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HP-β-Cyclodextrin/TPGS Composite Complexes for Enhanced Resveratrol Solubility, *In- Vitro* Pulmonary Deposition and Cytotoxicity

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ABSTRACT

Resveratrol (RES), a non-flavonoid polyphenol, has been extensively studied for its anticancer effect. However, its poor solubility and low oral bioavailability hampered its use. Hence, the aim of the current work was to enhance RES solubility and to develop lung targeted delivery system with enhanced cytotoxicity for treatment of lung cancer. The combinatorial effect of both complexation and micellization approaches was tried for the first time, aiming to enhance RES solubility. In this context, composite complexes of hydroxypropyl- β-cyclodextrin (HP-β-CD) and the vitamin E derivative (TPGS), at different mass ratios with the drug, were prepared using the solvent evaporation technique. The selected formulations were subjected to spray-drying in order to develop a dry powder for inhalation. Morphology, particle size, drug crystallinity and *in-vitro* pulmonary deposition of the spray-dried powders (SDP) were assessed. *In-vitro* cytotoxicity of the selected SDP was assessed on A549 lung cancer cell line and compared to that of the free drug. A statistically significant improvement in drug solubility was attained using the prepared HP-β-CD/TPGS composite complexes compared to HP-β-CD alone for the same mass ratios. The results suggested that both RES/ HP-β-CD /TPGS ratios of 1:12:0 and 1:12:1 were optimum. The amorphization of the drug was confirmed by X-ray diffraction. The selected SD formulations exhibited a volume mean diameter smaller than 6 µm indicating their suitability for deep lung delivery. A high respirable fraction reaching 52% was achieved using RES/CD/TPGS composite complex. The *in-vitro* cytotoxicity was significantly enhanced in case of composite complex compared to free drug and CD complex. This study suggests the suitability of HP-β-CD/TPGS composite complexes as a novel promising carrier for enhancing the solubility of RES as well as the deposition and cytotoxicity of SD powders after inhalation.

1. INTRODUCTION

Resveratrol (*trans*-3,4′,5-trihydroxystilbene), RES, a non-flavonoid polyphenol belonging to the stilbenes, has been extensively studied for its anti-carcinogenic activity, in addition to other health benefits [1,2]. In plants, the molecule exists in two isomers, *trans*-resveratrol (RES) and *cis*-RES (**Figure 1**), and their glucosides, *trans*-piceid and *cis*-piceid. The unconjugated (aglycone) *trans*-isomer is the most associated with health benefits and the most stable from the steric point of view[3]. RES is found in low quantities in our diet, especially red wine and peanuts.

RES is effective in preventing all stages of cancer development and has been found efficient in most cancers including prostate, breast, stomach, colon, lung, thyroid and pancreatic cancer cell lines[4]. Bishayee and colleagues had shown that RES can influence carcinogenesis by modulating signal transduction pathways that control cell division and growth, apoptosis, inflammation, angiogenesis and metastasis[5]. *In-vivo*, RES has shown efficacy in preventing and treating skin, esophageal, intestinal, and colon tumors[6]. In addition, RES demonstrated well tolerability and modulation of enzyme systems involved in carcinogen activation and detoxification[7]. Hence, this drug possesses a promising anti-cancer prospective but, due to its poor bioavailability, it becomes more efficacious when it can come in direct contact with tumor cells e.g., skin, gastrointestinal tract or lung cancers.

RES suffers from a low bioavailability following oral administration as it is rapidly metabolized in intestine and liver by phase II enzymes[8]. Also, RES has low water solubility (log P = 3.4) and stability making its clinical success a challenge. To improve its bioavailability, different formulation approaches had been developed such as microencapsulation, nanoparticles, complexation and micellization[9]. Microencapsulation of RES was shown to increase its solubility in aqueous medium thereby improving bioavailability and also inhibited light and heat-induced degradation[10]. Various RES-loaded microparticles (MPs) either based on pectin or chitosan were developed using zinc ions or vanillin as crosslinking agent, respectively, with encapsulation efficiencies exceeding 90% [11,12]. The prepared formulations exhibited a controlled drug release coupled with an enhanced stabilization.

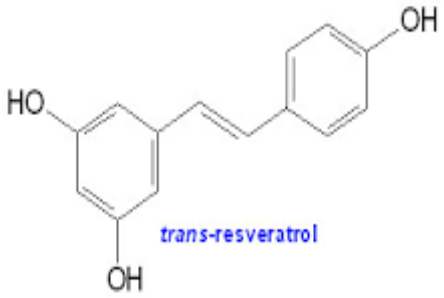
Nanoparticles (NPs) loaded with RES were also formulated with the potential to increase drug bioavailability, target delivery and stability. Teskac *et al.* formulated solid lipid nanoparticles (SLNs) as a colloidal carrier for RES by melt emulsification process. RES loaded SLNs

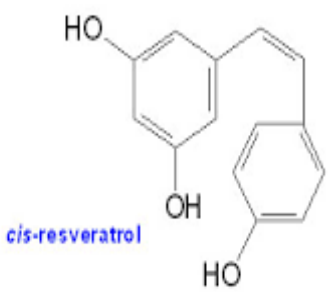
improved drug cellular fate through enhanced particle uptake by cells. SLNs released the drug in sustained manner improving its bioavailability and stability[13]. Gokce *et al.* conducted a comparative study between SLNs and nanostructured lipid carriers (NLCs) loaded with RES prepared by high shear homogenization technique using compritol 888ATO, miglyol, poloxamer 188, tween 80. Both formulations showed potent antioxidant activity at a low concentration of 50 µM but NLCs containing RES penetrated deeper into the skin than SLNs[14].

