RESEARCH ARTICLE

PRELIMINARY FORMULATION STUDY OF LYCOPENE SELF-ASSEMBLE NANOPARTICLES VIA BOX-BEHNKEN DESIGN IMPARTING AN EFFICIENT DRUG DELIVERY: STATISTICAL AND ILLUSTRATIVE APPROACH

Mohammad Yusuf

Department of Clinical Pharmacy, College of Pharmacy, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia.

……………………………………………………………………………………………………....

Manuscript Info

………………….

Abstract

…………………………………………………………………

Lycopene, a structurally polyene conjugated antioxidant, is frequently utilized to treat ailments induced by augmented oxidative stress. Its colossal structure and poor water-solubility failed to show the requisite clinical outcomes. To overcome these drawbacks, a formulation study introducing Lycopene as Chitosan-phosphatidylserine self-assembled nanoparticles (LNP)to correlate the dependent and independent variables employing Box-Behnken design has been performed. LNP formulation was achieved by injection technique, using chitosan's intrinsic property of towards Phosphatidylserine for self-assembly. Independent variables, i.e., chitosan concentration (X_1), Phosphatidylserine (X_2), and injecting rate (X_3) were correlated against dependent variables (responses) nanoparticle size, cumulative release, and lycopene encapsulation. Prepared nanocrops were stable, under 300 nm particle size, Zeta potential below +30 mV, close PDI to 0.3, and exhibited *invitro* 69-76% cumulative release. Overall, the formulation strategy was fruitful to develop a formulation in the future for efficient drug delivery.

……………………………………………………………………………………………………....

Introduction:-

Pharmaceutical industries are enforcing well to introduce natural drugs by augmenting their bioavailability. Several studies are going on to overcome poor bioavailability and solubility of these drugs, but only Nano-drug delivery has given hope for natural drugs to be at par with the currently in use chemical drug molecules. Nanoparticles have several advantages i.e., imparting solubility, good bioavailability, high payload and accurate organ targeting of any drug with controlled mechanism, especially to brain [1-3]. Recently, nanoparticles of numerous antioxidants like piperine, curcumin, beta-carotene, and thymoquinone formulated and augmented the bioavailability and solubility of the original compound [4-7]. Lycopene known for its variety of biological activity, mainly acting via Oxidative stress mechanisms[8]. Lycopene's giant molecular structure, weight, volume, and geometrical isomerism implicates poor pharmacokinetics, these factors often impart poorer water-solubility affecting its bioavailability [9-11].

Formulation of nanoparticles is done using several techniques, including varied raw material concentrations, giving rise to chances of several biohazards posed by the byproducts formed in the design process [12, 13]. Self-assembly approach is the best remedy to deal with this problem, as the technique is devoid of any byproduct or intermediate formation[14, 15]. Natural polysaccharides like Chitosan are safe with higher bio-acceptability and easily processed

Corresponding Author:-Mohammad Yusuf
Address**:-**Department of Clinical Pharmacy, College of Pharmacy, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia.

inhuman gut[16], Otherwise, phosphatidylserine also approved by FDA as a safer dietary product [17]. Hence, we intended to prepare and optimize LNP from chitosan and phosphatidylserine as no such study reported until now.

Materials:-
Lycopene, ethyl-acetate, Chitosan, Phosphatidylserine (PS), hexyl hydride, filter (WF), 33 mm Cellulose dialysis tube-membrane (CD), 0.45μm Whatman, phosphate buffer (PBS), ethanol, HCl, were obtained from Sigma Aldrich.

Methods:-
Lycopene Nanocrops Formulation:
PS (X_2) and Lycopene (25 mg) dissolved in 90% ethanol (10 ml) to get Solution A. Stock solution of acidified (0.2 M) Chitosan (X_1) was ready and put overnight to get solution B (93cP). Solution A (4 ml) progressively was interspersed in solution-B (46 mL), by a steady injecting rate (X_3) under 1450 rpm homogenization and filtered to obtain Lycopene nanocrops. All nanocrops were lyophilized and stored. Size, charge, and distribution were computed by Zetasizer (Nano-Z90, Malvern, UK) and surface morphology was evaluated by TEM.

Experimental design:
Box-Behnken Design optimized all nanocrops via Design-Expert® Version (V.12, Stat-Ease, USA). Independent variables, i.e., chitosan concentration (X_1), Phosphatidylserine (X_2), and injecting rate (X_3) (levels coded −1,0,+1, Table 1) were designated to relate with dependent variables (responses) i.e. nanoparticle size, lycopene Encapsulation, and cumulative release were studied. Design-Expert® performed 17 hits(Table 2)

Table 1:-Experimental design with Independent variables.

