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FABRICATION, CHARACTERIZATION AND PHARMACOLOGICAL ACTIVITY OF USNIC ACID LOADED NANOPARTICLES

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ABSTRACT: Usnic acid is lichen metabolite. Lichen species are the source of usnic acid. It has Antitumor and Anti-proliferation activity. Usnic acid is poorly water soluble drug. So its dissolution and bioavailability is poor. To enhance dissolution and bioavailability of usnic acid nanotechnology has been adopted. So in this study usnic acid powder was formulated as a nanoparticle with the help of PLGA polymer. Nano-precipitation with sonication method was adopted to formulate usnic acid loaded nanoparticles. Obtained nanoparticles were undergone to characterization and evaluation studies. SEM study revealed that particles are in the range of nanometer level and it is in spherical shape. FTIR and DSC studies have shown that no interaction between drug and polymer. *In vitro* drug release was maximum for optimized formulation. Optimized formulation also exhibited more antitumor activity than free usnic acid. Kinetic study revealed that the drug release is zero order. It concludes nano-precipitation with sonication is effective method for preparation of usnic acid loaded nanoparticles.

INTRODUCTION: Usnic acid (2, 6 - diacetyl -7, 9-dihydroxy-8, 9h-dimethyl-1, 3(2H, 9Bh)-dibenzofurandione) is a lichen metabolite ^{1,2}. Usnic acid is a yellow pigment and occurs in two enantiomeric form is found in lichens ^{3, 4, 5}. Most of literature survey has shown usnic acid exhibit antitumour and anti-proliferation activity. Kumar and muller has found out usnic acid has cytotoxic activity against human keratinocyte cell culture ⁶ and lot of researches have been carried out study on usnic acid to reveal the cytotoxic activity ⁷.

In addition to the antitumour activity, usnic acid has been shown to exhibit antiviral, antiprotozoal, antimicrobial, anti-inflammatory and analgesic activities ^{8, 9, 10}. Since usnic acid has hydrophobic nature it has poor dissolution. Nanoparticles are good platform to increase solubility and bioavailability of hydrophobic drug ¹¹. So nanotechnology was adopted to bring up the good dissolution and better bi-availability.

Nanoparticles are referred as nanosized colloidal particle ranging in size from 10 - 1000 nm in which drug is encapsulated by polymers. These small size nanoparticles can be easily absorbed into RES and other system of body ¹². Among various types of nanomaterials, polymeric nanoparticles have the advantages of relatively high biocompatibility, stability, and flexibility of conjugating ligands on their surface for targeted drug delivery ^{13, 14}.

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The objective of this current project was to develop the usnic acid loaded nanoparticles with PLGA polymer and evaluate physico chemical and pharmacological activities.

MATERIALS AND METHODS:

Materials: Usnic acid was procured from sigma Aldrich. Tween 80, Poly Ethylene Glycol, Chloroform, Acetone were purchased S. D. fine Chem Ltd., Mumbai. Solvents Methanol (HPLC) grade, HPLC water (lichrosolv) were purchased from Merck, India. Other reagents were purchased from local supplier.

Method of Preparation: All batches of formulations were prepared by nano precipitation method by sonication method^{15, 16}. The drug and polymer as mentioned in the **Table 1** were dissolved in acetone (6 ml) and chloroform (4ml) respectively 0.4 ml of Tween 80 was added into drug solution and then organic phases were mixed by using vortex mixer. The organic phase was added dropwise (0.5ml/min) into aqueous phase (10 ml phosphate buffer of pH 7.4) containing 0.06 g PEG. Then it was sonicated using probe sonicator and the organic phase was removed by evaporation under reduced pressure.

TABLE 1: COMPOSITIONS OF USNIC ACID LOADED NANOPARTICLES

S. no.	Batch code	Amount of Drug in mg	Amount of Polymer in mg	Drug : Polymer
1	G ₁	40	2	1:0.05
2	G ₂	40	4	1:0.1
3	G ₃	40	8	1:0.2
4	G ₁ S	40	2	1:0.05
5	G ₂ S	40	4	1:0.1
6	G ₃ S	40	8	1:0.2

G –formulations not consist of surfactant, GS – formulations consist of 0.4ml Tween 80

Characterization and Evaluation Studies:

Morphology: The usnic acid loaded nanoparticles were subjected to scanning Electron Microscope (SEM) for determining its size and shape. The nanoparticle size and shape were to be characterized and photographed¹⁷.

