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Formulation and evaluation of solid–lipid nanoparticle based 0.1% Soy isoflavone dermal gels

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Abstract

Solid – Lipid Nanoparticles (SLNs) based 0.1 % topical gel of soy isoflavones was formulated with the aim of better penetration through stratum cornea and efficient deposition of soy isoflavones in dermal matrix so as to aid in reversing postmenopausal skin aging. Isoflavoneaglycones were extracted from whole herbal soy extract and were characterized with respect to physical, spectral and antioxidant activity. SLNs prepared by microemulsion template technique were characterized by photon correlation spectroscopy for size and by Scanning Electron Microscopy for morphology. The nanoparticulate dispersion was suitably gelled and characterized with respect to pH, spreadability, rheology, drug content, in-vitro permeation using pig ear skin model and its safety was assessed using primary skin irritation studies. The developed SLN-based gel showed about 60% deposition of soy isoflavones in dermal matrix and showed no skin irritation on intact rabbit skin.

Keywords: *Nanostructured Nanomaterial, Solid – Lipid Nanoparticles, Topical Gel, Soy Isoflavones, Draize Patch Test*

INTRODUCTION

After menopause, the skin undergoes changes that include thinning, decrease in elasticity and wrinkle formation. This abrupt skin aging is the consequence of a lower production of collagen and elastin, the supportive and elastic protein of the skin. The loss of hormonal activity reduces the skin tone and its hydration. This deterioration is based on the reduction of the metabolic activity of skin cells and results in wrinkles and dry skin. Oral and topical estrogen applications have shown to preserve collagen content, elastic properties, and thickness of the skin in postmenopausal women. However, human hormones cannot be used in cosmetic formulations and due to the potential adverse effects should only be considered in severe cases in dermatological products. The treatment of skin during and after menopause with an active aglycone preparation from soy is a completely new approach in the battle against the aging process in the skin. Therefore topical application of isoflavones is beneficial in postmenopausal skin, where it replenishes the loss of endogenous estrogen [1].

Over the last decade, plant derived ingredients have gained a lot of interest in cosmetic formulations. However, in many cases, these products do not offer the proposed activity since the preparations does not contain the active molecules in an appropriate concentration. There are several soy isoflavone products on the market that are sold as dietary food supplements. In most cases, these food supplements contain only isoflavone glycosides, the molecular form that is biologically not active. However, after ingestion the glycosides are transformed by intestinal glucosidases and intestinal bacterial metabolism into the estrogenically active form. Since the skin does not harbor such bacteria and enzymes, the active isoflavone preparations for skin care must be in the form of aglycone. Unfortunately, these aglycones have a poor solubility in water and oil. Thus, a special galenic form is necessary to introduce these isoflavone preparations into cosmetic formulations. Isoflavones aglycones either encapsulated into suitable matrix can be applied in different formulations such as gels, lotions and creams since the product can easily be formulated into the water phase of cosmetics [2 - 3].

Solid lipid nanoparticles (SLNs) are colloidal carrier systems providing a number of features advantageous for topical route of application [4 – 7]. The small size of the lipid particles ensures close contact to stratum corneum and can increase the amount of drug penetrating into skin [8 – 10].

Hence present research work focuses on development

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and evaluation of Solid – lipid nanoparticle based 0.1% Soy isoflavone gel for dermal care of menopausal women. So far liposomal preparations of Soy isoflavone aglycones have been reported but developing aqueous based dermal gels containing SLNs of Isoflavone aglycone is a novel approach as it has not been explored earlier.

MATERIALS AND METHODS

Whole soybean extract containing 40% total isoflavones was obtained as a gift sample from, Biocon, (India). Solid-lipids Softisan 601 from Sasol Olefins and Surfactants GmbH, (Germany) while Tefose 63 from Gattefosse, France were obtained as gift samples. Tween 20 and Brij 35 were of analytical grade and were purchased from S.D. fine Chem Ltd, India. Methocel® A4M, Methocel® E5 LV and Methocel®E15 LV were obtained from Colorcon Asia Pvt. Ltd, India while Polyplasdone®K90D, Polyplasdone®S630, Polyplasdone®K25/32 were obatined as gift samples from BASF, India. Different grades of Carbopol were obtained as gift samples from Noveon, Mumbai, India. Xanthan gum and Carrageenan were gifted by Signet Chemical Corporation, Mumbai, India. 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest grade commercially available. The amount of solvent used had no effects on the assays.

Extraction and characterization of Isoflavoneaglycone from dry herbal whole Soy extract

Isoflavoneaglycones were extracted from the whole herbal soy extract by refluxing the whole extract with 4M Ethanolic Hydrochloric acid for 2 h followed by precipitation of the aglycones using water as non solvent. The crude product obtained was dried at 50°C for 9 hours followed by recrystallization using ethanol as solvent and water as non-solvent to obtain pure product [11]. The extracted product was identified by performing its Physical and spectral characterization and determining its Antioxidant Activity using various assays such as Total Phenolic content by Folin – Ciocalteu method, DPPH. (2,2-diphenyl-1-pycrylhydrazyl) Free radical scavenging assay and Total reduction capacity by Oyaizu method [12 – 14]. Ultraviolet/ Visible Spectrum of a 10 µg / ml solution of the extracted product in methanol was recorded on a UV Spectrophotometer, Shimadzu (UV – 1650).An Infrared spectrum of the extracted aglycones was recorded on a Perkin Elmer-FTIR, Spectrum RXI spectrophotometer LM 500 model.