Codes	Independent Variables	Levels		
		-1	**0**	**+1**
X_1	Chitosan (w/v)	5	10	15
X_2	PS (mg/ml)	2.5	5.00	7.5
X_3	Injection rate (ml/min)	5.0	10.0	15

Table 2:-Box-Behnken Design with level values.

Nanocrops	Box-Behnken		
	Chitosan (w/v)	PS (mg/ml)	Injection rate (ml/min)
NC-1	**-1**	**-1**	**0**
NC-2	**+1**	**-1**	**0**
NC-3	**0**	**-1**	**-1**
NC-4	**0**	**-1**	**+1**
NC-5	**0**	**0**	**0**
NC-6	**+1**	**0**	**-1**
NC-7	**0**	**0**	**0**
NC-8	**+1**	**0**	**+1**
NC-9	**0**	**0**	**0**
NC-10	**-1**	**0**	**+1**
NC-11	**0**	**0**	**0**
NC-12	**0**	**0**	**0**
NC-13	**-1**	**0**	**+1**
NC-14	**-1**	**+1**	**0**
NC-15	**+1**	**+1**	**0**
NC-16	**0**	**+1**	**-1**
NC-17	**0**	**+1**	**+1**

Lycopene% Encapsulation Efficiency (%EE):
100 μL of Lycopene Nanocrop was extracted in hexyl hydride (3 ml, 3000 rpm, 4 minutes), floating layer of hexyl hydride was parted and diluted to quantify free Lycopene at 471 nm by Ultraviolet spectroscopy equations:

$$EE\% = \frac{(\text{Total LYC} - \text{Free LYC})}{\text{Total LC}}\ 100 \qquad\qquad (1)$$

TEM LNP surface Morphology:
Optimized Nanocrop was visualized via TEM (268D, Philips, USA). Nanocrops kept on 300 size copper grid, with dropping phosphotungstic acid (10 sec), observed under 70-80 KV, magnification 30000x.

Invitro Lycopene cumulative Release (CR) from Lycopene Nanocrop:
Lycopene CR from Nanocrop was detected in PBS (pH 7.4) at 37˚C. 1 ml Nanocrop was sandwiched in dialysis bag (Sigma-Aldrich, CA, USA), hanged in PBS (10 ml)kept with constant agitation(150 rpm) at 25°C. At set time intervals, 1 ml was reinstated with fresh volume for 24 hrs and quantified by Ultraviolet spectroscopy (471 nm).

Results and Discussion:-
Statistical assessment of nanocrops:
All the formulation results were examined by Design-ExpertV.12. Numerical optimized method was chosen to access independent variables in-relation to the responses. All the responses followed Quadratic polynomial equations (Table 3) and significant findings are reported as per ANOVA analysis.

Table 3:-Polynomial equations as coded factors for Particle size, % EE and Cumulative Release.

Actual Factors	Coded	Particle size	% EE	Cumulative Release
	Intercept	+220.14	+30.64	+72.20
Chitosan	A	+38.58	-0.0500	-3.50
PS	B	+3.79	+7.66	+0.0000
Injection rate (ml/min)	C	-2.74	+0.1375	+0.0000
Chitosan * PS	AB	+1.43	-0.3000	+0.0000
Chitosan * Injection rate (ml/min)	AC	-3.17	+0.7500	+0.5000
PS * Injection rate (ml/min)	BC	+6.45	-0.1250	+0.5000
Chitosan ²	A²	+4.30	-0.8075	-0.1000
PS ²	B²	+8.58	+0.6675	+0.4000
Injection rate (ml/min)²	C²	+7.08	+1.07	+0.4000

Table 4:-Box-Behnken Design with all dependent and independent variables.

