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Isolation of ROS scavengers and development of its delivery system

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Abstract

The present work was aimed at unlocking the pharmacologically active antioxidant molecules from the plant source and preserving their reactive oxygen species (ROS) scavenging activity in a well-planned environment with improved efficacy. Curcuminoids found in Curcuma species are multifaceted compounds containing curcumin, demethoxycurcumin, bisdemethoxycurcumin. In the present work, these were extracted efficiently from the rhizome of *Curcuma longa* L. with the help of statistical tools. The critical parameters like drug solvent ratio, extraction time were optimized. The identity of the compounds was confirmed with the help of instrumental techniques like HPTLC, FT-IR and HPLC. To improve solubility of sparingly soluble curcuminoids, without losing antioxidant properties was a challenge. Self-micro emulsifying delivery system (SMEDDS) was chosen as it has high drug loading capacity and is thermodynamically stable. The excipients were chosen based on solubility studies. The phase diagrams were constructed to define the formulation space. The developed delivery system was further evaluated for its permeability and ability to maintain antioxidant contents. The developed SMEDDS were able to achieve above desired quality attributes and can be marketed in future.

1. Introduction

For centuries, turmeric is being recognized and used worldwide in many different forms like curries, tea, drinks, sauce, cheese, chips and as a preservative and a coloring agent (Hewlings and Kalman, 2017). Turmeric, *Curcuma longa* (*C. longa*), is a perennial herb of Zingiberaceae family. It is grown in the various regions of world with tropical climate, but extensively in India and China, where it is part of not only food and medicine but sociocultural life. The rhizome portion of the plant is used predominantly as it can be dried, preserved for long time.

Turmeric powder, the powder of dried rhizomes, is about 60-70% carbohydrates, 6-13% water, 6-8% protein, 5-10% fat, 3-7% dietary minerals, 3-7% essential oils, 2-7% dietary fiber, and 1-6% yellow pigment. This golden yellow colour of the turmeric powder is due to curcuminoids, agent responsible for diverse health promoting effects (DiSilvestro *et al.*, 2012). Curcuminoids also possess therapeutic properties like anticancer (Liu and Ho, 2018), antiulcerative colitic (Lahiff and Alan, 2011), antiarthritic (Chandran and Goel, 2012), antidiabetic (Pivari *et al.*, 2019), anti-alzheimeric (Ringman *et al.*, 2005). To get maximum benefits, these curcuminoids are separated from turmeric and used. Since the turmeric rhizome contains multiple components, extraction of curcuminoids becomes tedious.

Curcuminoids is a mixture of three principal compounds: 77% of curcumin (curcumin I), 17% of demethoxycurcumin (curcumin II), and 3% of bisdemethoxycurcumin (curcumin III) (Figure 1) (Srinivasan, 1953). These compounds are practically insoluble in

water at acidic and neutral pH, and soluble in methanol, ethanol, dimethyl sulfoxide and acetone and unstable in alkaline pH.

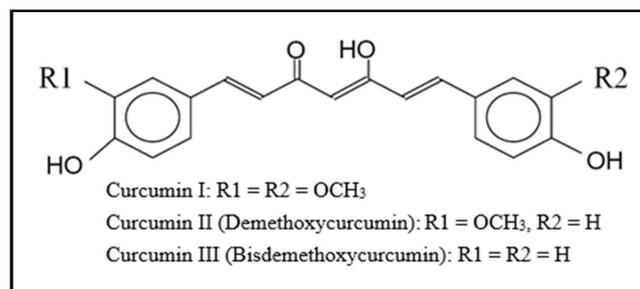


Figure 1: Chemical structure of curcuminoids.

The pharmacokinetics of curcumin, on oral administration was studied in Sprague-Dawley rats. Measurements of blood plasma levels and biliary excretion showed that curcumin was poorly absorbed from the gut. About 75% was excreted in the feces (Wahlstrom and Blennow, 1978). In this sense, it is comparable to class IV drugs of Biopharmaceutics Classification System.

To improve the bioavailability of curcumin, numerous approaches like complexation (Celebioglu and Uyar, 2020), novel particulate dosage forms (Chen *et al.*, 2012; Acevedo-Guevara *et al.*, 2018) were developed. The major disadvantage of these techniques is high cost, due to use of specialised excipients or manufacturing equipments. There is a need to develop stable dosage form with high drug loading efficiency and easy scalability at low cost. In the present work, curcuminoids were extracted to achieve maximum yield using statistical analysis and then were formulated into a self microemulsifying drug delivery system (SMEDDS). SMEDDS is a mixture of oil, surfactant and cosurfactant which on dilution with aqueous media forms transparent emulsion without the aid of mechanical equipment.