Total polyphenolic content in the extracted product was determined using Folin – Ciocalteau colorimetric method in three different solvents viz. methanol, methanol – water mixture (1:1) and plain water. 100 mg of extract was stirred with 100 ml of each of above mentioned solvents for 30 min to ensure complete extraction of the phenols in the respective solvents. The suspensions were then centrifuged at 1660g for 10 min and aliquots of supernatant fraction were withdrawn and were analyzed for total polyphenolic content. About 100 µl of the extract solution was mixed with 900 µl of distilled water. To each of these solutions 2 ml of distilled water was added followed by addition of 1 ml of FolinCiocalteu reagent that was diluted in the ratio of 1:1 with distilled water and 1 ml of Sodium Carbonate (Na2CO3, 2 g/L). The final volume was made up to 10 ml with distilled water and the solutions were cyclomixed on a vortex mixer, incubated at 40ºC for 30 min and its absorbance was recorded at 760 nm. The content of total polyphenols in the extract was expressed as mg/g of Gallic acid equivalents [12].

DPPH• Free radical scavenging assay

For radical scavenging measurements, appropriate aliquots of the solution of whole soybean extract, crude product and of the recrystallised product dissolved in methanol were withdrawn so as to make solutions with concentrations ranging between 50 – 500 µg/ml and were further diluted with methanol to make final volume of 1 ml. Two such sets were prepared and to one of this set 1 ml of methanolic solution of DPPH (200 µM) was added while to the other set 1 ml of methanol was added. Control solution was prepared by mixing 1 ml of plain methanol to 1 ml of methanolic solution of DPPH (200 µM). The samples were incubated at room temperature for 20 minutes and the absorbance was recorded at 517 nm.

The percent radical scavenging activity was determined from the difference in absorbance (A) of DPPH between the control and samples using following formula:

Radical scavenging (%) = (OD of CONTROL − OD of SAMPLE) / (OD of CONTROL)x 100, Where OD is Optical Density. Inhibitory concentration (IC50) was used as a measure of comparison of antioxidant activity of the Whole extract and isoflavoneaglycone. It is calculated from the plot of percent DPPH inhibition V/S Concentration [13].

Total reduction capacity by Oyaizu Method

The total reduction capacity of the extracted product was determined by diluting an appropriate aliquot of the test extract dissolved in different solvents (viz. methanol, methanol – water (1:1) and water) with concentration of 1500 µg/ml with water to make final volume of 1 ml. To this 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$; 10 g/L) were added. The mixture was incubated at 50ºC for 30 min. After incubation, 2.5 ml of tri chloro acetic acid (100 g/L) was added and the mixture was centrifuged at 1650 g for 10 min. Finally 2.5ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride ($FeCl_3$; 1g/L). The absorbance of the solution was measured at 700 nm. High absorbance indicates high reducing power [14]. The antioxidant activity of the extracted product was compared with the dry herbal whole Soy extract from which the aglycones were extracted.

Analysis of Extracted Isoflavoneaglycones

A High Performance Liquid Chromatography (HPLC) method was developed for the analysis of extracted isoflavoneaglycones. The liquid chromatographic system consisted of Agilent Quaternary pump (G1311A) coupled with a variable wavelength Detector (G1314B), Column Thermostat (G1316A) and a thermostated auto sampler (G1329A). Data integration was performed using Agilent Chemstation version Rev. B. 0201 – SRI [260] software for LC peak integration. Chromatography was performed on Waters Spherisorb® 5µm ODS2 4.6 x 250mm Analytical column (Waters Corporation, Massachusetts, U.S.A).The mobile phase was prepared by mixing Acetic Acid (0.033 M) - Methanol- Acetonitrile (48:40:12, v/v/v), respectively. The pH of the mobile phase was 3.7. Chromatography was performed at room temperature under isocratic conditions at a flow rate of 1.0 ml/min and injection volume of 100 µl. Detection was done at 254 nm [15].

Formulation development

Screening of Components: The solubility of the isoflavoneaglycones was determined in different solid lipids, surfactants, and solubilizers. An excess of drug was added individually to surfactants, and solubilizers (5 ml each) in screw-capped tubes. After 24 h, each sample was centrifuged, and 0.5ml clear supernatant layer was diluted suitably and analyzed by HPLC. For the solubility studies in solid lipids, 100 mg of the drug was taken in a test tube. The solid lipid was added in increments of 0.5g and the test tube was heated in a controlled temperature water bath. The amount of lipid required to solubilize the drug in molten state was noted [16 – 18].

Formulation of Microemulsion:

Selection of a microemulsion system was based on the drug-solubilizing capacity of the excipient. The selected components were as shown in Table 1. The components selected for the formulation of microemulsion system were GRAS-listed [16 – 18].

Table 1.Selected components for microemulsion formation

Components	Formulation 1	Formulation 2
Solid – Lipid	Softisan 601 [Glyceryl Cocoate (and) Hydrogenated Coconut Oil (and) Ceteareth – 25]	Tefose 63 [PEG-6-32 stearate (and) glycol stearate]
Surfactant	Tween 20	Tween 20
CoSurfactant	Brij 35	Brij 35
Aqueous Phase	Double-distilled water	Double-distilled water

Construction of Pseudo-ternary phase diagrams:

Pseudo-ternary phase diagrams of solid – lipid, surfactant, co-surfactant and water were developed using titration method at 25±2 °C [16]. Phase behavior of systems was studied at various ratios of surfactant to co-surfactant (Km) viz. 1:0.5, 1:1 and 1:2. Mixtures of surfactant and co-surfactant (at a specific Km) with Solid - Lipid were prepared at ratios (w/w) of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10. The mixtures were heated slowly at 60°C to melt the lipid, vortexed to form a monophasic system with the added surfactant and a small amount of water increment maintained at 60°C was added in to it; vortexed and allowed to equilibrate. Resulting mixtures were evaluated visually for transparency and flow properties; and by polarizing microscope for optical isotropy. Endpoint of titration was the point, where mixture became turbid or phase separation was observed [18]. At this point, amount of water, oil, surfactant and cosurfactant added was noted. Monophasic, clear, low viscous and non-birefringent systems were considered as microemulsion (ME) and shown as ME region.

Characterization of Micro emulsion

Freeze-thaw cycling

Microemulsion was subjected to freeze-thaw cycles (-4 to 40°C) of 24 h for a period of one week and assessed for physical instabilities such as phase separation and precipitation [19 – 20].