FTIR Study: The spectra of the samples were recorded using an IR spectrophotometer. KBr pellets were prepared by gently mixing sample with KBr. Fourier transform infrared spectrum were recorded in the range of 400 - 4000 cm⁻¹.

Differential Scanning Calorimetry (DSC)

Analysis: DSC experiments were carried out to determine the possible interactions between drug and polymers. Therefore in the present study DSC analysis were carried out on samples of pure drug and formulated nanoparticles^{18, 19}. Weighed quantity of pure drug and formulation were taken in aluminum pans. The pans were sealed with aluminum caps. The samples were heated at 20 °C/min up to 300 °C. The location of the thermal transition and surface area of the peaks were obtained from the thermo grams.

Entrapment Efficiency:^{20, 21} The nanosuspension with known amount of drug incorporated was centrifuged at 15,000 rpm for 15 minutes. The

supernatant was separated and 1ml of supernatant was suitably diluted with phosphate buffer (pH 7.4) and the absorbance was measured spectrophotometrically at 290 nm. The drug entrapment efficiency was calculated by the following equation.

$$\text{Entrapment Efficiency} = \frac{\text{Amount of drug added} - \text{Amount of free drug in supernatant liquid}}{\text{Amount of drug added}} \times 100$$

In vitro Drug Release Studies:

By UV Spectrophotometric Method:²² The *in vitro* drug release studies were performed using the dialysis bag diffusion technique. The release studies of usnic acid from nanoparticles were performed in phosphate buffer (pH 7.4). Total quantity of prepared formulations were placed in a cellulose dialysis bag and tied at both ends. The dialysis bag was immersed in the receptor compartment containing 50 ml of phosphate buffer (pH 7.4) at 37 °C under magnetic stirring. An aliquot of receptor media (1ml) was withdrawn at regular intervals upto 24hr and the same volume was replaced by fresh dissolution medium (phosphate buffer pH 7.4) and analyzed spectrophotometrically at 290 nm. The percentage of drug released at various time intervals was calculated from calibration curve.

In vitro Drug Release for the Optimized Formulation (G₂S) by HPLC Method:²³

Procedure: The total quantity of optimized formulation was placed in the dialysis bag and bag immersed in the receptor compartment containing 50 ml phosphate buffer (pH 7.4) at 37 °C under magnetic stirring. The samples were withdrawn at the time intervals of 6, 12, 18 and 24 hrs with replacement of equal volume of dissolution medium. The withdrawn samples were filtered using sartorius membrane filter (0.45µ).

Sample Analysis: The filtered samples were suitably diluted and analyzed by HPLC method using Kromosil C8 column (250 x 4.6 mm internal diameter 5µm particle size) and a mobile phase composed of methanol / 20 mM phosphate buffer solution pH 7.4 (70:30 v/v). Sample aliquots (20µl) were injected and eluted with the mobile phase at a flow rate of 1ml/min. The usnic acid peak was verified at a wavelength of 280 nm. The amount of drug released at various time intervals was determined from the standard calibration curve.

Cytotoxic Evaluation of Usnic Acid and Usnic Acid Loaded Nanoparticles: In this present study usnic acid isolated from lichens and usnic acid loaded nanoparticles were evaluated for their cytotoxic activity^{24, 25}.

Induction of Cancer using DAL: Dalton's Ascites Lymphoma (DAL) was supplied by Amla cancer research center, Trissur, Kerala, India. The cells were maintained *in vivo* in swiss albino mice by intraperitoneal transplantation. While transforming the tumour cells to the grouped animal the DAL cells were aspirate from peritoneal cavity of the mice using saline.

The cell count was done and further dilutions were made, So that total cells should be 1x 10⁶ cells/ml/mouse. This volume was given intraperitoneally and the tumour was allowed to grow in the mice for minimum of seven days before starting the study.

Treatment Protocol: Swiss albino mice were divided into 5 group of six each. The animal belonging to groups 2 - 5 were injected with DLA cells (1 x 10⁶ cells/ml/mouse). Intraperitoneally, While the remaining group 1 served as the normal

control group. After the inoculation, the groups were treated with compounds as given below.

Evaluation of Clinical Parameter:

Cancer Cell Count: The fluid (0.1 ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8 ml of ice cold normal saline or sterile Phosphate Buffer Solution (PBS) and 0.1 ml of trypan blue (0.1 mg/ml) and total number of the living cells were counted using heamocytometer.

