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Herbosome an approach to deliver *Aegle marmelos* extract: Formulation, characterization and stability study

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ABSTRACT

Aegle marmelos fruit has tremendous potential to treat the various ailments for instance; diabetes, microbial diseases and many more. Despite, having abundant therapeutic activity poor lipid solubility and gastric instability limits the efficacy of *Aegle marmelos* extract (AME). With an idea to overcome these hurdles, AME was formulated as Herbosome in this study. The concept of Herbosome is gaining popularity, as it provides a sheet of lipid on the outer part of drug or extract, which helps in the bioavailability enhancement. In the present work, AME was prepared by cold extraction method and percentage yield was found to be 20% w/w. Here, Marmelosin one of the major phytoconstituent in *Aegle marmelos*, was used as a marker for standardization of the prepared extract. Further, preliminary phytochemical screening and thin layer chromatography (TLC) was carried out. Whereas, quantitative estimation of Marmelosin in AME by High-performance thin layer chromatography (HPTLC) was conducted. In further steps, pre-formulation parameters (effect of several processes and formulation parameters) were studied and Herbosome loaded AME was formulated by conventional technique i.e. thin film method and soyalecithin were used as phospholipids. Several parameters including, percent entrant efficiency (%EE), particle size, polydispersity index (PDI) and zeta-potential were taken into consideration for the evaluation of AME Herbosome. Later on, followed by, *In vitro* release of optimized formulation was studied and the formulation was able to release up to 92% even after 12 hours. After developing successful AME Herbosome, stability study was performed as per the International Conference on Harmonisation (ICH) guidelines up to the period of a month. Herbosome was found to be stable at room temperature, even between 2-4ºC. Henceforth, to confirm the efficacy of optimized formulation, *In vitro* studies (antidiabetic) are going on in the laboratory.

Keywords: *Aegle marmelos*, *Aegle marmelos* extract, Herbosome, *In vitro* release, Marmelosin, Thin film method, soyalecithin, and stability study

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List of Abbreviations

AME	*Aegle Marmelos* Extract	min	Minute
cm	Centimeter	MLVs	Multi-lamellar Vesicles
cm^{-1}	Centimeter inverse	M	Molar
ºC	Degree Celsius	nm	Nanometre
DSC	Differential Scanning Calorimetry	%EE	Percentage Entrapment efficiency
FT-IR	Fourier Transform Infrared	%	Percentage
HPTLC	High-Performance Thin Liquid Chromatography	PBS	Phosphate Buffer Saline
Hr	Hour	PDI	Poly Dispersity Index
ICH	International Conference on Harmonization	R_f	Retention factor
µg	Micro-gram	R_t	Retention time
µg/mL	Microgram per milliliter	RPM	Rotation Per Minute
mg	Milli-gram	SD	Standard deviation
mL	Milli-litre	TLC	Thin Layer Chromatography

1. INTRODUCTION:

Aegle marmelos, eminently known as Bael family Rutaceae; is used as home remedies since the antiquity across the world. It is one of the famous Ayurvedic medicinal plants especially in India [1]. As per data, almost every part of *Aegle marmelos* for example; leaves, fruits, bark, seeds, and roots have numerous medicinal values [2]. Literature shows that the fruit part of the plant is enriched with the number of secondary metabolites (Fig 1).

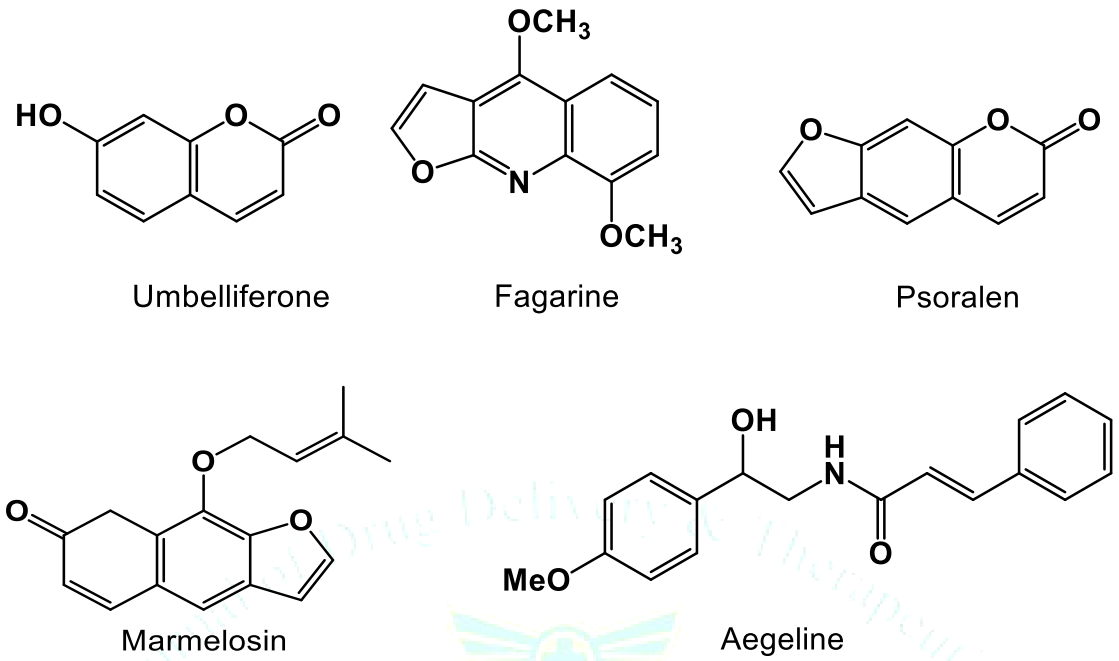


Fig 1 Chemical structure of secondary metabolites in *Aegle marmelos*

Till date, many applications of *Aegle marmelos* fruit in the management of pathophysiology have been explored by researchers. Due to its significant potential, scientists have tried to develop many formulations but poor lipophilicity of extract, resulting in the terms of low bioavailability [3]. That ultimately leads to a decline in the desired therapeutic actions. By keeping in mind these issues, we have tried to develop the Herbosome of AME in the current discussed study.

