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Design and Development of Dry Powder Sulfobutylether-β-Cyclodextrin Complex for Pulmonary Delivery of Fisetin

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ABSTRACT

This study has investigated complexation of fisetin, a natural flavonoid, with three types of cyclodextrins to improve its solubility. Sulfobutylether-β-cyclodextrin (SBE-β-CD) showed the highest complexation efficiency while maintaining the *in vitro* antioxidant activity of fisetin. Addition of 20 % v/v ethanol in water improved the amount of solubilized fisetin in the complex 5.9-fold compared to the system containing water alone. Spray drying of fisetin-SBE-β-CD complex solution in the presence of ethanol produced a dry powder with improved aerosolization properties when delivered from a dry powder inhaler, indicated by a 2-fold increase in the fine particle fraction (FPF) compared to the powder produced from the complex solution containing water alone. The pitted morphological surface of these particles suggested a more hollow internal structure, indicating a lighter and less dense powder. Incorporation of 20 % w/w leucine improved the particle size distribution of the powder and further increased the FPF by 2.3-fold. This formulation also showed an EC_{50} value equivalent to fisetin alone in the A549 cell line. In conclusion, an inhalable dry powder containing fisetin-SBE-β-CD complex was successfully engineered with an improved aqueous solubility of fisetin. The dry powder may be useful to deliver high amounts of fisetin to the deep lung region for therapeutic purposes.

KEYWORDS

Fisetin, sulfobutylether-β-cyclodextrin, pulmonary delivery, spray drying, leucine, A549 cell line.

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1. Introduction

Fisetin (3, 3’, 4’, 7-tetrahydroxyflavone) is a flavonoid with antiproliferative, apoptotic, antioxidant, anti-inflammatory and neuroprotective activities. In recent years, fisetin has attracted widespread interest as many studies have shown its anticancer effect *in vitro* using many cancer cell lines, including lung, colon and prostate cancers [1]. It has shown synergistic activities with other anticancer agents (i.e. cisplatin and cyclophosphamide) in different carcinoma cell lines [2,3] and acts as an adjunct in cancer therapy to alleviate specific adverse effects related to cytotoxic agents or other therapeutics, including protection against ovariectomy-induced bone loss in mice [4] and has a renoprotective effect in cisplatin-induced nephrotoxicity in rats [5].

The low aqueous solubility of fisetin (< 1mg/ml) [6] hinders its delivery; this has led to a number of formulation approaches aiming to improve its delivery. Fisetin has been formulated in nanoemulsions [6], nanocochleates [7], liposomes [8,9] and cyclosophoroase dimer complexes [10].

Complexation of fisetin with cyclodextrins has been studied previously to improve its solubility. One study found that fisetin could only form a stable complex with β-cyclodextrin, but not with the α-cyclodextrin [11]. Complexation of fisetin with another type of cyclodextrin (γ-cyclodextrin) was also undertaken in order to understand the behaviour of fisetin in the nano-cavity, and confirmed the suitability of the nano-vehicle for parenteral administration of fisetin [12]. Another study investigated inclusion of fisetin into β- and γ-cyclodextrins which resulted in a better aqueous solubility of fisetin and higher cytotoxic activity of fisetin against Hela and MC-7 cells [13]. However, none of the studies has yet explored the formulation and application of fisetin complex for pulmonary delivery.

Sulfobutylether-β-cyclodextrin (SBE-β-CD) is a modified β-CD which has been approved by the US Food and Drug Administration (FDA) as a pharmaceutical excipient. Its parent form (i.e. β-CD) causes renal toxicity after parenteral administration. Although hydroxypropyl-β-cyclodextrin (HP-β-CD) and SBE-β-CD are considered safe at relatively high doses for the parenteral route [14], the latter has a safer profile for parenteral delivery, with low kidney toxicity and low tendency to cause haemolysis. In general, cyclodextrins that are considered to be the safest for parenteral formulation (i.e. γ-CD, HP-β-CD and SBE-β-CD) are also considered safe for pulmonary application [15].

This study explores the complexation of fisetin with SBE-β-CD with the aim of increasing the solubility of the compound. The complex was further engineered into a dry

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powder inhaler (DPI) formulation with optimized aerodynamic properties and tested against the human lung adenocarcinoma cell line (A549). Compared to other routes, such as oral and parenteral, the final DPI preparation will potentially be useful in the local delivery of fisetin in lung-associated diseases, including acute lung injury and lung cancer, allowing dose reduction. The study may also provide an insight into the feasibility of a cyclodextrin complex as inhalable particles to deliver other insoluble anticancer agents via the pulmonary route.

2. Materials and Methods

2.1 Materials

Fisetin (3,3',4',7-tetrahydroxyflavone) was purchased from Carbosynth Ltd. (Berkshire, UK) and SBE-β-CD (Captisol®) was a gift from Cydex Pharmaceuticals (La Jolla, USA). β-CD (Kleptose® DC, MW: 1135) and HP-β-CD (Kleptose® HP, MW: 1480) were gifts from Roquette Pharma (Northamptonshire, UK). L-leucine was purchased from EMD Chemicals Inc. (San Diego, USA) and absolute ethanol (99.9%) was procured from Fisher Scientific (Loughborough, UK). Acetonitrile, water, DMSO and trifluoroacetic acid (TFA) of HPLC grades and DPPH• (2,2-diphenyl-1-picrylhydrazyl) free radical, L-ascorbic acid and methanol of analytical grades were obtained from Sigma-Aldrich (Dorset, UK).

The RPMI-1640 medium, fetal bovine serum and penicillin-streptomycin solution were purchased from Gibco (USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (USA).

2.2 Phase solubility study

A phase solubility study was conducted according to the method of Higuchi and Connors [16] to investigate the influence of cyclodextrins on the solubilization of fisetin. An excess amount of fisetin was added into 5 mL deionized water containing different concentrations of β-CD, HP-β-CD and SBE-β-CD (0.156 to 10 mM). The mixture was mixed in a bath sonicator for 15 min and left for further mixing at 100 rpm and 37°C for 24 h in an orbital incubator shaker. The uncomplexed fisetin was filtered from the solution using a 0.45 µm syringe filter. The filtrate was diluted accordingly and measured using HPLC.

The concentration of solubilized fisetin (mM) was plotted against different concentrations of cyclodextrin (mM). The slope and y-intercept (S_o) of the straight lines were used to calculate the complexation parameters as follow;

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$$\text{Stability constant, } K_s = [\text{slope}/ S_o (1\text{-slope})] \quad \text{Eq. (1)}$$

$$\text{Complexation efficiency, CE} = \text{slope}/(1\text{-slope}) \quad \text{Eq. (2)}$$

2.3 Investigation of different complexation methods

Complexation of fisetin with SBE-β-CD was carried out at a 1:1 molar ratio using two different methods of complexation; namely an aqueous method and a co-solvent method.

In the aqueous method, fisetin and SBE-β-CD were dissolved in 10 mL deionized water and mixed in a bath sonicator for 15 min. The mixture was further mixed for 24 h in a shaking incubator at 80 rpm and 37°C.

The co-solvent method involved solubilization of fisetin in 2 mL absolute ethanol and SBE-β-CD in 8 mL deionized water. The solutions were mixed and sonicated in a bath sonicator for 15 min prior to solvent evaporation under vacuum at 80°C, using a rotary evaporator (RC900, KNF Neuberger Ltd., UK). The dried complex was rehydrated with rotation at 80°C using 10 mL deionized water and left to cool to room temperature. In both methods, final solutions were filtered using a 0.45 µm syringe filter to separate the uncomplexed fisetin, and the filtrates were used for further studies.

2.4 Investigation of different feed solution compositions for spray drying

In Section 2.3, the co-solvent method was able to increase the amount of solubilized fisetin in the preparation. As the preparation was in solution, it was imperative to remove the organic solvent (i.e. ethanol) by rotary evaporation, and the complex was then rehydrated with water. As spray drying was introduced later to produce an inhalable powder, the ethanol could be removed by the process. An omission of evaporation and rehydration steps was considered an interesting approach, worth of investigation, to simplify the preparation steps. Therefore, the physical characteristic and *in vitro* aerosol deposition profile of the dry powders produced from two types of feed solutions (i.e. feed solution containing water and feed solution containing 20 % v/v ethanol in water) were investigated.