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2. Materials and Methods

2.1 Chemicals and plant material

Rhizomes of turmeric were collected from Palghar, Maharashtra, India. Vegetable oils were obtained from the local supermarket. Tween 80 and glycerol of LR grade were purchased from SD Fine Chem Industry. Ethyl oleate, hexane and propan-2-ol were from Loba Chemie. The standard curcumin (total curcuminoids 97% and curcumin 84%) was purchased from Sigma-Aldrich Chemie GmbH. Crodamol GTCC was gifted by Croda India. Kollisolv PEG 400 was gifted by BASF, India. HPLC grade water was purchased from J.K. Labs, Thane, Maharashtra. All other solvents of AR grade and HPLC grade were purchased from E. Merck.

2.2 Extraction of curcuminoids

The fresh turmeric rhizomes were cleaned with water, sliced in thin pieces and dried in shade. The semi-dried material was further reduced to size less than 500 micron. The material was further dried in an oven to get the moisture content below 10 %w/w and reduced to size less than 250 micron. This turmeric powder was defatted using hexane. The solvent was removed with decantation and turmeric powder was dried at room temperature. This defatted turmeric powder was used for extraction.

In Soxhlet extraction, turmeric powder was placed in a “thimble” made of strong filter paper. The required quantity of methanol was added in distillation flask as a solvent. The turmeric powder and methanol were taken in the ratio of 1:10 to 1:30. The extraction was carried for 3 to 6 h. Upon completion of the extraction, the solvent was separated from the extract under reduced pressure using rotary vacuum evaporator (Superfit Continental Pvt. Ltd, Mumbai, India). The resultant precipitate was washed twice with equal amount of cold propan-2-ol: hexane (1:1) to get purified curcuminoids. The weight of resultant curcuminoids was recorded.

2.3 Identification of curcuminoids

The standard and sample (extracted) curcuminoids were separately dissolved in methanol and optical density was recorded in the region 200 to 600 nm on UV-Vis Spectrophotometer (Evolution 300™, Thermo Fisher Scientific, Hemel Hempstead, UK). HPTLC studies were performed using TLC Silica 60 F 254 plates. 1 mg/ml standard and sample curcuminoids solutions in acetone were applied in bands of 8 mm width. The plates were developed using solvents chloroform: methanol: formic acid in the ratio of 96:04: 01. CAMAG, Linomat 5, Switzerland, software was used for scanning the plates. The FTIR spectral studies for the standard and sample curcuminoids were carried out using potassium bromide as a substrate in the range of 400-4000cm⁻¹ on Bruker Alpha T (Bruker Optik GmbH, Ettlingen, Germany). The HPLC analysis was carried out on Agilent 1260 Infinity II (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) equipped with UV/Vis Detector. Mobile phase used for analysis was composed of acetonitrile: 0.1 % orthophosphoric acid in HPLC water (35:65 % v/v). The standard and sample curcuminoids were dissolved in methanol. Column Inertsil ODS-3V, 150*4.6 mm, 5 µm (GL Sciences Inc, Tokyo, Japan) was used for analysis. The detection was carried out at wavelength of 420 nm.

2.4 Formulation development for curcuminoids

2.4.1 Solubility study for curcuminoids

Curcuminoids solubility studies in various oils, surfactants, and co-surfactants were carried out. In a glass vial, 5 g of vehicle were taken to which curcuminoids were added in increments of 10 mg. The samples were stirred. The procedure was repeated till the solution was saturated and undissolved curcuminoids were visible. Each sample was stirred at room temperature for 24 h. After reaching equilibrium, each vial was centrifuged at 7000 rpm for 20 min. The supernatant was filtered through a 0.45 µm syringe filter membrane and diluted with methanol for quantification by UV-Visible spectrophotometer.

2.4.2 Construction of phase diagrams

Tween-80 and Kollisolv PEG 400 were mixed in the ratio 1:1, 2:1 and 4:1 to form surfactant mix (Smix). Oil (Crodamol GTCC) and Smix were mixed in the ratio of 1:9 to 9:1. Water was added in each test tube dropwise and mixed using vortex mixer. The observations were recorded. The clear transparent homogenous solution was considered as a microemulsion and phase diagrams were prepared.

2.4.3 Preparation of curcuminoids SMEDDS

On the basis of ternary phase diagrams SMEDDS components were selected for drug incorporation. Three batches F1 (10% oil and 90% Smix) F2 (12.5% oil and 87.5% Smix) and F3 (16.67% oil and 83.33% Smix) were prepared. The curcuminoids were added to it at the concentration of 2% w/w and stirred at room temperature for 30 min.

2.5 Characterization of curcuminoid SMEDDS

2.5.1 Clarity and particle size

After preparation, the SMEDDS were observed visually. Further, the SMEDDS were diluted with 250 ml of 0.1 N HCl and stored at 37°C to see the effect of dilution. The resultant solutions were examined spectrophotometrically at wavelength of 652 nm and per cent transmittance were recorded. Formulations F1 and F2 were further studied as they showed high transmittance. F1 and F2 were diluted 250 times and then droplets size was measured using Nano Sight NS500 (Malvern Instruments, Amesbury, UK).