Herbosome is the latest technology, especially in herbal drug delivery. Herbosome is composed of two words "Herbs" meaning plant and "some" meaning cell-like. Scientists working in this field have evaluated that Herbosomes are sufficiently able to deliver the drug in an appropriate manner inside the body and makes it bioavailable [4]. The inclusion of phospholipids with the phytocompound helps in the enhancement of lipid solubility and ultimately permeability [5]. As per literature search and our present knowledge, this is the first attempt made on to prepare Herbosome using *Aegle marmelos.* Additionally, to check the medicinal potential (antidiabetic) of developed AME Herbosome, *in vitro* study is going on in our laboratory and soon we will publish elsewhere.

2. MATERIALS AND METHODS:

2.1 Solvents, chemicals & Instruments used:

Soyalecithin was purchased from Sigma Aldrich. Cholesterol was procured from Himedia, Mumbai. Marmelosin was procured from Natural Remedies. Chloroform, Toluene, Ethyl acetate was of analytical grade and obtained from Spectrochem Mumbai, INDIA. Methanol was the product of Qualigens, India. Water obtained from Milli- Q Biocel, Millipore (USA) was used throughout the study. Basic unit Differential scanning calorimetry (DSC) 214 Intra-cooler (NETZSCH, Germany), FT-IR spectrophotometer ALPHA, OPUS 7.5 (Bruker, Germany), UV Spectrophotometer (Shimadzu UV-1800, Japan), Magnetic Stirrer (Remi, India), pH meter (pH Tutor, Eutech, Singapore), Micro-pipette (Eppendorf, India), Centrifuge (R-24,Remi, India), Analytical Balance (JB1603-C, Mettler Toledo, India), U. V. Chamber, Rotavapor (R-300, Buchi), and HPTLC (CAMAG) Linomat IV sample applicator.

2.2 Collection of the plant material:

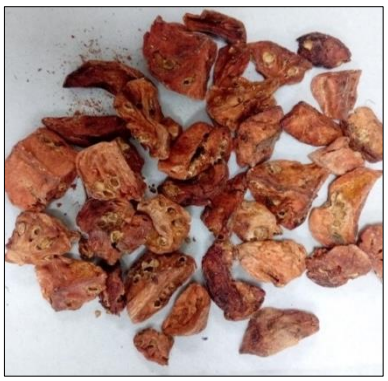


Fig 2 Fruit of *Aegle marmelos*

Fruit of *Aegle marmelos* (Fig 2) was procured from LVG (Lalubhai Vrijal Gandhi), Ahmedabad, India.

2.3 Preparation of *Aegle marmelos* fruit extract:

Aegle marmelos fruit was powdered and dried at room temperature. 100 gm of powder was extracted with methanol at room temperature using a cold extraction

process. Further, the percentage of yield value was calculated. The extract was allowed to dry at room temperature and stored in the proper conditions.

2.4 Chemical characterization of prepared AME:

2.4.1 Standardization of fruit extract: After successful preparation of AME it was screened to identify the presence of various secondary metabolites by following discussed techniques [6].

2.4.1.1 Preliminary phytochemical analysis:

Extract of *Aegle marmelos* was subjected to chemical tests using standard procedures.

2.4.1.1.1 Detection of Alkaloids: Dragendroff's test:

In 2mg of extract, 5ml of 2M HCl was added. To this, 1ml of Dragendroff's reagent was added. Formation of orange or orange-red precipitate indicated the presence of alkaloids.

2.4.1.1.2 Detection of Terpenoids: Liebermann-Burchard's test:

2mg of the extract was dissolved in acetic anhydride; allowed for cooling, followed by heating and then 1ml of concentrated sulphuric acid was added. Presence of pink color confirmed triterpenoids presence.

2.4.1.1.3 Detection of Flavonoids: Shinoda's test:

In 2mg of extract, 10 drops of dilute Hcl was poured followed by a small piece of magnesium ribbon were added. Formation of pink, reddish or brown color indicated the presence of flavonoids.

2.4.1.1.4 Detection of Saponins: Foam test:

The extract was diluted with 20ml of distilled water in measuring cylinder and shaken about 15 minutes. Formation of 1cm foam indicated the presence of Saponins.

2.4.1.1.5 Detection of Coumarins:

10mg of the extract was dissolved in methanol and alcoholic KOH was added. The appearance of yellow color got decolorized while adding concentrated HCl showed the presence of Coumarins.

2.4.1.1.6 Detection of Phenolic compounds:

The extract was dissolved in alcohol and 1 drop of neutral ferric chloride was added to this. The appearance of an intense blue color indicated the presence of Phenolic compounds.

2.4.1.2 Thin layer chromatography (TLC) of fruit extract:

TLC was performed to characterize the AME using Marmelosin (Fig 3) as a standard. Solvent system Toluene (9.3): Ethyl acetate (0.7) was used. Detection was carried out under the Ultraviolet (UV) at 365nm.