Derived Parameters:

Body Weight: All the mice were weighed, from the day 1 to day 15 of the study. Average increase in body weight on the 15th day was determined.

Percentage Increase in Life Span (ILS): % ILS was calculated by the following formula:

$$\% \text{ ILS} = \frac{\text{Life span of the treated group}}{\text{Life span of normal control group}} - 1 \times 100$$

RESULT AND DISCUSSION:

Preparation of Nanoparticles: Usnic acid loaded nanoparticles were prepared by nano-precipitation method with sonication technique using biodegradable polymers namely PLGA. This method was based on the interfacial deposition of polymer followed by diffusion of a semi-polar and miscible solvent in the aqueous medium in the presence of surfactant. Usnic acid is hydrophobic drug and for this type of drug nanoprecipitation method is ideal method for preparing nanoparticles^{26, 27}.

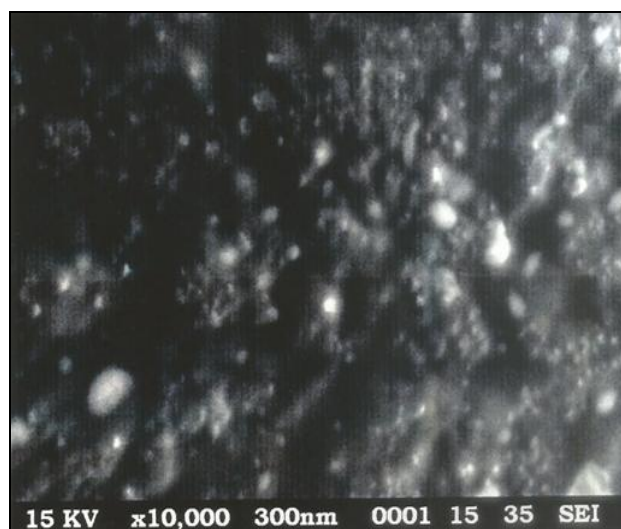


FIG. 1: SEM PHOTOGRAPH OF USNIC ACID LOADED NANOPARTICLES

Evaluation of Prepared Nanoparticles:

Morphology: The morphology of nanoparticles was examined by SEM. SEM images revealed that all the nanoparticles prepared displayed good morphology with spherical structure and smooth surface. Average size of optimized nanoparticle range is 200 nm to 300 nm respectively²⁸.

Drug - Polymer Interaction Study: FTIR spectrum of drug - PLGA optimized formulation (Fig. 2)

showed peaks such as C-O stretching (1190.79 cm^{-1}), C = O stretching (1631.6979 cm^{-1} , 1692.35 cm^{-1}) and C-H stretching 2929.58 cm^{-1} . The absorption bands observed for the formulation of drug and polymer indicated were closed to the absorption bands of pure Usnic acid (Fig. 2). Therefore the interaction between usnic acid and polymer was negligible.

