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Original Article

DEVELOPMENT AND CHARACTERIZATION OF PLANT OIL-BASED POTENT ANTICHOLINESTERASE MICROEMULSION CONTAINING *WITHANIA SOMNIFERA* EXTRACT WITH ENHANCED TRANSDERMAL DELIVERY OF PHYTOCONSTITUENTS FOR THE TREATMENT OF COGNITIVE DISORDERS

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ABSTRACT

Objective: This work was carried out to develop *Cymbopogan Citratus* (lemon grass)oil based microemulsion formulation loaded with the extract of *Withania somnifera* which possess enhanced transdermal delivery of phytoconstituents with anticholinesterase activity useful in treating Alzheimer's disease.

Methods: Methanolic extract of *Withania somnifera* roots were prepared and it was investigated for the inhibition of acetylcholinesterase (AChE) activity by Ellman's assay. Based on the acetylcholinesterase activity, the specific amount of extract was loaded on to the microemulsion formulation. The *Cymbopogan Citratus* oil, tween 20, ethanol was used as oil phase, surfactant, and cosurfactant, respectively, for the preparation of microemulsion. Pseudo ternary phase diagram was constructed using a water titration method. The microemulsion formulations were characterized for droplet size, PDI, zeta potential and drug content. The optimized formulation was subjected to *in vitro* drug release and permeation studies and compared with the extract.

Results: IC_{50} value of ashwagandha extract for anticholinesterase activity was found to be 68.73 µg/ml. The optimized microemulsion formulation had droplet size of 199.9±0.3 nm with PDI 0.029±0.2, zeta potential of-19.49±0.7mv and drug content was found to be 97.5±1.3%. The optimized microemulsion formulation showed 85±1.02% release of withaferin A after 24 h of *in vitro* drug release study. The prepared microemulsion loaded with ashwagandha extract showed excellent permeation of withaferin A(1.4µg/cm^2/min) than the flux obtained from extract solution (0.7µg/cm^2/min).

Conclusion: Optimised microemulsion formulation is suitable for transdermal delivery of anticholinesterase phytoconstituents from ashwagandha extract hence useful in the treatment of Alzheimer's disease

Keywords: Alzheimer, Ashwagandha, Microemulsion

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INTRODUCTION

Cognitive disorders such as Alzheimer's disease (AD) is characterised by impairment of memory and behavior due to progressive degeneration of neurons [1]. Most of the drugs used for the treatment of cognitive disorders provide symptomatic relief by showing the effect on neurotransmitter. Use of Acetylcholinesterase inhibitors (Ach E) for enhancing the acetylcholine (Ach) levels in the brain is one of the most important approaches used for treating cognitive disorders. The principal role of AchE is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of ACh. Food and Drug Administration (FDA) has approved drugs such as rivastigmine, tacrine, donepezil and galantamine for the treatment of AD, which are cholinesterase inhibitors [2]. But these drugs have side effects such as nausea, abdominal pain, vomiting, anorexia [3] and bioavailability problems which necessitates the finding of suitable Acetylcholinesterase inhibitors with less side effects with improved bioavailability.

Withania somnifera, which is known as Ashwagandha or Indian ginseng which, is popular in the traditional ayurvedic system of medicines and worldwide used as nutraceutical. Aqueous extracts of ashwagandha have been reported to increase acetylcholine transferase activity and acetylcholine content in animals and are hence useful in improving cognitive functions [4, 5].

Withania somnifera is useful in treating Alzheimer's disease by enhancing the acetylcholine (ACh) levels in the brain through the inhibition of the Acetylcholinesterase enzyme [6]. Withanolides and Withaferin A in *Withania somnifera* are responsible for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity [7]. Therefore, *Withania somnifera* is a beneficial natural source for the development of drugs for treating AD.

Currently approved treatment for AD involves the administration of cholinesterase inhibitor drugs through oral route shows gastrointestinal side effects. These side effect can be overcome by the administration of drug through transdermal route. Transdermal drug delivery system has several advantages over conventional oral administration, including controlled drug delivery while maintaining steady drug concentration [8], bypasses the first pass metabolism [9]., useful for elderly patients who have difficulty in swallowing.

