Formulation, characterization and in-vivo evaluation of curcumin-loaded organically modified (ORMOSIL) nanoparticles for cancer therapy

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Abstract: The potential health benefits of curcumin taken orally are limited due to its poor absorption and bioavailability. Therefore the aim of the present study was to improve the bioavailability and aqueous solubility of curcumin without compromising on its biological activities by preparing the silica nanoparticles doped with curcumin and to study the anticancer potential of the curcumin doped silica nanoparticles. Keeping in view its importance, the curcumin was embedded in the silica nanoparticles prepared by reaction of Tween-20, 1-Butanol, triethoxyvinylsilane and 3-aminopropyltriethoxysilane using water as solvent. After completion of reaction, the nanoparticles were obtained by dialysis of the reaction mixture. The nanoparticles were characterized by SEM , TEM , DLS and XRD analyses. The SEM, TEM and DLS analysis shows the average particle size to be 66 nm, 70 nm and 83 nm respectively, Further in-vivo studies were conducted on wistar rats to determine the maximum tolerance dose (MTD) of nanoparticles and study the anti-cancer potential by tumor regression analysis. The MTD was found to be 10 mg/kg body weight of wistar rats and curcumin-doped ORMOSIL nanoparticles in comparison with pure curcumin revealed the highly significant results in tumor regression in EAT induced tumor model.

Keywords: Silica nanoparticles, curcumin, EAT tumor model, cancer.

1. Introduction

Cancer is one of the most deadly diseases in terms of morbidity and mortality. The present approach to cancer treatment is based on modern medicine which is quite expensive[1]. Moreover, anti-cancer drugs available exhibit side effects and affect the normal function of

genes. Medicinal plants provide an alternative which is safe, effective and affordable. Turmeric (*Curcuma Longa*) has been used for centuries in India and China as traditional herbal remedy and victory spice[2]. Turmeric has been used traditionally for treatment of lives diseases, bacterial infections, skin disorders, cough, asthma etc[3]. The active ingredient of turmeric is curcumin (1E, 6E)-1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6- heptadiene-3,5-dione), a hydrophobic polyphenol derived from the rhizomes of the herb *curcuma longa*, which has well-established anti-inflammatory[4-7] property has shown great promise in prevention of cancer with proven anti-cancer activity[8]. Previous studies have shown the effect of curcumin in inhibiting cancer development and progression[9]. It has been observed that curcumin blocks transformation, inhibition and propagation of tumor. Curcumin has shown to inhibit the proliferation of tumor cells of wide variety. Various human studies[10-13] and animal models[14, 15] have proved that curcumin is safe even at very high doses. However limiting factor for widespread use of curcumin against cancer has been its poor aqueous solubility and bioavailability, which severely limits its clinical utility[10]. The absorption, biodistribution, metabolism and elimination studies of curcumin have confirmed the poor absorption, fast metabolism and elimination as prime reasons for poor bioavailability. The various nanoformulations, liposome's formulations[11], phospholipids complexation, solid-lipid nanoparticles have been synthesized with moderate success in improving the aqueous solubility, providing longer circulation, improved permeability and resistance to metabolic processes. Recent studies have shown that targeted and triggered drug delivery systems in coordination with nanotechnology are emerging as prominent solution to hydrophobic drugs. Curcumin doped Silica Nanoparticles, have lipophilic organic groups and can host active lipophilic molecules in their interior, as well as form electrostatic complex with therapeutic

agents such as genes on their surface. Owing to this fact, they have numerous potential applications in diagnostic imaging, as well as drug, and gene delivery[12,13]. This present study aims to improve the bioavailability and aqueous solubility of curcumin without compromising on its biological activity by preparing the silica nanoparticles doped with curcumin and to study the anticancer potential of the synthesized curcumin doped silica nanoparticles.

2. Materials and methods

2.1 Materials

Curcumin (1E,6E)-1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6- heptadiene-3,5-dione), Tween-20 (Polyoxyethylene (20) sorbitan monolaurate), n-butanol, chloroform, triethoxyvinylsilane (TEVS), 3-aminopropyltriethoxysilane (APTS) Dulbac oís Minimal Essential Medium (DMEM) were obtained from Sigma Chemicals, USA. Water was distilled in distillation assemble in laboratory. Ethylene diamine tetraacetic acid (EDTA), ethanol, were purchased from Sigma-Aldrich (St. Louis, USA). Fetal calf serum was procured from ICN Chemical Co. (CA, USA) and ascorbic acid from S. D. Fine Chemicals (Mumbai). Sterile filtered phosphate buffer saline (PBS: 145mM NaCl, 5mM KCl, 4mM $MgCl_2$, 7.6mM Na $2HPO_4$, 2.4mM NaH_2PO_4, 10mM glucose pH 7.4) was used for washing and incubation of cells and fluorescent measurement. Other chemicals used in this study were of analytical grade.