2.5.2 In vitro dissolution studies

The SMEDDS formulations were filled in hard gelatin capsules (size 00) and introduced in dissolution jars of USP dissolution apparatus II (EDT-208Lx, Electrolab India Pvt Ltd, Mumbai, India). Each jar contained 500 ml of 0.1 N HCl maintained at 37°C and stirred at 50 rpm. The aliquots were taken at 0, 10, 20, 30, 40 and 60 min and replaced with equal quantity of dissolution media. The aliquots were filtered through 0.22 µm syringe filter and curcuminoids content were analysed by HPLC. The determination was performed on a C18 column (4.6 mm × 150 mm, 5 µm, Intersil ODS) with a mobile phase of acetonitrile: 0.1% orthophosphoric acid (35:65). The analysis was carried out at the wavelength of 420 nm. The graphs were plotted for % cumulative drug release Vs time. The dissolution profile was compared with dissolution profile of capsule filled with equivalent quantity of the drug suspended in phosphate buffer of pH 5.5.

2.5.3 *In vitro* permeability studies

The small intestine of overnight fasted chick (male, 0.5-0.6 kg) was immediately collected after slaughtering. The small intestine was washed with phosphate buffer solution and was cut into 6.0 ± 0.5 cm pieces. One end of intestine was tied with the thread. Curcuminoids and curcuminoid SMEDDS equivalent to 4 mg of the drug were dispersed in water and then carefully introduced into the intestine using blunt needle and syringe. The other end of the intestine was closed. Each sac was placed in a glass tube containing 20 ml of equal mixture of ethanol and phosphate buffer pH 5.5. The entire system was maintained at 37°C and was mounted on rotary shaker. Aliquots were withdrawn from outside the sac and the medium was replaced by fresh medium. The concentration of curcuminoids which were able to cross the membrane was measured by HPLC.

2.5.4 Antioxidant activity (AA) stability studies

2.5.4.1 DPPH free radical scavenging activity studies

Curcuminoids and curcuminoids SMEDDS were diluted with methanol and mixed with 2 ml of 0.004% DPPH solution. The solution was stored in the dark for 30 min and absorbance were recorded at 517 nm. The per cent inhibitions in free radical scavenging activities were calculated by following formula:

$$\% \text{ Inhibition} = ((\text{Abs of control} - \text{Abs of sample}) / \text{Abs of control}) \times 100 \dots (\text{Equation 1}).$$

2.5.4.2 Hydrogen peroxide scavenging activity studies

Curcuminoids and curcuminoids SMEDDS were diluted with ethanol and mixed with 2 ml of 20 mM solution of hydrogen peroxide in phosphate buffer (pH 7.4). After 10 min, the absorbance was measured at 230 nm using Spectrophotometer. The per cent inhibitions in hydrogen peroxide scavenging activities were calculated by following formula:

$$\% \text{ Inhibition} = ((\text{Abs of control} - \text{Abs of sample}) / \text{Abs of control}) \times 100 \dots (\text{Equation 2})$$

Curcuminoid and curcuminoids SMEDDS were further stored at 60°C in glass vials for 14 days. AA of the curcuminoids and curcuminoids SMEDDS stored at 60°C were compared with initial AA. The per cent reduction in antioxidant activity inhibition was calculated by the following equation:

$$\% \text{ Reduction} = ((AA_{\text{initial}} - AA_{\text{on 14 days at 60°C}}) / AA_{\text{initial}}) \times 100 \dots (\text{Equation 3})$$

2.5.5 Statistical analysis

The experiments were performed in triplicate. The data were recorded as mean \pm standard deviation and analysed by Microsoft office Home and Student edition of 2016. Significant differences between means for unformulated curcuminoids and curcuminoids SMEDDS were determined by student t test, and $p < 0.05$ was regarded as significant, and $p < 0.01$ was very significant.

3. Results

3.1 Extraction of curcuminoids

The extraction conditions were optimized by using statistical software Design Expert ® 8.0 software (Stat-Ease, Inc., Minneapolis). Central Composite design with three center point at

alpha value equal to 1 was used in the study. Drug solvent ratio and time were taken as independent variables and % yield as a dependent variable. Total 11 batches were prepared (Table 1).

Table 1: Design matrix for extraction of curcuminoids

		Factor 1	Factor 2	Response
Std	Run	A:A (Herb solvent ratio)	B:B (Time)	yield
				%
11	1	22.5	4.5	5.83
7	2	22.5	3	5.6
8	3	22.5	6	6.1
4	4	30	6	6.12
1	5	15	3	5.11
6	6	30	4.5	6.02
5	7	15	4.5	5.25
3	8	15	6	5.56
10	9	22.5	4.5	5.7
9	10	22.5	4.5	5.75
2	11	30	3	5.88

The central composite model used in the study was found to be significant with f-value of 46.67. There was only a 0.03% chance that an f-value, this large could occur due to noise. The response surface plot showed the effect of solvent quantity and time on yield (Figure 2). The optimized equation for the yield was:

$$\text{Yield} = +2.79487 + 0.191877A - 0.001725B - 0.004667A * B - 0.002760A^2 + 0.026550B^2 \dots (\text{Equation 4})$$