Transdermal delivery of drugs requires penetration through the skin which acts as a barrier to reach the systemic circulation [10]. Various formulation strategies has been applied in order to reach the target site [11, 12] and optimisation of formulation parameter was done by using computer tools [13, 14]. To improve transdermal delivery, one of the strategies includes use of a microemulsion system which is suitable for hydrophilic and lipophilic drugs. The development of microemulsion formulation is economical since there is no usage of sophisticated technology and it is a thermodynamically stable system. Microemulsion formulation can include various permeation enhancers for improving drug penetration through the skin.

In the current study, plant oil (lemongrass oil) is used in microemulsion formulation which has a dual role. It acts as a permeation enhancer and has anticholinesterase activity which will show synergistic activity with phytoconstituents present in ashwagandha extract.

Therefore, the development of microemulsions loaded with *Withania somnifera* extract, as a transdermal delivery system would be an attractive and worthwhile option that has better anticholinesterase activity for the treatment of Alzheimer's disease.

MATERIALS AND METHODS

Ashwagandha roots, lemon grass oil was purchased from Yarrow Chem Products Mumbai, India. Methanol (HPLC grade), Acetonitrile (HPLC grade), Water (HPLC grade), Galantamine Hydrobromide, Acetylcholinesterase (AChE, specific activity 222U/mg) from electrophorus electricus, Acetyl thiocholine iodide, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Withaferin A was procured from Natural remedies, Bangalore.

Methodology

Plant material and preparation of extract

Roots of *Withania somnifera* were collected from the local market and identified taxonomically (voucher no: 19GN802E). Dried roots were pulverized and subjected to soxhlet extraction using 200 g of powdered plant material with 1000 ml of methanol as the solvent at 60 °C and the extract was filtered, and concentrated to get dried extract [15].

HPLC analysis for estimation of withaferin A

Withaferin-A has been used as biomarker which is one of the constituents responsible for anticholinesterase activity. Methanol extract of the roots of *Withania somnifera* was subjected to reverse phase HPLC analysis to determine the Withaferin-A content.

HPLC analysis was carried out by method reported by Gurav *et al.* [16]. HPLC system consists of SHIMADZU LC-10 AD pump, rheodyne injector, and ultraviolet, visible detector. Hypersil ODS C18 column (24× 4.6 mm i. d, 5 µm particle size) and acetonitrile: water (50:50) is used as mobile phase. The solvent flow rate is maintained at 1 ml/min and the wavelength is set at 220 nm. Standard Withaferin A of concentration 0.02, 0.04, 0.08, 0.16, 0.24, 0.32 and 0.40 mg/ml were prepared in methanol. Extract solution was prepared by taking 25 mg of the extract and dissolved in 10 ml of methanol. Solutions were filtered through 0.45 µm membrane filter, and a 10 µl was injected to HPLC column and peak area was determined. Standard plot was made by plotting the peak area of standard withaferin A versus concentration and amount of withaferin A in the extract was determined by the linearity equation.

Formulation and characterization of microemulsion

Selection of surfactant and cosurfactant

For the formulation of microemulsion, tween 20 (HLB value-16) was used as surfactant because it is known that the higher the HLB value of surfactant, the easier it forms o/w emulsion [17]. Since the use of surfactant alone is unlikely to reduce the interfacial tension between oil and water, ethanol is added as a cosurfactant in order to form stable microemulsion. Ethanol is hydrophilic; therefore, it partitions over the aqueous and oil phase. Further, it has been reported that ethanol is able to insert into the interfacial layer and forms a tight interfacial film [18]. The essential oils were commonly used as an oil phase in the microemulsions for transdermal applications since they composed mainly of terpenes, which could be act as skin penetration enhancers [19] and possess anticholinesterase activity which is attributed to the citral content [20]. Hence in the present study, lemongrass oil is chosen as oil phase.

Construction of the phase diagram

In order to select the suitable blend of surfactant and cosurfactant, different weight ratios of Smix (surfactant and co-surfactant) such as 1:0, 1:1, 2:1, 3:1 were prepared. Construction of Pseudo ternary phase diagram was done by using the water titration method (25 °C) [21]. For each phase diagram at specific Smix, the ratio of lemon grass oil and Smix were varied as 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 respectively and the mixture obtained was subsequently titrated with water under moderate stirring at room temperature. Microemulsion region was assigned to samples when they are clear and transparent. Pseudo-ternary phase diagrams were plotted by using CHEMIX School Ver.10, from which microemulsion region were identified, which is shown in fig. 2.