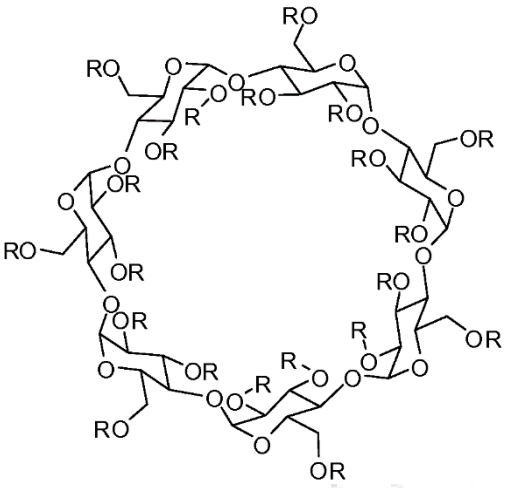
As a lipophilic drug, RES bioavailability was improved after incorporation in vesicular system bilayers. Liposomes, niosomes, transfersomes and ethosomes were prepared and showed enhanced drug protection against environmental and biological degradation thus improving the drug bioavailability and penetrability through the skin.[15-19].

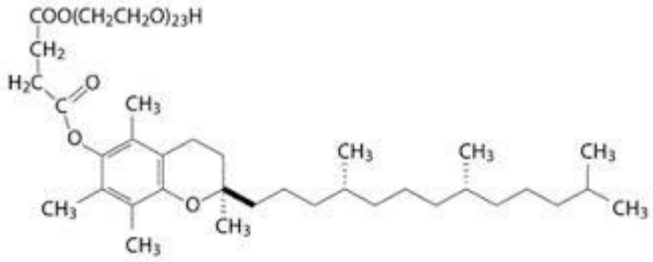
A mixed micelle system composed of methoxy poly (ethylene glycol)-β-polycaprolactone (mPEG-PCL) and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) loaded with RES has also been formulated. The drug loaded mixed micelles enhanced the cytotoxicity of RES against MCF-7/ADR cells and inhibited the activity of P-GP receptors as well [20].

Aiming at improving the solubility of poorly water-soluble drugs and enhance stability of labile drugs, cyclodextrins (CDs, **Figure 1**) have been playing a very important role through inclusion complexation or solid dispersion [21] and by providing a molecular shield, thus insulating drugs against various degradation processes[22]. CDs are cyclic oligosaccharides of a glucopyranose, containing a relatively hydrophobic central cavity and hydrophilic outer surface[23]. As a result of their molecular structure and shape, they possess a unique ability to act as molecular containers by entrapping guest molecules in their internal cavity. No covalent bonds are formed or broken during drug CD complex formation, and in aqueous solution, the complexes readily dissociate and free drug molecules remain in equilibrium with the molecules bound within the CD cavity. The parent or natural CDs consist of 6, 7 or 8 glucopyranose units and are referred to as alpha, beta and gamma CD, respectively. Hundreds of modified CDs have been prepared and shown to have research applications, but only a few of these derivatives, those containing the hydroxypropyl (HP), methyl (M) and sulfobutyl ether (SBE) substituent have been commercially used as new pharmaceutical excipients[24].









TPGS

R = H β-cyclodextrin

R = $CH_2CH(OH)CH_3$

HP-β-cyclodextrin

Figure 1: Chemical structures of *trans*- and *cis*- resveratrol, cyclodextrin and TPGS

TPGS or Vitamin E TPGS (**Figure1**) is a water-soluble derivative of natural Vitamin E, which is formed by esterification of Vitamin E succinate with polyethylene glycol 1000. It has an average molecular weight of 1,513, an amphiphilic structure of lipophilic alkyl tail and hydrophilic polar head with a hydrophilic/lipophilic balance (HLB) value of 13.2 and a relatively low critical micelle concentration (CMC) of 0.02% w/w[25]. TPGS has a melting point around 37–41°C and is heat stable at temperatures up to 199°C[26].As one of the novel nonionic surfactants, TPGS displayed a significant surface activity and hence has been widely used in wetting, emulsification, solubilization and detergency[27]. It can solubilize a variety of both water-soluble and water-insoluble compounds. As the water content increases, TPGS forms lamellar reverse micellar phase, hexagonal phase, and normal micellar phase. TPGS was found to increase the

apparent solubility and stability for some unstable drugs by incorporation into TPGS micelles[28]. TPGS significantly enhanced the aqueous solubility of paclitaxel, celecoxib, corticosteroids, capuramycin analog SQ641 and propofol[29-34]

Hence, the aim of this work was to improve RES solubility, for the first time, through the combination effect of CD and TPGS, respectively. The selected formulations were subjected to spray-drying technique aiming to the development of dry powder for inhalation (DPI) suitable for lung delivery. The morphology, particle size and *in-vitro* pulmonary deposition of the spray-dried powders were assessed. The *in-vitro* cytotoxicity of RES in the selected SD powders was investigated on A549 lung cancer cell line compared to the free drug.

2. MATERIALS AND METHODS

2.1. Materials:

Trans-resveratrol (RES) was supplied from Hangzhou FST pharmaceutical Co., Ltd. (Hangzhou, China). Hydroxypropyl (HP)-β-cyclodextrin (Kleptose® HP, KL), maltodextrin (Glucidex®) and xylitol (Xylisorb®) were kindly supplied from Roquette Pharma, Lestrem, France. (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma–Aldrich Co., (St. Louis, USA). D-α-tocopheryl polyethylene glycol succinate (TPGS) was generously provided by Isochem (France). Absolute ethanol was supplied from El Nasr Pharmaceutical Chemicals (ADWIC) (AbouZaabal, Cairo, Egypt). Leucine (Leu) and methanol (HPLC grade) were obtained from Fluka (Switzerland). Lung epithelial cancer cell line A549 was obtained from the American Type Culture Collection (ATCC, USA). All other chemicals and reagents were of analytical grade.