Box-Behnken						Independent Variables			Dependable Variable		
Nanocrops	STD	Run	X_1	X_2	X_3	Chitosan (w/v)	PS (mg/ml)	Injection rate (ml/min)	Size	%EE	Cumulative Release
NC-1	1	4	-1	-1	0	5	2.5	10	192.1	21.3	76
NC-2	2	8	+1	-1	0	15	2.5	10	267.7	23.5	69
NC-3	9	1	0	-1	-1	10	2.5	5	242.1	24.6	74
NC-4	11	17	0	-1	+1	10	2.5	15	220.6	25.7	72
NC-5	17	11	0	0	0	10	5	10	221.1	29.2	70
NC-6	6	14	+1	0	-1	15	5	5	273.8	29.4	68
NC-7	16	16	0	0	0	10	5	10	220.4	29.9	73
NC-8	8	9	+1	0	+1	15	5	15	265.1	30.6	70
NC-9	14	2	0	0	0	10	5	10	218.5	30.9	73
NC-10	7	10	-1	0	+1	5	5	15	198.3	30.9	76
NC-11	13	6	0	0	0	10	5	10	221.4	31.1	72
NC-12	15	3	0	0	0	10	5	10	219.3	32.1	73
NC-13	5	12	-1	0	+1	5	5	5	191.6	32.7	76
NC-14	3	7	-1	+1	0	5	7.5	10	195.5	38.1	76
NC-15	4	15	+1	+1	0	15	7.5	10	276.8	39.1	69
NC-16	10	13	0	+1	-1	10	7.5	5	238.1	39.3	73
NC-17	12	5	0	+1	+1	10	7.5	15	242.4	39.9	73

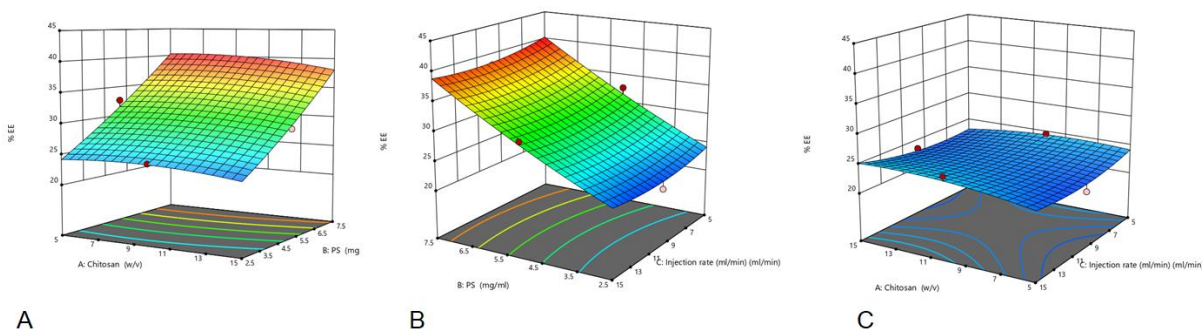**Particle size reliability on Independent Variables:**
Particle size affects bioavailability and physical stability of any drug. Smaller particle size offers high surface area to provide better tissue dispersal and cellular access. Nanoparticles over 230 nm trapped by spleen and decreases the bioavailability of drug[18, 19]. Particle size and responses are shown in Table 4. As per quadratic paradigm, F-value of 311.08 proved a high significance (p=0.0001), whereas P values for chitosan concentration (p=0.0001), Phosphatidylserine (p=0.0016) and injecting rate (p=0.0087) with regression coefficient (R^2) of 0.9667 adjusted to 0.9943 representing nearly no variation in experimental model (Figure 6). Adequate precision of 51.351 signifying appropriate navigation for design space. Chitosan concentration increases the particle size, where phosphatidylserine concentration or and injecting rate not affected much. Increase particle size (189-287 nm) may be of high viscosity or chitosan in medium. Relationship between particle size and dependable factors is shown in response surface graphs. (Figure 1)
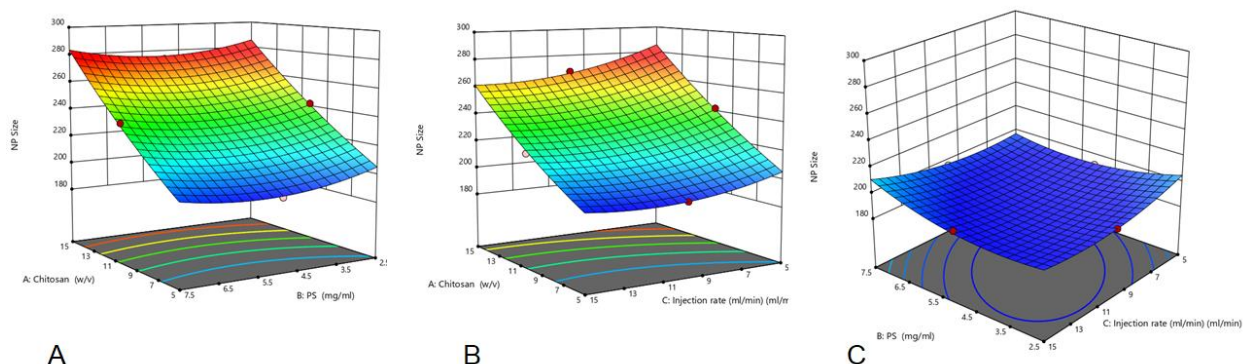
Figure 1:- Response surface graphs for chitosan concentration, Phosphatidylserine (PS) and injecting rate on particle size