Fisetin-SBE-β-CD complex was prepared using the co-solvent method containing 20 % v/v ethanol in water and a 1:1 molar ratio of fisetin to SBE-β-CD. Fisetin and SBE-β-CD were dissolved in ethanol and deionized water, respectively, prior to a mixing in a bath sonicator for 15 min. The mixture was then treated differently to produce the two feed solutions for spray drying. For the feed solution containing 20 % v/v ethanol in water, the mixture was directly subjected to spray-drying. For the feed solution containing water alone, the mixture containing 20 % v/v ethanol in water was subjected to solvent evaporation under

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vacuum at 80°C using a rotary evaporator, to remove the ethanol. Then, the dried complex was rehydrated with deionized water for 10 min at 80°C and left to cool to room temperature. Both solutions in a final total powder mass of 0.4 %w/v were spray dried and the dried powder produced by both methods was immediately transferred to a desiccator at room temperature until further analysis.

2.5 Incorporation of leucine as dispersibility enhancer

Fisetin-SBE-β-CD complex solution containing 20 %v/v ethanol in water was prepared and leucine was added at a concentration of 5, 10 and 20 %w/w of the complex. The solution was stirred with magnetic stirring for 10 min, producing a final total powder mass of 0.4 %w/v in every formulation. The formulations were spray dried and stored in a desiccator at room temperature.

2.6 Spray drying of the complexes

Fisetin-SBE-β-CD complex solutions (0.4 %w/v) were spray dried using a mini laboratory spray dryer, model B-290 (Büchi Labortechnik, Switzerland) in an open-loop mode configuration using compressed air as the drying gas. The process was done under the following conditions: Inlet temperature (T_{in}): 120°C, corresponding to an outlet temperature (T_{out}): 66°C, spray gas flow: 536 L/h, pump: 20% (4.83 mL/min), aspirator: 100% (35 m^3/h).

2.7 Characterization of the preparations

2.7.1 Determination of the amount of solubilized fisetin

The amount of solubilized fisetin was determined by dissolving an accurately weighed sample of spray-dried powder in deionized water. The solution was vortexed for 5 s and filtered using 0.45 µm syringe filter, to remove the uncomplexed fisetin. An aliquot of 30 µl of the appropriately diluted samples, before and after filtration, were injected into the HPLC to determine the amount of fisetin. The amount of solubilized fisetin was calculated according to the following equation;

$$\text{Solubilized fisetin (\%)} = \frac{\text{Amount of solubilized fisetin}}{\text{Total amount of fisetin in the formulation}} \times 100\% \quad \text{Eq. (3)}$$

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2.7.2 HPLC analysis

The analysis was performed using an Agilent 1260 series HPLC system (Agilent Technologies, USA) equipped with quaternary pump VL, standard autosampler, thermostatted column compartment and variable wavelength detector VL. The data were acquired and analysed using OpenLab CDS ChemStation Edition (B.04.03) software. The chromatographic separation was performed using an ether-linked phenyl column with polar end-capping, Synergi^{TM} Polar-RP 80°A (250 x 4.6 mm i.d, 4 µm particle size).

30µL samples were injected onto the column with a mobile phase comprising acetonitrile and 0.1 %v/v TFA in water, in the ratio of 30:70 (v/v). The chromatographic conditions were maintained at 40°C at a flow rate of 1.0 mL/min and a detection wavelength of 362 nm. The retention time of fisetin was found to be 8.45 min. The method was validated according to ICH guidelines [17] with a limit of detection (LOD) of 0.4 µg/mL and limit of quantification (LOQ) of 2 µg/mL. The method was linear in the range 2-60 µg/mL with a mean linear regression equation of $y = 79.40 (\pm 2.53)x - 22.93 (\pm 15.50)$ from five calibration curves. The coefficient of determination, R^2 was 0.9998 (±0.0002). The intra-day precision ranged from 0.34 to 1.26% while the accuracy ranged from -1.62 to -2.52%. The inter-day validation showed that all results were within the limit of ±5% [18], with the precision ranging from 2.94 to 3.20% and accuracy from 1.20 to 2.21%.

2.7.3 Determination of *in vitro* antioxidant activity

The antioxidant activity of free fisetin and the complex solutions was determined using the DPPH assay described previously [19,20]. Each sample was prepared in 5 or 6 different concentrations prior to the assay. Then, 1 mL of each concentration was added into 1 mL methanolic solution of DPPH• (100 µM) and the solutions were mixed by aspiration. An equal amount of deionized water was added into 1 mL DPPH• solution to serve as a control. The mixtures were incubated in the dark for 40 min prior to measurement in the UV spectrophotometer (Cary 100 UV-Vis, Agilent Technologies, USA) at 517 nm. The percentage inhibition was calculated as follows;

$$\text{Inhibition (\%)} = [(\text{Abs Control} - \text{Abs Test})/\text{Abs Control}] \times 100\% \qquad \text{Eq. (4)}$$

The concentration at which the absorbance decreased by 50% (IC_{50}) was determined from a linear correlation between concentration of fisetin (mM) against the inhibition (%) for all tested samples. The antioxidant assay for each sample was conducted in triplicate and the IC_{50} was compared to ascorbic acid as a standard.

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2.7.4 Determination of particle size distribution

The particle size distribution of the dried powder was determined using a Sympatec HELOS BF laser diffraction analyzer (Sympatec GmbH, Clausthal-Zellerfeld, Germany) with RODOS/M dry powder disperser. The sample was loaded into a sealed sample tube and the tube was inserted into the micro-dosing device (ASPIROS). Sufficient sample was loaded to obtain an obscuration of more than 1 and each sample was measured in triplicate at an air pressure of 4 bar. Results were analysed using Windox 5 (version 5.7.0.0) software.

Results were analysed based on Mie Evaluation Extended (MIEE) algorithm for spherical, isotropic and homogenous particles, which transferred the scattered light data into particle size information. The complex refractive index value used in the analysis was 1.550, with air as continuous phase.

Results were expressed as the volume mean particle size and percentage undersize at 10% (X_{10}), 50% (X_{50}) and 90% (X_{90}). The X_{50} is also known as the volume median diameter (VMD) of the particles. The width of distribution was expressed as Span according to following equation;

$$\text{Span} = (X_{90} - X_{10})/X_{50} \qquad \text{Eq. (5)}$$

2.7.5 Assessment of aerosol properties using the next generation impactor (NGI)

The aerosol properties of the dry powders were evaluated using the NGI (Copley Scientific Limited, Nottingham, UK) conducted under pharmacopoeial conditions (Apparatus E, European Pharmacopoeia, Chapter 2.9.18). Air flow through the apparatus was adjusted to be at 60 ± 3 L/min using vacuum pump and two-way solenoid valve timer. The air flow rate was tested using a flow meter (DFM2000, Copley Scientific Limited, Nottingham, UK) prior to the testing.

The central cup of the pre-separator insert was filled with 15 mL deionized water and the impaction cups were coated with 1 %v/v silicone oil in hexane before analysis. Powder samples (30 ± 1 mg) were accurately weighed and filled into no. 3 hard gelatine capsules and were individually loaded into the dosage chamber of an Aerolizer® device (Novartis, Surrey, UK). The capsule was pierced and the Aerolizer® was inserted into a mouth-piece adaptor. The powder was drawn into the NGI and tested for 4 s at 60 L/min. This was carried out using 3 capsules for each sample. After all three actuations, powder in capsules, device, mouthpiece, induction port, and stages 1 to 8 of the NGI were collected with thorough rinsing using deionized water into separate volumetric flasks. The solutions were mixed in a bath sonicator for 15 min and appropriately diluted prior to fisetin determination using HPLC.

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Under these conditions, the cut-off diameter for each NGI stage are; Stage 1: 8.06 µm, Stage 2: 4.46 µm, Stage 3: 2.82 µm, Stage 4: 1.66 µm, Stage 5: 0.94 µm, Stage 6: 0.55 µm, Stage 7: 0.34 µm and Stage 8 as terminal Micro-Orifice Collector (MOC) (Apparatus E, European Pharmacopoeia, Chapter 2.9.18).

The aerosolization parameters, including fraction recovered (FR), emitted dose (ED), fine particle dose (FPD) and fine particle fraction (FPF) were calculated as follows;

$$FR = \frac{\text{Mass of fisetin from capsule to Stage 8}}{\text{Initial mass of fisetin loaded into the capsule}} \times 100\% \quad \text{Eq. (6)}$$

$$ED = \frac{\text{Mass of fisetin from device to Stage 8}}{\text{Mass of fisetin from capsule to Stage 8}} \times 100\% \quad \text{Eq. (7)}$$

$$FPD = \text{Mass of fisetin on Stage 2 to Stage 8} \quad \text{Eq. (8)}$$

$$FPF = \frac{\text{Fine particle dose}}{\text{Initial mass of fisetin loaded into the capsule}} \times 100\% \quad \text{Eq. (9)}$$

Mass median aerodynamic diameter (MMAD) is determined as particle size at the 50% of cumulative fraction by mass for the aerosolized powders. It is calculated from the graph of cumulative fraction against effective cut-off diameter on log probability axes. Geometric standard deviation (GSD) is a measure of the width of an aerodynamic particle size distribution. It is calculated using the same plot as for calculation of MMAD using following formula;

$$GSD = (d_{84}/d_{16})^{1/2} \quad \text{Eq. (10)}$$

2.7.6 X-ray powder diffraction (XRPD)

XRPD analysis of samples was performed using a Rigaku MiniFlex 600 X-ray diffractometer (Rigaku, Japan) at room temperature. The Cu anode X-ray tube was operated at a generator voltage of 40 kV and a current of 15 mA. Diffraction patterns were recorded over diffraction angle (2θ) of 5-37°, a scanning rate of 5°(2θ)/min and scan step 0.05° (2θ).