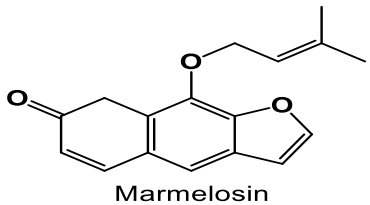


Fig 3 Chemical structure of Marmelosin

2.4.1.3 Quantitative analysis of Marmelosin using HPTLC:

2.4.1.3.1 Preparation of standard Marmelosin solution: A standard solution of 100µg/ml was prepared by dissolving 1 mg of Marmelosin in methanol. A calibration curve between peak areas vs. concentration of Marmelosin was obtained.

2.4.1.3.2 Estimation of Marmelosin in the extract: 50 µl of the extract was applied on a TLC plate. The peak area of the test solution corresponding to standard Marmelosin was determined [7].

2.5 Pre-formulation study: Herbosome was prepared through a thin film or a so-called solvent evaporation method. As a lipid carrier, Soyalecithin was used. AME was prepared in the two different solvents i.e. water and phosphate buffer saline (PBS) to form a thin film. A calibration curve was obtained using these two solvents and extract in the water phase was found significantly more stable [8]. Hence, water containing extract was chosen for the preparation of AME Herbosome.

2.5.1 Calibration curve of extract in water:

2.5.1.1 Preparation of stock solution:

A stock solution of 100µg/ml was prepared by dissolving 2.5mg of extract in distilled water in a 25ml volumetric flask.

2.5.1.2 Preparation of working solution:

Appropriate aliquots of stock solution were pipette out and diluted with water to get a working solution in the range of 10-60 µg/ml. A calibration curve was plotted between concentrations vs. absorbance.

2.5.1.3 Analysis of working solution by UV-Visible spectrophotometer:

A calibration curve in water was obtained by measuring the absorbance of working solution against water as a blank. All the reading was recorded in triplicates.

2.5.2 Calibration curve of extract in PBS:

2.5.2.1 Preparation of stock solution:

A stock solution of 100µg/ml was prepared by dissolving 2.5mg of extract in PBS (pH-7.4) in a 25ml volumetric flask.

2.5.2.2 Preparation of working solution:

Appropriate aliquots of stock solution were pipette out and diluted with PBS (pH-7.4) to get working solution in the range of 10-60 µg/ml. And the calibration curve was plotted between concentrations vs. absorbance.

2.5.2.3 Analysis of working solution by UV spectrophotometer:

A calibration curve using water was obtained by measuring the absorbance of a working solution. All the reading was recorded in triplicates.

2.6 Extract-excipients compatibility study:

Differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) techniques were performed to evaluate the interaction between extract and excipients. Thermogram of an individual component was also recorded. A binary mixture of extract: Excipients in a ratio of (1:1) was prepared by physical mixing. The sample was weighed out and heated under dry nitrogen in the scanning range of 200°C-250°C. To perform IR, around 2mg of the mixture was kept on ATR. The samples were then scanned in the range of 400-4000cm^{-1}.

2.7 Formulation of Herbosome:

2.7.1 Herbosome was prepared through the conventional thin film hydration technique. Selected lipid soyalecithin was dissolved in organic solvents and the solvent was removed

by means of evaporation using Rotavapor. Then dry lipid film deposited on the flask wall was hydrated by the addition of hydrating fluid containing extract. Hydration of dry film yields a population of multi-lamellar vesicles (MLVs) heterogeneous both in size and shape.

2.7.2 Optimization of instrumental condition and formulation parameter for prepared Herbosome:

The formulation was exposed to different temperature conditions (40, 50 and 60ºC) and speed (100, 160, 200 rpm). The evaluation was done on the basis of film morphology and integrity, recovery of solvents and duration of film formation. Different extract and lipid ratio were screened on the basis of entrapment efficiency of extract, size, and PDI.

2.8 Characterization of AME Herbosome: Following-listed parameters were evaluated [9].

2.8.1 Microscopic evaluation and measurement of size, zeta potential, and polydispersity index (PDI) of Herbosome:

Microscopic characterization (optical microscopy) was performed to ensure formation and lamellarity of Herbosome. The mean particle size and PDI of AME loaded Herbosomes were recorded. Further, the zeta potential of Herbosome was determined using Zeta sizer.

2.8.2.2 Entrapment efficiency determination:

The entrapment efficiency of AME Herbosome formulation was determined by centrifugation method. The dispersed Herbosomes were centrifuged at 13,500 rpm, 4°C temperature for 45 min. The unentrapped drug was present in the supernatant of the centrifuge tube. It was collected and diluted up to 100 times and analyzed through UV visible spectrophotometer. The percentage entrapment efficiency (% EE) of liposome was calculated using the following equation:

$$\% EE = [(Wa - Ws) / Wa] \times 100$$

Where,

Wa = amount of extract loaded in formulation

Ws = amount of free extract

2.9 *In vitro* release study of AME:

In vitro release of prepared Herbosome was carried out using dialysis bag method and PBS (pH 7.4) was used as a medium. Dialysis bag was placed in 250 mL of PBS and stirred at 100rpm over a magnetic stirrer at 37°C (Fig 4).

Fig 4 *In vitro* release study

Aliquots were collected at 15, 30, 60, 120, 180, 240, 300, 360, 420, 480, 720 min time interval. Sink condition was maintained by replacing the aliquots with the same amount of fresh PBS. Samples were analyzed using UV visible spectrophotometer at 280.2 nm. Experiments were carried out in triplicate and results presented as the mean of all three values with standard deviation (SD).