Polysorbate 20, a derivative of sorbitan monolaurate, is a polysorbate surfactant which is used as a detergent and emulsifier due to its well-established stability and relative non- in a number of domestic, scientific, and pharmacological applications. Polysorbate 20 is used as an excipient in pharmaceutical applications to stabilize emulsions and suspensions.

Cell line

Ehrlich Ascites Tumor (EAT) cells were obtained from National Center for Cell Science, Pune, India. The cell was maintained in rat by intraperitonial inoculation.

Animals

The animals were housed in polyethylene cages in groups of four rats per cage and were kept in room temperature maintained at 25±2°C with a 12-h light/dark cycle. Experiments were performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India after approval from ethical committee from the Jamia Hamdard, New Delhi, India (173/CPCSEA). They were acclimatized for one week before the start of the study and were allowed free access to standard laboratory feed (Hindustan Lever Ltd, India) and water ad labium. Healthy Wistar rats (55 animals) of either sex weighing between 250-300 g were used for the present study. The rats were divided into 11 groups of 5 rats each.

2.2 Methods

2.2.1 Synthesis of organically-modified silica (ORMOSIL) nanoparticles

The curcumin doped-ORMOSIL nanoparticles and void nanoparticles were synthesized by procedure given by Dinda et al.,[16] with minor modifications of replacing Tween 80 with Tween 20. Typically 0.44gm Tween 20 and 800µl 1-Butanol were mixed by vortexing at room temperature, which was added dropwise to 20ml of distilled water. The solution was stirred for 2.30 hrs, after which the solution of 5µl (?) of curcumin in chloroform (10mg/ml) was added to it. After stirring this solution for 1hr, 200µl triethoxyvinyl silane was added and the solution was further stirred for 1 hr. 10µl 3-aminopropyltriethoxysilane was added to this solution and

the solution was left to stir for 48 hrs at room temperature. After completion of reaction, the nanoparticles were obtained by dialysis of the reaction mixture.

2.2.2 Analysis of Curcumin incorporation

A high performance liquid chromatography (HPLC) assay was used to determine the Curcumin in the nanoparticles. A Waters HPLC system having four pumps, an autosampler and a PDA detector with Empower software were used for analysis. The mobile phase consisted of Acetonitrile: Tetrahydrofuran: 2% Acetic acid in the ratio 50: 30: 20 v/v. Mobile phase was used as diluent. The HPLC column used was inertsil ODS 3V, 250 x 4.6mm, 5µ. Column temperatue was kept at 25˚C and autosampler was operated at 10˚C. Curcumin elution was monitored at 425nm.

2.2.3 Characterization of nanoparticles

Dynamic light scattering (DLS) and Zeta potentialParticle sizes were determined using dynamic light scattering (DLS) with help of Malvern zeta sizer for curcumin doped-ORMOSIL nanoparticles and void ORMOSIL nanoparticles. For the experiment, 5 ml of the each synthesized nanoparticles solution were prepared in ddH_2O. The particle sizes were determined from the autocorrelation function using the Stokes–Einstein equation:

r ¼ kT/ D6pZ,

where r is the particle radius, k is the Boltzmann constant, T is the absolute temperature, D is the diffusion coefficient, and Z is the viscosity of the liquid in which the particles are suspended .

Transmission electron microscopy (TEM): The curcumin doped-ORMOSIL nanoparticles and void ORMOSIL nanoparticles were suspended in ddH_2O and one drop of sample was put onto a formvar carbon coated grid (Ted Pella, Inc.) and allowed to dry for 5 min. The grid was then

stained with 2% uranyl acetate, rinsed in 95% ethanol and allowed to air dry for 10 min. Images were taken using a JEOL 1011 transmission electron microscope (TEM) to an accelerating voltage of 80 kV.

Scanning electron microscopy (SEM): The shape of the ORMOSIL nanoparticles was observed by Hitachi scanning electron microscope (Model No. S-4700)

X-ray diffraction of the nanoparticles (XRD): The curcumin doped-ORMOSIL nanoparticles and void ORMOSIL nanoparticles developed were determined using X-ray diffraction (XRD). The nanoparticles were determined using a Philips X-ray diffractometer (X'pert Pro). This diffractometer, with an X'celerator detector (Philips), used Cu-Ka radiation (l ¼ 1.5418A˚) and was operated at 45 kV and 40 mA.