Optical birefringence

The microemulsion was checked both visually and using cross-polarizers for optical isotropy to confirm absence of other phases [19 – 20].

Formulation of solid – lipid nanoparticles from microemulsion templates

Solid lipid nanoparticles (SLNs) of isoflavoneaglycone were prepared from warm microemulsion templates for the preparation of SLNs [16 – 18]. The oil phase consisted of melted solid – lipid in which the isoflavoneaglycone was dissolved at 60°C. The aqueous phase consisted of surfactant, co-surfactant and water. Temperatures for both phases were maintained above the melting point of the lipid (60°C). The oil phase was added to the aqueous phase, and both phases were mixed using a cyclomixer at this temperature to form a microemulsion. This warm microemulsion was diluted in cold water (2–3°C) under mechanical stirring to form SLN dispersion such that the concentration of Soy isoflavoneaglycone in the final dispersion remains as 0.1%w/w.

Formulation of SLN based gel containing 0.1% w/w soy isoflavoneaglycone

The nanoparticulate dispersion obtained after diluting the warm microemulsion templates was gelled using different gelling agents like carbopols, xanthan gum, and carrageenan. Based on compatibility with the nanoparticulate dispersion, ease of preparation, and aesthetic appeal; carbopol (971 P) was selected as the gelling agent. Carbopol was dispersed using an overhead stirrer at the speed of 700rpm for 3h. Different concentrations of carbopol (971 P) were used for gelling (0.5–1%) and the one giving optimum viscosity was chosen for further studies. Carbopol (971 P) 1% was added to the nanoparticle dispersion under overhead stirring at 1000rpm. Stirring was continued until carbopol was dispersed. The carbopol dispersion was neutralized using 50% w/w triethanolamine.

Stability studies

Hydrophilic polymers such asPolyplasdone K90D, Polyplasdone S630, Polyplasdone K25/32, Methocel® A4M, Methocel® E5 LV and Methocel®E15 LV were incorporated in the formulation as crystal growth inhibitors. They were incorporated in the formulationat 5% concentration level by dissolving them in the aqueous phase before coarse emulsion formation. The formulations were filled in plain glass vials and stored at room temperature and in refrigerator. They were observed forclarity or development of turbidity and time within which the turbidity developed.

Characterization of Nanoparticulate Dispersion

Determination of Particle Size and Polydispersity Index:

All measurements were performed in triplicate on a Beckman N4 plus submicron particle size analyzer at a temperature of 25 + 2°C and at 90° to the incident beam applying the principal of photon correlation spectroscopy (PCS) on samples diluted fivefold (v/v) in water and filtered through 0.22µ filter. All data obtained were analyzed by Contin program [19 – 20].

Determination of Drug Encapsulation Efficiency:

The percentage of incorporated Isoflavones i.e. entrapment efficiency was determined by spectrophotometric determination at 260 nm on Shimadzu UV-1650 PC UV-VIS Spectrophotometer (using methanol as solvent), after centrifugation (Eltek TC4100D Research Centrifuge) of the aqueous dispersion. The amount of free drug was detected in the supernatant and the amount of incorporated drug was determined as a result of the initial drug minus the free drug. The entrapment efficiency was calculated using following equation:Entrapment Efficiency (%)= $\{(W_{initial\ drug} - W_{free\ drug)/} W_{initial\ drug}\} \times 100$, where "$W_{initial\ drug}$" is the mass of initial drug used for the assay and the "Wfree drug" is the mass of free drug detected in the supernatant after centrifugation of the aqueous dispersion [19 – 20].

Particle morphology

Sample was analyzed in the form of aqueous dispersion using a Cameca SU-SEM probe (Resolution: upto 40 oA; Magnification: upto 40,000 x; Accelerating Voltage: upto 30 kV; fully integrated EDS/WDS system) at 25+ 2 °C. No special sample preparation was required.

Characterization of the Gel

Determination of drug content, spreadability, and pH

For determination of drug content, 1 g of the formulation was taken in a 50 ml volumetric flask, and volume was made up with methanol. The samples were mixed thoroughly and filtered using a 0.45 µm membrane. About 5 ml of this filtered solution was taken in a 10 ml volumetric flask and the volume was made up with mobile phase. The solution was then injected in the HPLC system and the content of the three isoflavonesviz, Daidzein, Glycetin and Genistein was ssessed.

The spreadability of the gel was determined using the following technique: 0.5 g gel was placed within a circle

of 1 cm diameter pre-marked on a glass plate over which a second glass plate was placed. A weight of 500 g was allowed to rest on the upper glass plate. The increase in the diameter due to spreading of the gels was noted [21].The pH of the 10%w/w gel was determined using Equip-tronic Digital pH meter Model EQ 610, standardized using pH 4.0 and 7.0 standard buffers before use [19 – 20].

Rheological Studies on the Gel:

Brooke field Synchro-Lectric Viscometer (Model RVT) with helipath stand was used for rheological studies. The sample (30g) was placed in a beaker and was allowed to equilibrate for 5 min before reassuring the dial reading using T-C spindle at 0.5, 1, 2.5, and 5 rpm. At each speed, the corresponding dial reading on the viscometer was noted. The spindle speed was successively lowered and the corresponding dial reading was noted. The measurements were carried in duplicate at ambient temperature. Direct multiplication of the dial readings with factors given in the Brooke field viscometer catalogue gave the viscosity in centipoises.

The consistency index and flow index were calculated from the Power law equation: $\tau = K\ rn$, where: "τ" is shear stress; "r" is shear rate; "K" is consistency index; "n" is flow index.Taking log of both sides, $\text{Log } \tau = \log K + n \log r$. Shear stress (dynes/cm2) = Viscosity (cps) X Rate of shear (sec-1). Thus, from the plot of log of shear stress v/s log of shear rate, the slope of the plot representing flow index and antilog of the y-intercept indicating consistency index was calculated [21].

