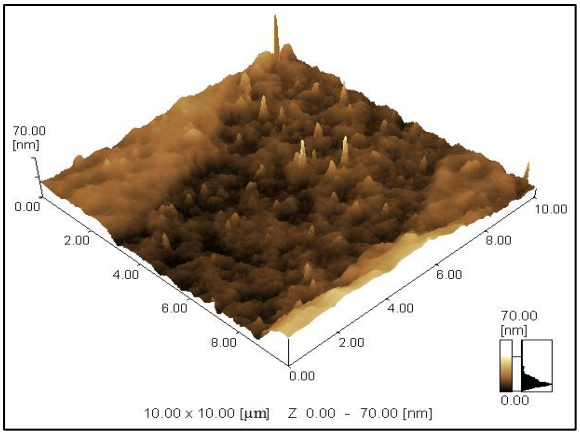

Fig. 5: Three-dimensional (3D) AFM image of *T. arjuna* nanosuspension

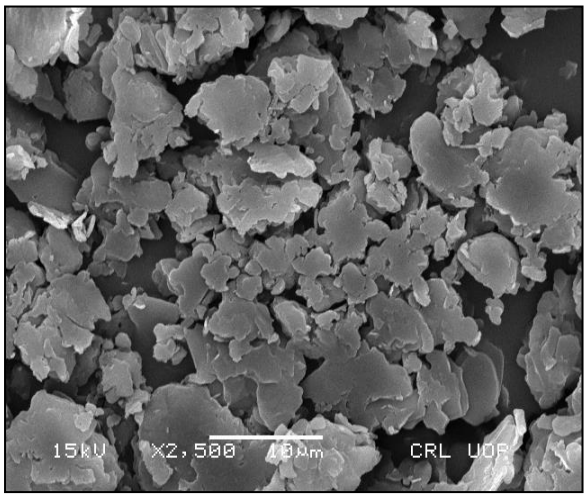

Fig. 6: SEM potograph of *T. arjuna* nanosuspension

nanosuspension with desired responses in a single experimental run. Furthermore, it optimized the responses and determined the relationship between response variables and the interactive effects of independent variables. To explain the relationship between independent and response variables two quadratic models were developed.

Analysis of variance (ANOVA) showed the significance of almost all the model terms in reducing the particle size and PDI values of *T. arjuna* nanosuspensions. The goodness of fit for the quadratic model was evaluated by coefficient of determination (R^2). Greater R^2 value (close to 1.0) for both response parameters showed that the quadratic model presented the system well over the given experimental domain as found previously (Sudhakar *et al*., 2015; Selvam *et al*., 2017).

The adjusted R^2 value corrected the R^2 value for the sample size and the number of runs. The values of predicted R^2 for both response parameters were in exact