%Encapsulation Efficiency reliability on Independent Variables:
Encapsulation efficacy offers real drug payload to any nanoformulations, it directly affects the drug bioavailability. %EE and responses are illustrated in Table 4. As per quadratic paradigm F-value of 28.81 proves a high significance (p=0.0001) whereas P values for chitosan concentration (p=0.9203), Phosphatidylserine (<0.0001) and injecting rate (p=0.7836) with regression coefficient (R^2) of 0.9737 adjusted to 0.9399 representing nearly no variation in experimental model (Figure 6). Adequate precision of 16.70 signifying appropriate navigation for design space. With increase of Phosphatidylserine concentration, this specifies that due to lipophilic nature of lycopene it was solvated in phosphatidylserine core via making electrostatic interaction or conjugated interaction with PS structure, Which has given encapsulation efficiency ranging 21-39%. Relationship between %EE and dependable factors is shown in response surface graphs. (Figure 2)
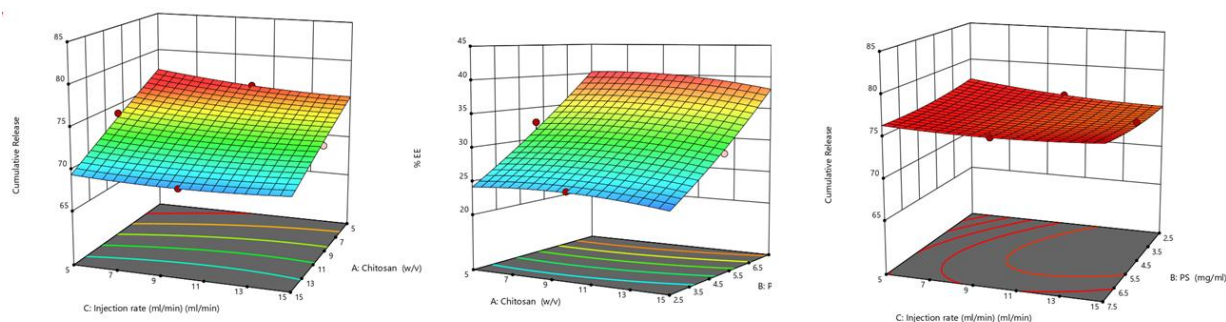

Figure 2:-Response surface graphs for chitosan concentration, Phosphatidylserine (PS) and injecting rate on %EE

Cumulative release reliability on Independent Variables:
Cumulative release gives prediction of drug deliverance in a similar biological system. The superiority of any formulation directly depends upon the drug amount released and affecting the bioavailability of any drug. Drug-

release is a direct polymer attribute, which affects both stability and therapeutic effectiveness of any drug. Mostly polymer offers a rapid initial release called the burst mode, which also depends upon polymer matrix offering drug encapsulation[20]. Cumulative release and responses are illustrated in Table-4. As per quadratic paradigm, F-value of 8.97 proves a significance (p=0.0043) whereas P values for chitosan concentration (p<0.0001), Phosphatidylserine (p=1), and injecting rate (p=1) with regression coefficient (R^2) of 0.9202 adjusted to 0.8175 representing nearly no variation in experimental model (Figure 6). Adequate precision of 9.3 signifying appropriate navigation for design space. Cumulative release was found dependent on all the dependable factors and ranged from 67-76%. Relationship between Cumulative release and dependable factors is shown in response surface graphs[21]. (Figure 3)


Figure 3:-Response surface graphs for chitosan concentration, Phosphatidylserine (PS), and injecting rate on Cumulative release

Optimization and validation:

According to desirability, separate optimized Batch of LNP was fabricated (Table 5). The findings are illustrated through digitally generated contour graphs (Figure 4). The relative error (<5%) was calculated between the predicted and actual values showing negotiable variation in the group (Figure 5 and 6).