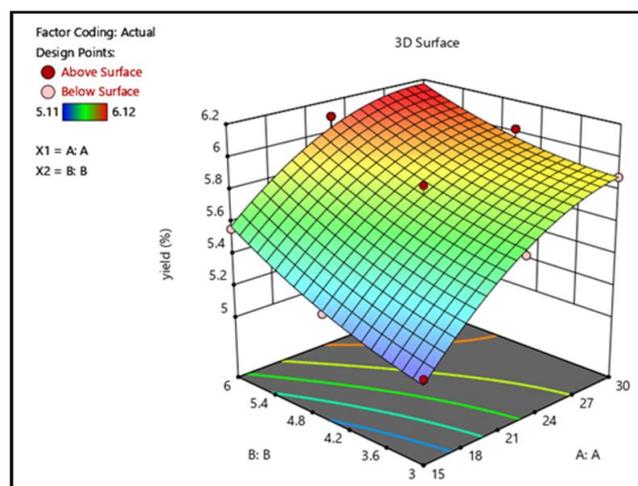


Figure 2: Response surface plot showing the effect of A:A (Herb: solvent ratio) and B:B (Time) on % yield.

3.2 Identification of curcuminoids

UV-VIS spectrum of methanolic solution of standard and sample curcuminoids matched with each other and showed maximum absorbance at a wavelength of 420 nm. In HPTLC studies,

bisdemethoxycurcumin, demethoxycurcumin and curcumin were detected at R_f of 0.203, 0.272 and 0.444, respectively (Figure 3) (Paramasivam, 2009). The percentage of curcumin and total curcuminoids were found to be 78.54% and 95.45%, respectively. FT-IR spectra of standard and sample curcuminoids (Figure 4) showed vibrations due to various chemical groups like -OH vibrations at 3508 cm^{-1} , C-H aromatic stretching vibration at 3057 cm^{-1} , -CH₃-

asymmetric stretching at 2947 cm^{-1} , -C = O stretching at 1627 , 1602 cm^{-1} , -C = C aliphatic stretching at 1510 cm^{-1} , -C = C aromatic stretching at 1456 cm^{-1} and phenol group at 1281 cm^{-1} . HPLC chromatograms (Figure 5) showed 3 distinct peaks corresponding to bisdemethoxycurcumin, demethoxycurcumin and curcumin at RT of 27.97, 32.41 and 37.39 for standard and sample curcuminoids. The total curcuminoids content was 98.3% and curcumin content was 81.8%.

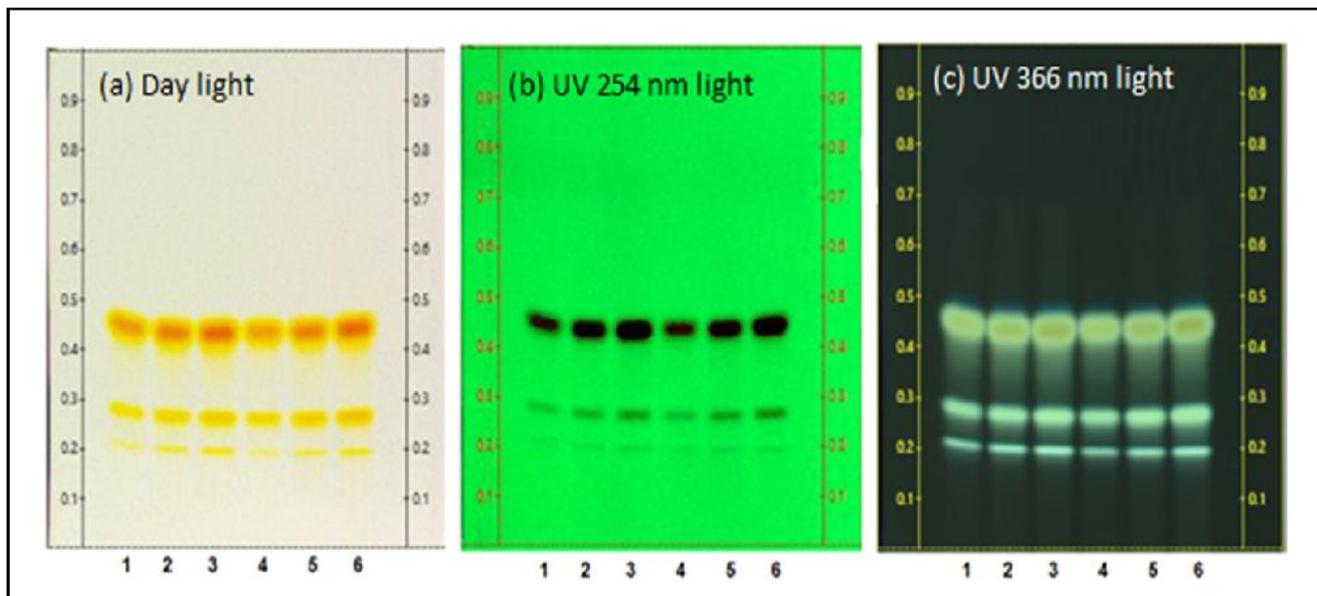


Figure 3: HPTLC chromatograms of curcuminoids (Track 1-3 of Standard and Track 4-6 of sample curcuminoids).