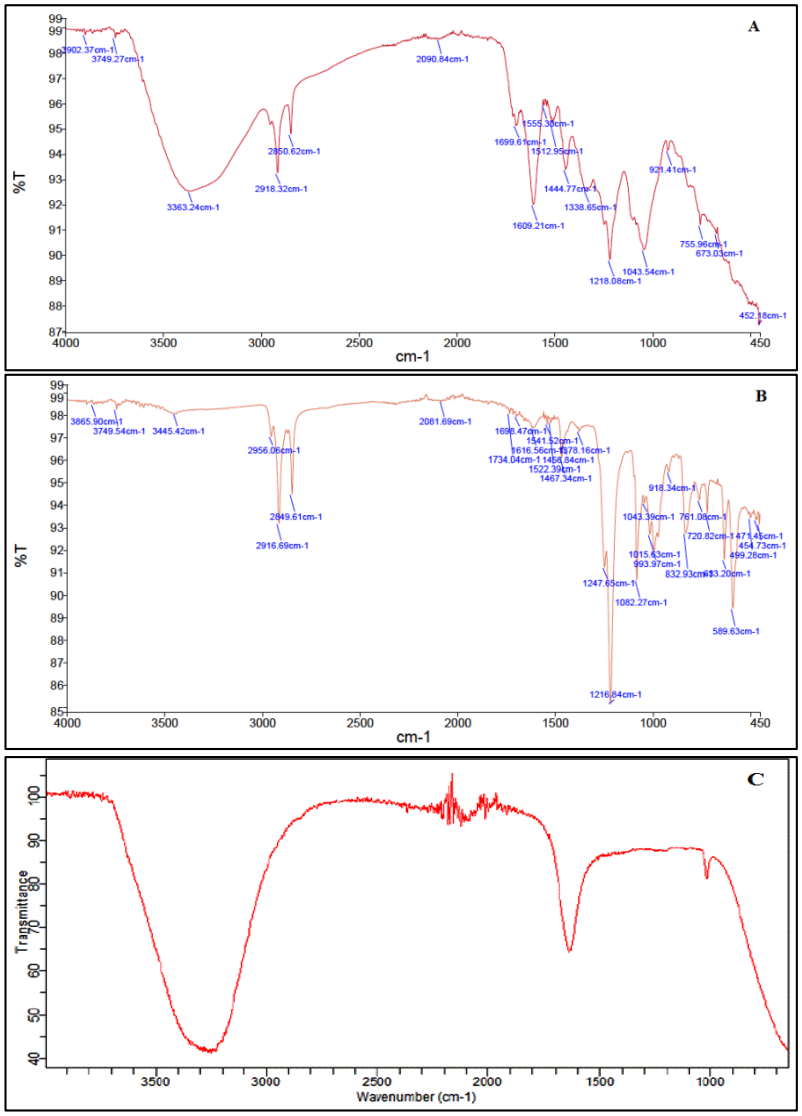

Fig. 7: FTIR spectrum of **(A)** *T. arjuna* native plant extract **(B)** *T. arjuna* nanosuspension and **(C)** stabilizer (Polysorbate-80)

agreement with their respected adjusted R^2 values that were close to their respective R^2. Present results illustrated that the regression model provided a good fit to the data. Signal to noise ratio was used to determine the adequate precision. Adequate precision should have value greater than 4. In the present study, greater value of adequate precision demonstrated that the selected model was precised for both responses. Moreover, lower CV (less than 10%) for both responses indicated the accuracy and reliability of the experiments conducted.

Three-dimensional response surface plots illustrated that the particle size and PDI values of *T. arjuna* nanosuspensions were greatly influenced by varying the amount of plant extract and concentration of stabilizer. Minimum particle size was observed at smaller amount of plant and higher concentration of stabilizer. Present results were supported by the findings of Hong *et al.* (2014) in

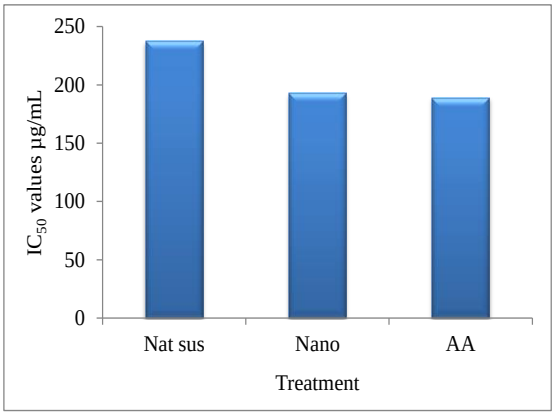

Fig. 8: Comparative antioxidant potential of *T. arjuna* nanosuspension and native suspension
Nat sus= Native suspension, Nano= Nanosuspension, AA = Ascorbic Acid

which particle size was sharply decreased and PDI was slightly decreased by increasing the concentration of stabilizer indicating that stabilizer has a positive effect on particle size reduction. These results may be because, large amount of stabilizer completely covered the surface of the nanoparticles and prevented their aggregation during the process of nanoformulation (Hong *et al*., 2014). Excellent correlation between experimental and predicted results proved the validity of statistical experimental design of RSM in optimization studies.

The particle size measured by AFM was almost same as provided by DLS technique (zeta sizer). This was because, particles of *T. arjuna* nanosuspension were monodispersed, having very small value of PDI. Good correlation between particle size (obtained from both techniques) confirmed the successful particle size engineering of *T. arjuna* plant extract. Present results were supported by the findings of Saxena *et al*. (2004) in which good correlation was noted in the size of PLGA nanoparticles analyzed by DLS and AFM because of very small particle size distribution (PDI values ranging from 0.01–0.06). No previous study was found in literature in which AFM was used to specifically characterize plant nanosuspension.

SEM images revealed good surface characteristics of *T. arjuna* nanosuspension, however, presence of larger and non-uniform particles at certain places may be due to the adhesion and aggregation of individual particles during freeze drying (Gang *et al*., 2016). Comparable results were also reported by Afifi *et al.* (2015) where polysorbate 80 was used as a stabilizer and SEM images revealed the presence of aggregates and individual particle association.