**Table 5:-Optimized Levels of independent Variables**

| Codes	Independent Variables	Optimized levels
X_1	Chitosan (w/v)	12.10
X_2	PS (mg/ml)	3.78
X_3	Injection rate (ml/min)	9.31
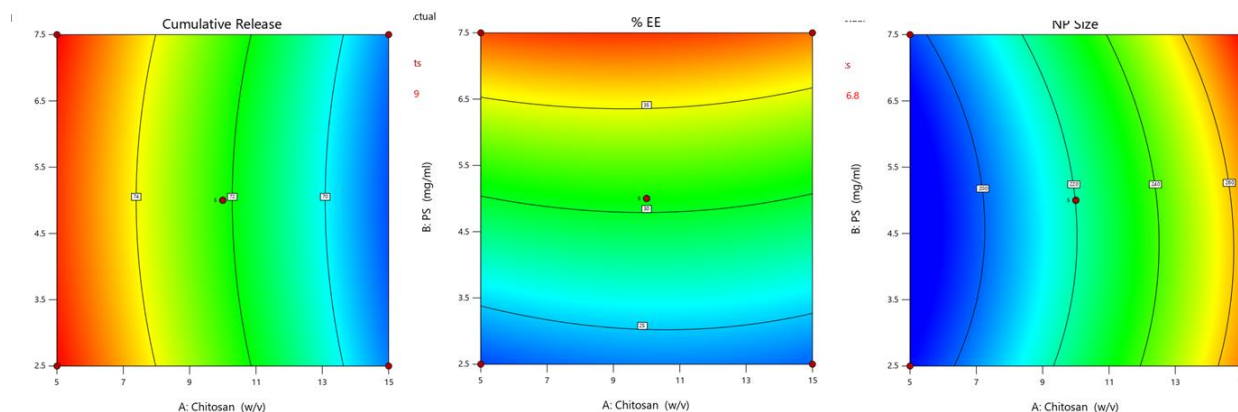
Fig. 4:-Desirability outcomes for chitosan concentration, Phosphatidylserine (PS) and injecting rate