In vitro skin permeation studies

The drug deposition potential of SLN based gel formulation was assessed using pig ear skin model. For the purpose of this study, pig ears were obtained within 2 h after slaughter of the animals. The whole skin membrane was then carefully removed from the underlying cartilage with the help of a scalpel. The subcutaneous tissues were removed and the skin was stored at -4^0C for a maximum period of 30 days before use [22].

The skin specimens prepared were then individually placed in KesharyChien type diffusion cells (n = 6) set at 32°C with thermostated water bath. About 10 ml of phosphate saline buffer pH 7.4 was used as the receptor phase. Assurance was made that the skin is in full contact with the receptor phase, leaving out any air bubbles. Next, about 300 to 350 mg/cm2of gel formulation was uniformly placed in the donor phase, in contact with the excised section of skin. Receptor phase was stirred constantly throughout the experiment and the temperature kept at 25°C. At set intervals of 0, 1, 3, 6, 12 and 24 hrs, 1 ml aliquot from the receptor phase was removed and immediately replaced by the same volume of phosphate buffer saline pH 7.4. The amount of isoflavones released into the receptor phase from the gel formulations was determined by HPLC and the cumulative % of drug permeated versus time (hours) graphs were plotted. The amount of formulation remaining on the skin was diluted suitably with mobile phase and amount of isoflavones remained on the skin (i.e. in donor compartment) was calculated by gently wiping off the formulation remaining on the skin. The skin was minced, transferred to a test tube and subjected to vortexing for 15 min in a mobile phase using a cyclomixer. The resulting solution was filtered through 0.45 µm membrane, injected into the chromatographic system and concentration was determined [15].

A comparative evaluation of the drug deposition potential of SLN nanoparticles and plain isoflavoneaglycone was performed wherein plain isoflavones dissolved in propylene glycol were dispersed in Carbopol 971P gel. The deposition ability of this was compared with Formulation 1 and Formulation 2 [23].

Skin irritation studies

Formulation 1 was tested for primary skin irritation using Draize patch test in rabbits [24 – 25].

Investigations using experimental animals are conducted in full compliance with local, national, ethical, and regulatory principles and local licensing regulations, per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International's expectations for animal care and use/ethics committees. The protocol for the animal study was approved by the Institutional Animal Ethical Committee (IAEC) no. ICT/PH/IAEC/0909/18.For this study, three white New Zealand rabbits weighing 2.5–3 kg were used. The back and sides of the rabbits were clipped free of hair 24 h prior to the application of formulation. The following formulations were applied to the skin of the rabbits after 24 hrs:

Group I: Drug loaded SLN based gel formulation (Soy isoflavones 0.1% w/w SLN based gel)

Group II: Drug free SLN based gel formulation (placebo SLN based gel)

Group III: Positive Control (No application)

About 1g of SLN based gel was applied on the hair free skin of rabbits by uniform spreading within the area of 4

cm2. The skin was observed for any visible change such as erythema (redness) or oedema (swelling). Evaluation was done by using the scale given by Draize. Scores between 0 and 4 were used to grade erythema and oedema, which range from no response to a severe response.

Table 2: characterization of extracted soy isoflavoneaglycone

Sr No	Parameters	Observation		
1	Description	Buff colored powder		
2	pH of 1% solution	4.88		
3	Bulk density	0.15 g / ml		
4	% LOD: (105°C for 10 min)	2.06		
5	λ max obtained from Ultraviolet/ Visible Spectrum	261.5 nm		
6	Total Phenolic content	Methanol	Methanol – Water (1:1)	Water
		98.28	60.73	26.32
7	IC_{50} value for DPPH· inhibition (Methanol extractable)	125µg/ ml		
8	Total reduction capacity by Oyaizu Method (%)	Methanol	Methanol – Water (1:1)	Water
		2.54	1.68	0.78

RESULTS

Extraction and Characterization of Isoflavoneaglycone from dry herbal whole Soy extract

About 81.25 % extraction efficiency was obtained by using the extraction method with a yield of 0.65g of isoflavoneaglycone obtained from 2g of whole soy extract containing 40% isoflavones. The actives were characterized with respect to their physical properties, spectral properties and antioxidant properties. The results for physical characterization are summarized in Table 2.

An ultravoilet absorption spectrum for a 10 ppm sample of extracted isoflavone is shown in Fig 1. The spectrum showed a λmax at 261.5 nm a value almost equal to the reported λmax value of 262 nm for isoflavones [26]. IR spectrum showed the presence of all the functional groups commonly found in isoflavones Fig 2.Isoflavoneaglycone solution of 125 µg / ml strength was able to inhibit 50 % of DPPH in the assay and was found to have comparable IC 50 value with the original herbal extract of Soy isoflavones. The results are indicated in Fig 3.

The results of the total reduction capacity by Oyaizu method were consistent with those of the DPPH• Free Radical Scavenging Assay with isoflavoneaglycone showing total reduction capacity value similar to that of the original soybean extract in all the three extraction solvents. The results are indicated in Fig 4.