2.10 Stability study of AME Herbosome:

The purpose of the stability study was to provide evidence on drug quality. Stability study as per ICH guidelines was carried out at room temperature and at freeze temperature [10].

3 RESULTS:

3.1 The AME was successfully prepared and the percentage yield was found to be 20% w/w.

3.2 Standardization of fruit extract:

3.2.1 Preliminary phytochemical analysis: Following (Table 1) comprises the results obtained from the chemical test.

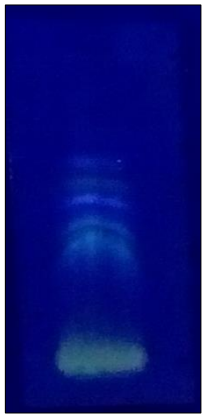
Table 1 Chemical characterization of Marmelosin

Class of compounds	Test	Observation
Alkaloid	Dragendroff's test	+ve
Steroids & Terpenoids	Liebermann Burchard's test	+ve
Flavonoids	Shinoda's test	+ve
Saponins	Foam test	-ve
Phenolic	-	+ve

Where, +ve = Positive, and –ve = Negative

3.2 TLC of fruit extract: TLC of AME was performed using Marmelosin as standard and R_f value was calculated and found to be 0.6 (Table 2).

Table 2 TLC of AME and standard Marmelosin

TLC Report			Chromatographic Condition
A	B	C	**Solvent system**: Toluene (9.3) :Ethyl acetate (0.7) **Detection**: UV long wavelength (365nm) **Rf value:** 0.60 **A**= Fruit extract **B**=Standard(Marmelosin) **C**= Comparison of standard (Marmelosin) and Methanolic extract
			

3.3 Quantification of Marmelosin in extract using HPTLC:

HPTLC fingerprint of different concentration for standard Marmelosin and AME was observed in long UV and short UV that is shown in Fig 5; A and B, respectively.

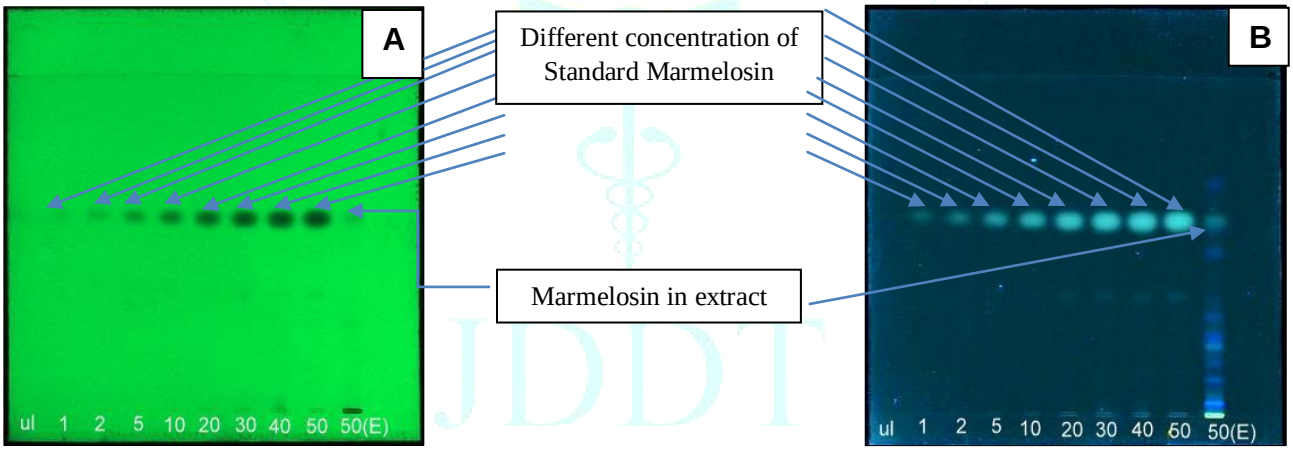


Fig 5A) TLC visualized under 254nm in UV chamber B) TLC visualized under 366nm in UV chamber

3.3.1 Calibration curve of standard Marmelosin using HPTLC:

Calibration curve of Marmelosin was recorded (Fig 6). This curve was further used to quantify Marmelosin in the AME.

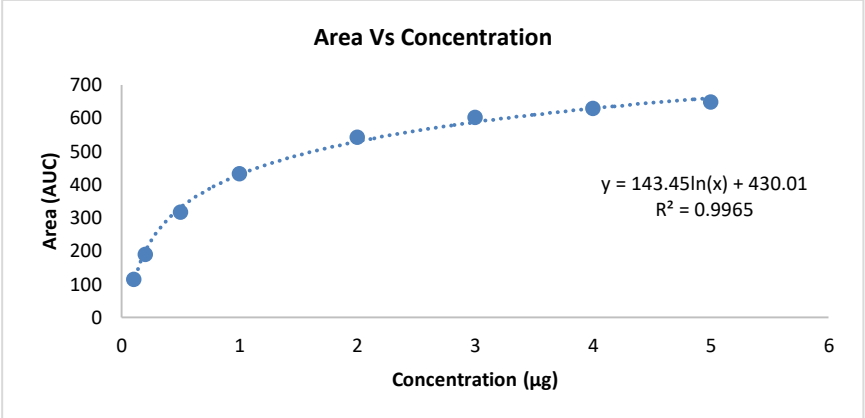


Fig 6 Calibration curve of standard Marmelosin

3.3.2 Estimation of Marmelosin in the extract:

The percentage of Marmelosin present in the extract was found to be 0.422%w/w (Fig 7).

