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Formulation and characterization of phospholipid vesicles from alkaloid rich fraction of *Alphonsea sclerocarpa*

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ABSTRACT

A Retrospective ethnopharmacological review on *Alphonsea sclerocarpa* belonging to Annonaceae family revealed the presence of alkaloids that confers Anti-fungal, Anti-oxidant and Anti-cancer activities etc. to the plant extract. Lower bioavailability of the alkaloids present in the plant and their enormous pharmacological potential has paved a path for this work. Therapeutic effectiveness of any compound with low bioavailability can be achieved by employing novel drug delivery techniques viz-a-viz phytosomes, Liposomes etc. In the present work novel, phospholipid vesicles from Alkaloid Rich Fraction of *Alphonsea sclerocarpa* were formulated for the enhanced delivery of therapeutically proven alkaloids and were accordingly characterized. Alkaloid Rich Fraction was prepared from the methanol extract by Acid/Base extraction process and was standardized against Boldine using High-Performance Thin Layer Chromatography (HPTLC). Phospholipid vesicles were prepared by varying the ratios of standardized alkaloid Rich Fraction to Soy Lecithin employing Solvent evaporation method. Among the phospholipid vesicles formulated the vesicles containing 1:1 ratio of Alkaloid Rich Fraction to Soy Lecithin was found to be the smallest and stable. The study has shown that the Phospholipid Vesicles of 1:1 ratio can be used for testing the Pharmacological activities further.

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INTRODUCTION

Alphonsea sclerocarpa also known by the local name Pulusumamidi was listed under the rare tree category found in Talakona Hills of Andhra Pradesh (Ram Babu M *et al.,* 2015). The understorey tree is also located along the western ghats in India as well in Srilanka and Malaysia (Rani VN *et al.,* 2017, Fries RE, 1959).In defiance of its availability, the tree is known to have pharmacologically active alkaloids possessing Antioxidant activity (Prasad DN, 2009),Anticancer activity (Joshi DSDS *et al.,* 2018), Antifungal activity (Indrani V *et al.,* 2015), Antimicrobial activity (Doddapaneni SJ *et al.,* 2018). Alkaloids of Aporphine class are proven for their cytotoxic activity and are the paramount type in *Alphonsea sclerocarpa*(Mohamed SM *et al.,* 2010). Aporphine alkaloids like boldine etc. are reported to have lower bioavailability. Undeterred by their pharmacological importance, poor oral bioavailability remained as a black hole in reaping the advantages of the phytotherapeutic (Cermanova J *et al.,* 2016). Conventional formulations cannot increase the bioavailability of the phytoconstituents and hence nanotechnology-based delivery systems such as Phytosomes (Phospholipid Vesicles), Liposomes, Ethosomes

etc. are employed (Bonifacio BV *et al.,* 2014). Contemplating this fact, the plant *Alphonsea sclerocarpa* has been chosen for the study after standardizing it against an Aporphine alkaloid Boldine. The aim of the present study was to develop the phospholipid vesicles of Alkaloid rich fraction of *Alphonsea sclerocarpa* to deliver the alkaloids at a therapeutic level. Phospholipids were known to enthral a hydrophilic head, and a hydrophobic tail part and are also considered to be biodegradable and hence in this study naturally occurring Phosphatidylcholine moiety like Soy lecithin has been employed (Nguyen TL *et al.,* 2017).

MATERIALS AND METHODS

Drugs and Chemicals

Soy lecithin was purchased from Lecico Gmbh Ltd, Germany. Boldine was procured from Sigma Aldrich, USA. All the other chemicals used were of analytical grade and were obtained from Merck Ltd, Mumbai.

Preparation of Alkaloid Rich Fraction

The In-house Methanolic extract from the bark of *Alphonsea sclerocarpa* (100g) was subjected to an acid/base extraction process. It was fractionated using petroleum ether (25ml) and water (25ml) initially. The aqueous layer was separated and extracted thrice with petroleum ether. The extracts were tested for the type of phytoconstituents present, and ether extract was found to possess waxes, steroids, Triterpenoids and other neutral and acidic compounds. The aqueous layer was found to contain Alkaloids, sugars and amino acids. The aqueous layer was then treated with 20ml of 0.2N HCl followed by stirring for 16hrs on a Magnetic stirrer at 100 rpm. The resulting solution was shaken with 20ml of chloroform to remove the non-basic material. The aqueous layer was then basified with ammonium hydroxide until it reached p^H 11 and extracted again with chloroform. The chloroform phase was dried in vacuum rotary evaporator, and the residue (11g) was tested for the presence of alkaloids. The residue so obtained is the Alkaloid Rich Fraction (ARF) (Singh S *et al.,* 2011). It was stored in a desiccator and utilised further. The yield was found to be (11%).