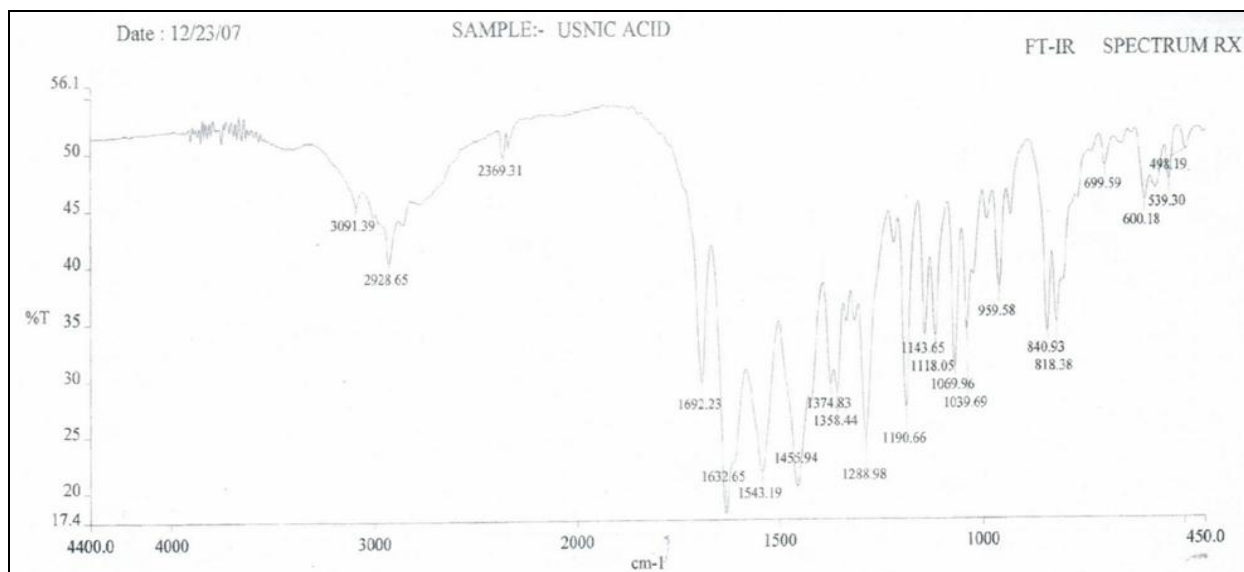


FIG. 2: FTIR SPECTRA OF USNIC ACID

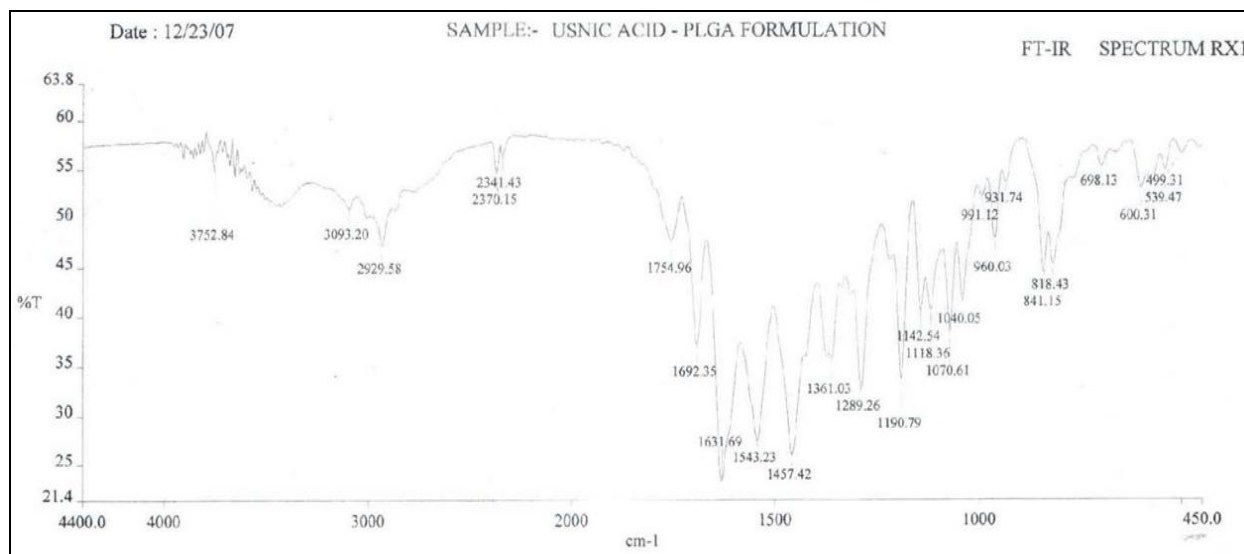


FIG. 3: FTIR SPECTRA OF USNIC ACID AND PLGA NANOPARTICLES

DSC Analysis: DSC thermo grams of free usnic acid, PLGA and usnic acid loaded nanoparticles were depicted in Fig. 4 and 5. DSC curve obtained for usnic acid showed exothermic peaks at $144.94\text{ }^{\circ}\text{C}$, $163.93\text{ }^{\circ}\text{C}$, $200.01\text{ }^{\circ}\text{C}$ and $277.7\text{ }^{\circ}\text{C}$. DSC curves of PLGA and usnic loaded nanoparticles

showed exothermic peaks at $45.80\text{ }^{\circ}\text{C}$, $67.83\text{ }^{\circ}\text{C}$, $169.64\text{ }^{\circ}\text{C}$, $178.3\text{ }^{\circ}\text{C}$ and $61.82\text{ }^{\circ}\text{C}$, $111.56\text{ }^{\circ}\text{C}$ and $153.28\text{ }^{\circ}\text{C}$ respectively. It was observed that pure usnic acid showed an exothermic peak of melting at $200.01\text{ }^{\circ}\text{C}$ and there was no peak obtained at this temperature for usnic acid loaded nanoparticles.