Preparation of Ashwagandha extract-loaded Microemulsion formulation

According to the phase diagram seven microemulsions (M1-M7) were selected with a surfactant: Cosurfactant weight ratio of 2:1 (table 1). Different Ashwagandha extract (0.5% w/w) loaded microemulsion formulation (M1-M7) were prepared with different concentrations of oil (5,10,15, 20%w/w) and Smix (20, 25, 30,35%w/w).

For the preparation of microemulsion, Ashwagandha extract (5 mg/ml) was dissolved in ethanol later it was mixed with Smix (2:1) and oil, respectively. Finally, water was added and mixed by vortex mixing.

Table 1: Composition of Ashwagandha extract-loaded microemulsion

Form code	Extract (mg)	Oil (%w/w)	Smix (2:1) (%w/w)	Water (%w/w)
M1	50	10	20	70
M2	50	10	25	65
M3	50	10	30	60
M4	50	10	35	55
M5	50	5	25	70
M6	50	15	25	60
M7	50	20	25	55

Characterization of ashwagandha extract-loaded microemulsion

Determination of pH of microemulsion

The pH of the microemulsion was measured using a calibrated pH meter (Systronics, India) in triplicate at 25 °C.

Determination of viscosity

Viscosity of the microemulsion was determined by using Brookfield R/S plus rheometer (Brookfield Engineering, Middleboro, MA) 100 rpm using spindle no 18.

Determination of % transmittance

The percent transmittance was measured using a UV-Visible double beam spectrophotometer (UV-1800 SHIMADZU). Keeping distilled water as blank at 650 nm

Determination of percent drug content

One ml of microemulsion was pipetted from the microemulsion and was diluted with methanol. It and the samples were analysed by HPLC as described above.

Determination of Globule size (PS), size distribution (PDI) and zeta potential

The globule size, size distribution and zeta potential of the microemulsion were determined by zeta sizer (Nano ZS, Malvern Instrument, UK).

Drug excipient compatibility studies by using fourier transform infrared spectroscopy (FTIR)

To find out the interactions between the extract and excipients; to identify the presence of standard compound withaferin A in extract

and microemulsion FTIR analysis was carried out using the instrument Bruker Alpha II

Scanning electron microscopy and transmission electron microscopy

To determine the morphological features (shape and size) of globules in microemulsion, optimised formulation was subjected to SEM (Zeis Sigma) and TEM (TEM-FEI, TECNAI T20, USA) studies.

Determination acetylcholinesterase (Ach E) inhibitory activity

Acetylcholinesterase (Ach E) inhibitory activity of the extract was determined by Ellman's assay [22]. Acetylthiocholine iodide was used as substrates to assay AChE activity. Ethanolic solution of Ashwagandha extract (10-250µg/ml) is used as a test compound and galantamine (1-20 µg/ml)) as the positive control. The reaction mixture consists of 10 µl test solution (extract), 150 µl of sodium phosphate buffer (pH 8.0), 10 µl of 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) and 20 µl of AChE solution. This mixture was incubated for 15 min at 25 °C. 10 µl of acetylthiocholine is added to initiate the reaction and absorbance is measured at 412 nm wavelength after 15 min. The reaction is carried out in 96-well microwell plate Spectra Max 340 (Molecular devices, U. S. A) and reading are taken in triplicate. The percentage inhibition of enzyme activity is calculated using the formula E-S/E×100, where E is the activity of enzyme with test and S is the activity of enzyme with test and IC_{50} values were determined.

***In vitro* drug release studies**

Withaferin A is used as a marker compound in ashwagandha extract for its quantitative determination of extract due to its acetylcholinesterase inhibitor activity. This compound was used as a marker for the quantitative determination of the extract in the formulation. An *in vitro* drug release from microemulsion formulations were performed using Franz diffusion cell across dialysis membrane (12 Kda, Hi Media). The membrane was soaked in deionized water for 12 h before use. 0.5 ml of microemulsion and 12 ml of phosphate buffer solution (pH 7.4) as dissolution medium (n=3), were placed in donor and receptor compartments, respectively. The dissolution medium was stirred at 50 rpm and maintained in a water bath at a temperature of 37±0.5 °C. At pre-determined time intervals (30 min, 1, 2, 3, 4, 6, 8, 12, and 24 h), one ml aliquots were withdrawn and Withaferin A content was estimated by HPLC.