Qualitative and Quantitative standardization of Alkaloid Rich fraction of Alphonsea sclerocarpa using Boldine as a biomarker by HPTLC

Parameters employed in Application

CAMAG Linomat 5 instrument was employed for the study. Nitrogen gas was used as the spray gas and methanol as a solvent. Dosage speed was set to 100nl/s and the syringe of 100 µl was employed. A total of six tracks were applied with 8.0 mm as the application position Y and a band length of 6.0mm. Silica gel 60F 254 precoated aluminium plate was employed as the stationary phase and Ethyl acetate: Methanol (8.5:1.5 v/v) as the mobile phase. Slit dimensions of the scanner were set to 5 mm x 0.45 mm and a scanning speed of 20mm/sec was employed.

Development

10 X 10 cm twin trough glass chamber was employed for the study. Length of the chromatographic run was 8 cm and the development time was about 15 min. The chamber was saturated previously for 15 min and then the plate was immersed in the mobile phase for development. The plate was dried and scanned using CAMAG Scanner.

Formulation of Phospholipid Vesicles

Phospholipid vesicles were prepared by employing Solvent evaporation method. The Vesicles were prepared by reaction of soy lecithin and Alkaloid Rich fraction in 1:1, 1:2 and 2:1 ratio at 55 rpm for 2 h at ambient temperature. The reaction is carried out by dissolving 50, 50 and 100mg of Soy lecithin and 50, 100, 50 mg of Alkaloid rich fraction in 15 ml of methanol correspondingly. The reaction was stirred for 2 h at 55rpm and the residual solvent was evaporated under reduced pressure using Rotavapor which resulted in the formation of a thin film. The thin film was separated and kept in an amber coloured glass bottle and stored at room temperature (Khan J *et al.,* 2014).

Evaluation of Phytosomes

The particle size and the Zeta Potential of the prepared vesicles were determined by Dynamic Light scattering using a Malvern pan analytical Instrument. Particle size was determined by diluting the vesicles with distilled water (1/100 v/v) and for the determination of zeta potential, the vesicles were suspended in 1mM NaCl Solution with the same dilution.

The yield of Phospholipid Vesicles

The prepared vesicles were dried thoroughly and weighed accurately. Percentage yield of vesicles is the ratio of Weight of Phytosomes obtained to the weight of drugs taken multiplied by 100.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR Spectra of the Alkaloid Rich Fraction, Soy Lecithin and the phospholipid vesicles were obtained by the ATR-FTIR (Bruker Optics, Alpha). The scanning range was 4000-400cm^{-1}.

Differential Scanning Calorimetry (DSC)

Thermal Curves of Alkaloid Rich Fraction, Soy lecithin and the formulated phospholipid vesicles were obtained in an Aluminium crimp pan. The pan was heated at a rate of 10°C/min from 30 to 300°C in a nitrogen atmosphere at a flow rate of 0.5ml/min. The curves were obtained with the help of a Differential Scanning Calorimeter (Thermal Analytics Q20, USA).

Scanning Electron Microscopy (SEM)

Surface topography of the prepared vesicles was observed by suspending them in water and placing them on an aluminium slab. The suspension was dried in vacuum at room temperature, and the micrographs were recorded at different magnifications from 50.0KX to 500X with a resolution limit of 200nm and EHT 10.0KV. The Micrographs were recorded on a Zeiss microscope.

RESULTS AND DISCUSSION

Qualitative and quantitative standardization of Boldine in Alkaloid Rich Fraction of Alphonsea sclerocarpa

The retardation factor (R_f) value for Boldine was found to be 0.49 and the quantity of boldine in alkaloid rich fraction of Alphonsea sclerocarpa was 2.571%w/w respectively. Chromatograms of both the standard boldine and Alkaloid rich fraction were shown in Figure 1. a and b respectively. This method is useful in establishing the plant's identity to differentiate it from the closely related other genera and therefore stands forefront in the quality control.

Formulation of Phospholipid Vesicles

Vesicles were found to be free flowing and they were formulated by the simple patented process. The formulated vesicles were then subjected to characterization.