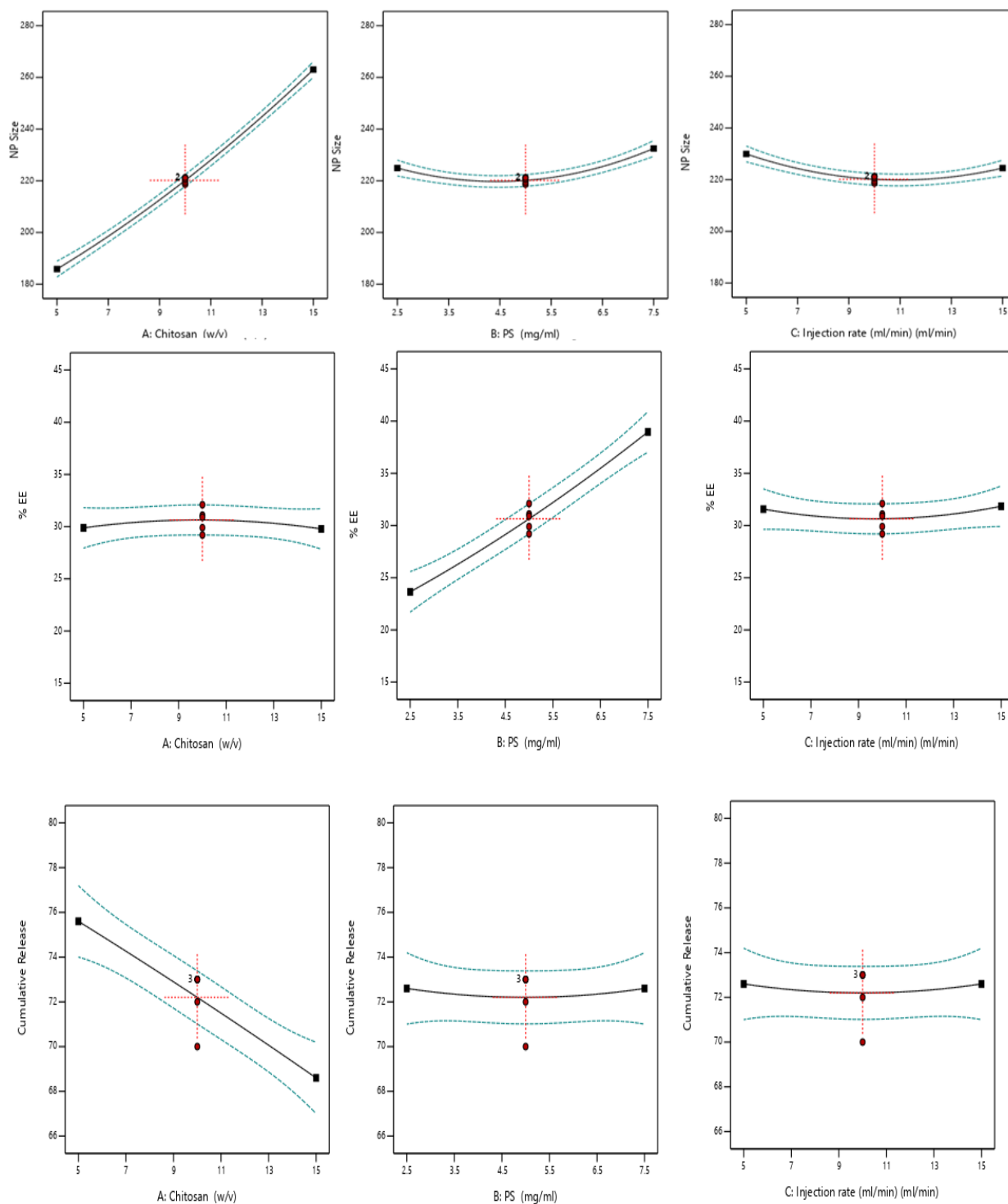
Fig. 5:-Desirability outcomes for chitosan concentration, Phosphatidylserine (PS), and injecting rate
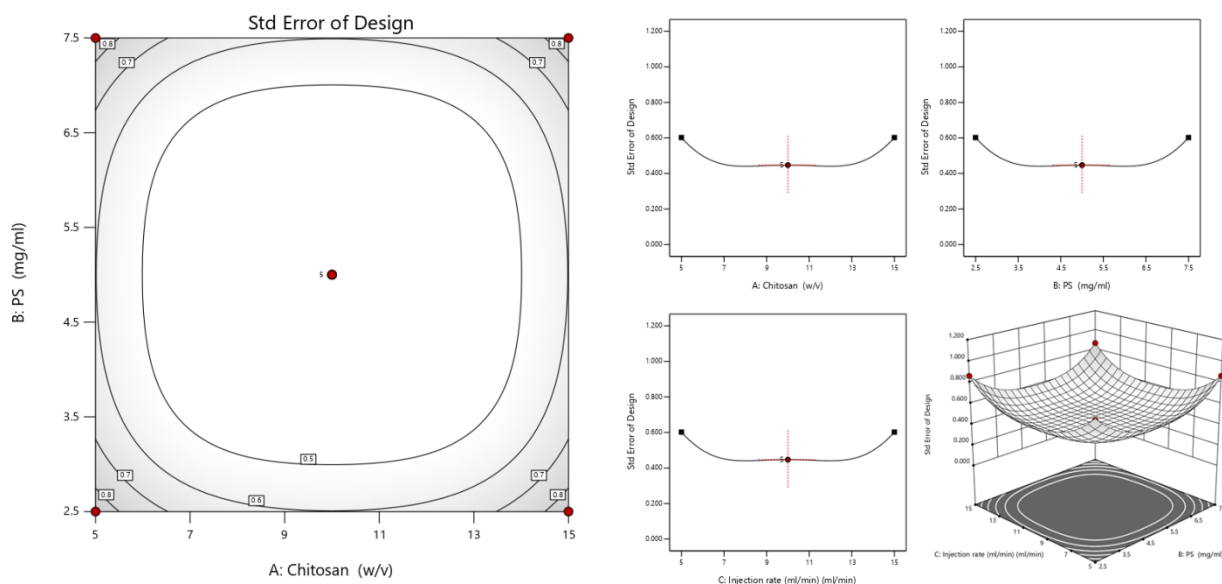
Figure 6:-Representation of Relative error among chitosan concentration, Phosphatidylserine (PS) and injecting rate
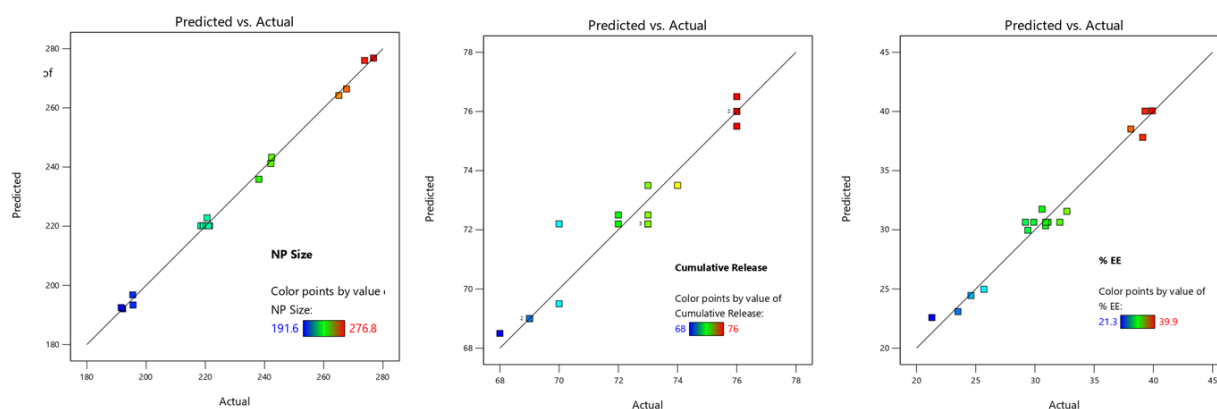

