3. METHODOLOGY

3.1. Analysis of curcumin

3.1.1. Analysis of curcumin by HPLC

Curcumin was analyzed by using HPLC technique according to USP-32/ NF-27[92]. The mobile phase was prepared by a filtered and degassed mixture of citric acid in water (1g/1000 ml) and tetra hydrofuran (THF) (6:4). The liquid chromatographic system was equipped with a UV lamp detector adjusted at wave length 420 nm and C18 column. The flow rate was 1ml/minute. Test solution was prepared by dissolving 20 mg curcuminoids in 50 ml acetone with sonication for 30 minutes, 5ml of the dissolved sample was diluted 10 folds with the mobile phase and passed through 0.45 µm filter before injection, 20 µl of the test sample was injected and is eluted for not less than three times. The retention time of each component of curcuminoids were detected and the percentage of each curve for each component from the total curves in the chart was measured[184]. Each peak was examined separately by exploring its UV spectrum via diode array option supplied with HPLC system.

3.1.2. Identification of curcumin by thin layer chromatography (TLC)

Curcmin was identified from total curcuminoids by application of TLC method. According to USP 32; test solution was prepared by dissolving 20 mg curcuminoid in 100 ml of acetone in a volumetric flask. Developing solvent system was prepared by mixing chloroform, methanol, and formic acid with ratio (96:4:1). 10 µl of the sample was applied on silica gel 60 F_{254} precoated TLC plate (6X7 cm) as a band. After elution, the plate was dried and examined under a UV-lamp at wave length 365 nm. Each spot of curcuminoid components were visually evaluated and compared to the reference stated in USP 32[92].

3.2. Solubility study of curcumin

Saturation solubility of CUR in water, (0.25%, 0.5% and 1%) SLS, 0.5% Tween 80, Soya bean oil, Miglyol 812, CRM EL and KLS P 124 using standard shake flask method. An excess quantity of CUR was added to solvent in a tightly capped conical flask. To achieve uniform mixing, samples were constantly agitated at conditions (100 rpm, 37℃ and 24 h) in a reciprocating water bath.

Samples were centrifuged at (4000 rpm, 15 min) after 24h equilibrium where aliquots of supernatant were diluted to appropriate concentrations with acetone. Aqueous samples were filtered through filter paper after centrifugation process and diluted with the same previous solvent. The samples were analyzed using spectrophotometer at wavelength 420 nm using acetone as a blank.

3.3. Preparation of curcumin - phospholipid complex (Curcumin phytosome)

Modification was taken place to the method expressed by Gupta *et al* [158] in the preparation of curcumin-phospholipid complex. Briefly, CUR and SPC in molar ratio of 1:1 were dissolved in 20 ml of dichloromethane in a round bottom flask. This mixture was refluxed for 2h at room temperature with application of magnetic stirrer and poured into a beaker containing colloidal silicon dioxide powder with the same ratio of SPC. The solvent was then evaporated by placing the beaker in a controlled temperature water bath with application of a mixer at 40 - 45℃ for 4 hours. The prepared flakes were grinded by using mortar and pestle into fine powder.

3.4. Characterization of the prepared curcumin-phospholipid complex powder in comparison to Meriva™.

3.4.1. Analysis of CUR content in the prepared CUR-SPC complex and Meriva ™

100 mg of test powder was sonicated in 100 ml of methanol in a volumetric flask for 15 mins. After filtration for test solution through a filter paper, 5 ml of the filtrate was diluted with 100 ml of the same solvent. Samples were analyzed spectrophotometrically at wave length 420 nm.

3.4.2. Differential scanning Calorimetry (DSC)

Thermal behavior of CUR-SPC complex was assessed using DSC analysis. The samples were sealed in the aluminum crimp cell and heated at the speed of 10℃/min from 30 to 300℃ in nitrogen atmosphere (60 ml/min). Thermograms of CUR, SPC (Lipoid S 100), prepared CUR-SPC complex and Meriva™ were examined[185].