2.7.7 Fourier transform infrared (FT-IR) spectroscopy

FT-IR analysis was undertaken using a Spectrum 100 FT-IR spectrometer (Perkin Elmer, Massachusetts, USA). Spectra were recorded over the range 4000-650 cm^{-1} with a resolution of 4 cm^{-1} and scanning speed of 0.2 cm/s.

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2.7.8 Thermogravimetric analysis (TGA)

Residual solvent content of the spray-dried powders was determined using a thermogravimetric analyser (Discovery TGA, TA instrument, USA). Samples were loaded onto platinum pans and placed in sample holders. The analysis was carried out under a nitrogen purge of 20 mL/min. A heating rate of 10°C/min in the range of room temperature to 400°C was applied to each sample and measurement was performed in triplicate.

2.7.9 Scanning electron microscopy (SEM)

A scanning electron microscope (SEM) was used to examine the surface morphology of produced powders. In the analysis, sample was placed on the surface of double-sided adhesive black carbon tabs which was attached to a 12.5 mm aluminium stub. The sample was sputter-coated with gold (Quorum Q150R; Quorum Technologies Ltd., Sussex, UK) prior to observation under SEM (FEI Quanta 200F SEM; FEI, Eindhoven, Netherlands) at an acceleration voltage of 5 kV.

2.8 *In vitro* cell toxicity of fisetin and the spray-dried formulation (MTT assay)

The human lung adenocarcinoma cell line (A549) was used in the study. The cells were cultured in RPMI1640 with L-glutamine supplemented by 10 %v/v fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin. The cell cultures were maintained in an incubator at 37°C with humidified environment of 5% CO_2 and 95% air.

Cell viability was evaluated using the MTT assay. In brief, the cells were seeded in a density of 2 x 10^4 cells per well in a 96-well plate and incubated for 24 h at 37°C (5% CO_2/95% air). Then, 200 µL treatment solutions were added at different concentrations in the treated wells and 200 µL complete media solution was used in the untreated wells. The treatment solutions included fisetin, the spray-dried powder containing 20 %w/w leucine (SD_20%Eth20%Leu) and the blank formulation (containing SBE-β-CD and leucine), at concentrations of fisetin ranging from 4.7 to 600 µM. A stock solution of fisetin was prepared in DMSO and diluted accordingly with complete media while the SD_20%Eth20%Leu and the blank formulation were prepared and diluted using complete media. As fisetin samples contained DMSO, the cells were also treated with 0.1 %v/v DMSO in the media to evaluate the DMSO-derived cytotoxicity. After 48 h incubation, the medium was replaced with 110 µL MTT solution (0.45 mg/mL) and further incubated for 2 h. Then, the MTT solution was removed and the generated formazan was solubilized in 150 µL DMSO. The plate was shaken for 10 min prior to the optical density (OD) measurement using a multi-mode microplate

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reader (SpectraMax® M2^e, Molecular Devices, USA) at 570 nm. The percentage of cell viability was calculated as follows;

$$\text{Cell viability (\%)} = \frac{\text{OD of treated cells}}{\text{OD of untreated cells}} \times 100\% \qquad \text{Eq. (11)}$$

The cell viability curve was plotted and the effective concentration to kill 50% of the cells (EC_{50}) was analysed using OriginPro® 2016 software (OriginLab Corporation, USA).

2.9 Statistical analysis

All parameters were statistically analysed using a t-test for independent group or one-way analysis of variance (ANOVA) with a post-hoc Tukey HSD test. For all analyses, differences were considered statistically significant when $p < 0.05$.

3. Results and Discussion

3.1 Phase solubility study

A phase solubility plot of fisetin in three different cyclodextrins is shown in **Fig. 1**. The plot depicts a Higuchi A_L type phase solubility behaviour for all complexes; a linear increase in solubility of fisetin with increasing concentration of cyclodextrins. Stability constant (K_s) is used to determine the solubilizing efficiency of cyclodextrins for a drug, as previously reported [19,20]. The solubilizing efficiency of fisetin in different cyclodextrins are in following order; SBE-β-CD > HP-β-CD > β-CD. The K_s value of fisetin in the β-CD system was in agreement with previous study of the complex in a neutral solution and 35°C which was 510 ± 30 M^{-1} [11]. The K_s values corresponded to a greater solubilization of fisetin at 10 mM SBE-β-CD (4.474 ± 0.239 mM) compared to the systems containing HP-β-CD and β-CD with values of 2.291 ± 0.046 and 0.628 ± 0.099 mM, respectively. All three complexes showed an increase in fisetin solubility in the aqueous solution compared to the free drug alone (0.1101 ± 0.0040 mM). **Table 1** shows complexation parameters for all fisetin-cyclodextrin complexes; 1:1 stoichiometry can be assumed as all slopes have values below 1 [21].

Complexation efficiency (CE) describes the ratio between cyclodextrin complex and free cyclodextrins. This parameter is more reliable as it is independent of both the intrinsic solubility and the y-intercept [22]. The CE values showed comparable results to that of the K_s

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values. The corresponding molar ratio of fisetin to respective cyclodextrins shows that the best ratio was in SBE-β-CD system (i.e. 1 out of every 2 SBE-β-CD molecules forms a complex with fisetin). Lower molar ratio of drug to cyclodextrin is favoured as less cyclodextrin will be needed to form a soluble complex; thus reducing the bulkiness of the formulation. Thus, SBE-β-CD was chosen for further optimization with fisetin.

3.2 Determination of antioxidant activity

The antioxidant activities of fisetin in the complexes formed by all three cyclodextrins were compared to that of the fisetin solution (**Fig. 2**). There was no significant difference in the IC_{50} values ($p > 0.05$) suggesting that complexation with all cyclodextrins maintained the antioxidant activity of fisetin. Further, the solutions of free fisetin and fisetin-cyclodextrin complexes showed significantly lower IC_{50} values ($p < 0.05$) compared to ascorbic acid, suggesting better antioxidant activity in all fisetin-containing solutions. The DPPH assay was also conducted on blank cyclodextrin solution. The cyclodextrins did not show any inhibition in the DPPH activity up to 50 mM concentration, confirming that the antioxidant activity of all formulations was solely a function of the fisetin.

3.3 Investigation of different complexation methods

In the preparation of drug-cyclodextrin complexes, ethanol has been used as a solvent [23-25]. In this study, ethanol alone could not be used as the solvent system as SBE-β-CD is not soluble in ethanol. Co-solvency (i.e. addition of solvent into aqueous solution) can be used to enhance the complexation process as the solvent can help to increase solubility of a hydrophobic drug [22]. In a preliminary study, different ratios of ethanol and water were studied to find an optimum composition that would facilitate and enhance the complexation process. The ethanol content affected the stability of the formed complexes, with sub-optimal compositions resulting in precipitation of fisetin within 3 h of preparation. It has been reported ethanol content should be optimized, as too high solvent content reduces the inclusion of drug, as solvent will competitively bind to the cyclodextrin molecules [26]. In this study, this may cause the expulsion of fisetin from the cavity of cyclodextrin, causing precipitation. Thus, 20% v/v of ethanol in water was selected for the co-solvent method, based on optimal drug inclusion and minimal precipitation.

The proportion of solubilised fisetin in complexes prepared using the aqueous and co-solvent methods was 15.2 ± 1.1 and 90.2 ± 3.9 %, respectively, indicating that complexes

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produced by the co-solvent method had a 5.9-fold higher ($p < 0.05$) content of solubilized fisetin.

3.4 Investigation of different feed solution compositions for spray drying

3.4.1 Particle size distribution and residual solvent content

The spray-dried powder produced from a feed solution containing water alone (SD_H_2O) showed a unimodal particle size distribution (**Fig. 3**A) with all particles below the size of 5 µm. The particles showed a narrow size distribution with an X_{50} value of 1.50 ± 0.18 µm and Span value of 1.55 ± 0.25.