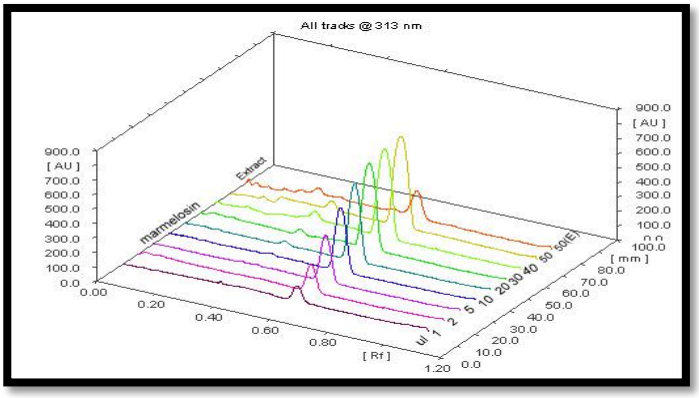


Fig 7 showed 3D surface graph Marmelosin and extract

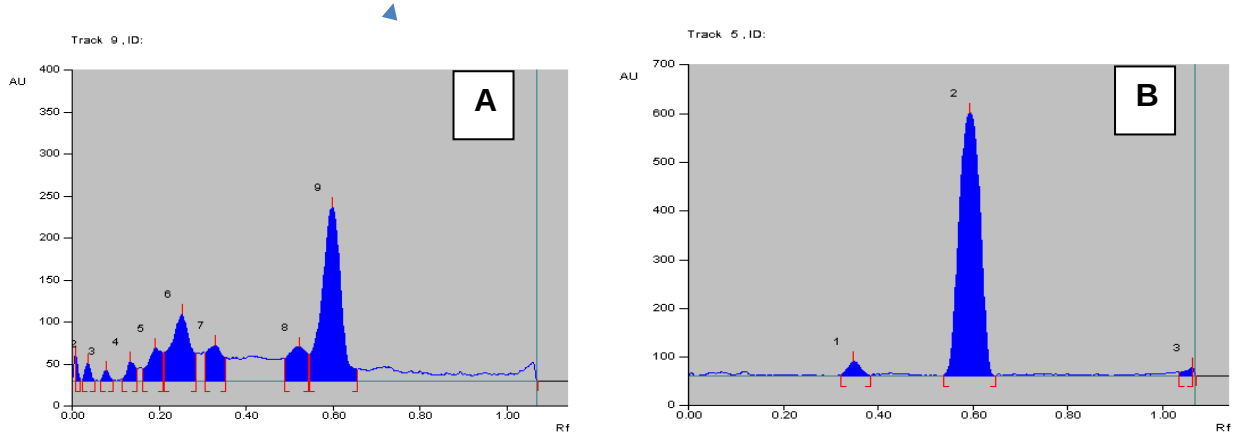


Figure 8. Calibration curve of Marmelosin using HPTLC

3.3.3.1 Determination of λ_{max} (absorbance maxima) in fruit extract:

3.3.3.2 Calibration curve of fruit extract in water: The calibration curve was developed using water as a medium at 280.2nm (Fig 9).

3.3.3.3 Calibration curve of fruit extract in PBS pH 7.4: The calibration curve was recorded using PBS as a medium at 280.2nm (Fig 10).

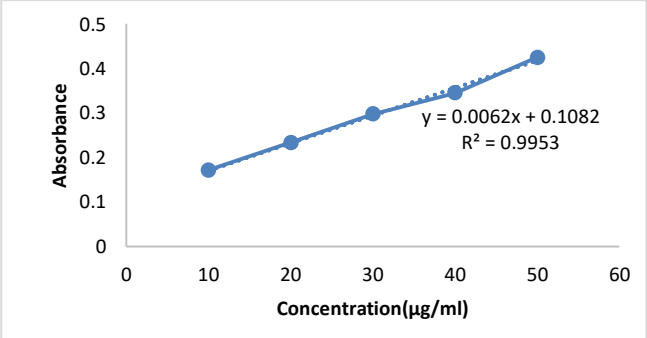


Fig 9 Calibration curve of fruit extract in water (n=3)

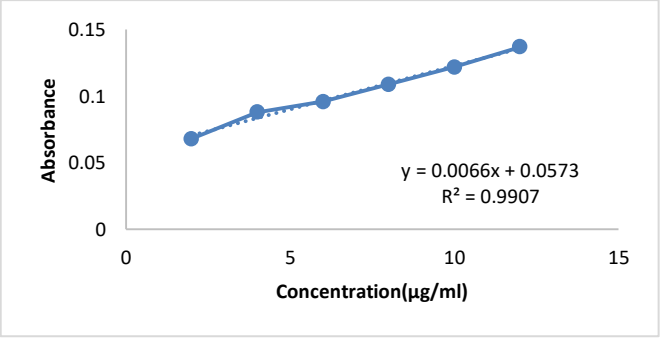


Fig 10 Calibration curve of fruit extract in phosphate buffer (n=3)

3.4 Extract – excipients compatibility study:

3.4.1 Extract – excipients compatibility study using DSC:

DSC curve of standard Marmelosin showed an endothermic peak at 102.7°C. Incorporation of cholesterol helps to maintain the rigidity of Herbosome vesicles. Additionally, AME containing Marmelosin along with the physical mixture of an extract with all excipients used in Herbosome formulation has shown a peak at the same position. This study showed that extract is not having any significant thermal interactions between extract and selected excipients (Fig 11).