3.4.3. Fourier Transform Infrared Spectroscopy (FT-IR)

3.4.3.1. FT-IR measurement for prepared complex and Meriva™

FTIR spectra were obtained using a FTIR spectrometer. Samples were mixed with dry crystalline KBr in a ratio 1:100 and tablets were compressed for measurements. A spectrum was collected for each sample within the wave number region 4,000 – 500 cm^{-1}. Samples assessed encompassed CUR, SPC, CUR-SPC complex and Meriva™[185].

3.4.3.2. FT-IR measurement for CUR-SPC complex in 1%SLS and 0.5% Tween 80

20 ml of 1% aqueous SLS and 0.5% aqueous Tween 80 was mixed separately with 100 mg of CUR-SPC complex powder was mixed using a magnetic stirrer for 20 mins. Samples were dried under stream of warm air until removing water from the powder prior to the measurement then mixed with dry crystalline KBr in a ratio 1:100 and tablets were prepared for measurements. A spectrum was collected for each sample within the wave number region 4,000-500 cm^{-1}.

3.4.4. Zeta-potential measurement

Zeta potential (ZP) was measured for the prepared CUR-SPC complex and Meriva™ by dynamic light scattering (DLS) technique (Malvern Zeta sizer, Malvern instruments, UK). Samples were dispersed in distilled water and sonicated for 15 mins. After sonication, samples were diluted with distilled water (1:10) before measurements.

3.4.5. Transmission electron microscopy (TEM)

Morphological examination of phytovesicles for prepared CUR-SPC complex and Meriva™ was carried out using TEM. Samples were diluted with aqueous 0.01% SLS (1:20) and were sonicated for 10 mins. A drop of the resultant phospholipids complex dispersions was placed onto a carbon-coated copper grid, leaving a thin liquid film. The film was dried by air then viewed. The mean particle size for vesicles was measured and photographed using TEM[(185)].

3.4.6. Scanning electron microscopy (SEM)

SEM photographs were taken to study the difference in morphology among CUR powder, prepared CUR-SPC complex and Meriva™. The dried sample was fixed on aluminium stubs using double-sided adhesive tape and coated with gold. A scanning electron microscope with a secondary electron detector was used to obtain digital images of the samples at an accelerating voltage of 10 kV[(184)].

3.4.7. Thin layer chromatography technique (TLC)

A modified TLC method from that used by Gupta *et al* [(158)] to evaluate CUR-SPC complex formation was performed. CUR powder, CUR-SPC complex powder and Meriva™ were weighed at 20 mg (in terms of CUR) and dissolved in 100 ml of methanol, 40 mg SPC S 100 was dissolved in 100 ml of dichloromethane. 10 µl was taken by a micro syringe from each sample and spotted on silica gel 60 F_{254} precoated TLC plate (6 X 7 cm) and allowed to dry by air. Elution was taken place by application of developing phase which composed of cholorform and methanol with ratio (6:4). After elution, the plate was dried by air and sprayed with 10% methanolic sulphuric acid. The plate was placed in electric oven at 110℃ for 10 min and the place of SPC spot was explored and compared among different samples by visual inspection.

3.4.8. *In vitro* dissolution studies

According to dissolution test for CUR at USP 32[(92)]; 900 ml 1% SLS were used as a dissolution medium using apparatus II at 100 rpm and because of the absence of a specified dissolution medium for CUR-SPC complex, different dissolution media (0.25%, 0.5% and 1%SLS and 0.5% Tween 80[(132)]) were used to study the dissolution pattern for CUR-SPC complex for 4 hours at the same pharmacopeial conditions. CUR powder, prepared CUR-SPC complex and Meriva™ were weighed to 40 mg (in terms of CUR powder) for the dissolution test.

10 ml sample was withdrawn at intervals; 60, 120, 180, 240 min and replaced by fresh medium and filtered through filter paper. The samples were diluted and analyzed spectrophotometrically at wave length 420 nm against corresponding medium as blank.