2.2.4 In-vivo anti-cancer activity

Maximum Tolerance Dose Determination: Maximum tolerated dose is defined as the highest dose of the chemical or drug that can be administered to an animal without causing toxicity or decreasing survival[17]. MTD for ORMOSIL nanoparticles administered intravenously was investigated in healthy wistar rats of either sex. Thirty wistar rats were divided into six groups of five rats each. The rats were administered i.v. with 0mg/kg only saline (void), 0.01mg/kg, 0.1mg/kg, 1mg/kg, 10mg/Kg and 100mg/kg of the curcumin doped ORMOSIL nanoparticles for three days respectively in the serial dilution of factor of 10 and injection volume was kept 200 µl when administered through intra venous route. The treated rats were observed for 10 days and percentage loss of body weight was recorded.

Tumor Regression analysis: Ehrlich Ascitic Tumor (EAT) model was developed according Valadares[18] et al. 2004 with changes, by injecting 4-6 weeks old wistar rats (25) subcutaneously with 0.1ml suspension containing 9 X 10^6 EAT cell lines on right flank above

forelimb. Experiments to study the tumor regression were done after tumor volume reached 0.7-1.4 cm^3 (app 7-10 days). To study the therapeutic effect of curcumin against cancer twenty five wistar rats were divided in five groups: (i) Group GI: Untreated/Control or no tumor induced; (ii) Group GII: Treated or tumor induced and this group is further divided into 4 subgroups; Group GIIa) 0.1ml suspension containing 9 X 10^6 cells given subcutaneously (s. c.) in right flank; Group GIIb): pure curcumin at the concentration 10 mg/kg body weight was administered. Group GIIc): was injected with ORMOSIL nanoparticles without curcumin (void) at concentration 10 mg/kg body weight. Group GIId: was injected with curcumin-doped ORMOSIL nanoparticles at MTD concentration (10mg/kg body weight) and the size of the tumor was observed at day 0 (first day of the treatment), day 10, day 12 and day 20 using Vernier caliper. Tumor volume was being calculated using the formula[19]

$$\text{Tumor volume } (mm^3) = (W^2 x L)/2$$

Where, W = tumor volume at the widest point

L = tumor dimension at the longest point.

Tumor % inhibition response was calculated using formula

Change in response = Day 0 – Day 20 /Day 0 X 100

2.2.5 Statistical Analysis

All the assays were done in triplicates and results were expressed as mean ± standard deviation of three measurements. Statistical analysis was executed using one way ANOVA by PRISM software and $P<0.05$ were considered significant.

3. Results and Discussion

3.1 Optimization of concentration of reagents for formulation of curcumin doped ORMOSIL nanoparticles

In the synthesis of curcumin doped ORMOSIL nanoparticles, curcumin as hydrophobic drug is dissolved in chloroform. The mixture of solvent and drug solution is then emulsified in an aqueous solution containing surfactant Tween 20 and Co-Surfactant n-butanol, acts as emulsifying agent, to form oil in water (o/w) emulsion. After the formation of stable emulsion, the chloroform is evaporated by continuous stirring. This continuous stirring is responsible for the formation of small particles. Triethoxyvinylsilane provides the effective surface modification which also makes them applicable to number of analysis such as adsorption of blood cells.[8]

Upon increasing the concentration of n-butanol, it was observed that the size of nanoparticles increased from 83.2nm to 94.13nm, whereas the decrease in the n-butanol concentration resulted in the decrease in size of nanoparticles to 58.66nm (Table 1). The concentration of TEVA used in the original protocol was found to be optimum as both increase and decrease in concentration resulted in increase in particle size (Table 1). ATPS is highly basic in nature and even a small change in concentration resulted in a very remarkable change in the pH of the reaction mass. An increase in the concentration resulted in increase in pH to about 10.2, resulting in degradation of curcumin, whereas the decrease in concentration resulted in increase in particle size. Hence it can be concluded that the concentration of APTS prescribed in the original protocol is optimum.

S.No.	Reagent	% Concentration of reagent altered	Particle Size (DLS,nm)
1	As per protocol	None	87.2
2	n-Butanol-1	-10%	58.66
3	n-Butanol-2	+10%	94.13

4	TEVS-1	-10%	136.7
5	TEVS-2	+10%	131.2
6	APTS-1	-10%	301.6
7	APTS-2	+10%	pH increases, curcumin degrades.

Table-1: Optimization of concentration of reagents for best particle size

3.2 DLS analysis of curcumin-doped ORMOSIL nanoparticles

Particle size of aqueous dispersed curcumin-doped ORMOSIL nanoparticles were measured in the nm sizes range a Malvern zeta-sizer, which revealed the z-average size 87.2 nm (Fig. 1).