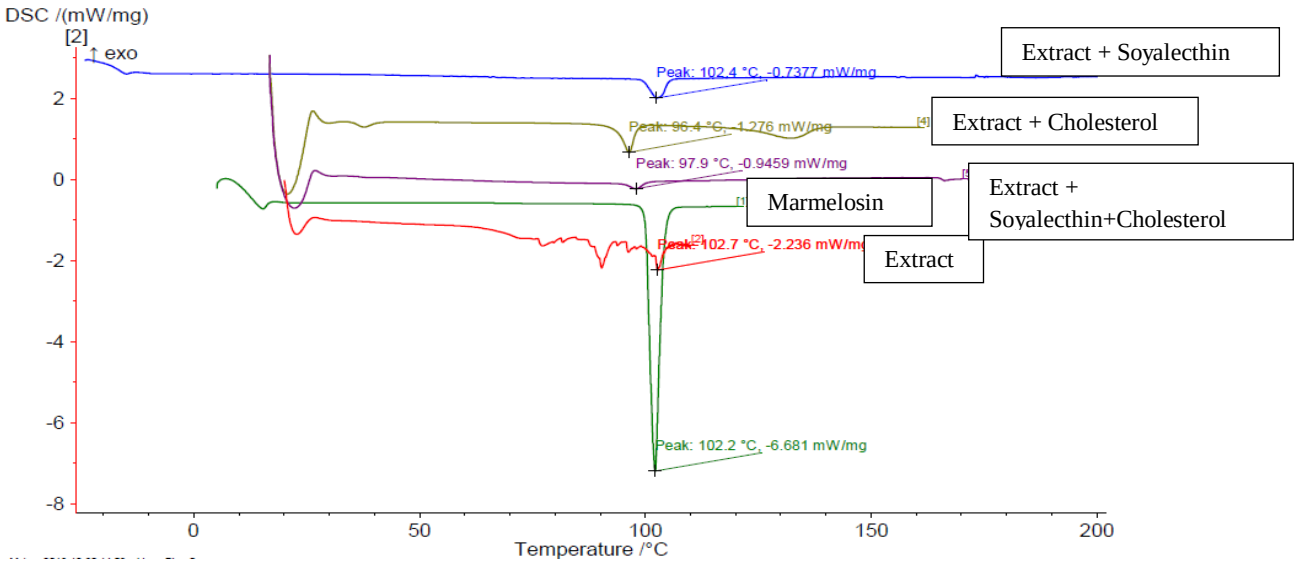


Fig 11 DSC overlay thermogram of a mixture of extract and excipients

3.4.2 Extract – excipients compatibility study using FTIR: To confirm the interaction, FTIR was performed and no interaction was found among between extract and excipients (Fig 12).

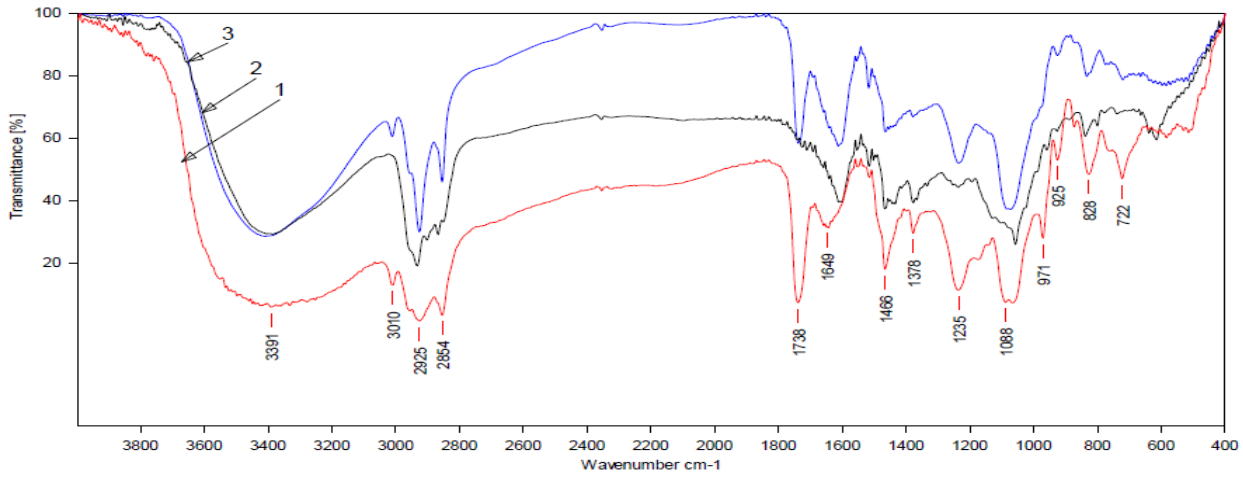


Fig 12 FTIR overlay spectra of the extract with soya lecithin and cholesterol

1) Cholesterol+soyalecithin+extract, 2) Cholesterol+extract, 3) Soyalecithin+extract

3.5 Screening of the process and formulation parameters for preparing Herbosome:

3.5.1 Temperature and speed: From the observation, it was concluded that F-2 and F-3 batches produced uniform film compared to all other batches. As the temperature increased, the duration of film formation was decreased but instead of a uniform film, a broken film was formed. That might be due to, the increased evaporation rate of the solvent because of high rotation per minute (rpm) and temperature in batches from F4-F9 leading to uneven film formation. However, F-2 batch took a long time for film formation in comparison to F-3 batch. Selected parameters i.e. the speed of rotation at 160 rpm and temperature at 40°C was considered for further investigation. Following (Table 3) summarizes about the optimized parameters.

