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Research Article

Preparation and evaluation of liposomal gel containing *Neolamarckia cadamba* leaves extract for anti-inflammatory activity

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Abstract

The objective of present research work is to develop liposomes as a carrier system for methanolic extract, its incorporation in to gel formulations and to characterize the prepared and develop liposomal gel formulation. Formulation was carried out by thin film technique. Scanning electron micrograph of the prepared nanosponges at 50.00 kx magnification showed that the liposome was porous with a smooth surface morphology and spherical shape. The porous nature of liposome was clearly observed in the SEM images. Particle size and zeta potential was determined by Malvern Zeta sizer. The particle size analysis confirmed that the prepared sample were in the nanometer range. Average particle size obtained for the formulations F1 to F5 were 162.7 nm to 195.6 nm. Zeta potential values of liposome indicated that the formulated liposomes are stable. The amount of drug being entrapped in liposome was calculated and all the prepared liposome was found to possess very high entrapment efficiency. The viscosity of liposome loaded gel is found to 6842±0.32cps. The pH of liposome loaded gel is 6.3 and spreadability is 12.11, indicating that liposome loaded gel has high release and permeability. The *in vitro* anti-inflammatory effect of *Neolamarckia cadamba* extract was evaluated against denaturation of egg albumin. The present findings exhibited a concentration dependent inhibition of protein (albumin) denaturation by *Neolamarckia cadamba* throughout the concentration range of 20-100µg/ml. Diclofenac sodium (at the concentration range of 20-100 µg/ml) was used as reference drug which also exhibited concentration dependent inhibition of protein denaturation; however, the effect of diclofenac sodium was found to be less active when compared with extract name. Moreover, the results demonstrated that the liposome-based drug delivery approach could be a valuable tool to improve the therapeutic efficacy of phytochemicals by improving their absorption, and bioavailability via altering their physicochemical and release properties.

Keywords: Liposomes, *Neolamarckia cadamba,* Qualitative phytochemical screening, Thin film technique, *In vitro* anti-inflammatory activity.

INTRODUCTION

Over the past century the modern and western medicine has revolutionized healthcare of humans and animals in the world, but still large percentage of populations in developing nations depends on phytomedicines[1]. Recently, Golden Triangle Partnership (GTP) program jointly by ICMR, AYUSH and CSIR has been initiated for validation of traditional Ayurvedic drugs/formulations and new drug development from Indian plant species[2]. *Neolamarckia cadamba* is one of evergreen tropical tree belongs to the Rubiaceae family, closely associated with the life of Lord Krishna (Hindu Deity) and has been used in folklore medicine to treat fever, uterine complaints, anaemia, blood diseases, skin diseases, eye inflammation, diarrhoea, leprosy, dysentery and stomatitis[3-8]. It contains the number of phytochemicals and secondary metabolites (viz., cadamine & isocadamine, 3β-dihydrocadambine & 3β-isodihydrocadambine, aminocadambine A & B, neolamarckines A & B, chlorogenic acid & β-sitosterol) responsible for its various biological and pharmacological activities such as antiplasmodial, analgesic, antidiabetic, antioxidant, antipyretic, anticancer and antimicrobial activities[9].The drug delivery system refers to a group of medicine delivery systems that are intended to improve therapeutic effect through controlled release. In addition to improving bioavailability and therapeutic impact, it is used for targeted pharmaceutical release[10]. Liposomes are one of the drug delivery techniques that is currently being developed. Liposomes are an excellent carrier for increasing the solubility and penetration of the chemicals they transport. Liposomes have both hydrophilic and lipophilic structures, which trap hydrophilic medications in the water core and lipophilic drugs in the lipid bilayer[11]. Along with their good solubilizing power, their manufacture is easier and makes liposomes an attractive drug carrier system[12]. Liposomes are biocompatible, biodegradable, and non-immunogenic due to their phospholipid composition (which is similar to cell membranes). Pharmaceuticals (active chemicals) are contained in liposomes with the goal of improving solubility, minimizing adverse effects, extending release, protecting drugs, targeting drugs, and increasing efficacy[13]. The researcher is interested in employing the thin film technique approach to formulate the methanol extract of *Neolamarckia cadamba* leaves into liposome dosage forms with the goal of enhancing the bioavailability and therapeutic impact of the methanolic extract of *Neolamarckia cadamba* leaves.

MATERIALS AND METHODS

Materials

Plant collection

The medicinal plant *Neolamarckia cadamba* (300 gm) was collected. After cleaning, plant parts (leaves) were dried under shade at room temperature for 3 days and then in oven dried at 45°C till complete dryness. Dried plant parts were stored in air tight glass containers in dry and cool place to avoid contamination and deterioration. Authentication of selected traditional plant *Neolamarckia cadamba* was authenticated by a plant taxonomist in order to confirm its identity and purity.