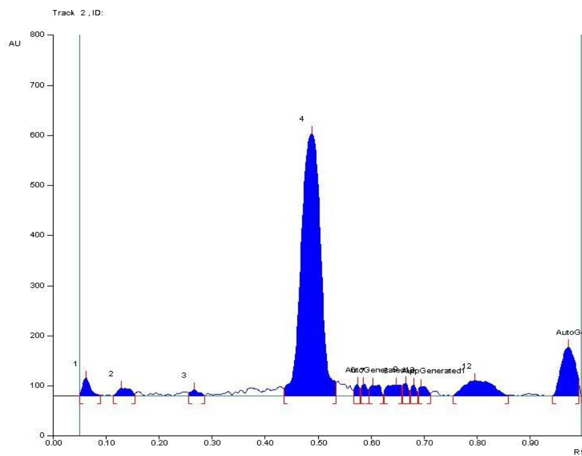

Figure 1a): High-Performance Thin-Layer Chromatogram of Standard

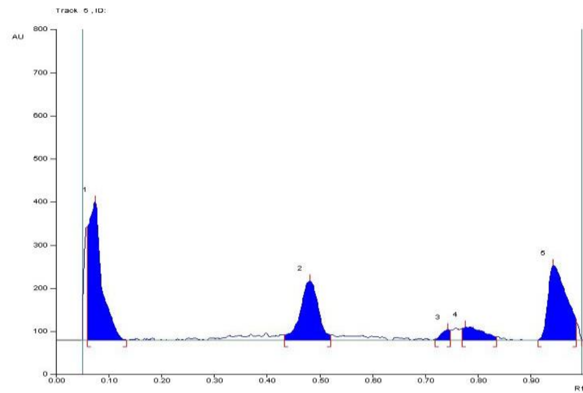

Figure 1b): High-Performance Thin-Layer Chromatogram of ARF of *Alphonsea sclerocarpa*

Evaluation of Phospholipid Vesicles

Particle Size, Zeta Potential and Polydispersity Index: The average diameter of the phospholipid vesicles was found to be 577.7nm and was shown in Figure 2. and the Polydispersity index was 0.77 as shown in Table 1. These vesicles can dodge out the bioavailability issues suffered by the traditional drug delivery systems owing to their nanosize. However, the Zeta potential value was found to be -23.5mV which is hindering the vesicles to be formulated as suspension or syrup.

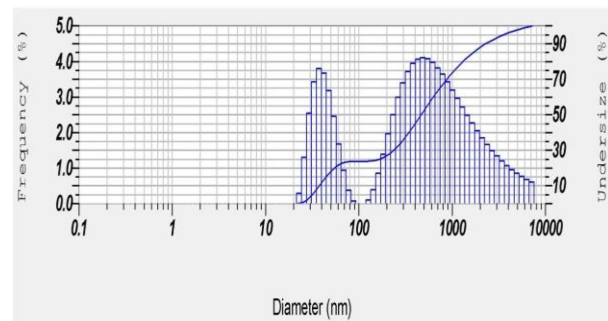

Figure 2: Particle size of the phospholipid vesicles of 1:1 ratio

Percentage Yield: The average percentage yield of all the three formulations was found to be 51.1%, 33.3%, 33.0% respectively Table. 2. The value varies from batch to batch.

Table 1: Average Particle Size and Polydispersity Index

Average Particle Size (nm)	Polydispersity Index
577.7	0.77

nm- Nanometres

Table 2: Percentage Yield of Phospholipid Vesicles

Alkaloid Rich Fraction(mg)	Soy Lecithin (mg)	Yield of Vesicles (%)
100	100	51.1.
100	200	33.3
200	100	33.0

mg- Milligram

Differential Scanning Calorimetry (DSC)

Thermal curves of DSC were obtained by plotting heat flux against temperature. In the present work DSC plots of Alkaloid Rich Fraction, Soy Lecithin and phospholipid vesicles were reported. Alkaloid Rich fraction has shown a moderately broad endothermal peak at 165.4°C and a narrow peak at around 190.5°C that was shown in Figure 3. The Phospholipid and ARF Complex has shown three peaks among which one appeared at 130.5°C that is almost identical to Soy Lecithin in Figure 4. and the other peak appeared at 192.1°C which clearly indicates that raise in temperature resulted in the melting of Soy lecithin and a new peak appeared at 220°C indicating the complex formation in Figure 5.

