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Formulation and evaluation of Annona squamosa hydroalcoholic extract loaded phytosomes

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Abstract

The objective of the present study was to prepare phytosomal formulation loaded with hydroalcoholic extract of *Annona squamosa* leaf. The extraction of leaf powder was done by maceration technique using 10:90, water: ethanol mixture with an extraction yield of 11.3%. The extract was found to contain alkaloids, glycosides, proteins, phenols, flavonoids and triterpenes. Phytosomes of the extract were prepared by solvent evaporation method using lecithin as the lipid molecule. The particle size of the phytosomes was from 678 nm to 2617 nm in size with a polydispersity index varying between 0.368 - 0.718. The phytosomes were visible as rigid, spherical vesicles in SEM image. The surface of the phytosome vesicles was found to be regular and smooth. Sharp and distinct endothermic peaks in DSC revealed the formulation of stable phytosomes due to molecular interactions between the extract and lecithin. The phytosomes exhibited anti-inflammatory action in the *in vitro* assays by inhibiting albumin denaturation and protease activity. The phytosomes were found to be stable under the storage conditions. The best formulation with respect to particle size and anti-inflammatory action was ASP 4 that contained 2:0.5 ratio of lecithin: extract.

Keywords: Phytosome, Annona squamosa, extract, lecithin, anti-inflammatory

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Introduction

Natural products, including plants, animals and minerals play a significant role in the discovery of new chemical entities for drug discovery and thus form the basis for treatment of human diseases. It is estimated that over 100 new natural product based leads are in clinical development. About 60% of anticancer and 75% of anti-infective drugs approved from 1981 to 2002 could be traced to natural origins. Thirty percent of the worldwide sales of drugs are based on natural products. A multidisciplinary approach combining natural product diversity with total, combinatorial synthesis and high-throughput screening may provide better results in drug discovery (Patwardhan et al., 2004).

Ayurveda often involves complex formulations which are prepared overseveral days and can contain many herbal and mineral components. Consequently, there is more dosage diversity than for Chinese medicine. Dosage ranges for individual non-toxic herbs are generally in the region of 1 to 6 g/day as powders or tinctures, with higher doses often recommended for decoctions (Nadkarni, 1976).

Phytosome one of the novel colloidal drug delivery system that holds great promise for reaching the goal as well as site specific drug release from the vesicles. Thus phytosome are formulated with natural phytoconstituent for targeting. The important technological advantage of phytosome as a drug carrier is high stability, high carrier capacity, feasibility of incorporation of phytoconstituent and feasibility of routes of administration. These properties of phytosome enable improvement of drug bioavailability and reduction of the dosing frequency and may resolve the problem of non adherence to prescribed therapy (Kidd, 2008). The phytosome technology involves the incorporation of phospholipids into standardized extracts improving their absorption and bioavailability (Bombardelli, 1989; Kidd et al., 2005).

Annona squamosa or Custard Apple is one of the most popular trees that are widely cultivated for its edible fruit. The fruit, bark and leaves of the plant are known to possess variety of pharmacological activities. The requirements of novel drug delivery system of herbal extracts than conventional herbal extracts are to enhance the bio-availability of herbal extract; to decrease the dose of herbal extracts; to ensure the localized delivery i.e. liver and so forth by novel carrier system.

Hence it was envisioned to formulate the phytosomal system as an efficienct method for oral delivery of *Annona squamosa* hydroalcoholic extract

Material and Methods

Bovine Serum Albumin and Trypsin were purchased from HiMedia laboratories, Mumbai. Ibuprofen was purchased from Yarrow Pharmaceuticals, Mumbai. Various chemicals and reagents used for preliminary phytochemical screening of extracts and other reagents for testing of anti-inflammatory activity were purchased from Oxford Fine Chemicals Pvt Ltd,

Mumbai. All the chemicals were used as received without any further processing or purification.

The leaves of *Annona squamosa* were collected from the local places of Gwalior, Madhya Pradesh in the month of October and authenticated at Saifia Science College, Bhopal.

Preparation of the plant material

The authenticated plant leaves were washed with distilled water, dried under shade and powdered using a blender at low speed. The powdered leaves were stored in air tight container until taken for use.

Physicochemical evaluation

Determination of foreign matter

Accurately weighed plant material was spread in a thin layer and inspected with the help of magnifying glass. Any foreign matter was removed manually. The leaves were reweighed and the percentage of foreign matter with respect to the air dried drug was determined (Indian Pharmacopoeia, 2007).