Chemical and reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals used in this study were of analytical grade.

Extraction

In present study, plant material was extracted by continuous hot percolation method using Soxhlet apparatus. Powdered material of *Neolamarckia cadamba* was placed in thimble of soxhlet apparatus. Soxhlation was performing at 60°C using petroleum ether as non-polar solvent. Exhausted plant material (marc) was dry and afterward re-extracted with methanol solvent. For each solvent, soxhlation was continued till no visual colour change was observed in siphon tube and completion of extraction was confirmed by absence of any residual solvent, when evaporated. Obtained extracts was evaporate using rotary vacuum evaporator (Buchitype) at 40°C. Dried extract was weighed and percentage yield for each extract was determined. Prepared extracts were observed for organoleptic characters (percentage yield, colour and odour) and was packed in air tight container and labelled till further use[14,15].

Phytochemical screening

Phytochemical screening to detect the presence of bioactive agents was performed by standard procedures[16, 17]. After the addition of specific reagents to the solution, the tests were detected by visual observation of color change or by precipitate formation.

Formulation of vesicle system (Liposomes)

Several parameters influence the final properties of liposomes. Major variables in the liposome properties include cholesterol and lecithin amounts and sonication time. Five different formulations with low and high values of cholesterol (100 to 300 mg), lecithin (100 to 300 mg), and sonication time (10 to 60 minutes) were used to prepare liposomal formulations. Liposomes were prepared by thin film method. Briefly, different concentrations of soya lecithin and cholesterol (Table 1) were dissolved in the chloroform-methanol (1:1) and 100 mg extract was added to the solution, then the mixture was evaporated in a rotary evaporator when the thin film was formed in the round-bottoms flask, it was hydrated with phosphate buffer (pH 7.4). The suspension was agitated by vortex for 30 minutes and then sonicated for ten to sixty minutes[18].

Table 1: Composition of liposome formulation

Ingredients	Liposome (F1)	Liposome (F2)	Liposome (F3)	Liposome (F4)	Liposome (F5)
Soy lecithin (mg)	300	250	200	150	100
Cholesterol (mg)	100	150	200	250	300
Extract (mg)	200	200	200	200	200
Chloroform-methanol (ml)	10	10	10	10	10
PBS (7.4)	10	10	10	10	10
Sonication time (min.)	30	30	30	30	30

Evaluation parameter of liposome formulation[19-21]

Particle size

The particle size is one of the most important parameters for the characterization of liposome. The size of liposome was measured using Malvern Zeta sizer (Malvern Instruments). The dispersions were diluted with Millipore filtered water to an appropriate scattering intensity at 25°C and sample was placed in disposable sizing cuvette.

Zeta potential

The zeta potential was measured for the determination of the movement velocity of the particles in an electric field and the particle charge. In the present work, the microspheres were diluted 10 times with distilled water and analyzed by Zetasizer Malvern instruments. All samples were sonicated for 5-15 minutes before zeta potential measurements.

Scanning electron microscopic (SEM)

The electron beam from a scanning electron microscope was used to attain the morphological features of the extract loaded liposome were coated with a thin layer (2-20 nm) of metal(s) such as gold, palladium, or platinum using a sputter coater under vacuum. The pretreated specimen was then bombarded with an electron beam and the interaction resulted in the formation of secondary electrons called auger electrons. From this interaction between the electron beam and the specimen's atoms, only the electrons scattered at 90°C were selected and further processed based on Rutherford and Kramer's Law for acquiring the images of surface topography.

Formulation of liposome loaded gel

Initially carbopol-934 was immersed in 50 ml of warm water (A) for 2hr and was homogeneously dispersed using magnetic stirrer at 600rpm. In separate container carboxy methyl cellulose and methyl paraben was added into 50 ml warm water (B) and stirred continuously to make stiff gel. Both the mixtures A and B were mixed with the continuous stirring. Then tri- ethanol amine (Drop wise) was added to neutralize the pH and liposome of optimized formulation was incorporated into the dispersion to obtained Gel. At this stage, permeation enhancer (Propylene glycol) was added. The final dispersion was agitated until smooth gel was formed without lumps[22].