FTIR analysis was carried out to evaluate the interaction of plant extract with stabilizer. Present results indicated that the plant extract in pure form or in the form of nanosuspension showed same structural features regarding functional groups as found previously (Mishra *et al.*, 2015; Shi *et al.*, 2016; Motawie *et al.*, 2017). Overall results of FTIR studies indicated the presence of H-bonding between plant extract and stabilizer and no chemical interaction was observed between these two.

Regarding the biological activities, *T. arjuna* nanosuspension showed enhanced antifungal activity than its native suspension against the fungal strain *A. niger*, which is a common fungus species of the genus *Aspergillus*. *A. niger* causes black mold diseases in certain vegetables and fruits such as onion, peanuts and grapes and is considered as a common food contaminant (Sharma, 2012). The black rot diseases caused by *A. niger* is responsible for huge loss of onion bulb in field and during storage (Saranya *et al*., 2017). Moreover, collar rot diseases of groundnut caused by *A. niger* is an important disease in various temperate countries (Kumari *et al*., 2017) which causes spoilage of mangoes, grapes and tomatoes (Sharma, 2012). Spoilage caused by *Aspergillus* species can be of nutritional, sensorial and qualitative nature like: discoloration, pigmentation, rotting, development of off-flavors and off-odors (Perrone *et al.*, 2007). Beside animal and plant pathogens, *A. niger* is also reported to produce ochratoxin A and fumonisin B_2 and aflatoxins (Sharma, 2012). However, the formulated nanosuspension of *T. arjuna* can prove to be a milestone to treat these fungal diseases owing to its enhanced antifungal potential.

Significantly enhanced antifungal activity of *T. arjuna* nanosuspension can be attributed to its greater dissolution rate and subsequently improved diffusion of nanosuspension in cultural media during the fungal growth. It may also be due the fact that extensive particle size reduction facilitated the diffusion of nanosuspension as compared to native suspension resulting in increased inhibition zone *(*Melkoumov *et al*., 2013*).* Another reason for better antifungal potential may be that, the unique physicochemical properties of nanosuspension (large surface to mass ratio, ultra-small size) leads to its high reactivity and unique interactions with biological systems (Shah *et al*., 2017). However, better antibacterial activity of native plant suspension of *T. arjuna* was in agreement with the previous findings of Das and Suresh, 2011 (2011) in which nanoformulation exhibited almost same or little inhibitory potential than coarse suspension.

Antioxidant potential of native plant suspension and nanosuspension was evaluated by employing DPPH radical scavenging assay owing to its simplicity, rapidity and cost effectiveness (Hassan *et al*., 2016; Mehmood *et al*., 2016). Results of antioxidant activity also demonstrated the enhanced DPPH radical scavenging potential of nanosuspension as compared to native plant suspension. As the radical scavenging potential of plants is mainly attributed to the presence of bioactive phytochemicals like phenolics and flavonoid contents (Zhang and Zuo, 2004; Ghimire *et al*., 2011; Ahmad *et al*., 2016), when we formulated the nanosuspension of *T. arjuna*, the enhanced antioxidant potential was observed for nanosuspension which might be attributed to the fact that, due to particle size reduction, solubility and dissolution rate of flavonoids was enhanced which ultimately improved the radical scavenging potential (Kakran *et al*., 2012a, b). It was noted that DPPH radical scavenging activity of native suspension and nanosuspension along with standard (ascorbic acid) was increased by increasing their concentration as found previously (Zafar *et al*., 2016; Bamidele *et al.*, 2017). Analogous results were noted in the finding of Sahoo *et al*. (2011), Tzeng *et al*. (2011) and Sonkaew *et al*. (2012) in which nanosuspensions showed enhanced radical scavenging potential than coarse water extracts. This enhanced radical scavenging potential of *T. arjuna* nanosuspension can be helpful to improve its therapeutic benefits.

Conclusion

In the present study *T. arjuna* nanosuspension illustrated significantly enhanced antioxidant and antifungal potential as compared to its native suspension which proves the importance of novel nanosizing technique in enhancing the biological activities of herbal extracts. The enhanced antifungal activity of *T. arjuna* nanosuspension against *A. niger* can prove to be a new horizon to treat fungal diseases of plants. The study also accentuated the effectiveness of CCD of RSM in optimizing the process parameters for the formulation of stable nanosuspension of *T. arjuna*.

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