Determination of ash values (WHO, 1998)

Total ash

The finely ground plant material was accurately weighed (5 g) and placed in a previously ignited and tarred silica crucible. The sample was ignited by gradual increase in temperature to 500-600°C until it was white, indicating absence of carbon. It was then cooled in a desiccator and weighed. The percentage of ash with respect to the air dried drug was calculated.

Acid-insoluble ash

25 mL of concentrated HCl was added to the crucible containing total ash. The beaker was covered with watch glass and boiled gently for 5 min. The watch glass was rinsed with 5 mL of hot water and the rinsing was added to the crucible. The insoluble matter was collected on an ashless filter paper and washed with hot water until the filtrate became neutral. The filter paper containing the insoluble matter was transferred to the silica crucible, dried on a hot plate and ignited to constant weight. The residue was allowed to cool in desiccator for 30 min and weighed without delay. The percentage of ash with reference to the air dried drug was calculated.

Water-soluble ash

To the crucible containing the total ash, 25 mL of distilled water was added and boiled for 5 min. The insoluble matter was collected in a sintered glass crucible or on an ashless filter paper, washed with hot water and ignited in a crucible for 15 min, at temperature not exceeding 450°C. The ash obtained was weighed and this weight was subtracted from the weight of total ash. The percentage of ash with reference to air dried drug was calculated.

Determination of loss on drying (WHO, 1998)

Accurately weighed powdered plant material was placed in a dry and tarred flat weighing bottle. The sample was dried in an oven at 100-105°C. The

operation was repeated until two consecutive weighing that did not differ by more than 5mg. The loss of weight in mg per g of air-dried material was calculated.

Alcohol soluble extractive value (WHO, 1998)

About 5 g of powdered plant material was weighed and macerated with 100 mL of the ethanol in a glass stoppered Erlenmeyer flask for 24 h with frequent shaking during the first 6 h and allowing to settle for 18 h. It was filtered rapidly and 25 mL of the filtrate was transferred in to a tarred petri dish and evaporated to dryness on a water bath. The residue was dried at 105°C for 6 h and cooled in desiccator for 30 min then weighed. The content of alcohol soluble extractable matter in mg per g of air-dried material was calculated.

Water soluble extractive value (WHO, 1998)

About 5 g of powdered plant material was weighed and macerated with 100 mL of the distilled water in a glass stoppered Erlenmeyer flask for 24 h with frequent shaking during the first 6 h and allowing to settle for 18 h. It was filtered rapidly and 25 mL of the filtrate was transferred in to a tarred petri dish and evaporated to dryness on a water bath. The residue was dried at 105°C for 6 h and cooled in desiccator for 30 min then weighed. The content of alcohol soluble extractable matter in mg per g of air-dried material was calculated.

Extraction of Plant Material (Khandelwal, 2005)

An accurately weighed quantity of 200 g of powdered plant material was taken in a 2 L glass jar. The jar was filled with 1000 mL of 10:90 ratios of water and ethanol and macerated for 24 h with intermittent shaking for first 6 h and allowing standing for 18 h. The macerate was filtered and the filtrate was evaporated on water bath. The thick syrupy residue obtained was subjected to rotary evaporation to remove all solvent. The dried extract was kept in desiccator until used for various tests.

Preliminary qualitative phytochemical screening (Ahmad, 2007)

The extract was qualitatively screened for the presence of secondary metabolites such as alkaloids, glycosides, phenolics and tannins, flavonoids and sterols.

Preparation of Phytosomes by solvent evaporation method (Karole et al., 2019)

The specific amount of leaves extract of *Annona squamosa* and soya lecithin (Table 4.1) were taken into a 100 mL round bottom flask and refluxed with 20 mL of acetone at a temperature 40 – 50°C for 2 h. The mixture is concentrated to 5-10 ml to obtain the precipitate which was filtered and collected. The dried precipitate phytosomes complex was placed in amber colored glass bottle and stored in refrigerator.

Table 1 Batch formula for phytosome preparation

Formulation Code	Ratio of Lecithin : Extract	Acetone (mL)
ASP 1	1:0.5	20
ASP 2	1:1	20
ASP 3	1:1.5	20
ASP 4	2:0.5	20
ASP 5	2:1	20
ASP 6	2:1.5	20

Evaluation of phytosomes (Dhase et al., 2015))

Visualization

Visualization of phytosomes was accomplished by utilizing scanning electron microscopy. Scanning electron microscopy has been utilized to decide particle size estimate appropriation and surface morphology of the complex. The samples were sputter-covered with gold/palladium for 120 s at 14 mA under argon air for auxiliary electron emissive SEM (Hitachi-S 3400N) and watched for morphology at voltage of 15.0 kV.