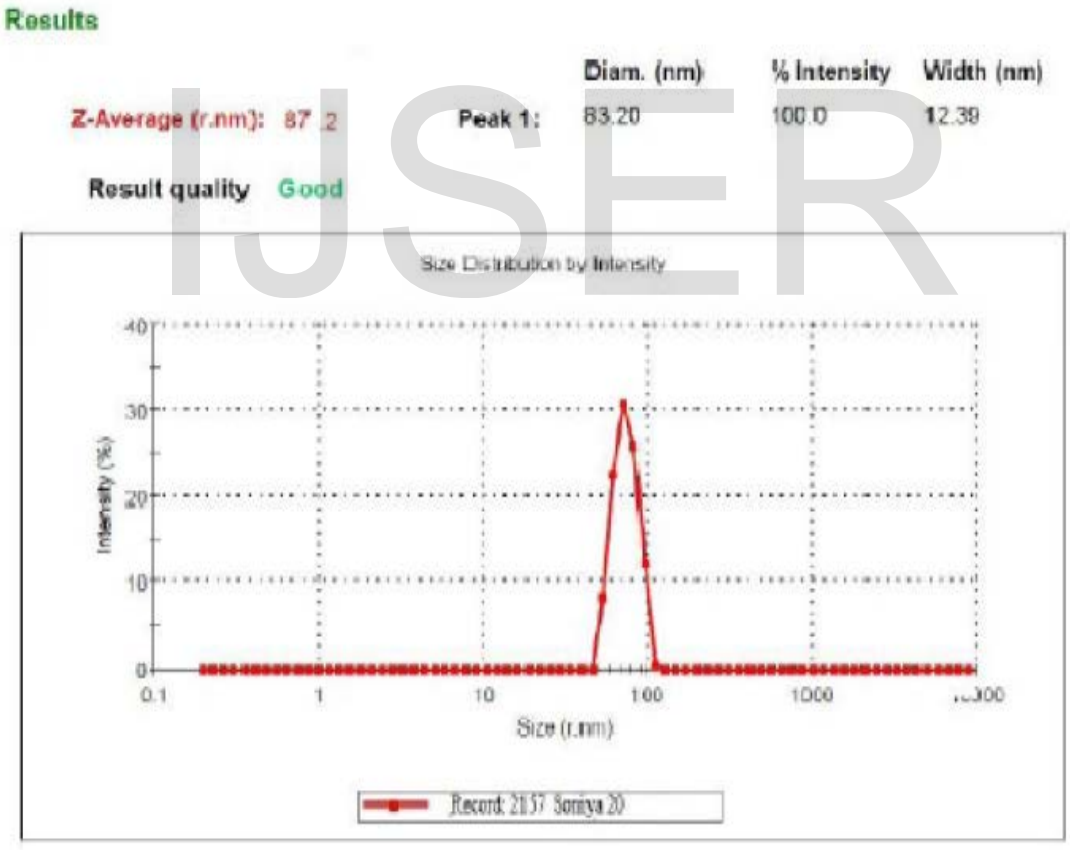

Fig. 1: Average size of curcumin doped ORMOSIL nanoparticles by DLS

3.3 TEM Analysis of curcumin-doped ORMOSIL nanoparticles

The nanoparticles were prepared in the form of reverse micelles. The Transition Electron Microscope (TEM) microphotograph of curcumin-doped ORMOSIL nanoparticles revealed that the particles were appeared to be symmetrically spherical in shape with an average particle size of 70nm (Fig. 2).