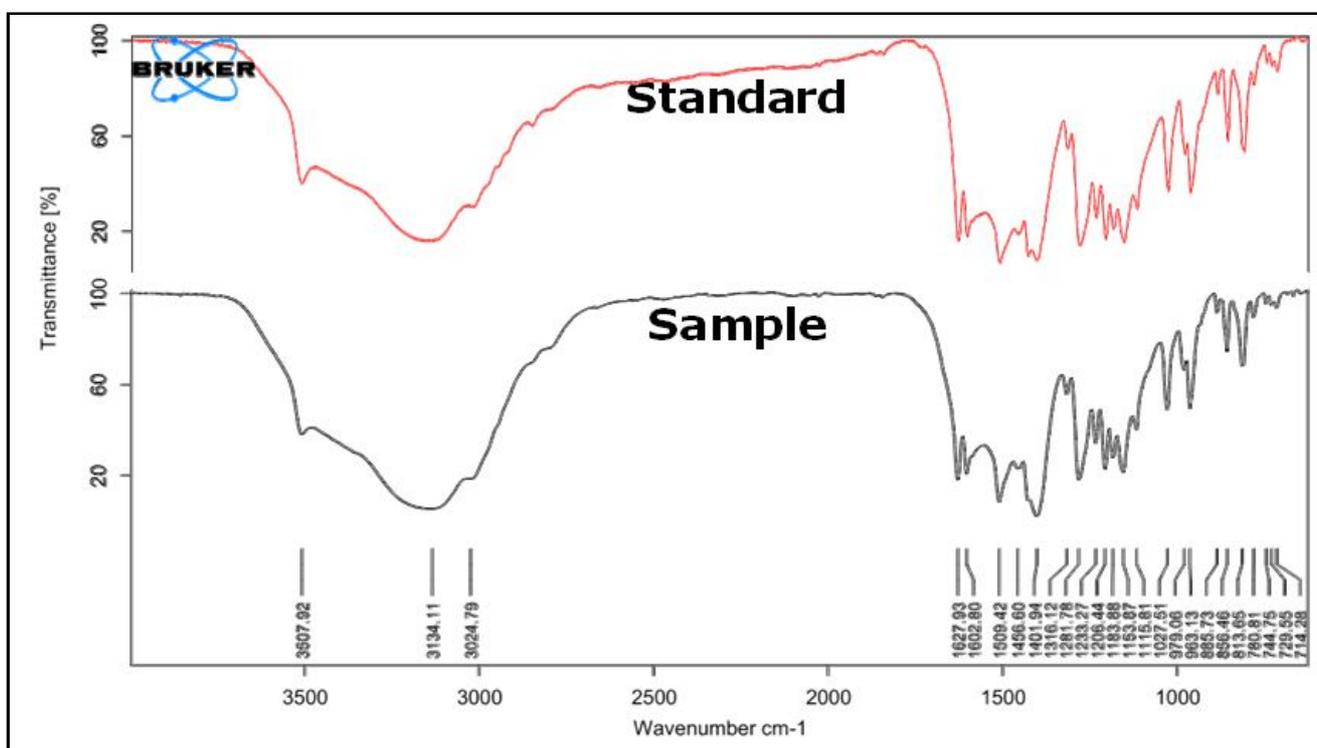


Figure 4: FT-IR spectra of standard and sample curcuminoids.