Figure 7:-Predicted and actual responses for optimized formulation

TEM characterization of optimized Lycopene nanocrop:
Fabricated LNP was evaluated for morphological examination. Actual and Predicted values are shown in Table 6, and Figure 7. TEM picture predicts regular, dispersible, and uniformly distributed formulation (Figure 8). Their size measured around 198.37±2.8 nm within the range PDI of 0.519, Zeta potential of 29.8±3.12 mV, sharing an encapsulation efficiency 39.13±1.25%, thru good cumulative release of 73%.

Table 6:-Predicted VS actual results of dependent factors

Dependent Variables	Predicted	Actual
LNP Size (nm)	196.1	198.3
EE (%)	39.9	39.1
Cumulative release (%)	73.8	73.0
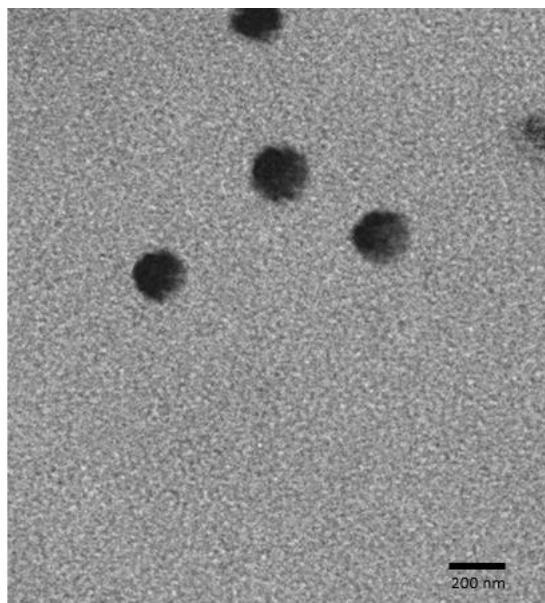
Figure 8:-TEM morphological illustration of optimized Lycopene Nanocrop

Conclusion:-
Self-assembled nanoparticles of lycopene (LNP) were effectively optimized and prepared from chitosan and Phosphatidylserine through Box-Behnken design to gain desired attributes. In the future LNP can be used for safe and effective delivery of the parent compound to the targeted biological system.

Disclosure:-
There are no conflicts of interest related to present research work.