Table 2: Composition of gel formulation

S. No	Excipients	Quantity
1.	Carbopol 934	1.00 gm
2.	Carboxymethyl cellulose	1.00 gm
3.	Propylene glycol	0.5 ml
4.	Methyl paraben	0.2 ml
5.	liposome	10 ml
6.	Tri-ethanolamine	q.s
7.	Water	100 ml

Characterization of liposome loaded gel[23,24]

Physical appearance

The prepared gel formulation was evaluated for appearance, colour, odour, and homogeneity by visual observation.

pH

pH of the formulation was determined by using digital pH meter (EI). The meter was allowed to stabilize as necessary and properly calibrated, begin by rinsing the probe with de-ionized or distilled water and blotting the probe dry with lint-free tissue paper. Immerse the sensing tip of the probe in the sample and record the pH reading and rinse the probe, blot dry and repeat step 2on a fresh portion of sample. The two readings should agree to within the accuracy limits of the meter. The samples were analyzed in triplicate. If slight deviations in pH were noted, it was adjusted to skin pH using drop wise addition of tri-ethanolamine solution.

Viscosity

The viscosity of the gel formulations was determined using Brookfield viscometer with spindle no. 61 at 100 rpm at the temperature of 25^{0}C**.**

Spreadability

An ideal topical gel should possess a sufficient spreading coefficient when applied or rubbed on the skin surface. This was evaluated by placing about 1g of formulation on a glass slide. Anotherglass slide of the same length was placed above that, and a mass of 50 mg was put on the glass slide so that the gel gets sandwiched between the two glass slides and spreads at a certain distance. The time taken for the gel to travel the distance from the place of its position was noted down. Spreadability was determined by the following formula.

$$S= M*L/T$$

Where, S-Spreadability, g.cm/s M-Weight put on the upper glass L-Length of glass slide T-Timefor spreading gel in sec.

Stability studies

The extract loaded gel formulation was packed and were placed in the stability test chamber and subjected to stability studies at accelerated testing (25^{0}C±2^{0}C and 60±5% RH) and (40^{0}C±2^{0}C and 70±5% RH) for 3months. The formulation was checked for evaluation parameter viscosity and pH at the interval of 30, 45, 60, 90 days (3 month) months. The formulation was tested for stability under accelerated storage condition for 3 months in accordance to International Conference on Harmonization (ICH) guidelines. Formulation was analyzed for the change in evaluation parameter viscosity and pH. All Results were compared against final formulation of 0 days as the reference.

Evaluation of *in vitro* anti-inflammatory activity of plant extract

The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of plant extractso that final concentrations become 20, 40, 60, 80 and 100µg/ml. Similar volume of double- distilled water served as control. Then the mixtures were incubated at (37±2) ^{o}C in Incubator (Universal) for 15min and then heated at 70^{o}C for 5min. After cooling, their absorbance was measured at 660 nm (Shimadzu 1700) by using vehicle as blank. Diclofenac sodium at the final concentration of (20-100µg/ml) was used as reference drug and treated similarly for determination of absorbance and viscosity. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ inhibition} = 100 \times (Vt / Vc - 1)$$

Where, Vt = absorbance of test sample, Vc = absorbance of control.

The extract/drug concentration for 50% inhibition (IC_{50}) was determined by plotting percentage inhibition with respect to control against treatment concentration[25].

RESULTS AND DISCUSSION

In phytochemical extraction the percentage yield is very crucial in order to determine the standard efficiency of extraction for a specific plant, various sections of the same plant or different solvents used. The yield of extracts received from the *Neolamarckia cadamba* is shown in Table 3. The results of qualitative phytochemical analysis of the crude powder of leaves of *Neolamarckia cadamba* are shown in Table 4. The particle size is one of the most important parameters for the characterization of liposomes. The average particle size of the prepared drug loaded liposomes was measured using Malvern zeta sizer. Particle size analysis showed that the average particle size of drug loaded liposomes was found to be range 162.7 nm to 195.6 nm Table 5& Figure 1. Zeta potential analysis is carried out to find the surface charge of the particles to know its stability during storage. If the particles in liposomes have a large positive zeta potential then they will tend to repel each other and there will be no tendency for the particles to come together. However, if the particles have low zeta potential values, then there will be no force to prevent the particles coming together and flocculating for liposomes. Zeta potential of all formulations was found to be range -1.9 mV to -12.2mV with peak area of 100% intensity. These values indicate that the formulated liposomes are stable Table 6 & Figure 2. SEM analysis was performed to determine their microscopic characters (shape & morphology) of prepared liposomes. Liposomes were prepared and dried well to remove the moisture content and images were taken using scanning electron microscopy. Scanning electron micrograph of the prepared liposomes at 50.00kx magnification showed that the liposomes were porous with a smooth surface morphology and spherical shape. The spongy and porous nature of liposomes was clearly observed in the SEM images Figure 3. An evaluation of the gel, including colour, odor, appearance and homogeneity, was conducted. Gel was discovered to have a dark green colour to it when tested. Gel does not have a distinctive odor and has a brown colour appearance, according to research conducted on its Table 7. The pH of the gel formulation was found to be 6.3, which lies in the normal pH range of the skin and would not produce any skin irritation. There was no significant change in pH values as a function of time. The physicochemical properties of prepared gel formulation were in good agreement. The viscosity was measured by the Brookfield viscometer spindle no. 61 at 100rpm. The result was shown in the Table 8. The viscosity of gel was found to be 6842 centipoises respectively. One of the essential criteria for a gel is that it should possess good spreadability. Spreadability depends on the viscosity of the formulation and physical