2.2. Methods:

2.2.1. Selection of the optimum hydrophilic carriers:

As a preliminary study, RES was mixed with one of the hydrophilic carriers, namely; maltodextrin, xylitol, KL and TPGS, in a mass ratio of 1:2. Both ingredients were mixed in a mortar and 50% alcoholic aqueous solution was added dropwise until a consistent paste-like mixture was formed. After storage in the dark overnight, the mixture was then allowed to dry under vacuum. Finally, the dried mixture was ground to a fine powder and stored in a desiccator until further analysis.

2.2.2. Preparation of RES-KL inclusion complexes

The complexes of RES and KL were prepared at different mass ratios (1:2, 1:3, 1:6 and 1:12). RES was dissolved in absolute ethanol then an aqueous solution of KLof equal volume was added portionwise to the previous drug solution and the mixture was left in dark overnight.

2.2.3. Preparation of RES/KL/TPGS composite complexes

In this study, TPGS was tried in conjunction with KL in the preparation of composite complexes. Hence, drug to KL ratios, mentioned above, were prepared with the addition of TPGS to RES alcoholic solution. TPGS was maintained at an equal mass ratio to the drug. The procedure was continued as described earlier in section 2.2.2.

2.2.4. Spray-drying conditions:

Spray-drying solutions were prepared as previously described with the addition of leu as dispersing aid at a double mass ratio to RES. The solutions with 2%w/v feed concentration were atomized using a Buchi mini spray dryer apparatus (Buchi-290, Switzerland) at a drying air flow 600 L/h, a pump rate of 4% and aspiration rate of 85%. The inlet temperature was adjusted at 80◦C and the outlet temperature, under these conditions, ranged from and 50-55◦C. SDP were collected by cyclone separation, transferred to glass vials and kept in a desiccator for further analysis.

2.2.5. Determination of the saturated aqueous drug solubility (DS)

An accurately weighed amount of RES, RES-carrier mixture or spray-dried (SD) drug-loaded inclusion/composite complex was added to 100 mL deionized water, stirred under magnetic stirrer overnight and then filtered through 0.22 mm membrane filter. The samples were then analyzed for their RES concentrations (µg/mL) by high-performance liquid chromatography (HPLC) assay according to the method reported by Juan *et al.*[35] with few modifications. This method was established to be linear, accurate, precise and selective for the measurement of RES in the prepared formulations. The chromatographic system consisted of Agilent Technologies 1200 series LC – G 1311 equipped with a solvent delivery pump and G1315D diode array detector using a Phenomex-C18 analytical column (5 mm particle size; 250×4.6 mm ID). The mobile phase consisted of methanol–water (70:30 v/v). The UV detector was set at 290 nm and

the sensitivity was set at 0.001 absorbance unit full scale. The flow rate and column temperature were adjusted at 1 mL/min and 30◦C, respectively. The data was recorded and calculated using ChemStation B.04.01 software. DS was determined as the concentration of drug dissolved in water, calculated by three determinations for each sample. The solubility enhancement ratio (SER) was also calculated as the ratio of DS in each complex to that of free drug.

2.2.6. Characterization of SD-composite complexes

2.2.6.1. Morphology of SD- composite complexes

The surface morphology of SD-composite complexes was investigated by a scanning electron microscope (SEM, JEOL Scanning Microscope, JSM-5500 LV, Tokyo, Japan) using a high vacuum mode. The powdered samples were mounted on round brass stubs and gold sputter-coated using SPI-Module gold sputter coater for 70 s under an argon atmosphere and then observed with SEM.

2.2.6.2.Determination of particle size of SD-composite complexes

The size of SD-composite complexes was determined using the dry dispersion unit of a laser diffraction particle analyzer at air pressure 4 bar (Mastersizer 2000, Malvern Instruments Ltd, UK). The size distribution was expressed in terms of D[v,10], D[v,50], D[v,90] and D[4,3] which are the diameters at 10, 50 and 90 percentile cumulative volume and the volume mean diameter (VMD), respectively. The span, defined as the difference between 90 and 10% cumulative volumes, divided by the 50% cumulative volume, was also recorded.

2.2.6.3.X-ray Diffraction (XRD)

XRD patterns of RES, selected plain and loaded SD formulations were acquired using an analytical X-ray Powder Diffractometer (Philips Analytical X-ray diffraction system, type PW 3710 with Cu tube anode) at 25°C. Samples were scanned from 4–70° 2θ.

2.2.7. *In-Vitro* aerodynamic deposition:

The aerosolization properties of the selected spray-dried (SD)composite complexes were investigated using a twin stage glass impinger (TSI) as previously described[36]. Briefly, volumes of 7 and 30 mL of methanol were introduced into stage 1 and 2, respectively. Aliquots of 20 mg

powders were loaded in hard gelatin capsules size (3) and placed in the Aerolizer1, attached to the throat of the impinge *via* the adaptor. The capsule was pierced and the powder was drawn through the TSI at an air flow rate of 60 L/min, using an electronic digital flowmeter, for 3×5 s aspirations. The *in-vitro* aerosolization properties of the powders were described in terms of: the emitted fraction (Em) defined as the percent of total loaded powder mass exiting the capsule determined gravimetrically and can be expressed as:

$$Em = \left(\frac{\text{m full} - \text{m empty}}{\text{m powder}}\right) \times 100 \quad (1)$$

Where, m_{full} and m_{empty} are the masses of the capsule before and after simulating the inhalation, respectively, and m_{powder} is the mass of the powder. Two other indices, the respirable particle fraction (RP) which is a percentage of stage 2 against emitted particles from the inhalation system and the effective inhalation index (EI), were also calculated using the following equations[37]:

$$RP\% = \left(\frac{St2}{Em}\right) \times 100 \quad (2)$$

$$EI\% = (Em \times St2)^{1/2} \quad (3)$$

Where, St2 is the fraction (%) distributed to stage 2 of the TSI.