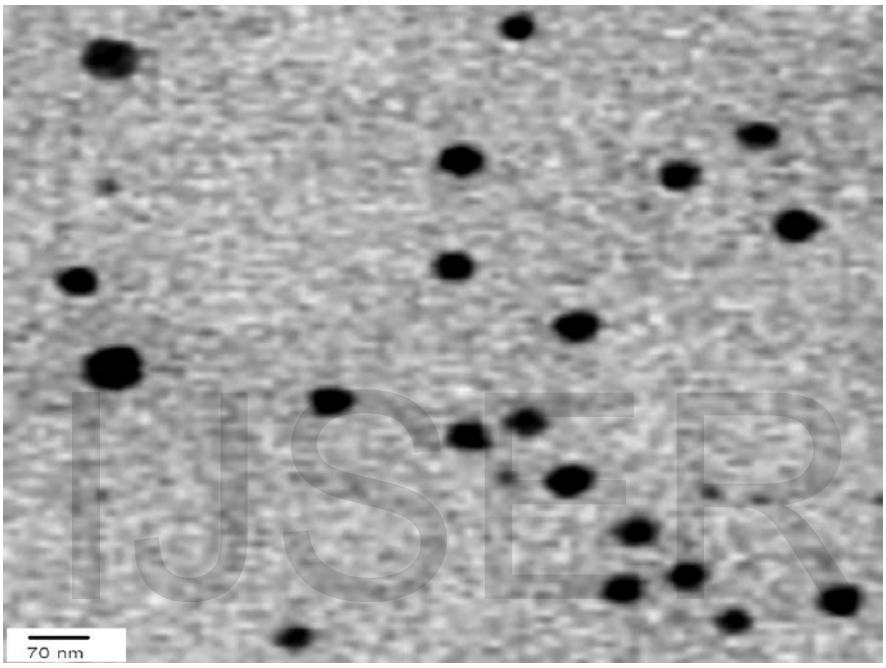

Fig. 2: Size determination of curcumin-doped silica nanoparticles by TEM

3.4 SEM analysis of curcumin-doped ORMOSIL nanoparticles

Surface morphology of curcumin-doped ORMOSIL nanoparticles is shown in SEM microphotograph, revealed the spherical shape and 68 nm size of nanoparticles (Fig 3).

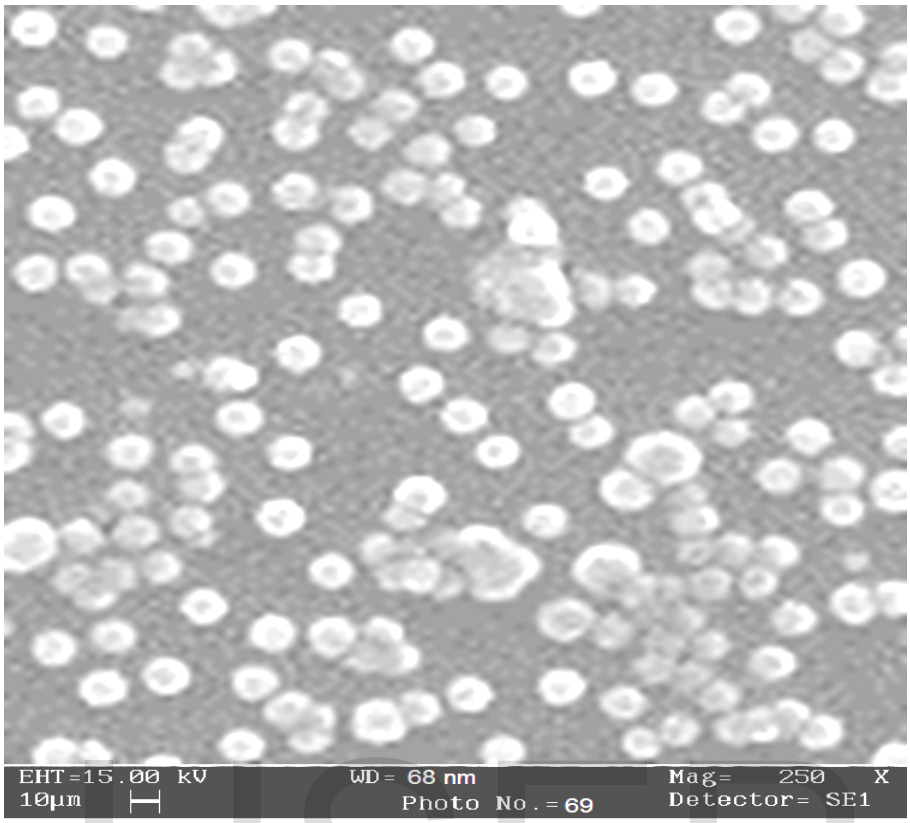

Fig. 3: Size determination of curcumin-doped silica nanoparticles by SEM

3.5 X-Ray diffraction (XRD) study of curcumin-doped silica nanoparticles

X-Ray diffraction was carried out to determine the crystalline characteristics of curcumin-doped silica nanoparticles. Fig. 4a showed the diffractograph of pure curcumin that exhibit the crystalline nature of powder form at 2º theta angel while curcumin-doped silica nanoparticles were examined by XRD, Fig 4b exhibited the amorphous nature of the formulated nanoparticles at 2° theta. The XRD of curcumin doped-ORMOSIL nanoparticles showed different pattern of peaks from that of pure curcumin, with no characteristic pure curcumin peaks observed in case of XRD of curcumin doped-ORMOSIL nanoparticles, indicating the absence of free curcumin in ORMOSIL nanoparticles.