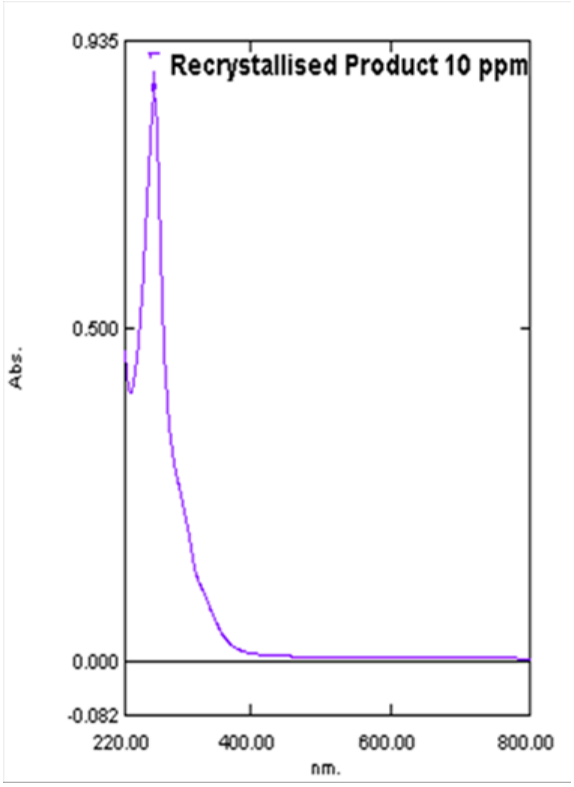
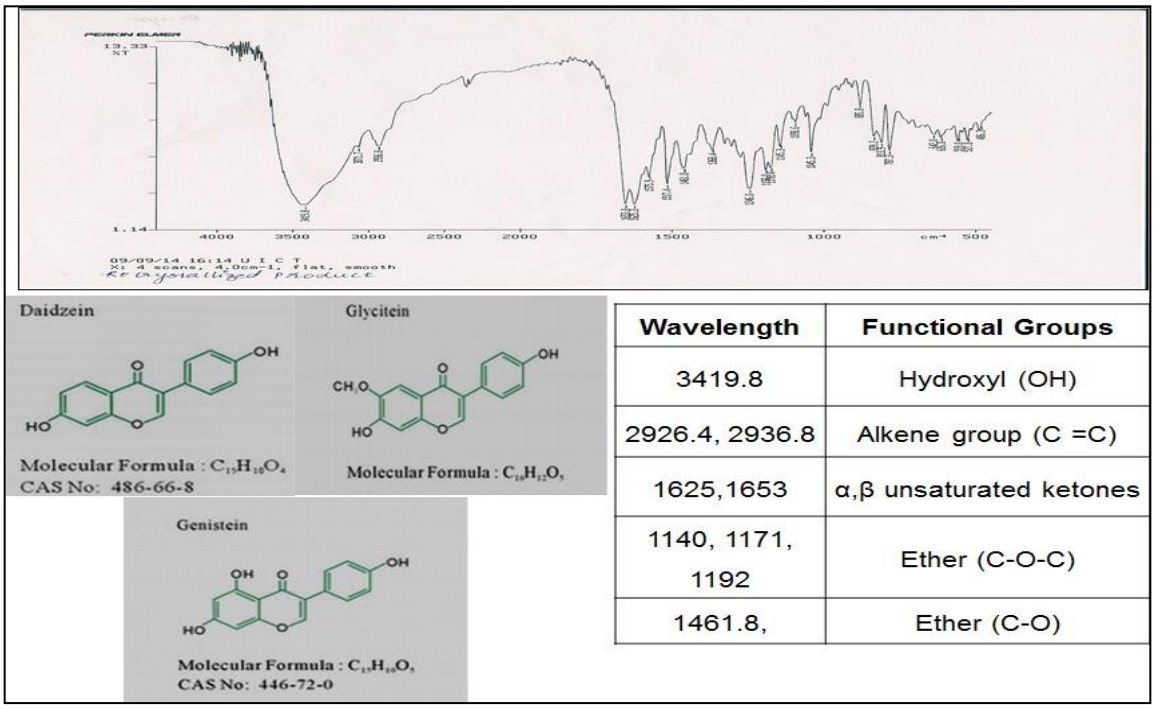


Figure 1: Ultravoilet absorption spectrum for a 10 ppm sample of extracted isoflavone



Wavelength	Functional Groups
3419.8	Hydroxyl (OH)
2926.4, 2936.8	Alkene group (C =C)
1625,1653	α,β unsaturated ketones
1140, 1171, 1192	Ether (C-O-C)
1461.8,	Ether (C-O)

Figure 2: IR spectrum showing the presence of all the functional groups commonly found in isoflavones

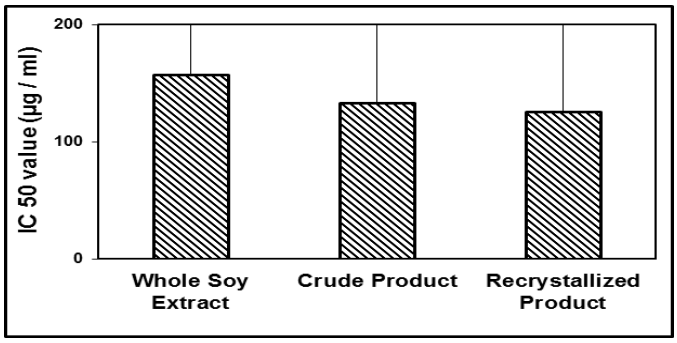


Figure 3: A comparative plot of 50 % Inhibitory Concentration value (IC 50) of DPPH vs. Concentration (µg / ml) of Methanol soluble extractives of Isoflavones in whole herbal extract, crude product and re-crystallized product

Formulation development

Solubility of soy isoflavoneaglycones in various solid – lipids and surfactants is shown in Fig 5a and 5b respectively.

Construction of Pseudo-ternary phase diagrams

Figures 6a and 6b show ternary phase diagrams for Softisan 601-Tween 20-Brij 35 and Tefose 63-Tween 20-Brij 35 systems, respectively. Tween 20: Brij 35 combination in the ratio of 1:1 at 10% concentration level was able to provide the desired particle size range.

Stability studies

Methocel® E5 LV was incorporated at 5 % concentration level by dispersing in the hot aqueous phase during microemulsion formation as crystal growth inhibitor to impart stability to the formulation.

Characterization of Nanoparticulate Dispersion

The average particle size of nanoparticulate dispersion formulated using Softisan 601 as solid lipid was found to be 126nm with a polydispersity index of 0.72 indicating homogeneity in particle size distribution while of the dispersion formulated using Tefose 63 was found to be 134 nm with a polydispersity index of 0.86. The drug encapsulation efficiency in the nanoparticles was found to be 57% for Formulation 1 while 46 % for Formulation 2.The image of particle morphology obtained from SEM of Formulation 1 is depicted in Fig 7.