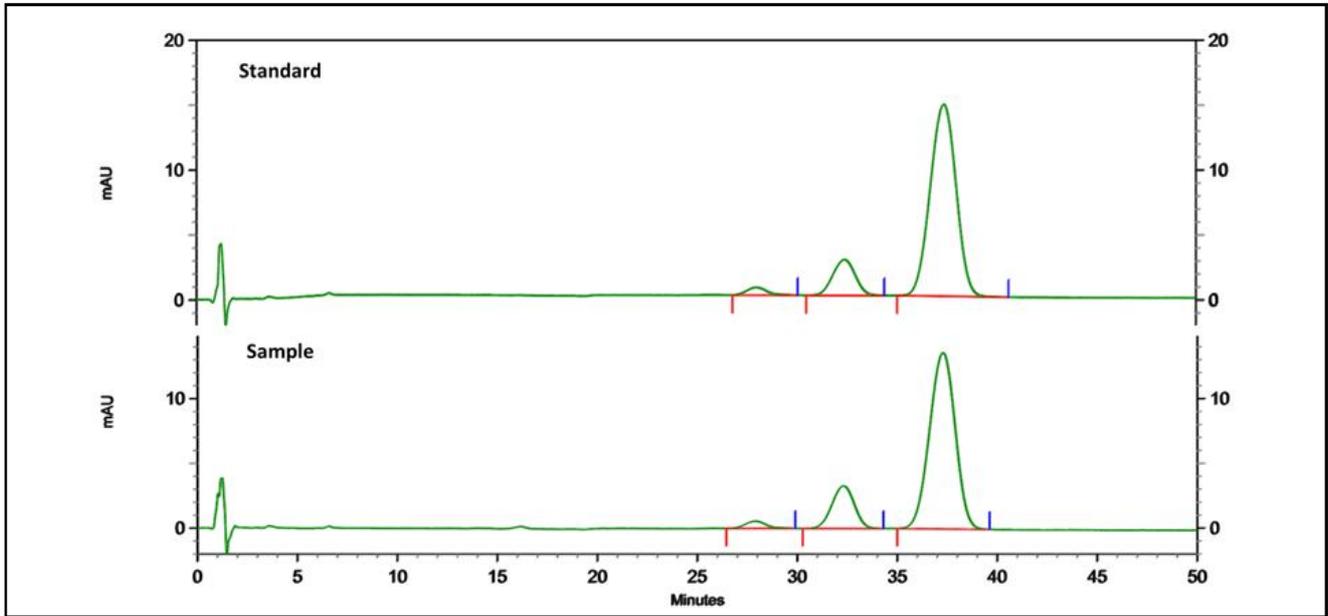


Figure 5: HPLC chromatograms of standard and sample curcuminoids.

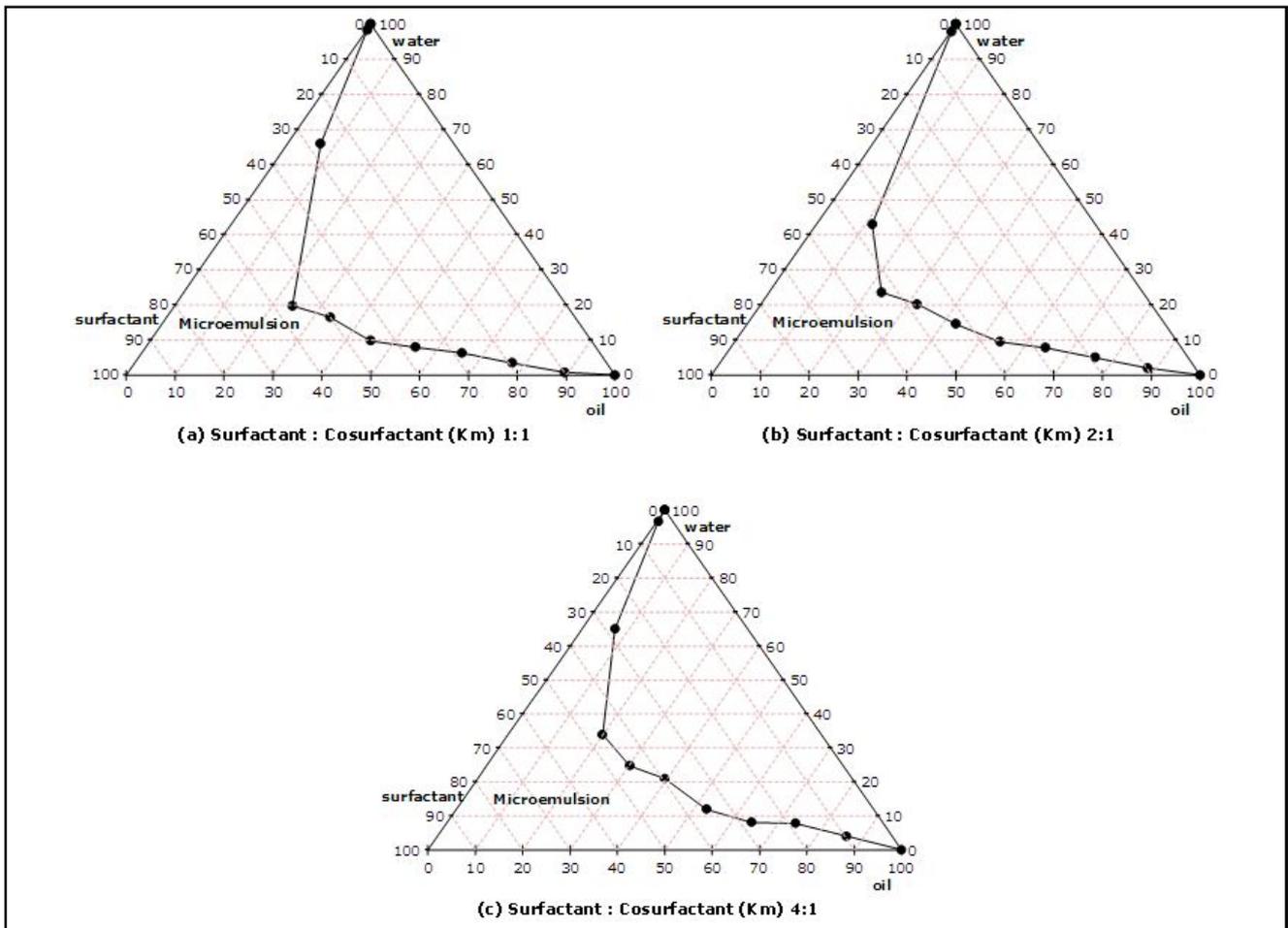


Figure 6: Pseudoternary phase diagrams indicating microemulsion regions for systems containing crodamol GTCC (oil), Tween 80: Kollisolv PEG 400 and water.