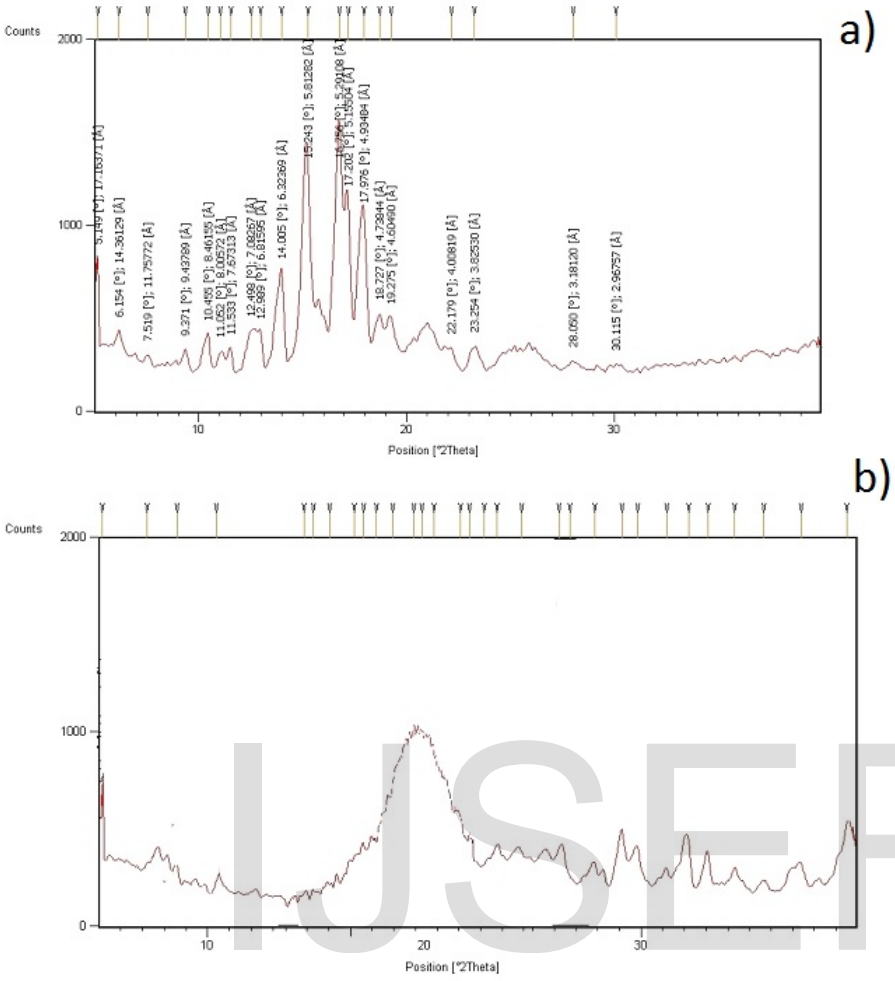

Fig.4a and 4b: 4a showed XRD diffractogram for pure curcumin; 4b showed XRD diffractogram for curcumin-doped ORMOSIL nanoparticles.

3.2 In-vivo anticancer activity

3.2.1 Maximum tolerance dose determination

The MTD studies for curcumin loaded silica nanoparticles were carried out in healthy wistar rats. In Group I loss in weight was observed while rats were measured weight loss was observed in silica nanoparticles treated animals. The loss in weight was concentration dependent with minimum loss at dose of 0.01mg/ml. In group treated with 100mgkg, mortality

was recorded on day 2. The animals treated with 10mg/kg showed no mortality. No apathy was seen in the formulations. As per the defination of MTD 10mg/kg was considered MTD for curcumin-doped silica nanoparticles. Finally group 4 which received the dose of 10 mg/kg of body weight did not show mortality till the 20th day but exhibit the 18 % body weight loss (Table 2). As per the defination of MTD 10mg/kg was considered MTD for curcumin-doped silica nanoparticles.