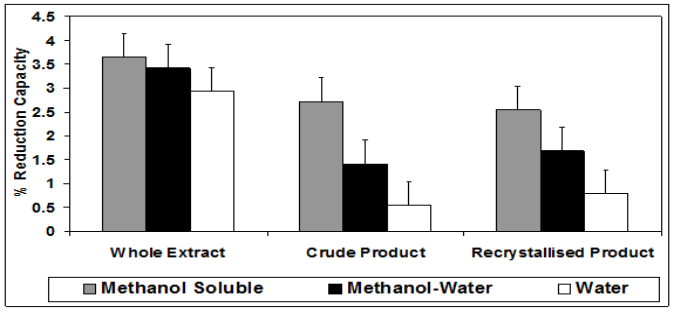


Figure 4: Plot of percent reduction capacity of the isoflavones vs. concentration (µg / ml) of methanol soluble, methanol – water soluble and water soluble extractives of isoflavones in whole herbal extract, crude product and recrystallised product

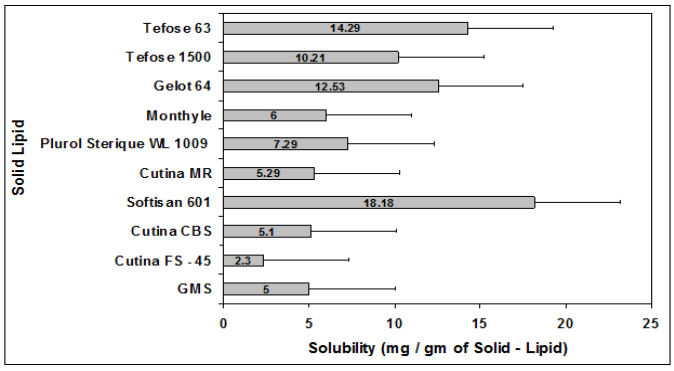


Figure 5a: Solubilization capacity of Isoflavone in various Solid – Lipids

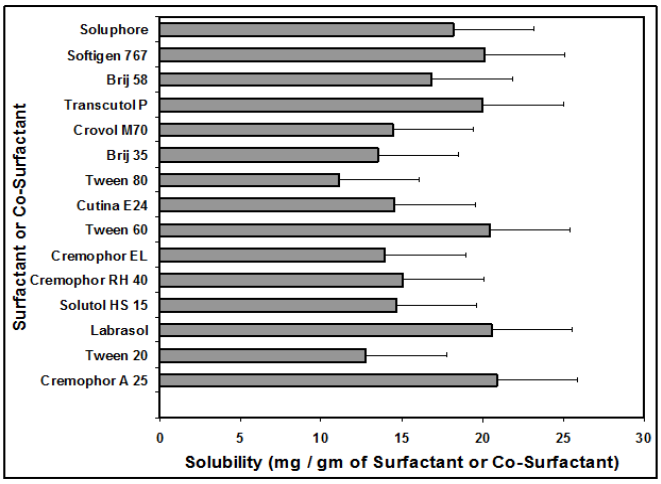


Figure 5b: Solubilization capacity of Isoflavone in various Surfactants and Co – Surfactants

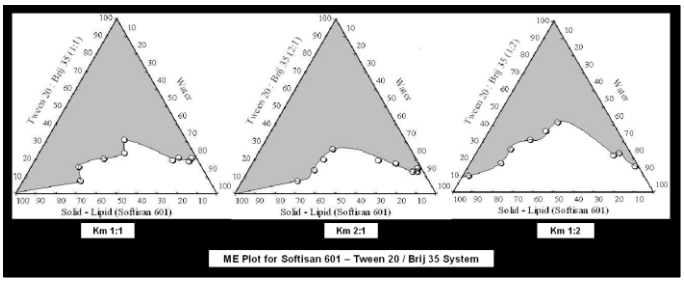


Figure 6a: Pseudo ternary Phase diagram for Softisan 601 - Tween 20 - Brij 35 system

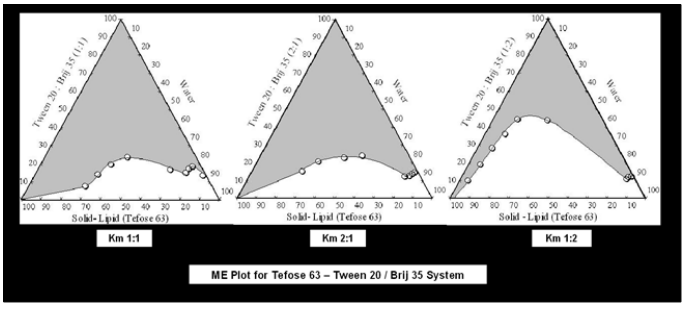


Figure 6b: Pseudo ternary Phase diagram for Tefose 63 - Tween 20 - Brij 35 system

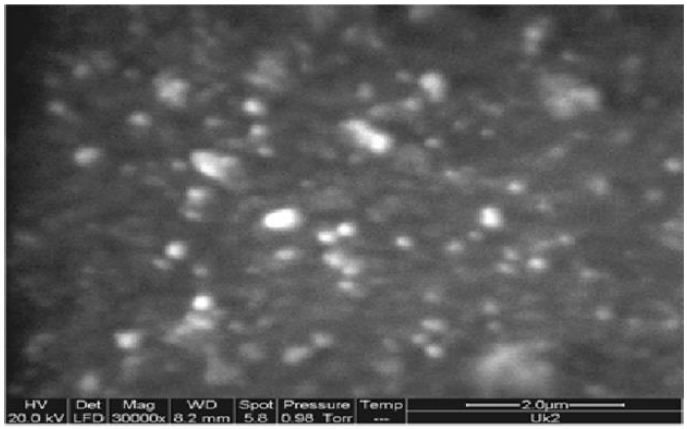


Figure 7: SEM image of aqueous SLN dispersion

Characterization of the Gel

The results of characterization of SLN particles and gel are summarized in Table 3.