2.2.8. *In-Vitro* cytotoxicity assessment using MTT assay:

Cytotoxicity of RES selected plain and RES-loaded SD-complexes were determined on lung epithelial cancer cells A549 using MTT assay previously described elsewhere [36]. Briefly, A549 cells were propagated in maintained in F-12Ham supplemented with 10% fetal bovine serum (FBS), 100 mg/mL of streptomycin and 100 Units/mL of penicillin in a humidified air atmosphere (5% CO2, 95% RH, 37◦C). Cells were then seeded in 96 wells plates at a concentration of 1 $\times 10^4$ cells/well. Serial two-fold dilutions of the tested formulae were added to confluent cell monolayers. The plates were incubated for 48 h at 37◦C in 5% CO2, 95% RH after which the media were removed and the cells were rinsed with 100 ml phosphate buffer saline (PBS). An amount of 20 ml MTT solution (5 mg/mL in PBS pH 7.4) was added to cells followed by incubation for 4 h the medium was removed and the formazan crystals were solubilized with 100 ml dimethyl sulfoxide for 15 min the absorbance was read on a microplate reader (Tecan

Sunrise1, Switzerland) at 570 nm. Cell viability was determined as a percentage of the negative control (untreated cells) using the following equation:

$$\% \text{ cell viability} = \frac{\text{A test}}{\text{A control}} \times 100 \quad (4)$$

Where, A(test) is the absorbance obtained for each of the concentrations of the test substance, A(control) is the absorbance obtained for untreated cells (incubated with medium only). The latter reading was assumed to correspond to 100% cell viability. The assay was performed on three occasions with six replicates at each concentration of test substance in each instance. The 50% cell cytotoxic concentration (IC50), the concentration required to kill 50% of the cells, was calculated according to the equation for Boltzmann sigmoidal concentration-response curve using the non-linear regression fitting models.

2.2.9. Data analysis:

All presented data are the mean of three replicates ±S.D. One-way ANOVA analysis was performed using MINITAB for Windows (release 15.1.30.0, 2007, State College, PA) software. Significant differences were considered at $p \leq 0.05$. Graph Pad prism was used for calculating IC50 values in cytotoxicity assay.

3. RESULTS AND DISCUSSION

RES is a BCS class II drug with low solubility (37 µg/mL, **Table 1**) and high permeability (log *P* 3.4). The poor aqueous solubility limits it's *in-vivo* bioavailability and thus it is a suitable candidate for applying different techniques and/or carriers aiming to increase drug solubilization in biological fluids.

3.1.Screening of different hydrophilic carriers:

As a preliminary study, the kneading method was applied using different hydrophilic carriers for enhancing the solubility of the hydrophobic RES. The naturally-occurring saccharides, maltodextrin and xylitol, are known to enhance the solubility of poorly soluble drugs by co-solvency effect due to the presence of hydrophilic domains in their structures [38,39]. It was also previously reported the ability of maltodextrin to improve the solubility of dimenhydrinate and to prevent the recrystallization of the drug substance[40]. Moreover, the CDs class and specifically

the modified water-soluble CDs .i.e. HP-β-CD (KL) is well-known to improve the solubility of hydrophobic molecules by host-guest system or inclusion complex formation, as reported by several studies [41,42]. Also, the non-ionic water-soluble surfactant, TPGS, became popular as a powerful solubility enhancer via micellization approach[43,44].

From **Figure 2**, it is obvious that all tested carriers showed an improvement in RES aqueous solubility. The effect was higher in case of KL and TPGS compared to maltodextrin and xylitol. The solubility of RES was increased by about 9-fold with the former 2 carriers compared to <2-fold while using the latter ones. This could be attributed to the mechanisms of solubilization applied that's to say that the inclusion complexation and the micelles formation exhibited the highest influential techniques for enhanced RES solubility.

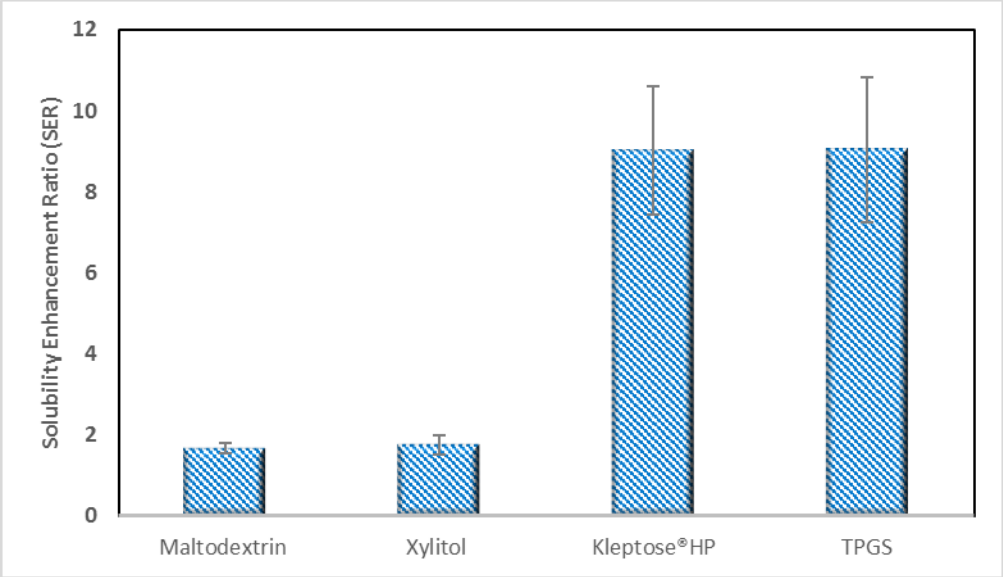


Figure 2. The SER of RES in combination with different hydrophilic carriers

3.2.Solubility studies:

Aiming at preparing a powdered formulation convenient for lung delivery, the solvent evaporation method coupled with spray-drying technique, one of the well-established method for inhalable powders, was used for the preparation of inclusion complexes [45]. It is worthy to mention here that spray drying RES alone resulted in a very fine powder (formula SD-RES) with a very low yield (about 20%) and the solubility of the SD-RES did not significantly differ from that of the pure drug, Table 1, due to the high hydrophobicity of RES (log *P* 3.4). Similarly,

spray drying RES with Kl at 1:2 resulted in a very fine powder with low yield (less than 30%). Hence, a dispersibility enhancer, Leu, was added to the formulae in a ratio of RES: Leu of 1:2 after preliminary optimization. The addition of Leu improved the significant yield which reached more than 70% in all formulae containing Leu (data not shown).