Body weight loss (%)						
Groups	**Dose**	Day 2	Day 4	Day 6	Day 8	Day 10
G1		0	0	0	0	0
G2	0.01 mg/kg BW	5.4 ± 0.8	5.6 ± 0.6	5.9 ± 0.8	6.4 ± 0.9	6.8 ± 0.4
G3	0.1 mg/kg BW	7.5 ± 1.2	8.4 ± 1	9 ± 0.9	9.2 ± 0.7	9.5 ± 1.1
G4	1 mg/kg BW	9.6 ± 1.1	10 ± 1.2	12 ± 1.5	12.5 ± 1.2	13.4 ± 2
G5	10 mg/kg BW	10.5 ± 0.9	12.2 ± 1.3	12.6 ± 1.1	14.3 ± 2	18 ±2.2
G6	100 mg/kg BW	18.25 ± 1.8	D	-	-	-

D= Death of rat. n = 5, values are expressed as Mean ± SD.

Table 2: Maximum Tolerance Dose (MTD) of curcumin-doped ORMOSIL nanoparticles

3.2.2 Tumor Regression analysis:

The maximum tolerance dose of curcumin-doped ORMOSIL nanoparticles selected was administered by Intratumoral injection which showed tumor regression from day 10 itself. The study was continued till 20 days as planned and it was observed that when doses at MTD were administered to the tumor bearing rats, marked reduction in the tumor volume was observed in comparison with pure curcumin and void ORMOSIL nanoparticles (Table 3).

Tumor regression analysis in relative tumor volume for pure curcumin, void ORMOSIL nanoparticles and curcumin-doped ORMOSIL nanoparticles were observed 0.57, 0.93 and 0.37 cm^3 at day 20 when started from the tumor size 0.785, 1.04 and 0.841 cm^3 respectively (Table 3). Total percentage change showed that void nanoparticles produced 10.5 % tumor inhibition, pure curcumin produced 27.3 % tumor inhibition while curcumin loaded silica nanoparticles produced 56% tumor inhibition (Table 3). The curcumin-doped ORMOSIL nanoparticles were much more effective than pure curcumin while void ORMOSIL nanoparticles did not exhibit any significant change.

Groups	**Relative tumor volume (cm^3)**				**Difference between tumor volume (cm^3)**		**% Change in tumor**
	Day 0	**Day 10**	**Day 12**	**Day 20**	**Day 0**	**Day 20**	
GI (Control)	0	0	0	0	0	0	-
GIIa (Tumor model)	0.956 ± 0.2*	1.1 ± 0.1*	1.41 ± 0.3*	1.72 ± 0.2**	0.956± 0.2*	1.72 ± 0.2**	-
GIIb (Curcumin)	0.785 ± 0.2	0.71 ± 0.04!	0.58 ± 0.03*	0.57 ± 0.02*	0.785 ± 0.2!	0.57 ± 0.02*	27.3
GIIc (Tween 20 void silica nanoparticles)	1.04 ± 0.2!	1.08 ± 0.1!	0.95 ± 0.2!	0.93 ± 0.09!	1.04 ± 0.2!	0.93 ± 0.09!	10.5
GIId (Tween 20 silica nanoparticles with curcumin)	0.841 ± 0.1	0.72 ± 0.08*	0.48 ± 0.05*	0.37 ± 0.04*	0.841 ± 0.1	0.37 ± 0.04*	56

Results are expressed in terms of mean ±SEM; n = 5 values are statistically significant at *$p < 0.05$; !denoted as not significant.

Table 3: Tumor Regression Analysis

4 Conclusion

In this study, curcumin-loaded ORMOSIL nanoparticles were prepared and the effect of concentration of various reactants was studied. Our study showed that round, spherical curcumin-doped ORMOSIL nanoparticles were prepared. The DLS, TEM and SEM analysis reveal that the size of nanoparticles are 87.2, 70.0 and 68.0 nm respectively. The in-vivo studies were conducted in two parts: first to determine the maximum tolerance dose and second was to determine the tumor regression analysis. The maximum tolerance dose was determined to be 10mg/kg of the body weight of the rat as no mortality was observed at this dose. This was the concentration given to the male wistar rats during tumor regression analysis and the regression of the tumor in the rats given curcumin-doped ORMOSIL nanoparticles was observed to be about 56% whereas the tumor regression in the rats treated with pure curcumin was observed to be about 27%. Hence this can be concluded that the effectiveness of curcumin against tumor has increased approximately two times when it is formulated in the form of ORMOSIL nanoparticles, thus making it a potential candidate for cancer therapy.

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Disclosure statement

The Author(s) declare(s) that they have no conflict of interest to disclose.