Table 3: Characterization of SLN based 0.1% soy isoflavone gel

Sr. No	Parameter	Formulation 1	Formulation 2
	Evaluation of SLN Dispersion		
1	Particle Size (nm)	126	134
2	Polydispersity Index	0.72	0.86
3	Entrapment Efficiency (%)	57	46
	Evaluation of SLN Based Gel		
4	pH	6.79	6.84
5	Spreadability	6.2 cm	
6	Rheology	9 x 106 cps at 5 rpm	
7	Drug Content	102.72 %	99.47 %

In vitro skin permeation studies

Fig 8 indicates cumulative % permeated through pig ear skin over a period of 24 hours. About 60% deposition of the isoflavons was achieved in the dermal matrix when formulated in the form of SLN based gel.

Primary skin irritation studies

The results of primary skin irritation test are depicted in Table 4with primary irritation index values on the skin at the end of 4, 24, 48, 72, 96 and 120 hours. The formulation i.e. drug loaded SLN based gel and drug free

SLN based gel (Placebo) showed no skin irritation on intact rabbit skin. In no case edema was observed.

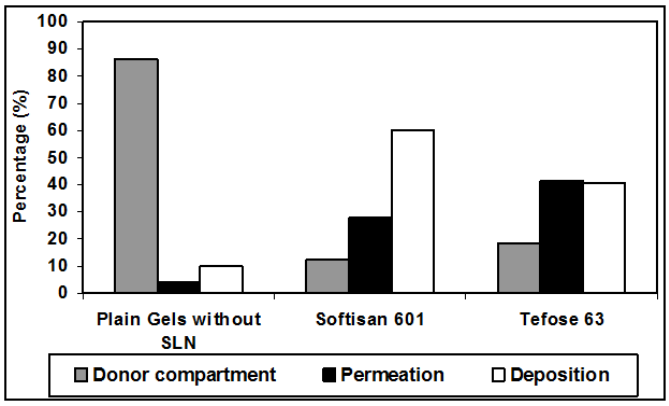


Figure 8: Comparative plot of % content of Isoflavones permeated or deposited in the pig ear skin and retained in the donor compartment form Plain Gel without SLN, SLN based Gel prepared using Softisan 601 and Tefose 63

DISCUSSION

Extraction and Characterization of Isoflavoneaglycone from dry herbal whole Soy extract

Owing to their hydrophobic nature, soy isoflavoneaglycones are highly soluble in organic solvents such as ethanol while are completely insoluble in water. Hence this solubility difference was used to isolate aglycones from the whole extract of Soy. Moreover the isoflavones exist in the form of glycosides in the whole extract. Hence the extract was refluxed with ethanolic hydrochloric acid so as to hydrolyse the glycosidic linkages and aid in complete extraction of the aglycones in ethanol.

Table 4: Primary irritation index values on the skin at the end of 4, 24, 48, 72, 96 and 120 hours

Formulation	Irritation Score					
	4 h	24 h	48h	72h	96h	120h
SLN based gel with drug	1	1	1	0	0	0
Placebo SLN based gel	1	1	0	0	0	0
Positive Control	0	0	0	0	0	0

The total antioxidant activities of herbal extracts cannot be evaluated by any single method, due to the complex nature of phytochemicals [27]. The DPPH• assay that measures radical scavenging capacity (RSC) is widely used for the measurement of free radical scavenging capacity in phytotechnology. In this method, the capacity of extracts to scavenge the lipid soluble DPPH• radical, which results in the bleaching of the purple color exhibited by the stable DPPH• radical is monitored at an absorbance of 517 nm. This bleaching occurs due to reduction of the antioxidants and lower absorbance of the reaction mixture thereby indicating higher free radical scavenging activity [13]. Gallic acid was taken as the standard antioxidant. Different studies have indicated that the electron donation capacity of bioactive compounds is associated with antioxidant activity. Thus in case of Oyaizu method the ability of extracts to reduce the ferric-ferricyanide (Fe+3) complex to the ferrous – ferricyanide (Fe+2) complex of prussian blue was determined by recording the absorbance at 700 nm after incubation and compared to that of gallic acid which is a known reducing agent [14].

The extracted active showed good antioxidant activity thereby confirming that the active have retained its activity even when subjected to harsh extraction conditions such as refluxing in 4M ethanolic hydrochloric acid and drying at 50ºC for 9 hours.

Formulation development

To achieve optimum drug loading solubility, the study was aimed to identify suitable solid – lipid components that possess good solubilizing capacity for the isoflavoneaglycone [28 – 30]. The isoflavones exhibited good solubility in modified lipids which are a blend of two or more lipids with emulsifiers. Thus appreciable solubility was observed in solid – lipid Softisan 601 (GlycerylCocoate + Hydrogenated Coconut Oil + Ceteareth – 25), Tefose 63 (PEG-6-32 stearate + glycol stearate) and Gelot 64 (Glyceryl stearate + PEG-75). Among surfactants, Tween 20 solubilized maximum amount of the isoflavoneaglycones; whereas in co-surfactants, Brij 35 exhibited good solubility for the aglycones. Selection of surfactants and co-surfactants was governed by their emulsification efficiency for selected solid – lipids rather than their ability to solublize the isoflavones [16 – 18]. Thus Softisan 601 and Tefose 63 gave more stable systems in comparison to Gelot 64. Hence these lipids were further screened for development of solid – lipid nanoparticles.