3.4.9. *Ex vivo* intestinal permeation study

3.4.9.1. Animals' protocol

Ex vivo permeation studies for CUR pure powder, Meriva™ and locally prepared CUR-SPC complex powder were carried out using non-everted gut sac technique[186-188]. A total number of 15 male wistar rats (weighing 200-250 g) were obtained from animal house of Faculty of pharmacy (Alexandria, Egypt). Experiments were performed in accordance with the European Community Guidelines for the use of experimental animals and were approved by the institutional ethics committee. The rats were housed in a temperature and humidity controlled room (23℃, 55%) with free access to water and standard rat chow. The rats were acclimated for at least 5 days and fasted overnight but supplied with water and libitum before the experiment. Animals were sacrificed by spinal dislocation and small intestine was immediately removed after sacrifice by cutting across the upper end of the duodenum and the lower end of the ileum and manually stripping the mesentery. The small intestine was washed out carefully with cold normal oxygenated saline solution (0.9% w/v, NaCL) using a syringe equipped with blunt end. The clean intestinal tract was prepared into 12 ± 0.5 cm long sacs having a diameter of 3 ± 0.5 mm.

3.4.9.2. Experimental protocol

3.4.9.2.1. Permeation experimental study

Each sac was filled via a blunt needle with 1 ml of test sample (equivalent 1 mg CUR) dispersed in a solution compromising from (0.9% w/v, NaCL), 100 µm itraconazole and 2% Tween 80.

Each sac was tied tightly with a thread and placed in a conical flask containing 50 ml of mixture of 2% ethanol and 98% saline solution containing 10% CRM EL. The entire system was maintained at 37℃ in a shaking water bath operated at 100 rpm and aerated with 5% CO_2 and 95% O_2 (10-15 bubble/min) using laboratory aerator. Samples were withdrawn from outside of the sac and replaced by a fresh medium for 3 hours [189] at time intervals 30, 60, 120 and 180 mins. Samples were analyzed spectrophotometrically at wave length 420 nm against used solution as a blank.

At the end of permeation test, each sac was withdrawn from its solution and cut from its two ends to wash the inner part of sac with a saline solution. The intestinal sac was opened longitudinally to isolate the mucosal layer by scratching by a spatula and 1 ml of acetone was mixed with mucous samples. The sample was centrifuged at 4000 rpm for 15 min prior to be analyzed by HPLC. Studies were performed in triplicates.

3.4.9.2.2. High-performance liquid chromatography (HPLC)

A validated HPLC method of analysis for CUR in mucosal cells was used by Marchzylo *et al*[130]. Standard CUR was prepared in acetone and diluted to appropriate concentration and samples were subjected for analysis by HPLC. The HPLC instrument (Agilant, USA) was equipped with C18 column (4.6 x150 mm, 3µm) with a guard (4.6 x 20 mm, 3 µm) kept at 35℃ and UV detector adjusted at 426 nm. Mobile phase was composed of two components: A; 10 mM Ammonium acetate and B; Acetonitrile. The mobile phase was gradient eluted at flow rate 1.5 mL per minute, initially adjusted at 95% of A progressing to 55% at 20 mins until reaching 5% at 33 mins.

3.5. Preparation of fills formulation of CUR-SPC complex in softgels.

According to previously discussed lipid formulation classification system (LFCS) in part one; class (III_a) was selected to formulate CUR-SPC complex in a soft gelatin capsule by application of a hydrophilic surfactant (i.e. HLB > 12) and triglyceride oil.

3.5.1. Preparation of CUR-SPC complex powder in softgels

Dispersion method[(91)] was applied to prepare lipid fill formulations where hydrophilic surfactant was mixed with triglyceride oil for 5 mins by using a magnetic stirrer. CUR-SPC complex powder was dispersed and mixed with dispersion medium for 2 mins till formation of viscous mass prior to encapsulation knowing that the ratio between the dispersed powder and the dispersion medium was examined experimentally and found to be (1:2). Compositions of lipid fill formulations were illustrated in Table 12.