References

1. Yadav D, Anwar MF, Garg V, Kardam H, Beg MN, Suri S, Gaur S, Asif M. Development of polymeric nanopaclitaxel, its cell proliferation comparison with free paclitaxel on carcinoma cells (MCF-7; B16F0). As Pac J Can Prev 2014; 15(5):2335-2340.
2. Sharma, O.P. (1976) "Antioxidant activity of curcumin and related compounds" Biochem. Pharmacol. , 25 (15), 1811-1812.
3. Shishodia S, Sethi G, Aggarwal BB (2005)"Curcumin: Getting back to the roots".Ann.N.Y.Acad.Sci.1056:206-217.doi: 10.1196/annals.1352.010.
4. Ruby, A.J, Kuttan G, Babu K.D, Rajasekharan K.N, Kuttan R (1995)" Antitumour and anti-oxidant activities of natural curcuminoids". Cancer Lett., 94(1), 79-83.
5. Sugiyama Y, Kawakishi S, Osawa T (1996) "Involvement of β-diketone moiety in the antioxidative mechanism of tetrahydrocurcumin". Biochem. Pharmacol., 52 (4), 519-25.
6. Srimal R.C, Dhawan B.N (1973) "Pharmacology of diferuloyl methane (curcumin), a non-steroidal anti-inflammatory agent". J.Pharm. Pharmacol., 25 (6), 447-52.
7. Aggarwal BB, Sundaram C, Malani N, Ichikawa H (2007)."Curcumin: The Indian solid gold". *Adv Exp.Med.Biol*.595:1-75.doi:10.1007/978-0-387-46401-5_1.PMID 17569205.
8. Aggarwal BB, Kumar A, Bharti A.C, 2003."Anticancer potential of Curcumin: preclinical and clinical studies". Anticancer research.23:363-398.
9. Cheng A.L, Hsu C.H, Lin J.K, et al, 2001." Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions". Anticancer research.21:2895-2900.

10. Lao D.C, Demierre M.F, Sondak V.K (2006) “Targeting evnts in melanoma carcogenesis for the prevention of melanoma”. Expert rev. Anticancer Ther. 6 (11), 1559-68.
11. Lao D.C, Ruffin M.T, Normolle D, Heath D.D, Murray S.I, Bailey J.M, Boggs M.E, Crowell J, Rock C.L, Brenner d.E (2006) “ Dose escalation of a curcumonoid formulation”. BMC Complement Altern. Med., 6,10.
12. Cheng A.L, Hsu C.H, Lin J.K, Hsu M.M, Ho Y.F, Shen T.S, Ko J.Y, Lin J.T, lin B.R, Ming-Shiang W, Yu H.S, Jee S.H, Che G.S, Chen T.M, Chen C.A, Lai M.K, Pu Y.S, Pan M.H, Wang Y.J, Tsai C.C, Hsieh C.Y (2001) “ Phase I clinical trials of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions”. Anticancer Res., 21 (4B), 2895-500.
13. Shoba G, Joy D, Joseph T, Majeed M,Rajedra R, Srinivas P.S (1998) “ Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers”. Planta Med, 64 (4), 353-6.
14. Shanker T.N, Shantha N.V, Ramesh H.P, Murthy I.A, murthy V.S (1980) “Toxicity studies on turmeric(Curcuma longa): acute toxicity studies in rats, guinepigs and monkeys”. Indian j. Exp. Biol. 18 (1), 73-5.
15. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB, (2007).” Bioavailability of curcumin: Problems and promises”. Molecular pharmaceutics, Vol.4, NO.6, 807-818.
16. Amit K. Dinda; Chandravilas K. Prashant; Saba Naqvi; J. Unnithan; Mohd. Samim; Amarnath Maitra “Curcumin loaded organically modified silica (ORMOSIL) nanoparticle; a novel agent for cancer therapy” Int. J. of Nanotechnology, 2012 Vol.9, No.10/11/12, pp.862 – 871.

17. Yadav D, Anwar MF, Garg V, Kardam H, Beg MN, Suri S, Gaur S, Asif M. Development of polymeric nanopaclitaxel, its cell proliferation comparison with free paclitaxel on carcinoma cells (MCF-7; B16F0). As Pac J Can Prev 2014; 15(5):2335-2340.
18. Valadares MC, Klein SI, Queiroz ML. Titanocene modulation of cytokine imbalance induced by Ehrlich ascites tumour rogression. Eur J Pharmacol 2004; 503:203–208.
19. Vos T, Caracoti A, Che J, Dai M, Farrer C, Forsyth N, et al. Identification of 2-{2-(2-(5-bromo-2-methoxyphenyl)-ethyl)-3-fluorophenyl}-4, 5-dihydro-1H-imidazole (ML00253764), a small molecule melanocortin 4 receptor antagonist that effectively reduces tumor-induced weight loss in a mouse model. J Med Chem. 2004; 47:1602–4.