3.3 Formulation development for curcuminoids

Curcuminoids showed up to 0.1% w/w solubility in sunflower oil, soybean oil, sesame oil and isopropyl myristate. Ethyl oleate, glycerine, polyglycerol oleate, span 80 showed intermediate solubility. Highest solubility was found in crodamol GTCC, kollisolv PEG 400 and tween 80.

Based on solubility studies excipients chosen for further studies were crodamol GTCC (oil), tween 80 (surfactant) and kollisolv PEG 400 (cosurfactant). The pseudo ternary phase diagrams showing microemulsion regions were constructed (Figure 6).

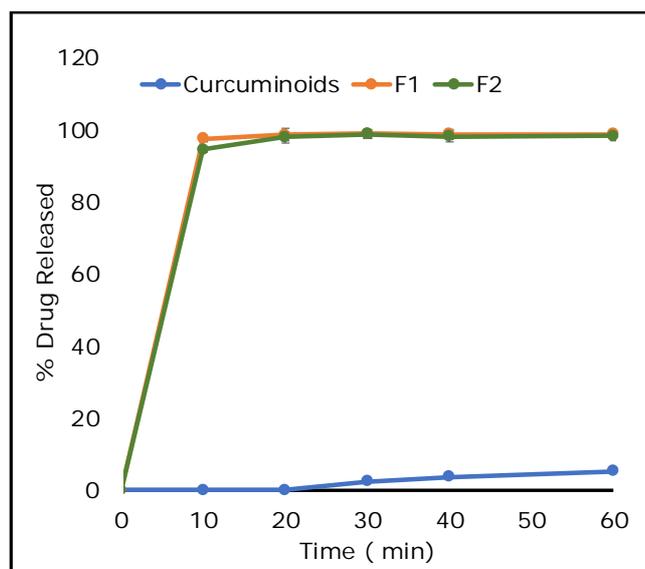


Figure 7: *In vitro* release profiles of curcuminoids and curcuminoid SMEDDS.

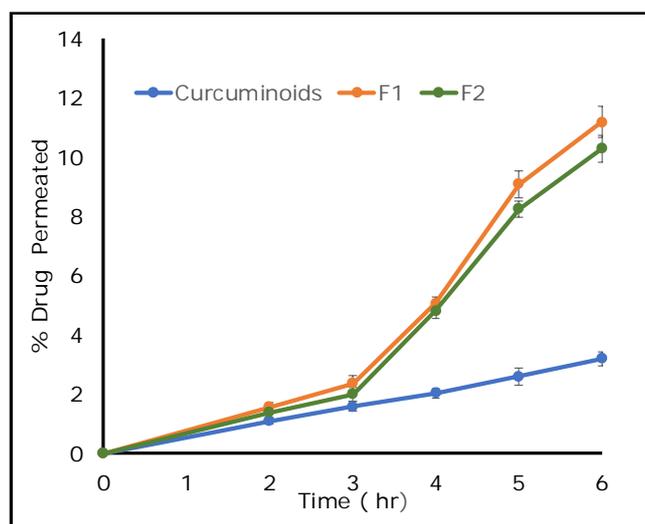


Figure 8: *In vitro* permeation profiles of curcuminoids and curcuminoid SMEDDS.

3.4 Characterization of curcuminoids SMEDDS

Among 3 selected formulations, F3 showed least % transmittance 91.54 ± 0 . Batch F1 and F2 showed good % transmittance (99.35 ± 0.30 and 97.69 ± 0.60 , respectively), hence, further studied for

droplet size analysis. Though F1 had smaller globule size (148 ± 3.51 nm) as compared to F2 (171 ± 3.05 nm), both batches showed globule size less than 200 nm. The dissolution profiles of curcuminoid SMEDDS F1 and F2 showed significantly high release as compared to curcuminoids (Figure 7). *In vitro* permeability studies showed 3.49 and 3.21 times increase in amount of drug permeated as compared non-formulated curcuminoids (Figure 8). Results of DPPH antioxidant stability studies showed that % reduction in antioxidant activity after 14 days on storage at 60°C were 2.23 ± 0.13 , 2.58 ± 0.36 and 2.65 ± 0.47 for unformulated curcuminoids, SMEDDS F1 and F2, respectively. Hydrogen peroxide scavenging activity stability studies showed that % reduction in antioxidant activity due to thermal effect were 2.86 ± 0.09 , 2.66 ± 0.36 and 2.73 ± 0.05 for unformulated curcuminoids, SMEDDS F1 and F2, respectively.