Acknowledgements:-

References:-
1. M. Yusuf, M. Khan, M.A. Robaian, R.A. Khan, Biomechanistic insights into the roles of oxidative stress in generating complex neurological disorders, Biological chemistry, 399 (2018) 305-319.
2. T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins: basic science and product development, Journal of pharmacy and pharmacology, 62 (2010) 1607-1621.
3. S. Kalepu, V. Nekkanti, Insoluble drug delivery strategies: review of recent advances and business prospects, Acta Pharmaceutica Sinica B, 5 (2015) 442-453.
4. M. Yusuf, M. Khan, M.M. Alrobaian, S.A. Alghamdi, M.H. Warsi, S. Sultana, R.A. Khan, Brain targeted Polysorbate-80 coated PLGA thymoquinone nanoparticles for the treatment of Alzheimer's disease, with biomechanistic insights, Journal of Drug Delivery Science and Technology, (2020) 102214.
5. M. Yusuf, M. Khan, R.A. Khan, B. Ahmed, Preparation, characterization, in vivo and biochemical evaluation of brain targeted Piperine solid lipid nanoparticles in an experimentally induced Alzheimer's disease model, Journal of drug targeting, 21 (2013) 300-311.
6. M. Yusuf, M. Khan, R.A. Khan, I.A. Maghrabi, B. Ahmed, Polysorbate-80-coated, polymeric curcumin nanoparticles for in vivo anti-depressant activity across BBB and envisaged biomolecular mechanism of action through a proposed pharmacophore model, Journal of microencapsulation, 33 (2016) 646-655.
7. M. Yusuf, R.A. Khan, M. Khan, B. Ahmed, Plausible antioxidant biomechanics and anticonvulsant pharmacological activity of brain-targeted β-carotene nanoparticles, International journal of nanomedicine, 7 (2012) 4311.
8. M. Kelkel, M. Schumacher, M. Dicato, M. Diederich, Antioxidant and anti-proliferative properties of lycopene, Free radical research, 45 (2011) 925-940.

9. E. Reboul, M. Richelle, E. Perrot, C. Desmoulins-Malezet, V. Pirisi, P. Borel, Bioaccessibility of carotenoids and vitamin E from their main dietary sources, Journal of Agricultural and Food Chemistry, 54 (2006) 8749-8755.

10. K. Gul, A. Tak, A. Singh, P. Singh, B. Yousuf, A.A. Wani, Chemistry, encapsulation, and health benefits of β-carotene-A review, Cogent Food & Agriculture, 1 (2015) 1018696.

11. P.J. Korytko, K.A. Rodvold, J.A. Crowell, M. Stacewicz-Sapuntzakis, V. Diwadkar-Navsariwala, P.E. Bowen, W. Schalch, B.S. Levine, Pharmacokinetics and tissue distribution of orally administered lycopene in male dogs, The Journal of nutrition, 133 (2003) 2788-2792.

12. S. Naahidi, M. Jafari, F. Edalat, K. Raymond, A. Khademhosseini, P. Chen, Biocompatibility of engineered nanoparticles for drug delivery, Journal of controlled release, 166 (2013) 182-194.

13. C. Vauthier, K. Bouchemal, Methods for the preparation and manufacture of polymeric nanoparticles, Pharmaceutical research, 26 (2009) 1025-1058.

14. H.-J. Cho, I.-S. Yoon, H.Y. Yoon, H. Koo, Y.-J. Jin, S.-H. Ko, J.-S. Shim, K. Kim, I.C. Kwon, D.-D. Kim, Polyethylene glycol-conjugated hyaluronic acid-ceramide self-assembled nanoparticles for targeted delivery of doxorubicin, Biomaterials, 33 (2012) 1190-1200.

15. H.S. Yoo, J.E. Lee, H. Chung, I.C. Kwon, S.Y. Jeong, Self-assembled nanoparticles containing hydrophobically modified glycol chitosan for gene delivery, Journal of Controlled Release, 103 (2005) 235-243.

16. T. Kean, M. Thanou, Biodegradation, biodistribution and toxicity of chitosan, Advanced drug delivery reviews, 62 (2010) 3-11.

17. M.J. Glade, K. Smith, Phosphatidylserine and the human brain, Nutrition, 31 (2015) 781-786.

18. P. Liu, Y. Sun, Q. Wang, Y. Sun, H. Li, Y. Duan, Intracellular trafficking and cellular uptake mechanism of mPEG-PLGA-PLL and mPEG-PLGA-PLL-Gal nanoparticles for targeted delivery to hepatomas, Biomaterials, 35 (2014) 760-770.

19. C. Greulich, J. Diendorf, T. Simon, G. Eggeler, M. Epple, M. Köller, Uptake and intracellular distribution of silver nanoparticles in human mesenchymal stem cells, Acta biomaterialia, 7 (2011) 347-354.

20. X.-Y. Lu, D.-C. Wu, Z.-J. Li, G.-Q. Chen, Chpater 7 - Polymer Nanoparticles, in: A. Villaverde (Ed.) Progress in Molecular Biology and Translational Science, Academic Press2011, pp. 299-323.

21. M.A. Alex, A. Chacko, S. Jose, E. Souto, Lopinavir loaded solid lipid nanoparticles (SLN) for intestinal lymphatic targeting, European journal of pharmaceutical sciences, 42 (2011) 11-18.