Particle size and size distribution

The particle size (z-average) and size distribution of the prepared phytosomes was calculated from the auto correlation function of the intensity of light scattered from the particles expecting a circular type of particles using Malvern Zeta sizer.

Differential scanning calorimetry

The thermograms were obtained for the phytosome and lecithin to ensure compatibility. Each sample was heated in the range of temperature 25°C to 300°C at a heating rate of 5°C per minute. The thermograms were observed for enthalpy changes, appearance/vanishing of peaks, and changes to a peaks onset time, shape, and relative area.

In vitro anti-inflammatory evaluation

Inhibition of albumin denaturation

Preparation of Phosphate Buffer Saline (PBS)

A solution of PBS was prepared by dissolving an accurately weighed quantity of 8 g NaCl, 0.2 g KCl, 1.44 g disodium hydrogen phosphate and 0.24 g potassium dihydrogen phosphate in deionized water to produce 1 L of solution.

The technique of inhibition of albumin denaturation reported previously (Singh et al., 2020; Kumari et al., 2015) was used with slight modifications. The volume of each component of the reaction mixture was reduced to half its volume.

The phytosomes were individually dissolved in DMSO and appropriately diluted to prepare solutions of 100, 200, 300, 400 and 500 µg/mL concentration. A solution of 1% BSA in deionized water was prepared for the test.

The reaction vessel was filled with 200 µL of BSA, 1400 µL of PBS and 1000 µL of the test solutions. Ibuprofen solution (1 µg/mL) was used in the positive control and distilled water was used in the negative control vessels instead of test solution.

The reaction mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. The mixtures were then allowed to cool to room temperature and

the absorbance of constituent of each vessel were analyzed in UV-Visible spectrophotometer at 660 nm. The inhibition of percent denaturation of albumin was determined using the following formula:

% Denaturation inhibition = (1−D/C) × 100%

Where D is the absorbance reading of the test sample, and C is the absorbance reading without test sample (negative control).

Antiprotease action

Preparation of Tris-HCl buffer

An accurately weighed quantity of 121.44 g of Tris was dissolved in 800 mL of distilled water. The pH of the solution was adjusted to 7.0 by addition of appropriate volume of concentrated HCl and the final volume of the solution was made up to 1 L with distilled water.

The technique of antiprotease action reported by Oyedepo *et al.,* 1995 and Sakat *et al.,*2010 was used with slight modifications. The reaction mixture was prepared with 0.06 mg trypsin, 1 mL 20 mM Tris-HCl buffer (pH 7.0) and 1 mL phytosome sample of different concentrations (100 - 500 µg/mL). The mixture was incubated at 37°C for 5 min followed by the addition of 1 mL of 0.8% w/v solution of casein in water. The mixture was incubated additionally for 20 min. In order to stop the reaction, 2 mL of 70% perchloric acid was added to the mixture. The turbid suspension obtained after the reaction was centrifuged and the absorbance of the supernatant was recorded at 210 nm against buffer as blank. The percentage inhibition of protease inhibitory activity was calculated by the following formula:

Percentage inhibition = (Abs control –Abs sample) x 100/ Abs control

Stability studies of optimized phytosome formulation

The prepared phytosomes were subjected to stability studies at 40±2°C/75±5% RH and 30±2°C/60±5% RH according to the ICH guidelines for a period of 3 months.

Results and Discussion

The preliminary investigations of the leaf, leaf extraction and qualitative phytochemical analysis was performed and the results are reported in table 2 and 3.

Table 2 Results of preliminary investigation of the leaf

Plant Part	Parameter	Value Obtained (%)
Annona Squamosa Leaf	Foreign Matter	1.2
	Total Ash	0.5
	Water Soluble Ash	1.5
	Acid Insoluble Ash	0.59
	Ethanolic Soluble extractives	12
	Water Soluble Extractives	8
	Loss on Drying	2.78
	Extraction Yield	11.3

Table 3 Qualitative phytochemical screening

Chemical Tests	Observation checked for	Inference
Alkaloids		
Mayer's reagent	cream colour precipitate	+
Hager's reagent	yellow colour precipitate	+
Wagner's reagent	reddish brown precipitate	+
Dragendorff's reagent	reddish brown precipitate	+
Glycosides		
Froth test	Frothing is seen	+
Kedde's Test	No color	-
Bontrager's Test	Rose pink or red color in the ammonical layer not found	+
Keller-Kiliani	No color in acetic acid layer	-
Phenols/Tannins		
Ferric chloride	Blue green color	+
Gelatin Solution	White precipitate	+
Alkaline reagent test	Yellow to red precipitate	+
Vanillin HCl test	Purplish red color	+
Flavonoids		
Shinoda test	red color	+
Alkaline reagent test	Yellow color that turns red on acidification	+
Zinc HCl reductino test	red color	+
Proteins		
Millon's Test	white precipitate, turns red on heating	+
Ninhydrin Test	Voilet color	+
Sterols/triterpenoids		
Liberman-Burchard Test	Brown ring at junction Upper layer turns green	+
Salkowski Test	Yellow color in lower layer	+