RES solubility was significantly increased ($p < 0.05$) with the addition of KL. The concentration of the water-soluble modified CD used to solubilize the drug was found critical. The addition of KL at only double mass portion that of drug increased RES drug solubility by about 28-fold.Further increase in HP-β-CD improved the solubilization of RES reaching the highest SER, which was 200-fold, at 1:12 drug:carrier ratio. This could be ascribed to the hydroxypropyl substitutions that enlarge the opening of native β-CD and destroy the strong intramolecular hydrogen bond network, which lets guest molecules access the HP-CD cavity easily and give a higher solubility [46]. Similar findings were previously reported Lu *et al.* [47].

Table 1: Saturated DS and SER data of RES and spray dried inclusion complexes.

Formula Code	RES:KL:TPGS (mass ratio)	DS (mg/mL) ±SD	SER ±SD
RES	1:0:0	0.037 ±0.015*	---
SD-RES	1:0:0	0.041 ±0.021*	---
C2	1:2:0	1.05 ±0.035	28.26 ±0.94
C3	1:3:0	1.25 ±0.078	33.72 ±0.21
C6	1:6:0	3.64 ±0.155	98.10 ±5.93
C12	1:12:0	7.55 ±0.032	203.56 ±1.23
T2	1:2:1	1.07 ±0.020	28.73 ±0.75
T3	1:3:1	3.12 ±0.265	84.15 ±7.14
T6	1:6:1	4.73 ±0.126	127.46 ±4.79
T12	1:12:1	12.48 ±1.616	336.27 ±4.54

*Non-significant difference at $p>0.05$

RES: free drug, SD-RES: spray-dried drug, DS: Drug solubility (saturated), SER: solubility enhancement ratio, SD: standard deviation. Formulae C2 to T12 contained Leu in a RES: Leu ratio of 1:2.

The water-soluble vitamin E derivative, TPGS, was selected in conjunction with KL aiming to study the combined effect on RES solubility. Due to its waxy sticky nature, TPGS was only maintained in equal portion with the drug in all composite complexes prepared fear from any inconvenience upon spray-drying. The post-hoc Tukey-HSD test showed a statistically significant difference in solubility between complexes with and without TPGS at the same drug:KL mass ratios. This could be attributed to the combinatorial enhancement of drug solubility based on two different mechanisms i.e. the complexation and the micellization. RES solubility showed an indifference before and after addition of TPGS only at the lowest CD content i.e. drug:CD ratio (1:2).

These results suggested that both RES/KL/TPGS ratios of 1:12:0 and 1:12:1 were optimum and hence selected for further investigations.

Table 2: Particle size of selected RES spray dried complexes.

Formula	D[4,3](µm)	Span	Distribution percentile volume (µm)		
			D(0.1)	D(0.5)	D(0.9)
C12	5.8±0.92	1.4±0.03	2.2±0.31	5.5±0.81	10.1±3.15
T12	3.2±0.75	1.6±0.02	0.8±0.24	2.9±0.67	5.6±1.35

Results are expressed as mean of 3 determinations ±SD.

3.3. Particle size

Table 2 Shows that both formulae C12 and T12, exhibited volume mean diameter (VMD) less than 6 µm indicating suitability for deep lung delivery. The small span values noted indicated homogenous particle size distribution; this can be seen from the volume based charts shown in **Fig. 3**.The smaller size of the particles of T12 compared to C12 could be attributed to the surface active properties of TPGS leading to the production of smaller droplet size during spraying. As

could be seen from the same table, 90% of the particles were less than 10.2 and 6 µm with C12 and T12 respectively, denoting the absence of aggregation in both formulae.

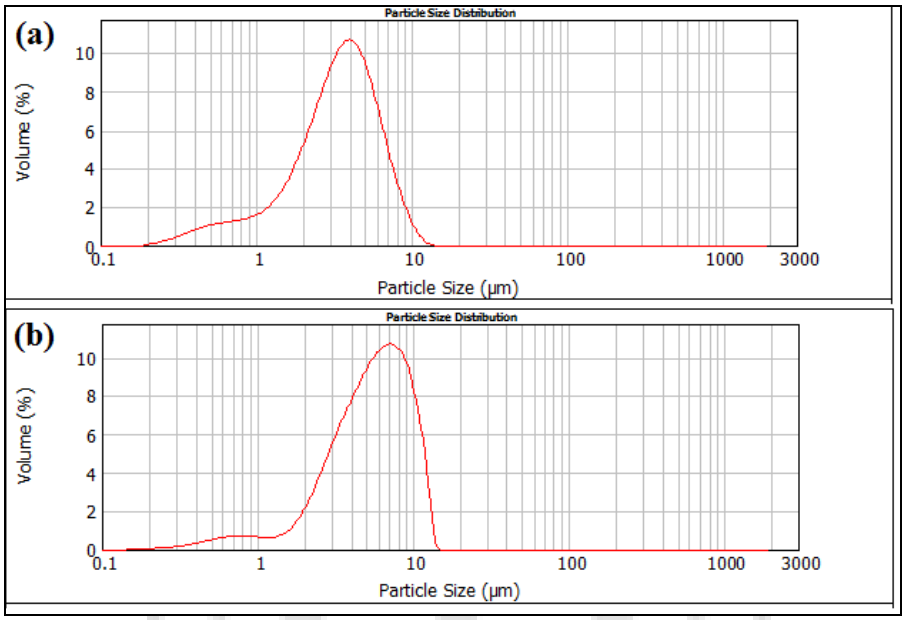


Figure 3: Volume-based particle size distribution of RESSD complex (a) C12 and (b) T12 measured by the dry dispersion method