***In vitro* permeation study**

An *in vitro* transdermal permeation study of microemulsion was conducted using Strat-M® membrane (Merck Millipore, Burlington, MA, USA) in Franz diffusion cell. This membrane is placed inside a diffusion cell between the donor and receptor compartment. The effective permeation area of the diffusion cell was 2.01 cm^2. The receptor media consist of 12 ml phosphate buffer (pH 7.4) which was stirred ta 50rpm and a volume of 0.5 ml of the microemulsion formulation and extract solution (containing an equivalent amount of withaferin A) were placed in donor compartment with temperature maintained at 32 °C. The samples are withdrawn from receptor media at predetermined time intervals and was analysed for the amount of withaferin A by HPLC.

The cumulative amount of withaferin A permeated through the membrane per unit area was plotted versus time and from the slope at the steady state flux (J) was calculated. From the ratio of flux and drug concentration, the permeability coefficient (Kp) was calculated.

***In vitro* release kinetics**

The *in vitro* drug release study data of microemulsion formulations were fitted to zero order and first order in order to establish the order for understanding the drug release kinetics. The mechanism of drug release was established with the help of Higuchi model and Korsmeyer Peppas model.

Stability studies

Stability studies of optimized microemulsion was done at temperature condition i. e 5 °C±3 °C (refrigerator) and room temperature of 25 °C±2 °C/60%±5% RH (thermostability chamber) for a period of 1 mo. Samples are withdrawn periodically (0,15,30 d). The samples were checked for physical stability, drug content, globule size and zeta potential

RESULTS AND DISCUSSION

Estimation of Withaferin A by HPLC

The reverse phase HPLC analysis of Ashwagandha extract was carried out for determination of Withaferin A. Standard plot of withaferin A showed good linearity at a concentration range of 20-240µg/ml with an acceptable regression coefficient (R^2) 0.9994, which is closer of 1. The amount of withaferin A in methanolic extract of Ashwagandha roots was found to be 1%.

Formulation and characterization of microemulsion

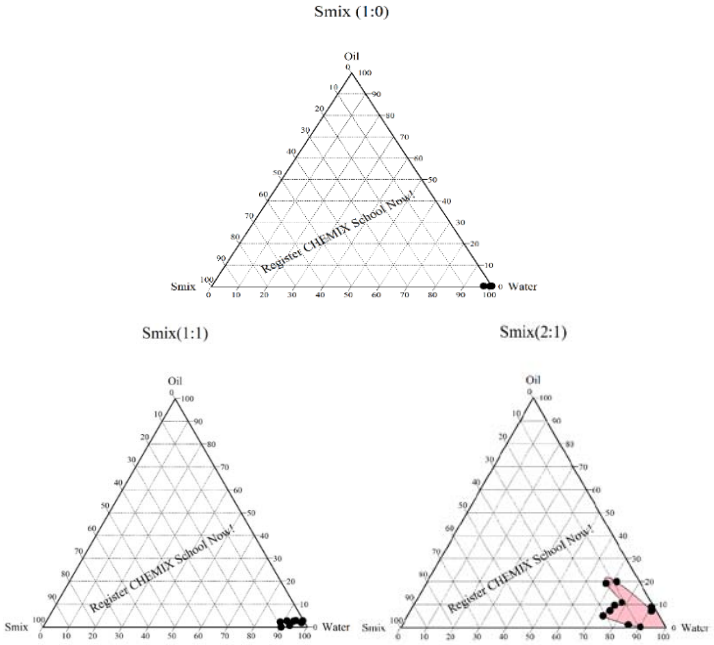

Fig. 1: Pseudo ternary phase diagrams using lemongrass oil as oil phase, water, Tween 20 (surfactant) and ethanol (cosurfactant) at different Smix ratio

Table 2: Formulation and characterization microemulsion loaded with Ashwagandha extract

Form. code	Drug content (%)	Size (nm)	PDI	Zeta potential (mV)	pH	Viscosity (centipoise)	% Transmittance
M1	97.3±1.0	223.4±0.2	0.248±0.4	-17.39±0.2	4.80	3.16	98.7
M2	98.0±1.6	227.8±1.2	0.35±0.7	-16.49±1.1	4.65	3.14	98.8
M3	96.5±0.9	255.0±1.2	0.447±0.1	-12.49±1.2	4.45	3.32	98.0
M4	97.8±1.6	259.0±0.7	0.490±0.2	-10.88±0.2	4.40	3.29	98.7
M5	96.6±0.8	202.3±0.11	0.200±0.2	-17.49±0.9	4.46	3.16	99.3
M6	96.9±1.6	208.4±0.9	0.191±0.3	-19.29±0.7	4.54	3.15	98.9
M7	97.5±1.3	199.9±0.3	0.029±0.2	-19.49±0.7	4.50	3.19	99.8