characteristics of the polymers used in the formulation. A more viscous formulation would have poor spreadability. Spreadability is a term expressed to denote the extent of area on which the gel readily spreads on application to the skin. The therapeutic efficacy of a formulation also depends upon its spreading value. The spreadability of gel formulation is found to be 12.11 g.cm/s. Formulation were found to be stable, both physically and chemically, for a period of 3 months at accelerated stability conditions ($25^0C\pm2^0C$ and 60±5%RH) and ($40^0C\pm2^0C$ and 70±5%RH). Evaluation parameters including viscosity and pH studies were not altered significantly. Results of assay and other evaluation criteria at periodic time points of stability studies are summarized in Table 9. No major changes were observed. In the present investigation, the *in vitro* anti-inflammatory effect of *Neolamarckia cadamba* extract was evaluated against denaturation of egg albumin. The results are summarized in Table 10&11.The present findings exhibited a concentration dependent inhibition of protein (albumin) denaturation by *Neolamarckia cadamba* throughout the concentration range of 20, 40, 60, 80 and 100µg/ml. Diclofenac sodium (at the concentration range of 20, 40, 60, 80 and 100 µg/ml) was used as reference drug which also exhibited concentration dependent inhibition of protein denaturation; however, the effect of diclofenac sodium was found to be less active when compared with extract name. This was further confirmed by comparing their IC50 values.

Table 3: Percentage yield of crude extracts of *Neolamarckia cadamba* extract

S. No	Plant name	Solvent	Theoretical weight	Yield(gm)	% yield
1	*Neolamarckia cadamba*	Pet ether	299	1.51	0.50%
2		Methanol	286.78	6.58	2.29%

Table 4: Phytochemical screening of *Neolamarckia cadamba*

S. No.	Experiment	Presence or absence of phytochemical test	
		Pet. Ether extract	Methanolic extract
1.	**Alkaloids**		
1.1	Dragendroff's test	Absent	Present
1.2	Mayer's reagent test	Absent	Present
1.3	Wagner's reagent test	Absent	Present
1.3	Hager's reagent test	Absent	Present
2.	**Glycoside**		
2.1	Borntrager test	Present	Absent
2.2	Legal's test	Present	Absent
2.3	Killer-Killiani test	Present	Absent
3.	**Carbohydrates**		
3.1	Molish's test	Absent	Present
3.2	Fehling's test	Absent	Present
3.3	Benedict's test	Absent	Present
3.4	Barfoed's test	Absent	Present
4.	**Proteins and Amino acids**		
4.1	Biuret test	Absent	Absent
5.	**Flavonoids**		
5.1	Alkaline reagent test	Absent	Present
5.2	Lead Acetate test	Absent	Present
6.	**Tannin and Phenolic compounds**		
6.1	Ferric Chloride test	Absent	Present
7.	**Saponin**		
7.1	Foam test	Present	Absent
8.	**Test for Triterpenoids and steroids**		
8.1	Salkowski's test	Present	Present
8.2	Libbermann-Burchard's test	Present	Present

Table 5: Particle size of all formulation

S No	Formulation code	Particle size (nm)	PI Value
1.	Liposome F1	170.5 nm	0.398
2.	Liposome F2	165.5 nm	0.295
3.	Liposome F3	195.6 nm	0.259
4.	Liposome F4	182.6 nm	0.359
5.	Liposome **F5**	**162.7 nm**	**0.160**