3.4. Spray dried Powder Morphology

Figure 4 shows hollow round, wrinkled non-aggregated particles with size averaging around 5 µm, in accordance with particle size measurement data. A similar deflated-ball like morphology had been previously reported for budesonide-CD spray dried powders and was attributed to CD, constituting the major proportion of the powder[48], associated with a high Peclet number, had been shown to lead to an evaporation rate at the droplet surface faster than the rate of diffusion of the dissolved components towards the center, forming a shell around the droplet [49]. The evaporation of the solvent inside the droplets caused the shell to crumple causing this wrinkled morphology.

On the other hand, SEM of T12, shown in **Figure 5**, reveals hollow, round particles with smooth surfaces. Due to these surface properties, TPGS migrated to the particles periphery, changing the wrinkled deflated ball appearance in spite of its presence in small amounts compared to CD. The notched appearance of the particles can be explained based again on the obvious hollow nature

of the particles, which under the effect of vacuum applied during SEM visualization, were deflated. Surprisingly, the particles were not fused, in spite of their agglomerated nature, observed visually. TPGS, with its lipid nature, could have acted as a soft binder, allowing agglomeration of the powder without fusion. Similarly, previous reports had shown that lecithin acted as a soft binder, for powders when combined with the sugar mannitol[50].

3.5. X-ray Diffraction (XRD)

The XRD spectra recorded for RES (a), RES-loaded SD-inclusion complexes (b, c) and RES-loaded SD-composite complexes (d, e) are presented in **Figure 6**. These studies are useful to investigate the crystallinity of the drug in complexes. RES is a crystalline drug showing characteristic intense peaks. Peaks corresponding to diffraction from drug crystal lattice are detected in SD-complexes only at low CD content irrespective to TPGS, as illustrated in **Figure 6(b and d)**. In accordance with previous studies, increasing CD proportion enhanced drug solubility due to better drug amorphization as confirmed in **Figure 6(c and e)** [51,52]. It is worthy to note that the enhancement of solubility was thus greatly correlated with the drug crystallinity. Using a RES:KL ratio of 1:3, SER of 33.72 ±0.21 and 84.15 ±7.14 were noted in presence and absence of TPGS, respectively. The partial amorphization seen at this ratio explained the enhancement of solubility. Increasing the ratio to 1:12, corresponding to a 1:2 RES:KL molar ratio, caused a notable increase in drug amorphization corresponding to the increase to the previously observed RES, 203.56 ±1.23 and 336.27±4.54 for formulae C12 and T12, respectively.

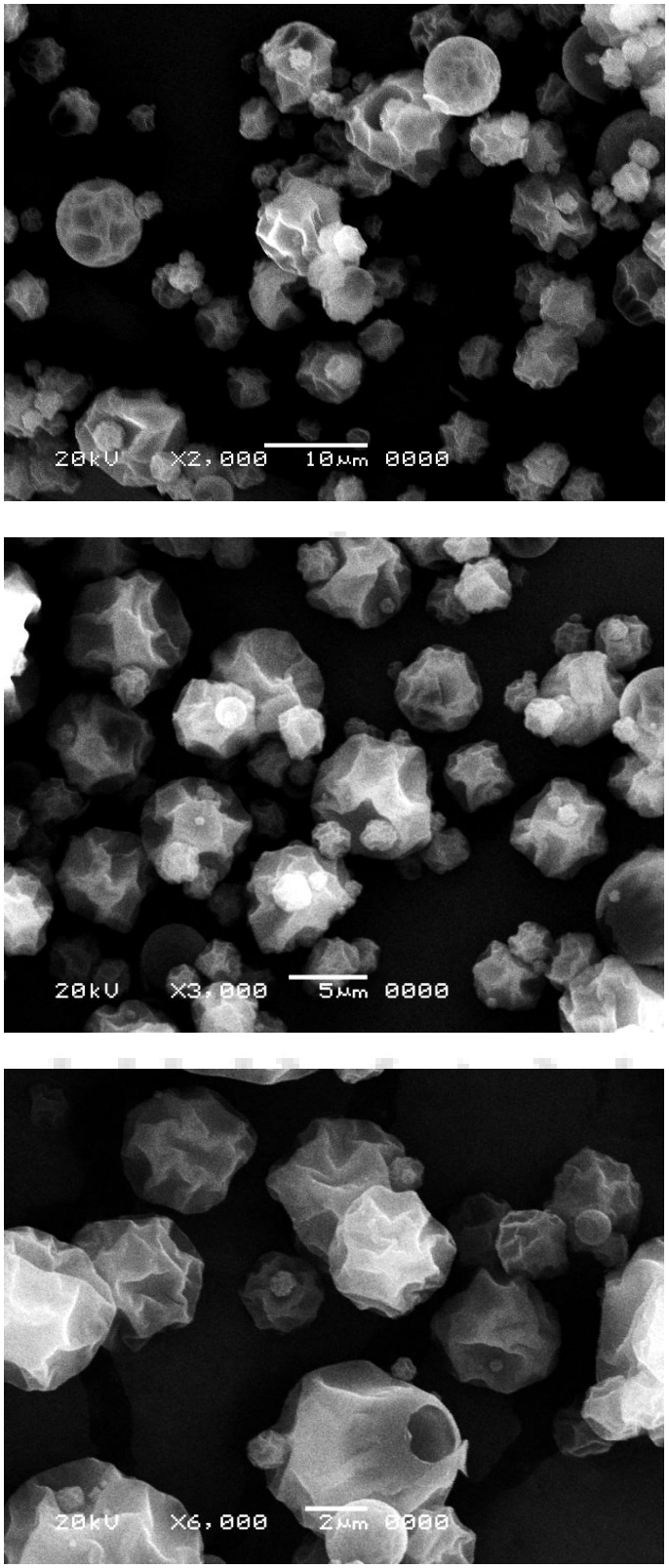


Figure 4: SEM of RES:KL SD-inclusion complex (formula C12)