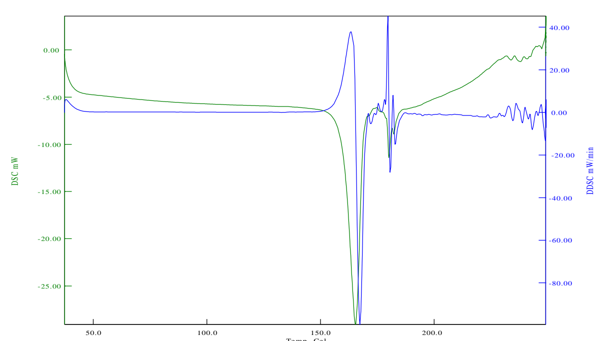

Figure 3: DSC Thermogram of Alkaloid Rich Fraction

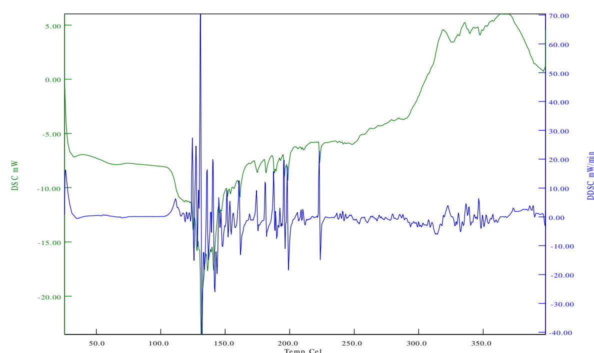

Figure 4: DSC Thermogram of the complex (Soy Lecithin & Alkaloid Rich Fraction)

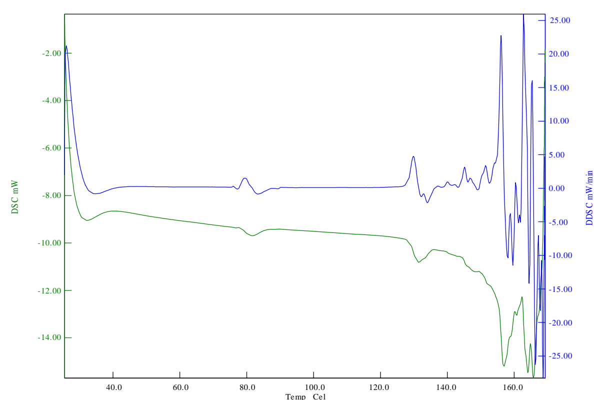

Figure 5: DSC Thermogram of Soy Lecithin

Scanning Electron Microscopy (SEM)

The Scanning Electron Microscopic images of all the three different vesicles are found to be in irregular shape with rough surface Figure 6. a-c.