It confirmed that usnic acid could be in an amorphous or solid - solution state in the polymer matrix²⁹. Thermograms of drug - polymer loaded nanoparticles showed that there was no melting peak of polymer (PLGA) observed and it

confirmed that polymer also in amorphous state³⁰. Hence DSC analysis results revealed that there was only physical interaction between drug and polymer, but there is no change in therapeutic action of drug.

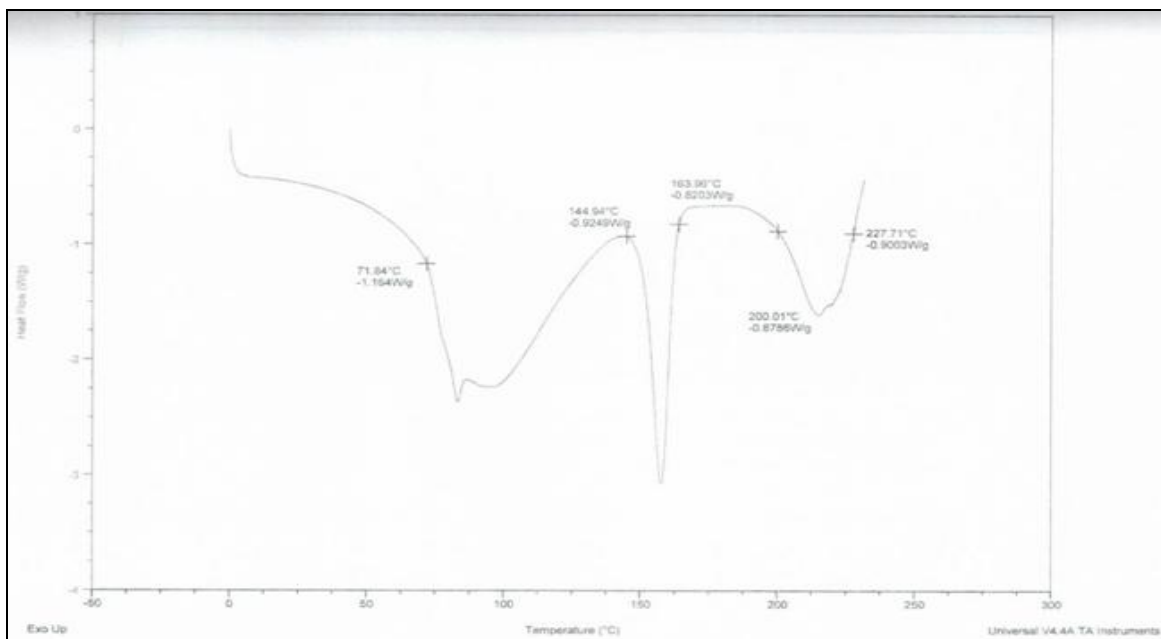


FIG. 4: DSC CURVE OF USNIC ACID

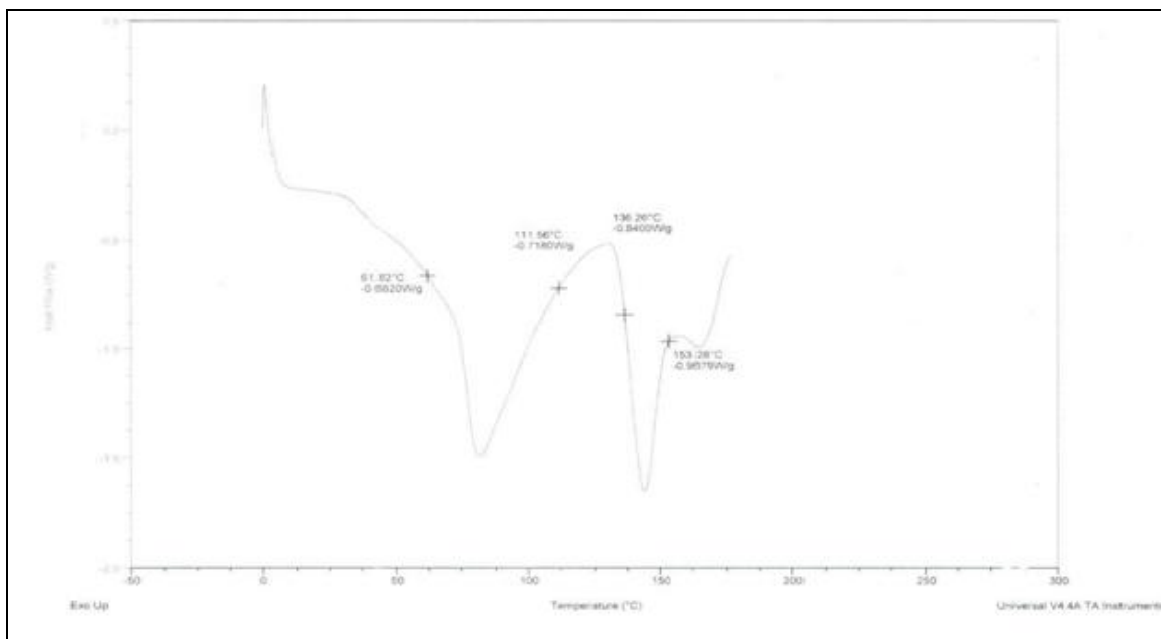


FIG. 5: DSC CURVE OF USNIC ACID LOADED NANOPARTICLES

Entrapment Efficiency: The entrapment efficiency of usnic acid with polymer PLGA in different ratio was determined and tabulated in **Table 2**. The entrapment efficiency of usnic acid loaded nanoparticles prepared by nanoprecipitation method with sonication was in the range of 74.95 to 95.27%. It showed that sonication technique

increased the entrapment of drug with polymer by decreasing particle size and increasing the surface area between drug and polymer. The results obtained also showed that increased entrapment efficiency of drug with increased concentration of polymer.

TABLE 2: ENTRAPMENT EFFICIENCY OF DRUG WITH POLYMERS

S. no	Formulation code	Amount of drug in supernatant	Percentage of entrapment
1	G ₁	13.39	66.50
2	G ₂	11.15	72.12
3	G ₃	7.67	80.82
4	G ₁ S	10.02	74.95
5	G ₂ S	5.25	86.87
6	G ₃ S	2.44	93.90

In vitro Drug Release Studies: The *in vitro* release profile of all prepared nanoparticles showed typical bimodal behaviour. In the present study nanoparticles prepared with lower polymer concentration were released faster than nanoparticles prepared with higher concentration of polymer. Drug - polymer ratio of 1:1 showed maximum cumulative drug release in 24 hours. Higuchi and korsmeyer Kinetic study equation confirms usnic acid nanoparticles release was zero order.