The spray-dried powder produced from a feed solution containing 20 % v/v ethanol (SD_20%Eth) displayed a bimodal size distribution with a group of particles having a mode less than 3 µm, and another population of particles in the size range: approximately 3 to 20 µm probably indicating agglomeration of smaller particles. The wider particle size distribution resulted in a Span value of 3.14 ± 0.51, though the X_{50} value was 1.48 ± 0.08 µm.

The formulations showed no difference ($p > 0.05$) in the residual solvent contents, with values of 7.75 ± 0.37% for the powder produced from feed solution containing water and 8.31 ± 0.53% for the powder produced from feed solution containing 20 % v/v ethanol. The residual solvent content of spray-dried powders varies between formulations, with some reports of ~3% [27] and others between 6 to 11% [28,29]. The residual solvent content depends on the hygroscopicity of the material. Evaporation of the solvent molecules in the drying chamber of the spray dryer will cause an increase in the vapour concentration within the instrument. A hygroscopic material will tend to take up more water during the spray-drying process causing a higher residual solvent content in the final powder formulation [28]. SBE-β-CD 7 that was used in the study has shown to be hygroscopic and adsorb more moisture compared to other substituted SBE-β-CD (i.e. SBE-β-CD 4 and 5; the number indicates the average number of substituted sulfobutyl ether groups per CD) [30]. Thus, giving a high residual solvent content.

Despite that, the powders were able to be dispersed during the measurement in laser diffraction analyzer and NGI, giving a sufficient particle size distribution and FPF.

3.4.2 FT-IR analysis

The infrared spectra of all samples are presented in **Fig. 4**. The fisetin crystal showed a characteristic O-H stretch at 3344.18 and 1205.94 cm^{-1}. These peaks are still evident in the

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spectrum of a physical mixture of fisetin and SBE-β-CD, showing that the drug remained in its crystalline state without any complexation. However, the peaks are absent on the spectra of spray-dried formulations indicating restriction of vibration and bending of fisetin molecules in the host cavity of the inclusion complex. This result is in agreement with a previous study of a SBE-β-CD-drug complex [31]. SBE-β-CD had a broad peak at 3382.94 cm^{-1}, which indicates O-H stretch of the alcoholic group in the molecule. It also has a C-H stretch of the alkane group at 2928.98 cm^{-1}. These peaks are less obvious in the spectrum of the physical mixture but can be clearly seen in the spectra of spray-dried formulations. No new peaks were seen in the spectra of the complex, indicating no chemical interaction between fisetin and SBE-β-CD molecules. The host-guest complex is reported to depend on hydrophobic interaction between lipophilic cavity of cyclodextrin and the drug, without formation or breakage of covalent bonds [32].

3.4.3 XRPD analysis

The diffractogram of pure fisetin (**Fig. 5**) indicated a typical crystalline nature with a large number of sharp diffraction peaks especially at 12.35, 15.50, 24.05, 26.25 and 28.25° (2θ). The spray-dried fisetin powder from 20 %v/v ethanol solution showed a lower degree of crystallinity and low intensities peaks, indicating the presence of a mixture of amorphous and crystalline state in the preparation. In contrast, broad and diffused peaks with low intensities were recorded for pure SBE-β-CD indicating its amorphous state. Some diffraction peaks from the crystalline fisetin are visible on the diffractogram of the physical mixture (labelled with arrows), but with lower intensities as fisetin was diluted with SBE-β-CD. The peaks are not present in the spray-dried formulation from both methods, suggesting the formation of fisetin-SBE-βCD complexes in the amorphous form.

3.4.4 *In vitro* aerosol deposition study

In the NGI, an air flow of 60 L/min was used, as in previous impactor studies of spray-dried powders [33,34]. **Fig. 6** shows the distribution of fisetin in the NGI. The spray-dried powder produced from a feed solution containing 20 %v/v ethanol showed improved aerosolization, based on lower deposition in the capsules to pre-separator stage but higher deposition in Stage 2 onwards compared to powder produced from a feed solution in water.

Table 2 shows the aerosol properties for both formulations calculated from the NGI deposition results. The powder which was produced from a feed solution containing 20 %v/v ethanol had a higher fine particle fraction (FPF) compared to spray-dried powder produced

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from a feed solution containing water alone. The powder produced from 20% ethanol also showed a 2-fold increase ($p < 0.05$) in the fine particle dose (FPD). In this study, almost 100% of fisetin loaded into the capsules was recovered which meets the requirements by European Pharmacopoeia (75 to 125%). Approximately 90% of fisetin was successfully emitted from the capsules for both formulations. The MMAD and GSD for both formulations were similar ($p > 0.05$). However, the MMADs for both formulations were significantly higher ($p < 0.05$) than the values of volume median diameter (X_{50}), suggesting that the particles may not be optimally dispersed during aerosolization.

3.4.5 SEM analysis

Fig. 7 shows scanning electron micrographs of all powders. The spray-dried fisetin powder from 20 % v/v ethanol solution (**Fig. 7**A) had spherical particles with the appearance of fisetin crystals on the surface. This corresponds with the XRPD result showing a mixture of both amorphous and crystalline state in the preparation. Particles of the spray-dried SBE-β-CD powder from the same solvent composition were spherical in form (**Fig. 7**B). The fisetin's crystals were not apparent in the SEM images of the spray-dried fisetin-SBE-β-CD powders (**Fig. 7**C and D), confirming the amorphosity of the preparations.

The spray-dried powders from the feed solution containing water alone comprised pitted spherical particles (**Fig. 7**C). The majority of the particles were approximately 2 µm in diameter. The spray-dried powders from the feed solution containing 20 % v/v ethanol also showed pitted spherical morphology, but with greater variation in size (**Fig. 7**D). The pitted morphology of the particles was more evident compared to the spray-dried powder from feed solution containing water, possibly suggesting a hollower internal structure. The difference in the evaporation rate between water and ethanol during spray-drying may contribute to the production of such particles. This may also indicate lower density of the particles. The powder produced was fluffier and lighter compared to the powder produced from feed solution containing water. This may explain the higher FPF observed in the formulation, as powder with lower density may move more readily to the lower stages of the NGI.

3.5 Incorporation of leucine as a dispersibility enhancer

3.5.1 Particle size distribution and residual solvent content

The spray-dried formulation from the feed solution containing 20 % v/v ethanol (SD_20%Eth) was chosen for further investigation by adding leucine as a dispersibility enhancer. This

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formulation was chosen as it gave better aerosolization properties compared to the spray-dried formulation from the feed solution containing water alone (SD_H_2O).

Addition of leucine into the spray-dried formulation changed the particle size distribution of the powder from bimodal to unimodal distribution, regardless of the amount of leucine added (**Fig. 3**B). The same change in the particle size distribution has been reported with the addition of 10 %w/w of leucine in the initial solution of a non-viral gene therapy formulation [35]. Incorporation of leucine as low as 5 %w/w has also been shown to reduce the cohesiveness and improve the flowability of spray-dried herbal extract powder [27]. The improvement in the flowability is shown by Span values presented in **Table 3**. Addition of leucine caused a reduction in Span value ($p < 0.05$) from 3.14 in the formulation without leucine, to the lowest value of 1.22 in the formulation with 20 %w/w leucine.

Table 3 shows that incorporation of leucine as low as 5 %w/w reduced the volume median diameter (X_{50}) from 1.48 ± 0.08 to 1.25 ± 0.01 µm ($p < 0.05$). Further, addition of leucine into the formulation did not affect formation of the fisetin-SBE-β-CD complex as shown by the same percentage of solubilized fisetin. At least 10 %w/w leucine was needed to significantly reduce ($p < 0.05$) the residual solvent content from 8.31 to 6.64%. The additional increase in the amount of leucine to 20 %w/w did not further reduce the residual solvent content ($p > 0.05$).

The spray-dried formulation containing 20 %w/w leucine, showed an increase in the aqueous solubility of fisetin from 31.51 ± 1.16 µg/mL to 1006.19 ± 37.08 µg/mL.

3.5.2 XRPD analysis

Fig. 5 shows diffractograms of raw materials and spray-dried powders with leucine. The diffractograms of spray-dried complexes containing leucine showed the disappearance of fisetin's and leucine's crystalline peaks, confirming that the formulations were in the amorphous form.

3.5.3 *In vitro* aerosol deposition study

An increase in the amount of leucine resulted in a significant reduction ($p < 0.05$) in the deposition of fisetin especially in capsules, device and mouthpiece stages (**Fig. 6**). An increase in the deposition of fisetin in Stage 2 to Stage 8 was also seen, though most of the increases for individual stages were not significant ($p > 0.05$), with the highest deposition seen in formulation containing 20 %w/w leucine.

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The fraction recovered and MMAD were not influenced by the presence of leucine (**Table 2**). However, the MMAD values of all preparations were significantly higher ($p < 0.05$) than their respective volume median diameters (X_{50}). This suggests that the particles act as aggregates during aerosolization, and were not completely dispersed.