3.5.2. Preparation of CUR-SPC semisolid complex in softgels

Based on solvent evaporation method of preparation of phytosome, 1 gm CUR powder was dissolved and refluxed with 2 gm SPC S 100 in 60 ml dichloromethane in molar ratio (1:1) in a conical flask for 2 hours. The prepared solution was placed in a controlled temperature water bath adjusted at 45℃ for 4-5 hours until formation of a semisolid form of CUR-SPC complex. The weight of the conical flask was recorded before and after the evaporation step to ensure the complete evaporation of dichloromethane from the formed complex. Hydrophilic or lipophilic vehicle was added to the prepared semisolid complex in ratio (1:1.5) and mixed mechanically with a glass rod at room temperature till formation of liquefied mass of the complex. Compositions of lipophilic/hydrophilic vehicle of CUR-SPC semisolid complex were illustrated in Table 13.

Table 12: Composition of CUR-SPC complex fill formulations in softgels

Material (mg)	F1	F2	F3	F4	F5	F6	F7	F8	F9
CUR-SPC complex powder	500	500	500	500	500	500	500	500	500
BHT	-	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
CRM EL	-	100	200	400	200	-	-	-	-
CRM RH 40	-	-	-	-	-	200	200	-	-
KOLLISOLV P 124	-	-	-	-	-	-	-	200	400
Soya bean oil	-	-	-	-	799.8	-	799.8	-	-
Miglyol 812	1000	899.8	799.8	599.8	-	799.8	-	799.8	599.8

Table 13: Composition of lipophilic/hydrophilic vehicle of CUR-SPC complex semisolid

Material (mg)	F10	F11	F12	F13
CUR	200	200	200	200
SPC S100	400	400	400	400
BHT	0.2	0.2	0.2	0.2
Kollisolv P- 124	360	360	360	-
CRM EL	-	-	-	360
Miglyol 812	539.8	-	-	-
Oleic acid	-	539.8	-	-
Castor oil	-	-	539.8	-
PEG 400	-	-	-	539.8

3.6. Characterization of fill formulations of CUR-SPC complex

3.6.1. Evaluation of *in vitro* dissolution test

In vitro dissolution test was carried out according to USP-32/NF-27 for dissolution of CUR capsule, where 900 ml of 1% SLS was used as a dissolution medium with application of apparatus II at 100 rpm. The amount dissolved after 60 min should not be less than 75% which represented the acceptance value for successful dissolution test. 40 mg of test sample (in terms of CUR) was manually injected in a hard gelatin capsule and attached to a sinker prior to the start of the dissolution test. After 60 mins; 10 ml aliquot was withdrawn and filtered through filter and diluted to a proper concentration with the corresponding medium and analyzed spectrophotometrically at wave length 420 nm.

F9, F10 and F13 were selected and proceeded to study dissolution pattern before and after shelf stability aliquots were withdrawn at time intervals; 30, 60, 120 and 180 min and were replaced by fresh medium. Samples were diluted and analyzed spectrophotometrically at above mentioned wave length.

3.6.2. Transmission electron microscope (TEM)

TEM for aqueous dispersion for F10 and F 13 was performed with same test procedures in experiment 3.4.5.

3.6.3. Shelf stability study

Selected formulations; F9, F10 and F13 were stored in a air filled soft gelatin capsule for 3 months at (25℃, 65% RH). Samples were monitored for any change in the physical state during the storage period.

3.6.3.1. *In vitro* dissolution test

F9, F10 and F13 were subjected to *In vitro* dissolution study with same procedures explained in experiment 3.6.1.

3.6.3.2. Transmission electron microscope (TEM)

TEM for aqueous dispersion for F10 and F 13 was performed with same test procedures in experiment 3.4.5

3.6.3.3. Zeta potential measurement (ZP)

ZP measurement was taken place for F 13 with the same test procedure in experiment 3.4.4.