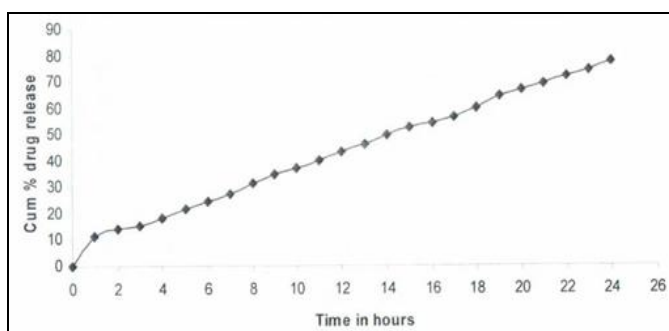


FIG. 6: IN VITRO RELEASE OF OPTIMIZED FORMULATION G₂S BY U.V. METHOD

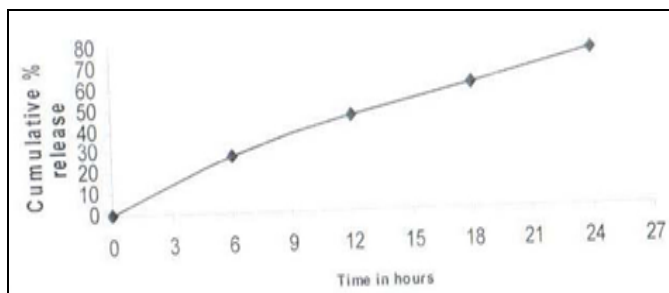


FIG. 7: IN VITRO RELEASE OF OPTIMIZED FORMULATION G₂S BY HPLC METHOD

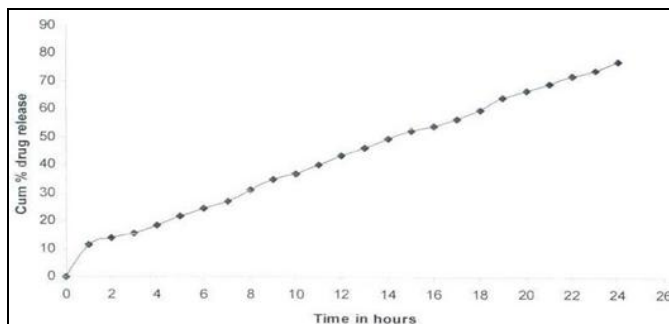


FIG. 8: ZERO ORDER PLOT OF OPTIMIZED FORMULATION G₂S

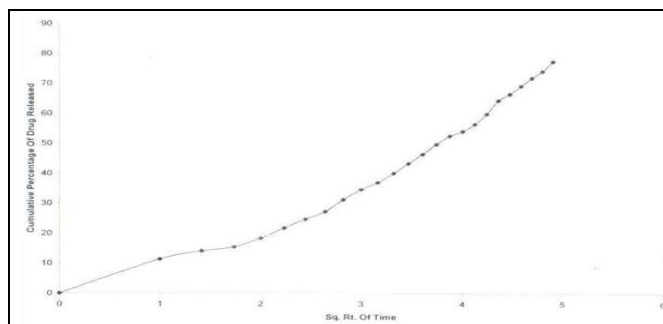


FIG. 9: HIGUCHI'S PLOT FOR OPTIMIZED FORMULATION

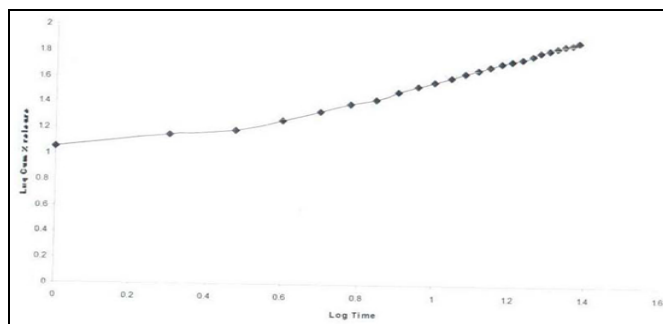


FIG. 10: KORSMEYER PLOT FOR OPTIMIZED FORMULATION

Effect of Usnic Acid and Usnic Acid Loaded Nanoparticles: The effect of usnic acid and usnic acid loaded nanoparticles on the survival of DAL tumour bearing mice is shown in **Table 4**. The percentage of ILS of the cancer control group was 44%, where as it was 90%, 67%, 78% for the group with 5FU 20mg/kg, usnic acid and usnic acid loaded nanoparticles respectively. The effect of usnic acid and usnic acid loaded nanoparticles on the average increase in body weight is shown in **Table 4**.

The average weight gain of tumour bearing mice was 8.16 ± 1.4gm, whereas it was 1.90 ± 0.09gm, 2.68 ± 1.32gm and 2.16 ± 1.11gm for the groups treated with 5FU, usnic acid, usnic acid loaded nanoparticles respectively. Usnic acid and usnic acid loaded nanoparticles were significantly reduced the packed cell volume and viable tumour cell count when compared to tumor control group (**Fig. 11, 12 and 13**).

TABLE 3: TREATMENT PROTOCOL CYTOTOXIC EVALUATION

Groups	Description	Dose	Treatments		
			Cancer Cells	Drug	Food / Water
1	Normal Control		-----		Access
2	Tumor Control		-----		Access
3	Positive Control	20mg/kg	Induced	5-Fluoro Uracil	Access
4	Treatment Control	5mg/kg	Induced	Usnic acid	Access
5	Treatment Control	5mg/kg	Induced	Nanoparticle	Access

TABLE 4: EFFECT OF USNIC ACID AND USNIC ACID LOADED NANOPARTICLE ON THE LIFE SPAN BODY WEIGHT AND CANCER CELL COUNT

Group	Treatment	No of Animals	% ILS	Increase in Body Weight (gms)	Cancer Cell Count MI X 10 ⁶
1	Normal control	6	≥30days	1.40±0.06	-
2	Cancer control	6	44% ^{**} (a)	8.16±1.4 ^{**} (a)	1.92±0.26
3	Positive control	6	90%	1.90±0.09	0.70±0.12
4	Usnic acid	6	67% ^{**} (b)	2.68±1.32 ^{**} (b)	1.12±0.30 ^{**} (b)
5	Nanoparticle	6	78% ^{**} (b)	2.16±1.11 ^{**} (b)	0.86±0.22 ^{**} (b)

All values were expressed as Mean ± SEM

Values were found out by using ONE WAY ANOVA. Followed by Newman keul's multiple range test.