The emitted dose from the capsules was improved in formulations containing 10 and 20 %w/w leucine. The value increased ($p < 0.05$) from 88.08% without the presence of leucine to 97.31% in the formulation containing 20 %w/w leucine. This may be caused by the low residual solvent content in both formulations compared to the formulation without leucine (**Table 3**). Low residual solvent content may reduce the adhesive interaction between capsule wall and particles thus increasing the number of particles emitted into the NGI. This may explain reduction in the amount of fisetin in capsules during the deposition study in NGI (**Fig. 6**).

An increase in FPF was found for formulations containing 10 and 20 %w/w leucine. The value increased from 32.49 ± 2.46% in formulation without leucine to 49.41 ± 9.01% in formulation with 10 %w/w leucine ($p < 0.05$). An increase in the amount of leucine to 20 %w/w caused further improvement in the FPF to 75.83 ± 3.34% ($p < 0.05$). Addition of 20 %w/w leucine increased the FPD from 3.76 mg in the formulation without leucine, to 7.06 mg ($p < 0.05$).

3.5.4 SEM analysis

The SEM images showed that incorporation of leucine into the formulation, at a concentration of as low as 5 %w/w caused an alteration in the morphology, giving a wrinkled spherical shaped particle. The wrinkled and rough surface becomes more pronounced with increased leucine content (**Fig. 7**E, F and G). The change in morphology can be explained by the enrichment of leucine on the surface of the particles. Leucine has a hydrophobic nature and has a tendency to act as a weak surfactant [36], which causes an accumulation of leucine at air-solution interfaces leading to enrichment of the amino acid on particle surfaces during spray-drying [27]. This forms a leucine film that collapses when solvent is removed by evaporation, thus forming the observed surface.

3.6 *In vitro* cell toxicity of fisetin and the spray-dried formulation

The A549 cell viability curve is shown in **Fig. 8**. The blank formulation, containing SBE-β-CD and leucine, showed no cytotoxic activity at all investigated concentrations (up to 495 µM for SBE-β-CD and 2313 µM for leucine). The cells also showed no DMSO-derived

cytotoxicity at a concentration of 0.1 %v/v DMSO in the media. Fisetin and the formulation containing 20 %w/w leucine (SD_20%Eth20%Leu) showed a similar growth inhibition curve with no significant difference ($p < 0.05$) in the cytotoxic activity. The EC_{50} value of fisetin and SD_20%Eth20%Leu preparation were 67.97 ± 14.48 and 75.86 ± 18.11 μM, respectively. The result shows that complexation with SBE-β-CD and spray-drying process did not alter the cytotoxicity of fisetin in the A549 cell line.

4. Conclusion

This study has shown that complexation with SBE-β-CD was able to enhance the solubility of fisetin without altering its *in vitro* antioxidant activity. Spray drying of the complex in an aqueous solution containing ethanol as a co-solvent produced a low density powder with better aerosolization properties than a powder produced from complexes in water alone. Further, addition of leucine into the preparation generated a powder with improved flowability, FPF and FPD. The spray-dried complexed preparations showed an unchanged cytotoxic activity of fisetin against the A549 cell line. In conclusion, the dry powder inhalation of fisetin-SBE-β-CD complex may provide an improvement in the solubility of fisetin and be capable of delivering high amount of fisetin to the deep lung region for therapeutic applications. Although fisetin may not be the best candidate for pulmonary delivery because of the relatively high doses needed, this study demonstrates how its formulation and delivery can be optimised and provides data that can be applied to other potential anti-cancer agents. Additionally, synergistic combination of fisetin with other anti-cancer agent may be explored for pulmonary delivery using a SBE-β-CD complex.

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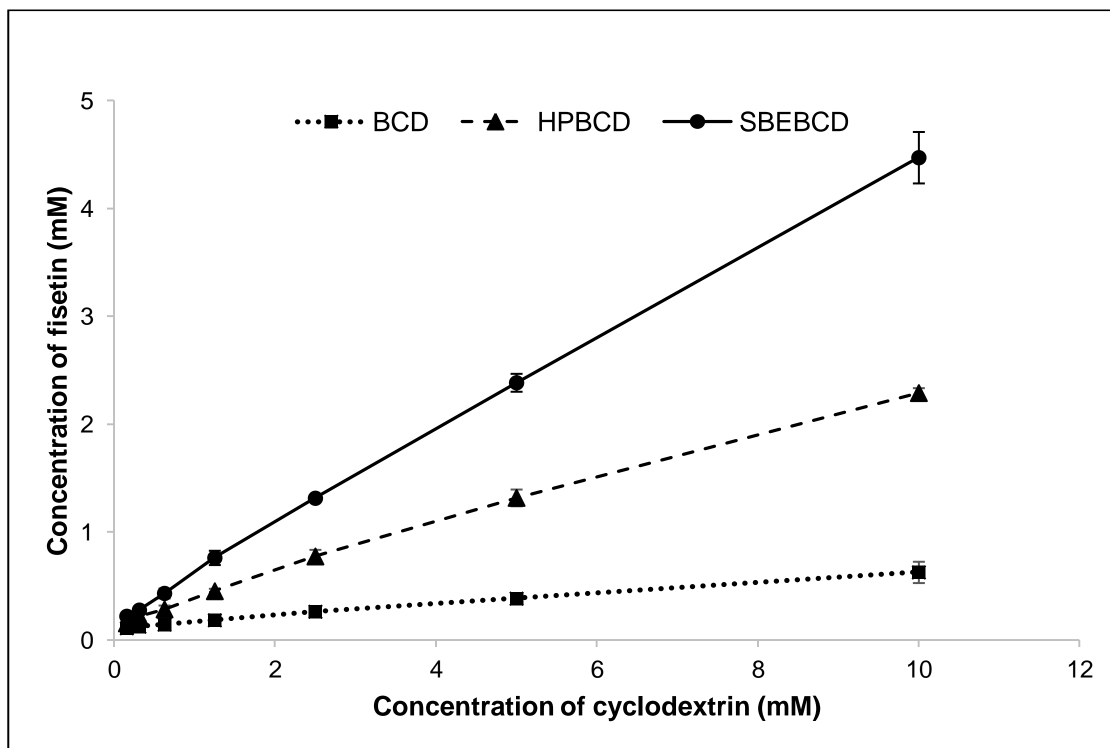

Fig. 1. Phase solubility plots of fisetin in different cyclodextrins, $n = 3 \pm$ s.d.

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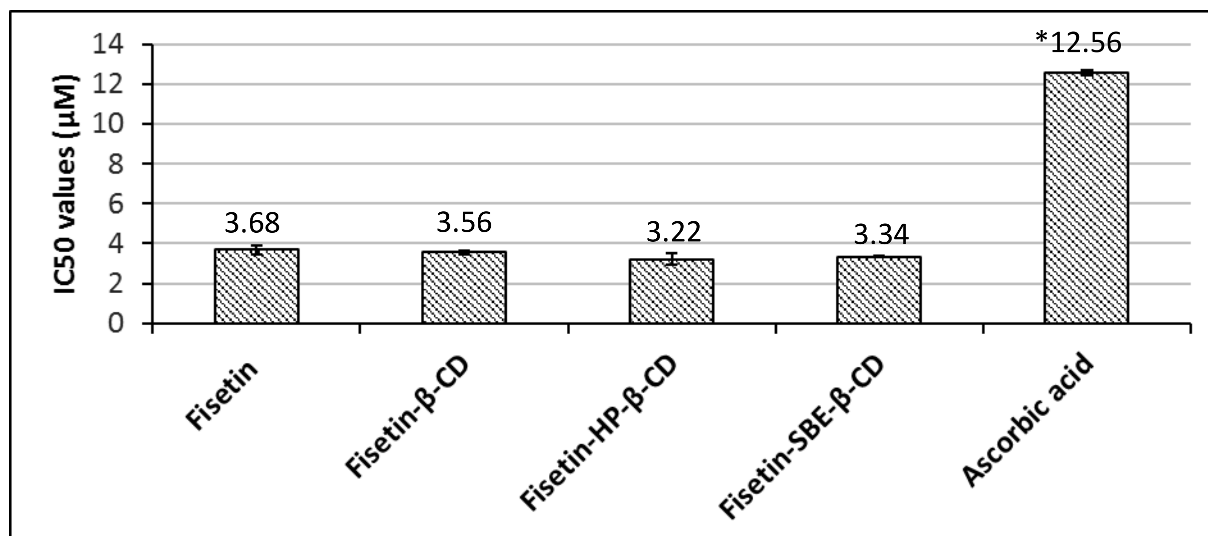

Fig. 2. IC_{50} values of fisetin, fisetin-cyclodextrin complexes and ascorbic acid, $n = 3 \pm$ s.d., *$p < 0.05$.