Preparation of phytosomes

The phytosomes loaded with *Annona squamosa* leaf extract were prepared using solvent evaporation method. In this technique, the phytoconstituents or extract and the lipid (lecithin) are kept in a flask containing organic solvent. This reaction mixture is kept at an optimum temperature usually 40°C for specific time period to attain maximum drug entrapment in the phytosomes formed. The organic solvent is then removed using rotary evaporator.

Particle size and size distribution

The particle size and size distribution for each batch of phytosomes was determined using zeta sizer. The formulations ranged from 678 nm to 2617 nm in size with a polydispersity index varying between 0.368 - 0.718 (table 4).

Table 4 Particle size and size distribution of various batches of phytosomes

Formulation Code	Particle Size (nm)	Polydisperisty Index (PDI)
ASP 1	927	0.528
ASP 2	1423	0.457
ASP 3	2617	0.611
ASP 4	678	0.718
ASP 5	1166	0.368
ASP 6	2107	0.529

It was evident from the results of the particle size that the amount of lipid and extract had a significant effect on the particle size of the phytosome. The phytosomes prepared with lower lipid were found to be of higher sizes whereas those with higher concentration of the lipid were small in size. The higher ratio of extract was found to increase the particle size of the phytosomes.

Surface morphology (visualization)

The phytosomes were visible as rigid, spherical vesicles in SEM image. The surface of the phytosome vesicles was found to be regular and smooth.

Differential Scanning Calorimetry

The thermogram of soya lecithin gives distinct peak at 57°C indicating melting. Sharp endothermal peak was found in the thermogram of the phytosome at 260°C.

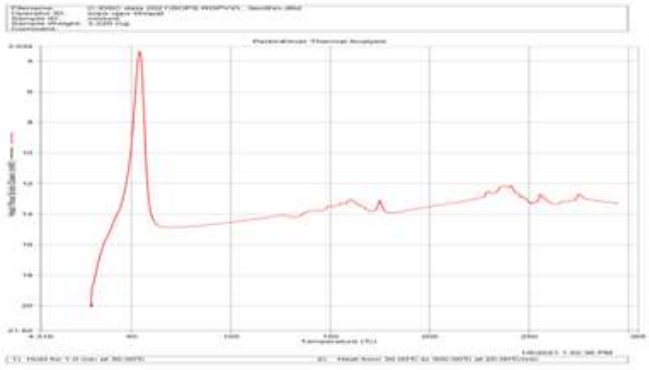

Figure 1 DSC thermogram of Lecithin

From the DSC it can be concluded that a stable formulation is formed by some molecular interaction that can be etiher van der waals forces or hydrogen bonding between extract and phospholipids that distributed the extract molecularly into phospholipid.

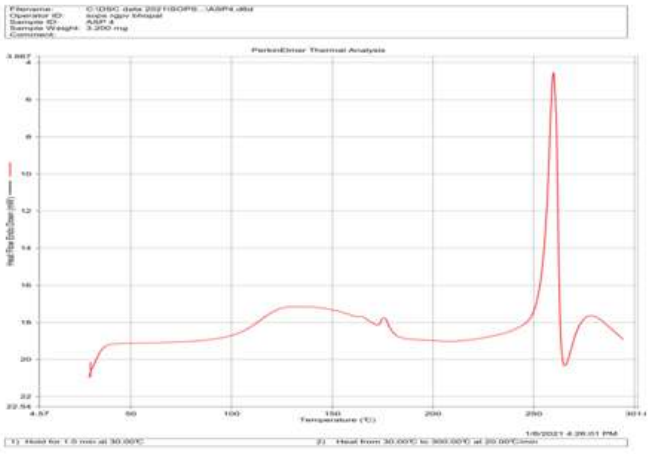

Figure 2 DSC thermogram of Phytosome (ASP 4)

Anti-inflammatory action of phytosomes

The anti-inflammatory activity for the phytosomes loaded with hydroalcoholic extract of *Annona squamosa* leaves was evaluated using protein denaturation methods. The results are reported in table 5 and 6.