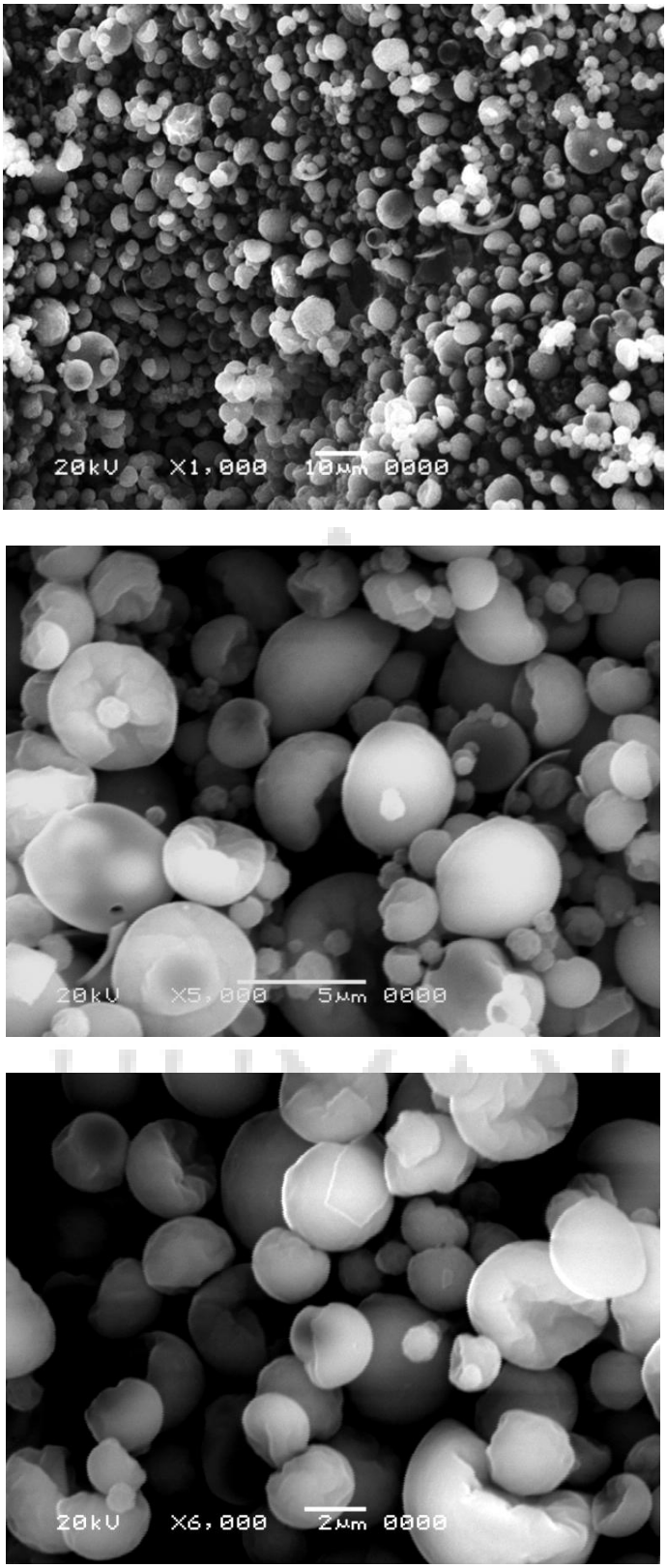


Figure 5: SEM of RES/KL/TPGS composite SD complex (formula T12)