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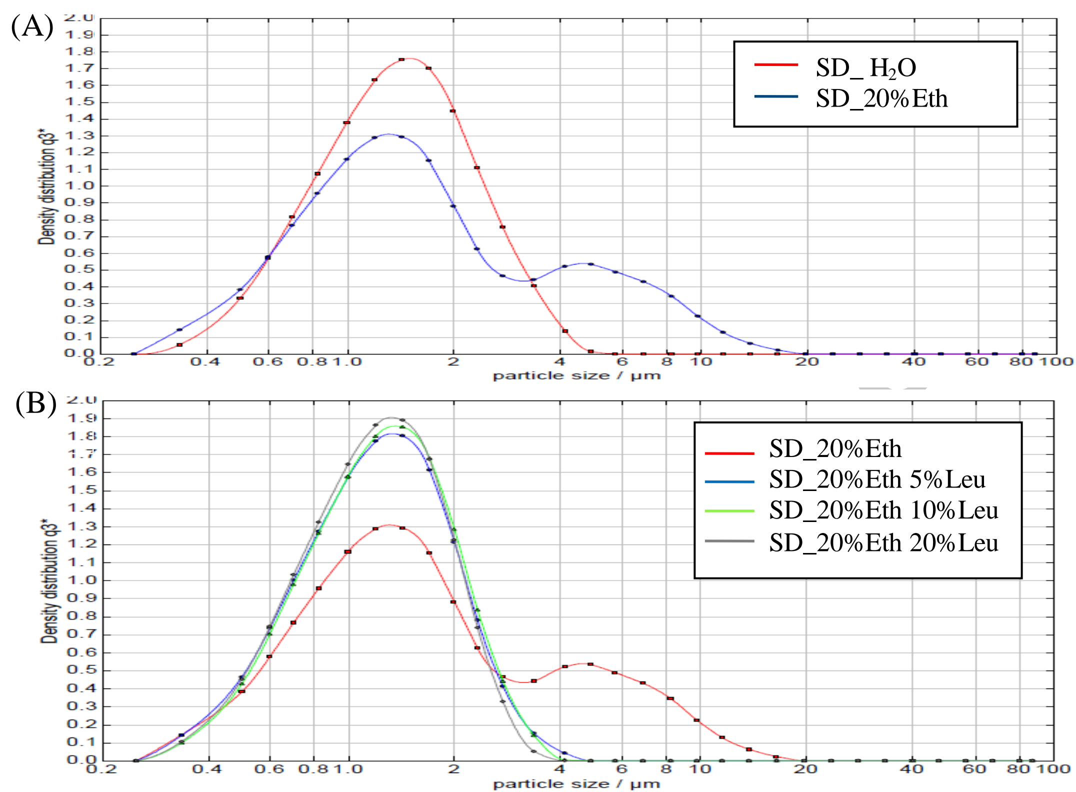

Fig. 3.

Particle size distributions of spray-dried powders (A) from different feed solutions and (B) in the presence of different contents of leucine.

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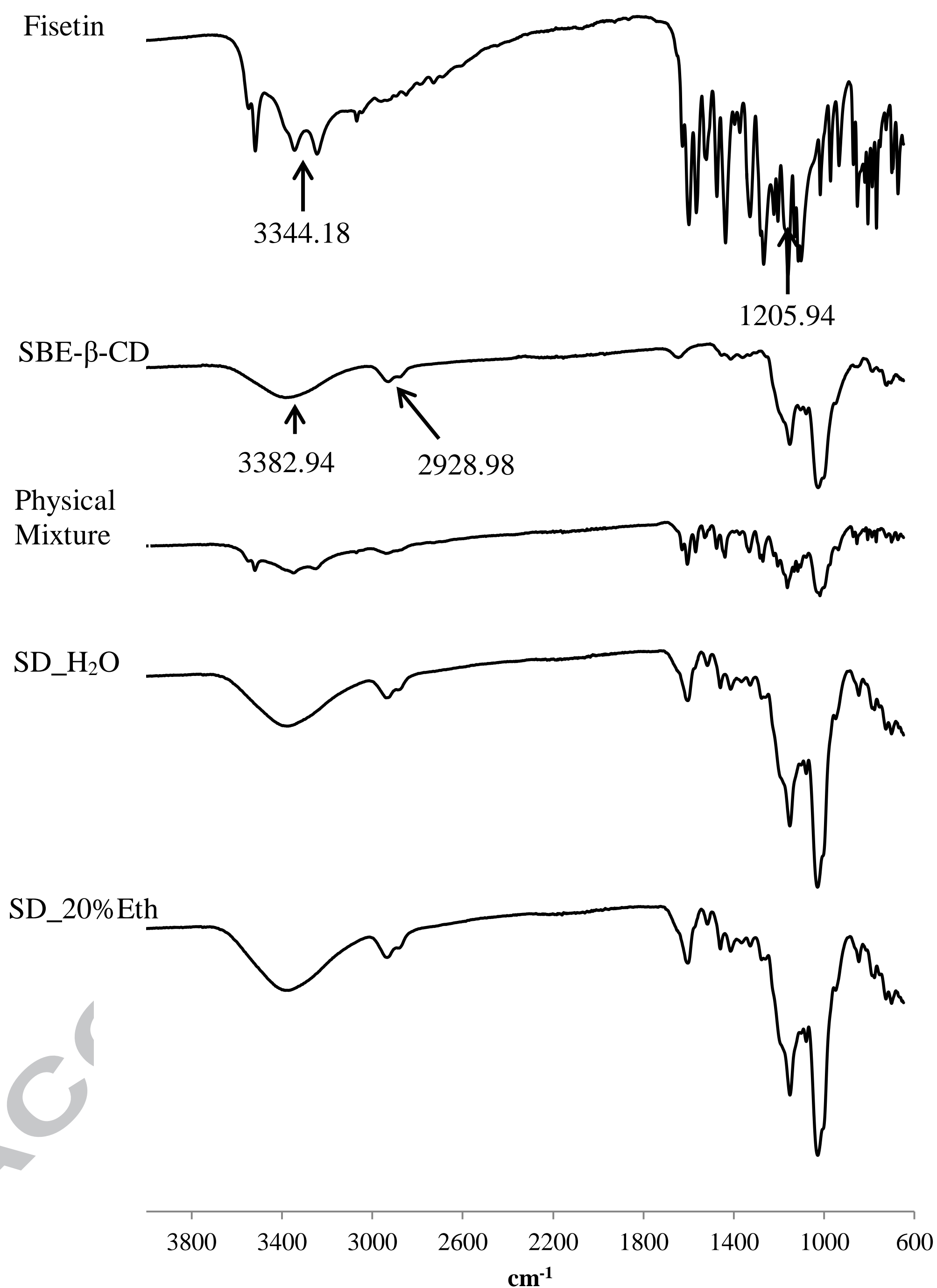

Fig. 4. FT-IR spectra of raw materials, physical mixture and spray-dried formulations of fisetin-SBE-β-CD complex (SD_H_2O and SD_20%Eth).