Table 5 Inhibition of albumin denaturation by phytosomes

Treatment	Inhibition of albumin denaturation (%)					
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL	10 µg/mL
ASP 1	7.5±2.291	16.1±2.156	27.3±1.194	33.5±2.167	39.6±3.869	ND
ASP 2	13.37±1.657	24.97±2.623	34.69±3.034	53.05±3.165	56.64±3.194	ND
ASP 3	5.29±1.163	11.24±1.196	18.06±1.695	22.37±2.068	30.06±3.162	ND
ASP 4	16.36±2.165	24.35±2.314	34.64±3.821	53.83±3.292	67.36±2.998	ND
ASP 5	11.01±1.659	20.18±2.617	32.86±3.009	44.29±3.616	53.93±4.209	ND
ASP 6	9.39±2.002	14.37±2.099	23.63±2.228	36.17±2.069	44.36±3.754	ND

Ibuprofen	ND	ND	ND	ND	ND	52.28± 3.261

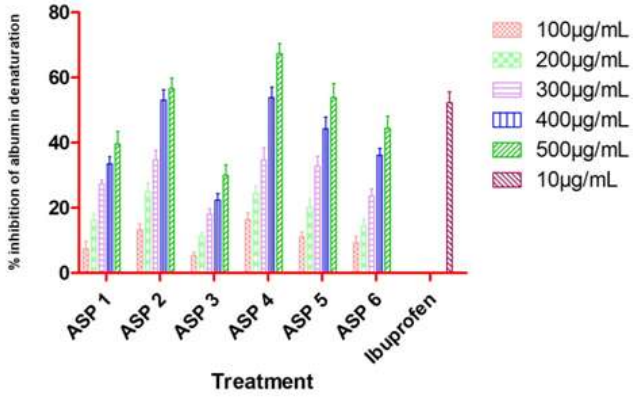

Figure 3 Comparative Inhibition of albumin denaturation by phytosomes

Table 6 Antiprotease action by phytosomes

Treatment	Inhibition of Protease Action (%)					
	10 µg/mL	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL
Ibuprofen	53.17± 2.146	ND	ND	ND	ND	ND
ASP 1	ND	4.12± 0.899	7.21± 1.033	13.43 ±1.066	15.08 ±1.066	20.18 ±1.523
ASP 2	ND	7.95± 1.011	13.50 ±1.066	19.87 ±2.666	26.77 ±3.033	38.96 ±3.033
ASP 3	ND	5.18± 0.911	8.14± 0.833	13.57 ±1.011	17.91 ±1.633	22.35 ±2.033
ASP 4	ND	9.63± 1.033	13.64 ±1.168	21.82 ±2.011	34.22 ±2.333	45.03 ±3.211
ASP 5	ND	7.29± 1.333	12.46 ±0.933	16.66 ±2.066	23.11 ±1.333	36.22 ±3.011
ASP 6	ND	6.04± 0.933	9.47± 0.666	14.55 ±1.033	19.28 ±2.011	28.24 ±2.988

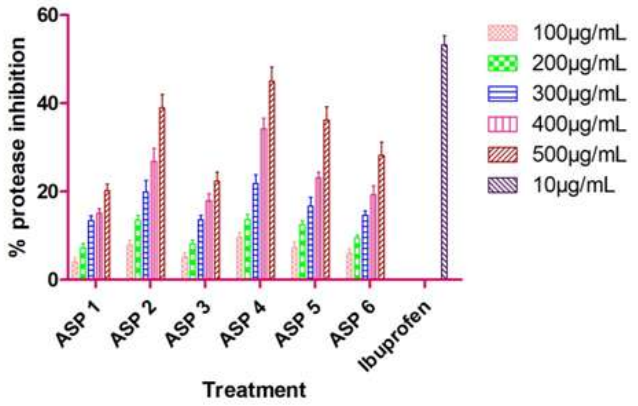

Figure 4 Comparative antiprotease action depicted by phytosomes

It was clear from the results that all the phytosomal formulations were able to exhibit anti-inflammatory action by virtue of their inhibitory action against protein denaturation. The formulation ASP 4 was having the highest ability to inhibit both albumin denaturation and protease activity.

Stability study of phytosomes

The formulation ASP 4 was subjected to stability studies according to ICH guidelines and the phytosomes were evaluated for particles size changes after 3 month. Stability of the formulation must be maintained until it reaches the targeted tissue. Lecithin plays a vital role in maintaining the physical stability by making the lipid bilayer flexible. It was found that at room temperature and accelerated temperature conditions very slight variation occurs in mean particle size. Hence the formulation could be considered to be stable in the storage conditions.

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