All values are expressed as mean±SD (n=3)

Effect of surfactant and cosurfactant ratio on microemulsion

The shaded region of phase diagrams indicates the microemulsion region. In fig. 1, a low-microemulsion area was observed at the Smix ratio 1:0 and 1:1. Higher nanoemulsion region was observed when the surfactant concentration was increased, i.e., at Smix 2:1. Phase diagram shows that at 20%w/w of Smix is needed to solubilise 5% w/w of lemongrass oil. Smix ratio 2:1 was selected for formulation of Ashwagandha extract-loaded microemulsion formulation.

Preparation and characterization of ashwagandha extract-loaded microemulsion

Different ashwagandha extract-loaded microemulsion formulations (M1-M7) were prepared with different concentrations of oil and Smix and all the formulations were subjected to physicochemical characterization, whose results are given below and tabulated in table 2.

The percentage drug content was in the range of 96.5-98.0%, which indicates that withaferin A is uniformly distributed in the globules of microemulsion and there is no much loss during the preparation and storage. The percentage transmittance was more than 98% indicating that the prepared microemulsion formulations were transparent in nature. The mean globule size of the microemulsion was found to be in the range of 199.9±0.3 nm to 259.0±0.7 nm and PDI is found in the range of 0.029±0.2 to 0.490±0.2. The globule size of the microemulsion was found to decrease as the concentration of Smix was increased. Increasing the Smix concentration caused the reduction in the interfacial tension which is one of the reasons for the formation of smaller size globules. Zeta potential was found in range between-10.88±0.2mV to-19.49±0.7mV. Zeta potential was found to be high for microemulsions with small globule size and as the globule size increased zeta potential reduced. The viscosity of the formulation was low (3.14cps to 3.29cps) due to the homogenous nature of the microemulsion. Small droplet size of drug loaded microemulsion plays a very important role in drug delivery as it determines the degree and intensity of drug release which is directly related the absorption of drugs. Transdermal preparations with small droplet sizes were found to be more efficacious than the larger droplet sizes [23]. Hence based on this fact, microemulsion formulation M7, which has small globule size and less PDI was selected as the optimised formulation.

Oil-in-water microemulsions offers considerable promise as a means to increase the apparent aqueous solubility of poorly water-soluble lipophilic drugs. In the ashwagandha extract, the marker compound is Withaferin A being lipophilic in nature, can easily pass through stratum corneum but in order to reach the dermal region, it has to pass through the aqueous layer under the horny layer, which is the biggest challenge [24] Ethanol is used as co-surfactant which acts as permeation enhancer by various mechanisms. Ethanol increases the solubility of water and oil in ethoxylated non-ionic surfactants like Tween thereby enhancing the formation of microemulsion [25]. Further, after the penetration of ethanol into the stratum corneum it changes the solubility properties of tissue resulting in an improvement of the partitioning of compounds through the membrane [26]. Lemon grass oil is used as oil phase which enhances the transdermal permeation and it improves diffusion, partitioning of lipophilic drugs compounds into the stratum corneum [27].

Hence O/W microemulsion is prepared and in order to enhance the transdermal permeation, lemongrass oil is used as oil phase which also acts as permeation enhancer.

Drug excipient compatibility studies by using Fourier transform infrared spectroscopy

Withaferin A shows broad peak at 3343.96 cm^{-1} due to O-H stretching shown by the hydroxyl groups and the peak at 1682 cm^{-1} due to C-H alkane bending vibrations. FTIR studies indicated that the presence of peak corresponding to the functional group of withaferin A is present in the extract as well as in microemulsion formulation [28].