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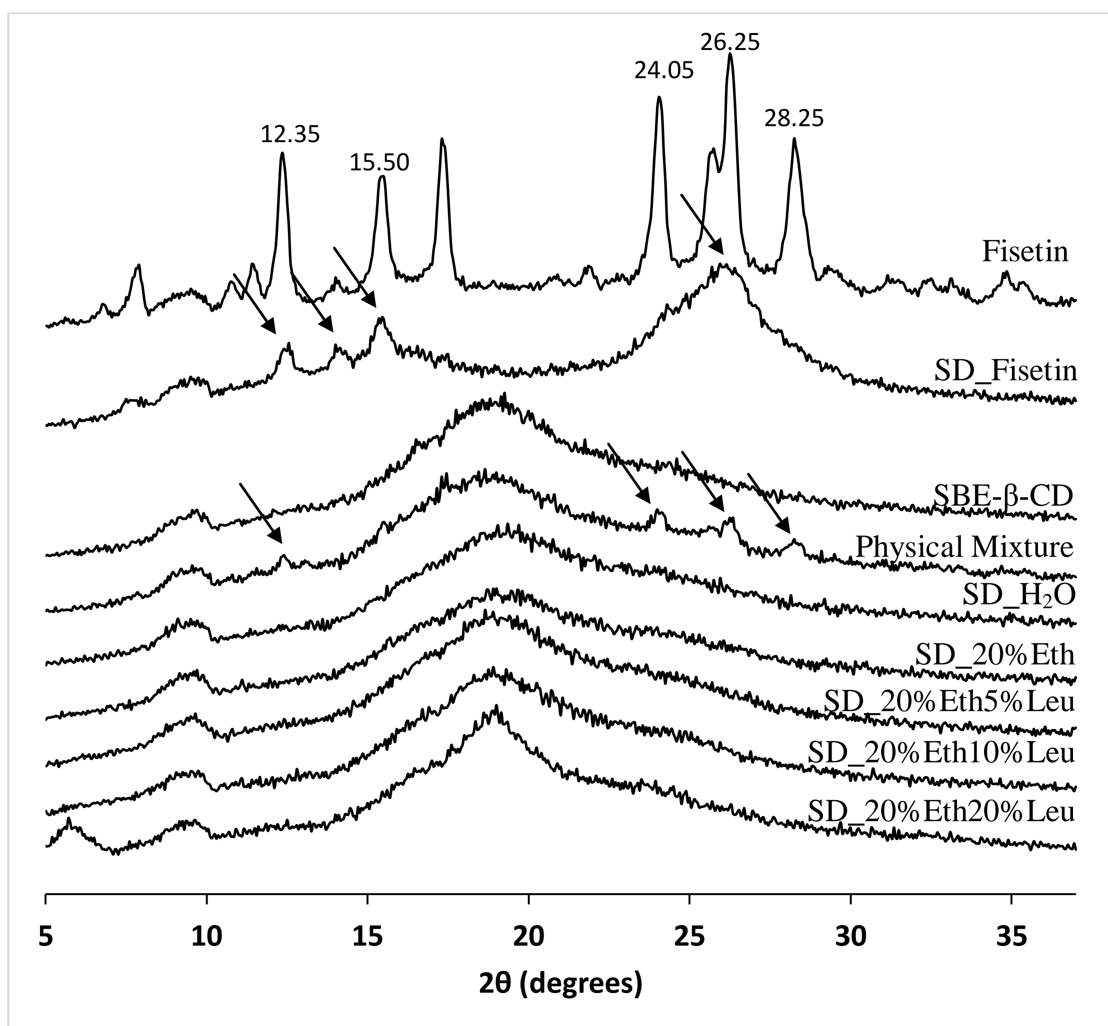

Fig. 5. XRPD diffractograms of raw materials, spray-dried fisetin (SD_Fisetin), physical mixture and spray-dried complexes.

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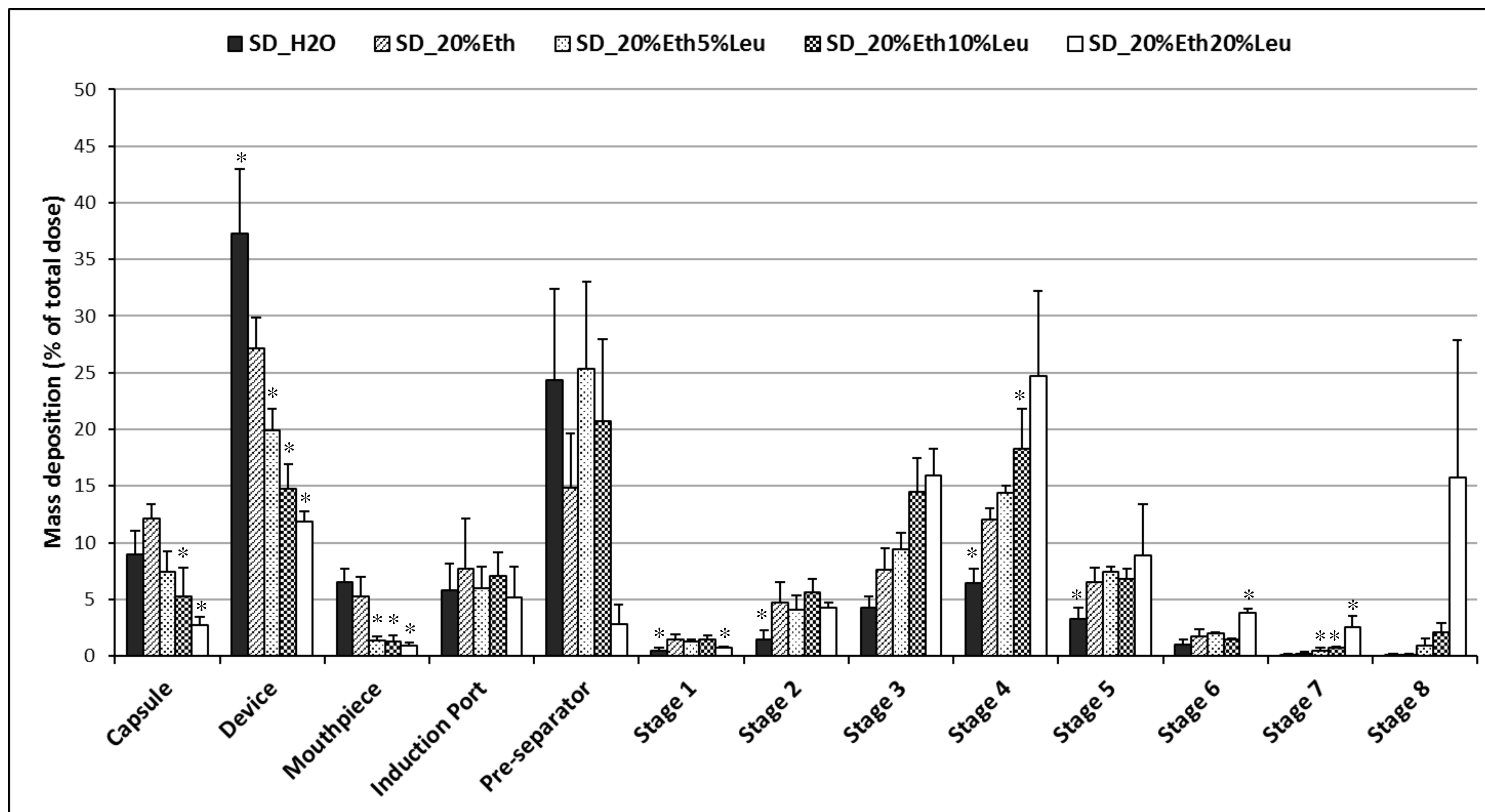

Fig. 6. Distribution of spray-dried powder preparations of fisetin in the NGI, $n = 3 \pm$ s.d, *significant difference compared to SD_20%Eth, $p < 0.05$.

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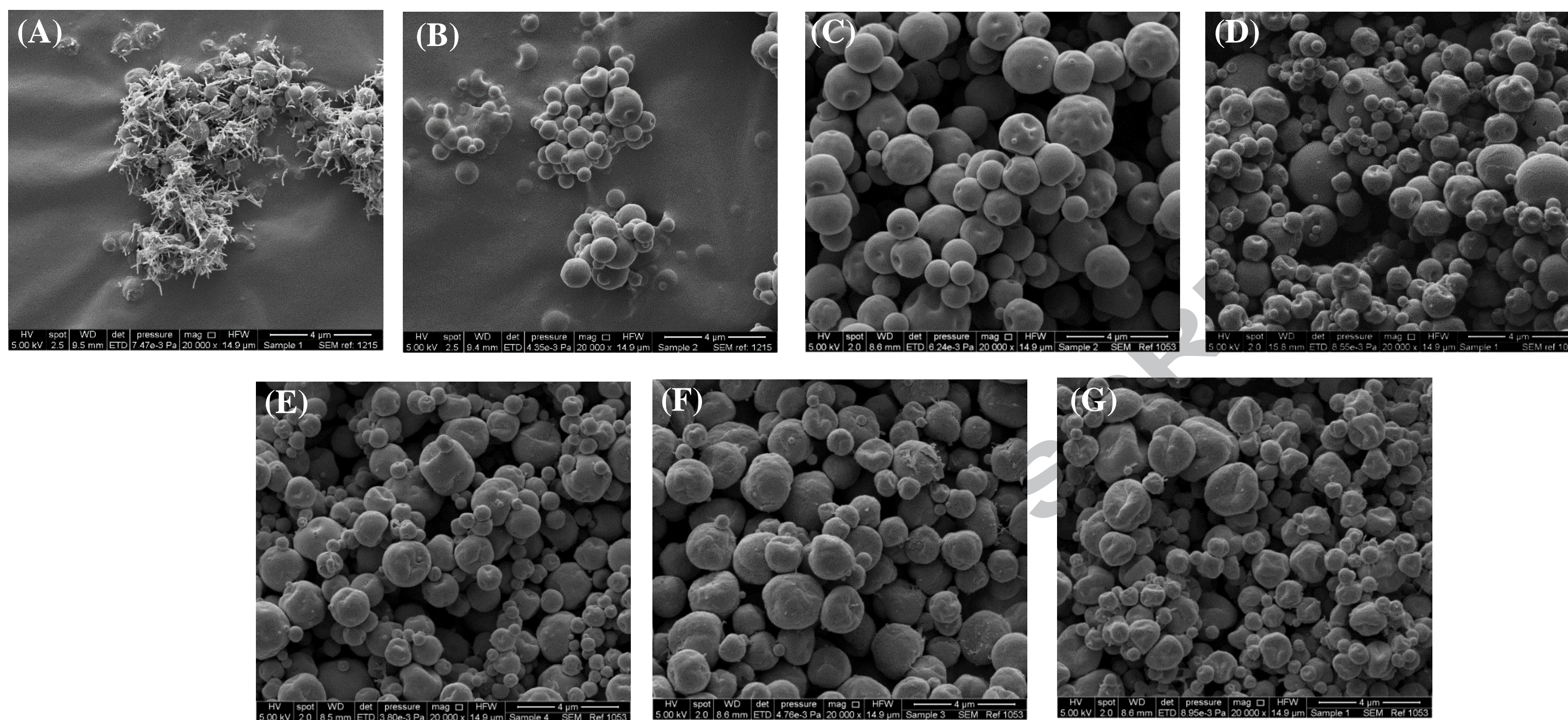