Table 3 Representing screening of process parameters Temperature and speed used to developed various batches

Formulation code	Temp. (°C)	Pressure (millibars)	Speed (rpm)	Uniformity of film	Recovery of solvent	Duration of film formation
F-1	40	600-800	80	No	Nil	8min
F-2	40	600-800	160	Yes	Nil	6min
F-3	40	600-800	200	Yes	Nil	4min
F-4	50	600-800	80	No	Nil	6min
F-5	50	600-800	160	No	Nil	5min
F-6	50	600-800	200	No	Nil	4min
F-7	60	600-800	80	No	Nil	3min
F-8	60	600-800	160	No	Nil	3min
F-9	60	600-800	200	No	Nil	3min

3.5.2 Optimization of formulation parameters:

Effect of extract and lipid molar ratio: Maximum entrapment efficiency, required particle size, and zeta potential was achieved using 1:4 extract to lipid ratio (Table 4) and Soya lecithin to cholesterol lipid molar ratio of 5.5:1.5 (Table 5). The screened ratios were carried forward for further studies.

Table 4 Effect of extract: lipid ratio (data represents mean ± SD, n=3)

Extract to lipid ratio E:L	Entrapment efficiency (%EE)	Size ± SD (nm)	PDI ± SD	Zeta potential ± SD (mv)
1:1	35.36	257 ± 6.4	0.592±0.21	-48.6±4.6
1:2	45.56	303.5 ± 6.71	0.458±0.41	-56.4±5.6
1:3	58.71	283.6 ± 4.6	0.497±0.36	-52.3±6.19
1:4	87.64	306.8±3.8	0.467±0.20	-48.3±5.98

Table 5 Effect of lipid molar ratio on Herbosome size, entrapment efficiency, and Zeta potential

S:C	Entrapment efficiency (%EE)	Size±SD	PDI±SD	ZP±SD
6:1	81.97	345.2±3.6	0.592±0.21	-50.6±4.6
5.5:1.5	87.64	306.5 ± 6.71	0.458±0.41	-48.3±5.6
5:2	58.71	283.6 ± 4.6	0.497±0.36	-52.3±6.19
4:3	45.56	249.8±3.8	0.467±0.20	-48.3±5.98
3:4	35.36	256.2±3.6	0.570±0.36	-43.6±6.71

3.6 Characterization of optimized Herbosome:

The optical microscopy confirmed the formulation of Herbosome (Fig 13). And, the optimized Herbosome formulation was found of an average size 306.3 nm and zeta potential of -48.7mV (Fig 14). Additionally, entrapment efficiency for an optimized batch of Herbosome was calculated from the above-listed formula and it was found to be 87.64±2.33%.

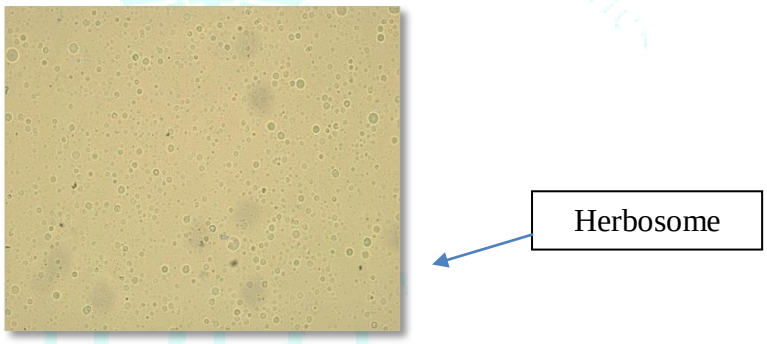


Fig 13 Herbosomes under the microscope

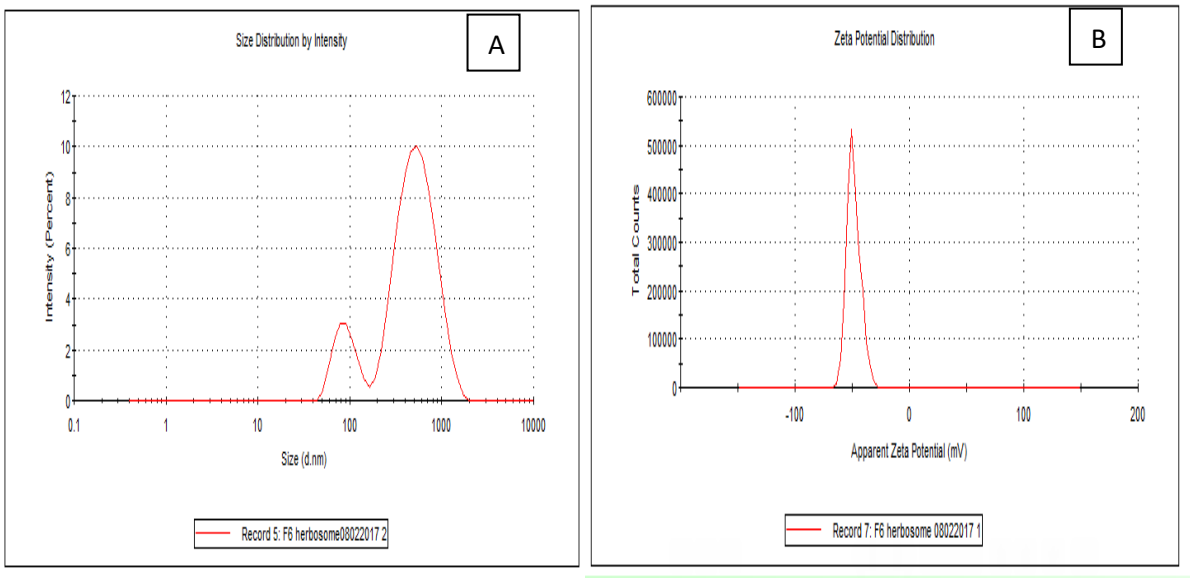


Fig 14A) Size and PDI graph B) Zeta potential graph of Herbosome

3.7 *In vitro* release of extract from Herbosome formulation: *In vitro* release of optimized formulation was carried out and release of the extract was found to be 91% in 8 hours whereas, 92% in 12 hours (Fig 15).