Construction of Pseudo-ternary phase diagrams

From the ternary phase diagrams for Softisan 601-Tween 20-Brij 35 and Tefose 63-Tween 20-Brij 35 systems it was observed that the microemulsion region for systems with Km = 1 was higher than those with Km = 0.5 but was comparable with systems with Km = 2. Hence, surfactant to cosurfactant ratio was maintained

at 1:1 (Km = 1). The amount of lipid incorporated in the system was governed by the solubility of the aglycone in the lipid and percent active loading. Thus to obtain a 0.1 % soy isoflavone gel about 5.5% of Softisan 601 and 7 % of Tefose 63 was required in the system. The surfactant-co-surfactant combination at 10% concentration was able to emulsify the required quantity of the solid lipid and provided clear and stable microemulsion systems with desired drug loading. The microemulsions prepared using the optimized quantity of selected components could survive the freeze-thaw cycling thus indicating the microemulsions to be thermodynamically stable. In the optical birefringence studies the micro emulsion was found to be isotropically clear. For optimum penetration through the dermal strata and efficient deposition in dermis of the skin a particle size range of 50 – 150 nm is desired. Thus the surfactant- co- surfactant concentration providing the said size range was considered optimum [5].

Stability studies

Stabilization of the SLN dispersions is usually accomplished by incorporating gelling agents or crystal growth inhibitors in the formulation. Use of gelling agent aids by increasing the viscosity of the formulation thereby decreasing the rate of particle agglomeration and provides stability to the formulation. The gelling agent was selected on the basis of ease of dispersion, safe for use for topical purposewith no dermal irritancy, compatability with other excipients, and capable of forming aesthetic gels with good feel and ease of application. Hence of the various screened gelling agents Carbopol 971P suited all the above mentioned criteria. But the gels showed turbidity after 35 days. It was observed that similar turbidity was observed in placebo formulation and was due to crystallisation of lipids in the formulation. Hence crystal growth inhibitors were incorporated to prevent this crytallisation of lipids. Of the different hydrophyllic polymers used Methocel® E5 LV was effective in inhibiting recrystallization of the lipids and providing stability to the formulation when stored in refrigeration [31 – 32].

Characterization of Nanoparticulate Dispersion

The Photon Correlation Spectroscopy (PCS) was used to monitor particle size and polydispersity of the SLN based dispersion. The basic principle of this method is that collections of illuminated scatters produce at any instant a net scattered light intensity, which results from the interference of the light scattered from each of them. Brownian motion causes the position and orientation of each scatter to change with time. Therefore, the phase and polarization from each scatter and the net scattered light intensity of a given polarization also fluctuate with time. This shift in wavelength is so small that it can be detected only for laser light beams, which are strictly monochromatic and very intense. These intensity fluctuations are thus related to the transitional diffusion coefficients of the illuminated scatters. The intensity fluctuations will be rapid for small rapidly moving particles than for more slowly diffusing particles. The diffusion coefficient, D is inversely proportional to the mean particle radius, r, as described by the Stokes-Einstein relationship: D = (KT / 6□□r) where: “k” is Boltzmann constant, “T” is absolute temperature, “□” is the suspending medium viscosity and “r” is the hydrodynamic radius of a spherical particle. There was a marginal increase (131nm + 10nm) in the particle size for Softisan 601 systems, while for Tefose 63 systems (140 nm + 10nm) after gelling of the SLN dispersion. Polydispersity is measure of particle homogeneity and it varies from 0 to 1. The closer the values to zero, more homologous are the particles [19].

Determination of the amount of drug incorporated in SLN's is of prime importance, since it may influence the release characteristics. The amount of drug encapsulated per unit weight of the nanoparticles is determined after separation of the free drug and solid lipids from the aqueous medium. This separation can be carried out using ultracentrifugation, centrifugation filtration or gel permeation chromatography [20]. In the current study ultracentrifugation method was employed.

Characterization of the Gel

The gel showed optimum viscosity at the concentration of 1% of Carbopol 791P. The drug content of the SLN based gel and pH was found to be within acceptable limits. Spreadability is an important property of topical formulation from a patient compliance point of view. Application of the formulation to skin is more comfortable if the base spreads easily, exhibiting maximum “slip” and “drag.” The large diameter indicates better spreadability. In general, the gels that possess a high consistency index are less spreadable. The release of the drug from the formulation is governed by its components as well as by the consistency of the formulation. Consistency index is a

measure of consistency and is equivalent to apparent viscosity at a shear rate of 1 sec−1. The consistency index of the formulation was found to be 3.14 X 106. Flow index (n) confers an idea of the flowability of the formulation from the container. It is a measure of the deviation of a system from Newtonian behavior (n = 1). A value of n < 1 indicates pseudoplastic flow or shear thinning; n > 1 indicates dilatants or shear thickening flow. The gel showed a flow index of 0.0408, indicating pseudoplastic flow behavior [21].

In vitro skin permeation studies

The advantages of in vitro experiments are lower cost, ability to test large numbers of formulations in relatively short time ability to identify the rate-limiting skin layer for a given compound [33]. In this regard, pig ear skin is considered as an excellent skin model, because the histological characteristics of pig and human skins have been reported to be very similar in terms of epidermal thickness and composition, pelage density, epidermal lipid biochemistry and general morphology [34]. Therefore, we used this pig ear skin model for in vitro permeation studies.

As indicated in Fig. 8 the formulation with Softisan 601 as lipid (Formulation 1) showed better deposition of the isoflavones in the dermal matrix in comparison to the formulation containing Tefose 63 as the lipid (Formulation 2).

CONCLUSION

Soy isoflavoneaglycones were extracted from the whole extract of soy bean containing 40 % Isoflavones. The aglycones were characterized using spectral methods and were found to possess all the required functional groups found in isoflavones. They were also characterized for their antioxidant activity, and were found to have optimum activity thereby indicating that the activity was retained in spite of being subjected to harsh extraction conditions. SLN based gel containing 0.1% soy isoflavones were successfully developed. The gel was found to be non-irritant in nature and afforded about 60% deposition of isoflavones in the dermal matrix of pig ear skin by incorporating them in solid – lipid base.

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