Fig. 7. Scanning electron micrographs of spray-dried (A) fisetin, (B) SBE-β-CD, spray-dried fisetin-SBE-β-CD complex from feed solutions containing (C) water and (D) 20 %v/v ethanol; in the presence of (E) 5 %w/w, (F) 10 %w/w and (G) 20 %w/w leucine; magnification: 20K.

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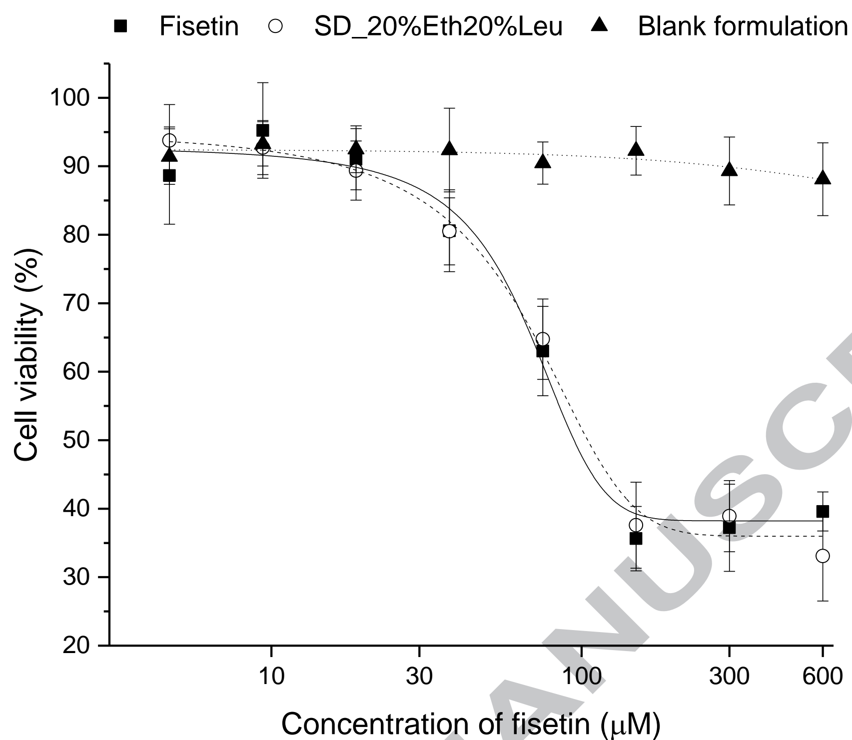

Fig. 8. Cell viability (%) of A549 cells after the treatment with fisetin, SD_20%Eth20%Leu preparation and blank formulation (containing SBE-β-CD and leucine) after 48 h, $n = 3 \pm$ s.d.

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Table 1

Complexation parameters of fisetin in different types of cyclodextrin. Mean ± s.d., *n* = 3.

System	Slope	y-intercept, S_o (mM)	Stability constant, K_s (M^{-1})	R^2	Complexation efficiency, CE	Molar ratio (Fisetin : CD)
β-CD	0.052 ± 0.009	0.114 ± 0.009	481.66 ± 67.38	0.9939 ± 0.0026	0.055 ± 0.010	1 : 19
HP-β-CD	0.217 ± 0.001	0.171 ± 0.029	1647.25 ± 264.88	0.9942 ± 0.0045	0.277 ± 0.002	1 : 5
SBE-β-CD	0.432 ± 0.019	0.184 ± 0.009	4138.84 ± 199.27	0.9985 ± 0.0007	0.763 ± 0.058	1 : 2

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Table 2

Aerosolization properties of the spray-dried powders. Mean ± s.d., $n = 3$.

Preparation	FR (%)	ED (%)	FPF[a] (%)	FPD (mg)	MMAD (µm)	GSD
SD_H_2O	96.49 ± 1.07	91.01 ± 2.12	16.59 ± 2.65*	1.88 ± 0.35*	2.35 ± 0.37	1.68 ± 0.03
SD_20%Eth	98.89 ± 5.43	88.08 ± 1.29	32.49 ± 2.46	3.76 ± 0.27	2.45 ± 0.32	1.75 ± 0.07
SD_20%Eth5%Leu	105.64 ± 4.81	92.54 ± 1.80	38.67 ± 2.73	4.60 ± 0.17	2.34 ± 0.16	1.75 ± 0.02
SD_20%Eth10%Leu	108.02 ± 2.95	94.73 ± 2.56*	49.41 ± 9.01*	5.66 ± 0.91*	2.54 ± 0.08	1.72 ± 0.02
SD_20%Eth20%Leu	98.80 ± 0.93	97.31 ± 0.74*	75.83 ± 3.34*	7.06 ± 0.30*	2.11 ± 0.01	1.73 ± 0.12

FR: Fraction recovered, ED: Emitted dose, FPF: Fine particle fraction, FPD: Fine particle dose, MMAD: Mass median aerodynamic diameter, GSD: Geometric standard deviation.

[a] fraction of particles less than 4.46 µm.

* significant difference compared to SD_20%Eth, $p < 0.05$.

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Table 3

Physical properties of spray-dried complexes in the presence of leucine. Mean ± s.d., *n* = 3.

Preparation	Particle size (µm)		Solubilized fisetin (%)	Residual solvent (%)
	X_{50}	Span		
SD_20%Eth	1.48 ± 0.08	3.14 ± 0.51	96.8 ± 5.0	8.31 ± 0.53
SD_20%Eth5%Leu	1.25 ± 0.01*	1.33 ± 0.05*	96.3 ± 0.9	7.27 ± 0.49
SD_20%Eth10%Leu	1.24 ± 0.05*	1.27 ± 0.02*	97.6 ± 0.4	6.64 ± 0.56*
SD_20%Eth20%Leu	1.23 ± 0.04*	1.22 ± 0.02*	98.8 ± 0.8	6.05 ± 0.66*

* significant difference compared to SD_20%Eth, $p < 0.05$.

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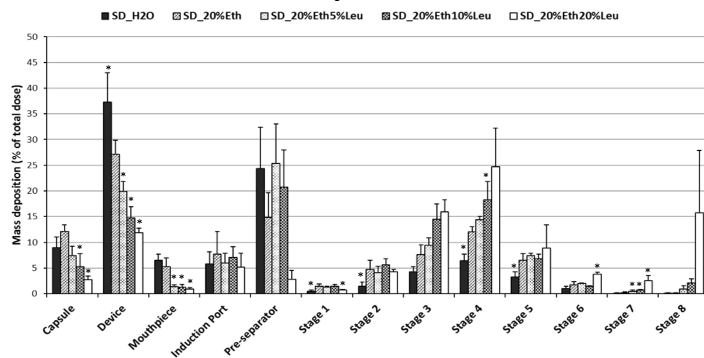
Fisetin-SBE-β-CD complex solution

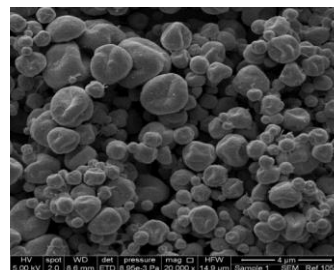
Fisetin-SBE-β-CD inhalable particles

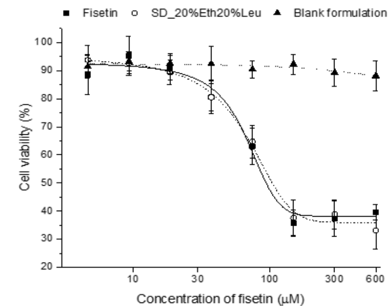
Aerosol deposition study (NGI)

SEM

MTT assay (A549 cell line)







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