4. Discussion

4.1 Extraction and identification of curcuminoids

In the present study, oil depleted turmeric was extracted with methanol, as curcuminoids have high solubility in methanol (Verghese, 1993). Though, various advanced techniques are available for extraction, solvent extraction method was followed as it is a simple method to work on laboratory scale. It was observed that as herb solvent ratio and time for extraction was increased, yield increased. To extract maximum quantity of curcuminoids using minimum solvent and minimum time was the aim of the optimization. When herb solvent ratio of was set at 1:25 and extraction was carried out for 4 h and 50 min, 5.76 ± 0.16 % yield was obtained as against predicted yield of 5.91%. This close agreement of values, confirmed suitability of design. The instrumental studies carried out using UV, HPTLC, FT-IR and HPLC confirmed identity and purity of curcuminoids.

4.2 Formulation development for curcuminoids

Self-microemulsifying drug delivery system (SMEDDS) has recently received attention due to its ability to enhance solubility and permeability. It is a useful technique for orally administered, poorly water-soluble drugs. SMEDDS (Gursoy and Benita, 2004) is a mixture of oil, surfactant, co-surfactant and on dilution with gastrointestinal fluid under the influence of gastric motility it forms a microemulsion (Rajpoot *et al.*, 2020; Hintzen *et al.*, 2013; Vithani *et al.*, 2019). Being isotropic in nature, it forms clear solution which is well accepted by patients. Liquid SMEDDS can be suitably colored and flavored for oral administration or can be filled in hard/soft capsules. Hence, it has convenience of dose adjustment and can be given as a unit or bulk dosage form. Tween 80 and PEG 400, the excipients with high curcuminoid solubility are high HLB nonionic surfactant and cosurfactant. Because of non-ionic in nature, it is more stable to presence of ions, change in pH and due to high HLB it easily forms o/w emulsion. Hence, the choice of excipients was in agreement with the aim of preparing oral formulation. It was observed that as when Km ratio was increased from 1:1 to 4:1, microemulsion region increased. Hence, Km ratio 4:1, was used in further studies.

4.3 Characterization of curcuminoids SMEDDS

Poor transmittance of F3 may be due to poor entrapment efficiency of the oil and Smix. For formulations F1 and F2, globule size was in

colloidal range and the formulations were able to withstand dilution effect. Solubilization of drug in oil and surfactant mix had helped to achieve 100% drug release in *in vitro* dissolution studies. Small globules of microemulsion provided effective environment for lipophilic curcuminoids to easily cross the membrane and formulations showed high permeability (Nipun and Islam, 2014). ROS is the term used for various reactive molecules and free radicals. Higher levels of ROS are formed during stress and can lead to cancer, diabetes mellitus, cardiovascular diseases, atherosclerosis, arthritis, *etc.* Curcuminoids regulate ROS production which helps in cellular homeostasis. Free radical scavenging activities for curcuminoids and curcuminoids SMEDDS were measured using DPPH (2, 2-diphenyl-1-picrylhydrazyl) reagent method introduced by Blois (1958) with suitable modifications. DPPH free radical scavenging assay is an easy, fast and precise method to determine antioxidant activity. The odd electron of nitrogen atom in DPPH is reduced by free radical to the corresponding hydrazine. Correspondingly purple color of DPPH is lost. The hydrogen peroxide scavenging activity was carried out based on the principle introduced by Ruch *et al.* (1989). The removal of hydrogen peroxide is very important for antioxidant defense of cells, as it causes oxidation of proteins, lipids and DNA. As antioxidant reacts with hydrogen peroxide, its concentration decreases and so is the absorbance. The percent reduction in DPPH free radical scavenging activity were not significant as compared to unformulated curcuminoids for both F1 and F2 with p value 0.33 and 0.17, respectively. Similar results were obtained for hydrogen peroxide scavenging activity with p values 0.36 and 0.27. This confirmed that curcuminoids were stable in SMEDDS and retained their antioxidant property.

5. Conclusion

This article describes a method for the extraction of curcuminoids by solvent extraction. The experimental design approach using response surface methodology was successfully applied to optimize solvent quantity and time. The isolated curcuminoids were identified through HPTLC, FT-IR and HPLC. Though, curcuminoids are therapeutically versatile, poor bioavailability demands higher dose, which is not convenient for the patient. The selected excipients were able to give preparations which spontaneously formed stable microemulsion with fine particle size. By incorporating curcuminoids in SMEDDS, inherent shortcomings of curcuminoids, *viz.*, low solubility and poor dissolution were effectively addressed while still preserving antioxidant activities. More than 3 times increase in the amount of drug permeated by curcuminoids SMEDDS as compared non-formulated curcuminoids was observed. This indicates that formulation with low dose will be effective and there will be high patient compliance. The formulations were stable to heat and retained antioxidant activity. The formulations can be further studied in animals and human beings.

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Conflict of interest

The authors declare that there are no conflicts of interest relevant to this article.

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