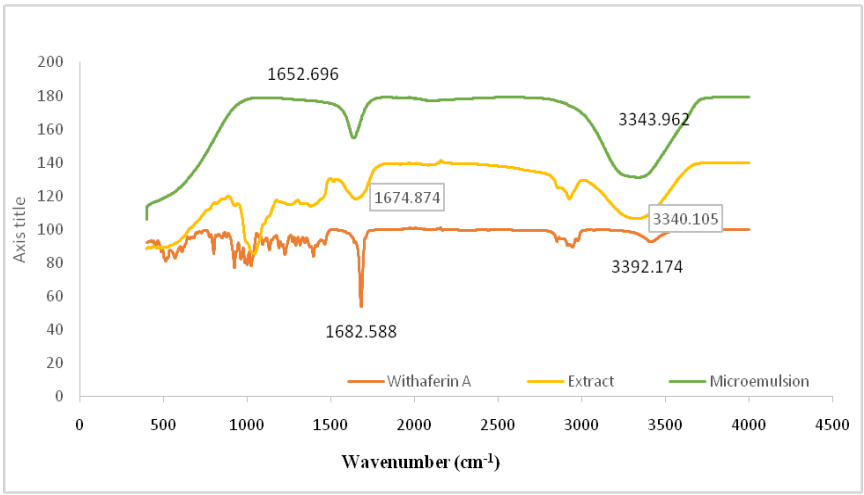

Fig. 2: FTIR spectrum of withaferin A, ashwagandha extract and optimised microemulsion formulation

Scanning electron microscopy and transmission electron microscopy (TEM)

To obtain more information about the surface and shape, SEM and TEM analysis were performed. The SEM photomicrograph (fig. 3a) revealed that the microemulsion are spherical in shape The TEM image (fig. 3b) reveals that microemulsions were uniform and spherical in shape and size in the range of 200 nm.

Determination acetylcholinesterase (Ach E) inhibitory activity

Ashwagandha extract showed IC_{50} value of 68.73 µg/ml, whereas standard drug galantamine showed IC_{50} value of 38.50 µg/ml. Based on the literature IC_{50} value of withaferin A is 84 µM [29]. Hence for the formulation of microemulsion, the amount of extract (equivalent to IC_{50} value of withaferin A) is taken.

***In vitro* release study of withaferin a from extract and optimised microemulsion formulation**

The cumulative percentage release of withaferin A from microemulsion (M7) and extract solution was found to be 85±1.02% and 92.0±1.0% after 24 h. The *in vitro* drug release from the optimized microemulsion formulations was found to be slow, gradual and spread over 24 h. The microemulsions formulation showed prolonged release with no burst effect when compared to drug release from extract solution. The release of withaferin A from microemulsion depends on interaction with the surfactant used and/or partitioning between oil and water phases.

The *in vitro* release kinetic study indicates that optimised microemulsion formulation follows first-order release kinetics as reflected by their correlation coefficient value. This may be due to the fact that drug release is dependent on the concentration of the oil and surfactant concentration. Microemulsion formulation followed Higuchi diffusion mechanism and type of diffusion is Fickian diffusion as 'n' value of koresmeyer peppas equation is 0.43. Hence rate-determining step for release of withaferin A from microemulsion is the diffusion of withaferin A from the oil droplet.

***In vitro* permeation study**

Optimized microemulsion (M7) formulation provided highest flux of withaferin A per unit area (1.4µg/cm^2/min) which is 2 times more than the flux obtained from extract solution (0.7µg/cm^2/min) which indicates that there is enhancement of delivery of withaferin A using microemulsion formulation. Permeability constant of withaferin A from microemulsion and extract was found to be 56 ×10^{-3} cm/h and 28×10^{-3} cm/h, respectively. The enhanced skin permeation of microemulsion may be due to the increased solubilization of drug, use of appropriate surfactant/cosurfactant mixture, oil phase which acts as permeation enhancer [30]. Optimized microemulsion showed excellent permeability due to the appropriate oil phase, surfactant/cosurfactant ratio, small globule size and low viscosity [31].