TABLE 5: EFFECT OF USNIC ACID AND USNIC ACID LOADED NANOPARTICLE ON SOLID TUMOR VOLUME

Group	Treatment	15 th day	20 th day	25 th day	30 th day
1	Tumor control	6.6±0.48	8.4±0.30	10.4±0.38	12.01±0.42
2	Usnic acid	5.42±0.42 ^{**} (a)	5.64±0.38 ^{**} (a)	7.22±0.26 ^{**} (a)	7.30±0.34 ^{**} (a)
3	Nanoparticle	5.06±0.39 ^{**} (a)	5.18±0.46 ^{**} (a)	5.13±0.36 ^{**} (a)	5.01±0.40 ^{**} (a)

Values are expressed as Mean ± SEM

Values were found out by using ONE WAY ANOVA. Followed by Newman keul's multiple range test.

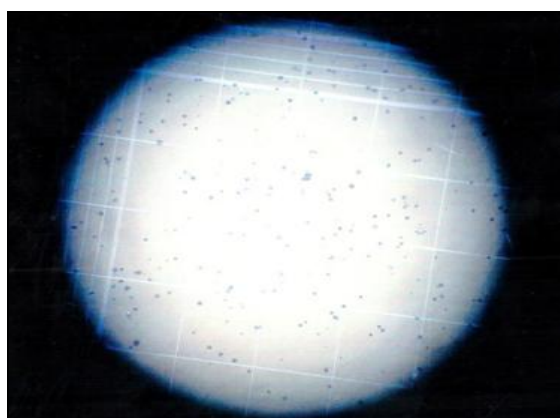


FIG. 11: VIABLE CANCER CELL IN DAL CELL LINE INOCULATED MICE

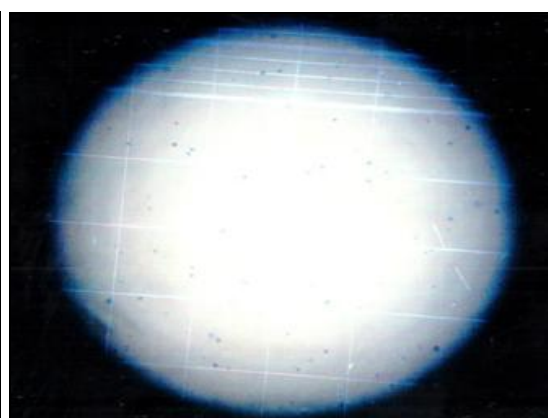


FIG. 12: VIABLE CANCER CELL IN USNIC ACID TREATED MICE

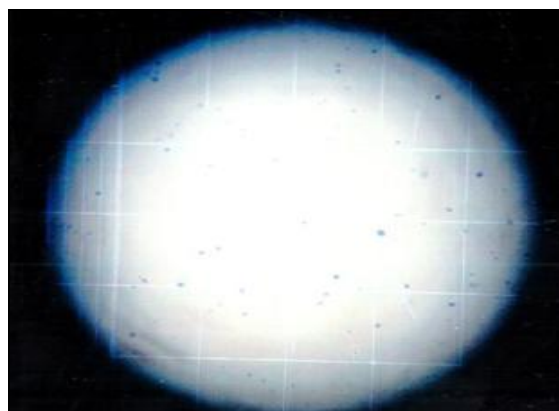


FIG. 13: VIABLE CANCER CELL IN NANO TREATED MICE

CONCLUSION: In this study usnic acid loaded nanoparticles were prepared by nanoprecipitation method with sonication technique with biodegradable polymer PLGA exhibited controlled drug release for 24 hours. Usnic acid loaded nanoparticles showed more antitumor activity and reduced the packed cell volume than unloaded free usnic acid. This study clearly showed that nanoparticulate system could be used to introduce usnic acid into chemotherapy and PLGA act as a better candidate to attain usnic acid in controlled manner at targeted site. Therefore developed usnic acid nanoparticles could be considered an effective anti-cancer drug delivery for cancer chemotherapy.

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CONFLICT OF INTEREST: The authors have no conflict of interest to declare.

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