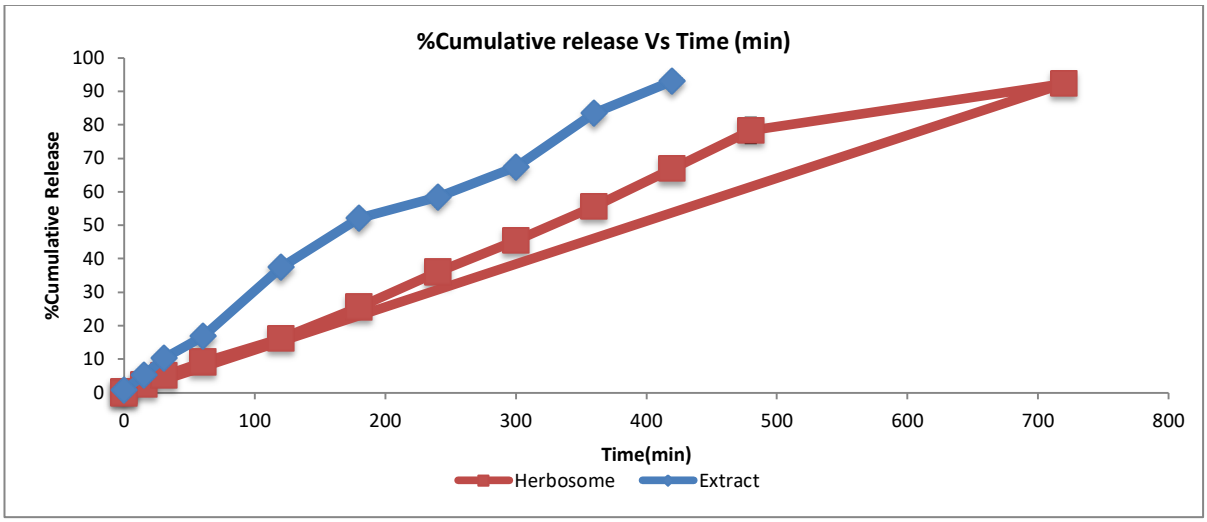


Fig 15 *In vitro* study of Herbosome in comparison with parent extract

3.8 Stability study: The data of stability studies indicated that the prepared Herbosome formulation was more stable at a frozen temperature (2-4ºC) (Table 6 & Table 7). And the optimized formulation was found to be stable over a period of one month.

Table 6 Stability study of Herbosome at room temperature

Parameters	Day of preparation	After one week	After fifteen days	After one month
Size(nm)	389±1.9	392.4±2.53	398.2±3.52	402.7±3.68
PDI	0.486±0.013	0.530±0.01	0.540±0.03	0.528±0.02
Zeta potential	-52.1±2.3	-41.9±1.97	-35.3±3.23	-38.2±2.71
Entrapment efficiency	84.83±1.9	82.15±0.71	78±1.20	72±0.91

Table 7 Stability study of Herbosome at freeze temperature (2-4ºC)

Parameters	Day of preparation	After one week	After fifteen days	After one month
Size(nm)	311.11±1.9	315.8±1.76	323.1±4.67	330.5±2.94
PDI	0.467±0.013	0.454±0.01	0.422±0.02	0.493±0.023
Zeta potential	-50.3±2.3	-49.3±2.61	-46.9±3.2	-50.3±2.71
Entrapment efficiency	87.64±1.9	85.45±0.71	82.23±0.89	80.6±1.60

4 DISCUSSION:

Extract of *Aegle marmelos* was prepared by the cold extraction process and standardization of extract was carried out using TLC (Rf = 0.6). Moreover, the concentration of Marmelosin in the extract was quantified using HPTLC which, found to be 0.422µg/mL. A calibration curve using extract was developed in both, water & PBS (pH 7.4). But, water was selected for the preparation of Herbosome due to its good solubility with the extract. Furthermore, interaction studies (extract – excipients) were investigated through DSC and FTIR analytical tools, and no significant interaction was observed. Herbosome was developed using "Thin film hydration technique" followed by, evaluated under the microscope and almost uniform Herbosome was found. The formulation was screened for its release and showed 92% of extract released in 12h. Besides it, AME Herbosome was evaluated for stability up to 1 month and it was found to be stable at room and freeze temperature 2- 4 °C.

5 FUTURE PERSPECTIVE:

AME loaded Herbosome was prepared successfully in the current study. As mentioned earlier, *Aegle Marmelos* has been reported for different beneficial effects; so the prepared formulation can be investigated to treat different ailments. And the exploration of *In vitro* and *In vivo* data will be helpful to ensure the safe and efficacious delivery of the formulation. This research will pave the way to scientists working in the field of either *Aegle Marmelos* formulations for its potent delivery or looking for the treatment of diabetes.

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