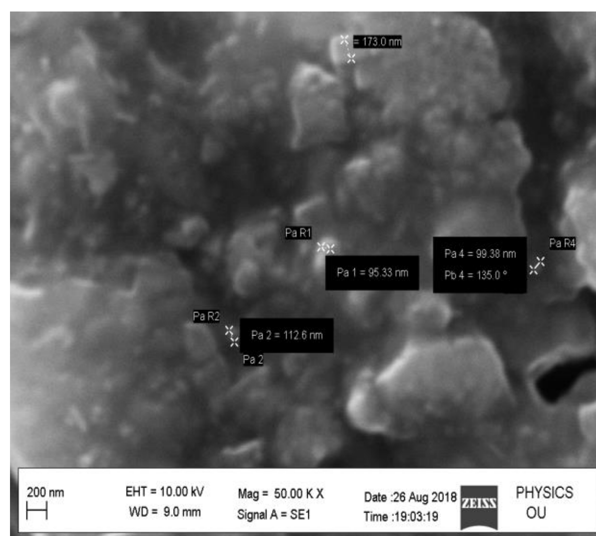

Figure 6a: SEM Micrograph of Vesicles of 1:1 ratio

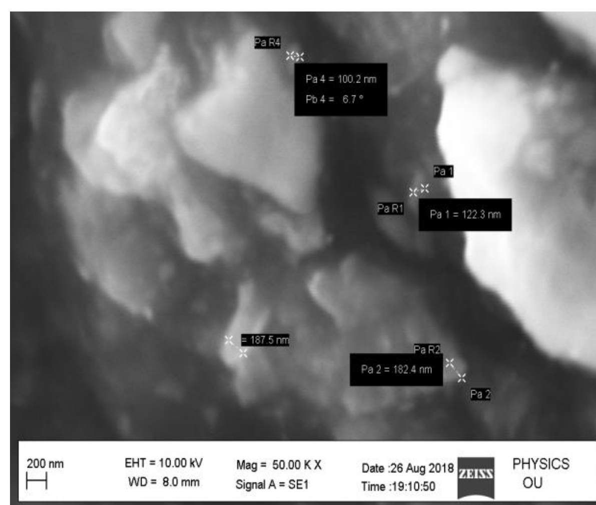

Figure 6b: SEM Micrograph of Vesicles of 1:2 ratio

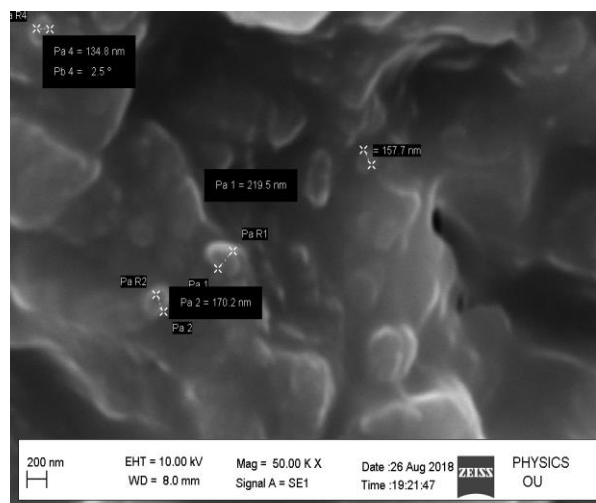

Figure 6c: SEM Micrographs of phospholipid vesicles of 2:1 ratio

Fourier Transform Infrared Spectroscopy (FTIR)

Complex formation can be confirmed by comparing the spectra of the physical mixture of Soy lecithin and ARF with the spectra of individual components. The presence of alkaloids in the complex can be revealed by the presence of absorption peak at 3471.28 cm^{-1} and 2932.20 cm^{-1}. The formation of hydrogen bond can be revealed by the presence of a peak at 3471.28 cm^{-1}. The presence of Soy lecithin in the complex was revealed by the peak at 2932.20 cm^{-1} Figure 7. a-c.

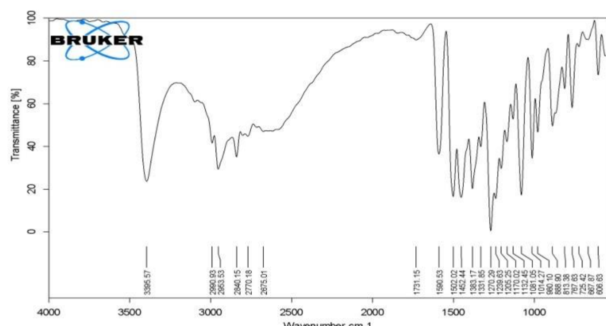

Figure 7a: FTIR Spectra of Alkaloid Rich Fraction

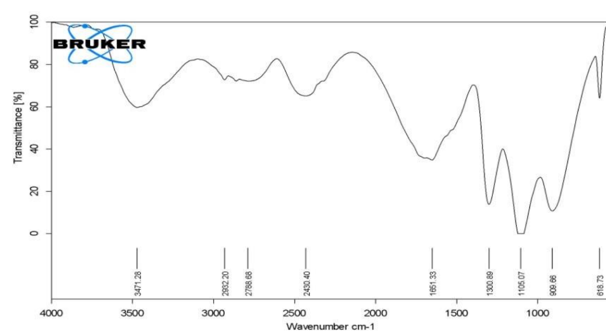

Figure 7b: FTIR Spectra of Phospholipid and ARF Complex

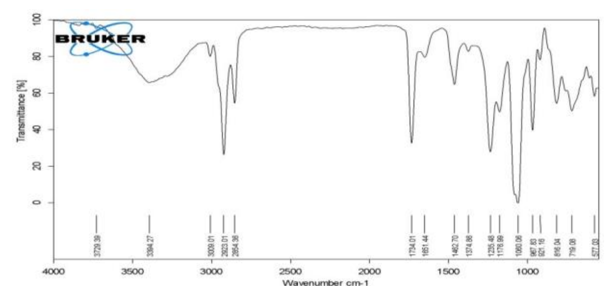

Figure 7c: FTIR Spectra of soy lecithin

CONCLUSION

Alphonsea sclerocarpa belonging to Annonaceae is found to possess huge amounts of Aporphine alkaloids. Presence of Aporphine alkaloid boldine is confirmed by standardizing the fraction using HPTLC. The alkaloid boldine is proven to have many pharmacological activities, but due to its poor therapeutic performance, the phytotherapeutic agent is least explored. Hence the alkaloid-rich fraction is formulated into a novel drug delivery system viz Phospholipid vesicles as they are known to enhance bioavailability of the agents that are poorly bioavailable. Among the phospholipid vesicles formulated the one containing 100mg of Alkaloid Rich Fraction and 100 mg of Soy Lecithin (1:1) was found to be very small and comparatively more stable than the other two ratios and hence the formulated vesicles can be utilised for testing the pharmacokinetic parameters further. The vesicles formulated must also be examined for various pharmacological activities.

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