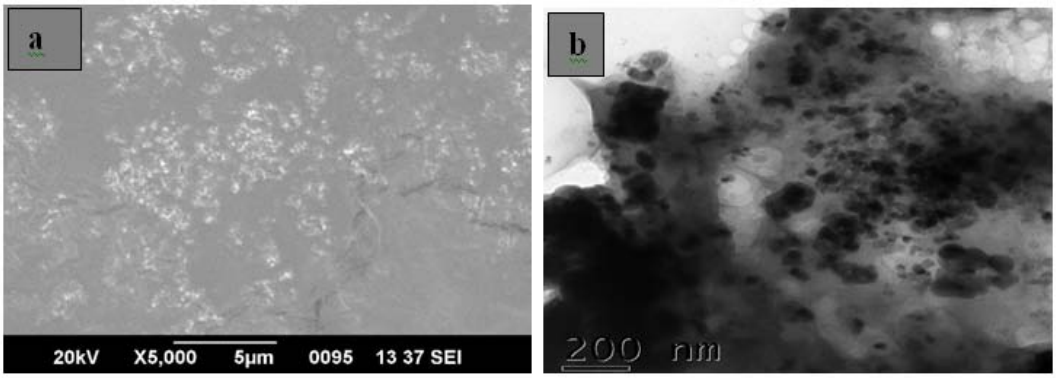

Fig. 3: SEM(a) and TEM (b) image of optimized microemulsion formulation

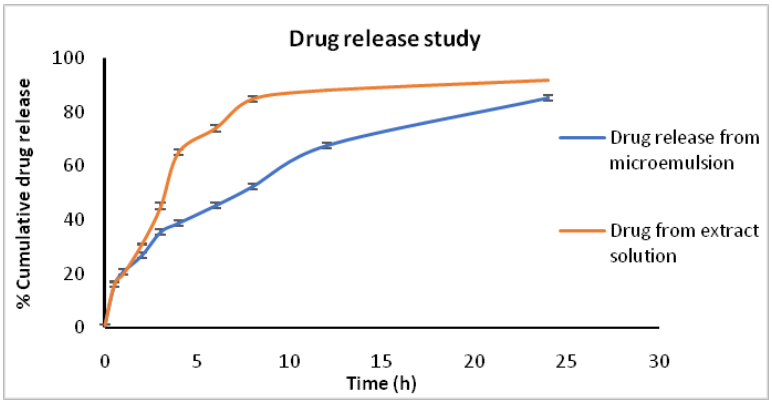

Fig. 4: Comparative *in vitro* drug release study of withaferin A from optimised microemulsion formulation and from extract solution, All values are expressed as mean±SD (n=3)

Table 3: *In vitro* drug release kinetics of optimised microemulsion

Form. code	Kinetic models								
	Zero order		First order		Higuchi		Korsmeyer-peppas		
Optimised formulation (M7)	R^2 0.9571	K -0.0789	R^2 0.9813	K -0.0005	R^2 0.9927	k 2.206	R^2 0.9543	K 1.3232	n 0.4300

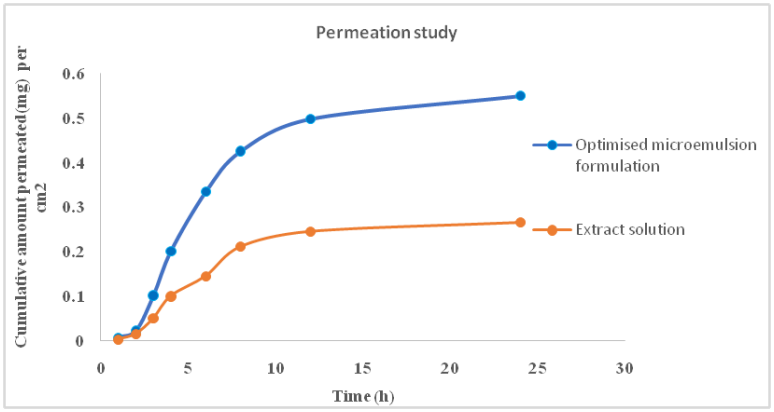

Fig. 5: Comparative *in vitro* permeation study of withaferin a from optimised microemulsion formulation and from extract solution, All values are expressed as mean, (n=3)

Stability studies

The results of stability studies indicate that there was no phase separation, drug precipitation was observed in optimised microemulsion formulation after 1 mo of stability testing. There were no significant changes in drug content, globule size and zeta potential, which indicate that the prepared microemulsion is stable

CONCLUSION

Loading the ashwagandha extract into microemulsions with permeation enhancers exhibited better solubilization and higher skin permeation of Withaferin A through the synthetic membrane in comparison to the extract. Hence formulating the extract containing multiple constituents with cognitive enhancement power into microemulsion with permeation enhancement has improved the delivery of drugs through skin, thereby expected to show enhanced activity against Alzheimer's disease. Overall, the present research will provide a formulation that is economical, has better efficiency and more patient compliance for the treatment of Alzheimer's patients.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

No conflict of interest

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