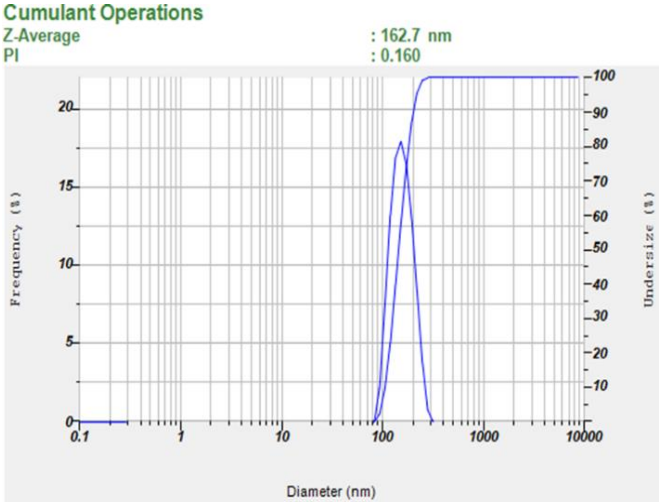

Figure1: Particle size of (F5)

Table 6: Zeta potential of all formulation

S.No	Formulation Code	Zeta potential
1.	Liposome F1	-1.9 mV
2.	Liposome F2	-12.2mV
3.	Liposome F3	-10.4 mV
4.	Liposome F4	-8.9mV
5.	Liposome**F5**	**-2.2 mV**

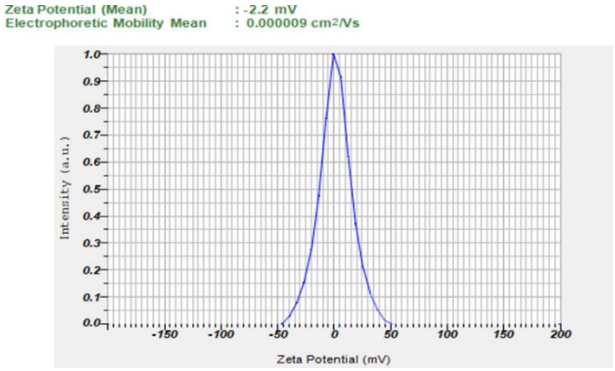

Figure 2: Zeta potential of (F5)

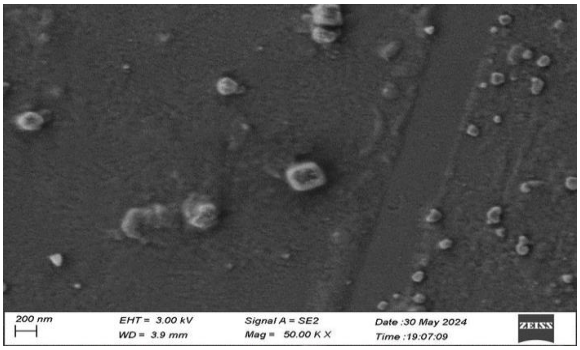

Figure 3: Scanning electron microscope of (F5)

Table 7: Physical appearance

S. No	Parameter	Result
1	Colour	Dark green
2	Odour	Odourless
3	Appearance	Green colour
4	Homogeneity	Homogeneous

Table 8: Characterization of gel-based formulation of liposomes

S. No	Formulation	Viscosity (cps)	pH	Spreadability (g.cm/s)
1	Gel	6842±0.32	6.3	12.11

Table 9: Stability study of formulation (Gel)

S. No	Time (Days)	25⁰C±2 ⁰C and 60 ± 5% RH		40⁰C±2 ⁰C and 70 ±5% RH	
		Viscosity	pH	Viscosity	pH
1.	0	6842	6.3	6842	6.3
2.	30	6848	6.5	6849	6.6
3.	45	6851	6.8	6852	6.9
3.	60	6853	7.0	6857	7.2
4.	90	6855	7.3	6860	7.5

Table 10: Effect of Diclofenac sodium (Standard) on protein denaturation

S. No	Concentration of extract (µg/ml)	Absorbance at 660nm	% Inhibition	IC50 Value
1.	Control	1.101	-	54.69µg/ml
2.	20	0.733	33.42	
3.	40	0.616	44.05	
4.	60	0.533	51.58	
5.	80	0.425	61.39	
6.	100	0.305	72.29	

Table 11: Effect of *Neolamarckia cadamba* extract on protein denaturation

S. No	Concentration of extract (µg/ml)	Absorbance at 660nm	% Inhibition	IC_{50} Value
1.	Control	1.054	-	66.7 µg/ml
2.	20	0.804	23.71	
3.	40	0.737	30.07	
4.	60	0.583	44.68	
5.	80	0.425	59.67	
6.	100	0.304	71.15	

CONCLUSION

Current study indicates for the first time, the potential of liposome formulation of *Neolamarckia cadamba* extract for enhancing the bioavailability. Moreover, the results demonstrated that the liposome-based drug delivery approach could be a valuable tool to improve the therapeutic efficacy of phytochemicals by improving their absorption, and bioavailability via altering their physicochemical and release properties.

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