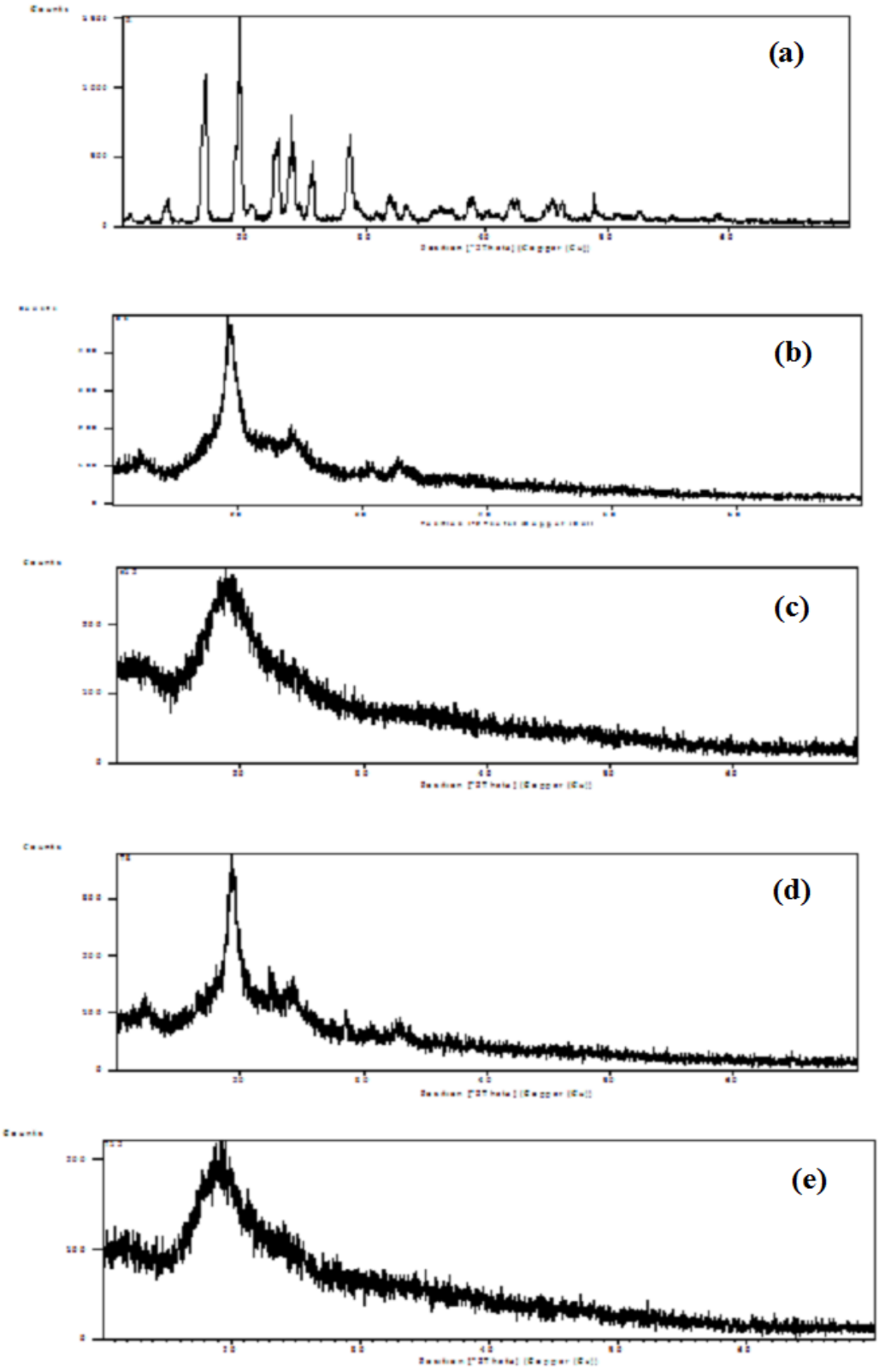


Figure 6: X-ray spectra of (a) RES, (b) C3, (c) C12, (d) T3 and (e) T12

3.6. *In-vitro* aerodynamic deposition

Using an airflow of 60L/min with both formulae (C12 and T12), almost all the SDP were withdrawn from the used inhalation device (*Aerolizer*®) and no powder traces were left in the capsule achieving almost an emitted dose (Em) of 100%. The presence of leu facilitated the delivery from the capsule due to its previously reported dispersibility enhancement effect[36].

As illustrated in **Figure 7**, formula T12 showed lower mouth and stage1 depositions with a significantly higher stage 2 deposition compared to formula C12. Similarly, significantly higher inhalation indices (RP and EI%) were noted with T12 compared to C12. These observations pointed to the significance of the addition of TPGS in T12. Its effects were seen on PS **(Table 2)** and morphology **(Figure 5)** were reflected on the improved *in-vitro* pulmonary deposition. It is worthy to mention at this stage, that the agglomeration of SDP of T12, visually noted and owed to the presence of TPGS with its lipidic nature, did not compromise the SDP deaggregation under the used pressure (60L/min) and on the contrary, it improved the powder performance probably due to improved flow properties.

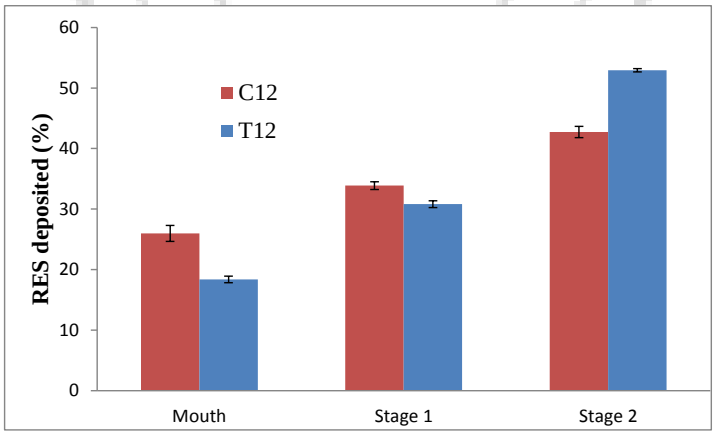


Figure 7: *In-vitro* pulmonary deposition of RES SD complexes using a twin stage impinger

Table 3: Calculated inhalation indices of RES SD complexes

Formula	RP (%)	EI (%)
C12	42.40±0.47	65.11±0.36
T12	52.93±0.26	72.62±0.09

Results are mean of 3 determinations ± SD

3.7. *In-vitro* cytotoxicity study

Figure 8 shows that pure RES caused a concentration dependent decrease in A549 lung cancer cell viability. Several studies have suggested that RES exerts anti-proliferative effects against A549 human non-small cell lung cancer cells by altering a large number of genes and proteins, inducing cell cycle arrest and apoptosis [53]. This was confirmed by the relatively high calculated IC50 value obtained after 48 h incubation with A549 cell line, 37 µg/mL, **Table 4.** Similarly, significant decrease in cell viabilities was seen after exposure to RES incorporated in the selected complexes (formulae C12 and T12). The calculated IC50 showed enhancement in RES anti-cancer effect in both formulations with 1.8 and 2.6 fold decrease in IC50 for C12 and T12, respectively. The significant increase in IC50, (p<0.05), compared to free RES could be ascribed to the effect of CD in improving drug permeability and anti-cancer efficiency. Similar findings were previously reported for CD complexes with curcumin that were shown to enhance the water solubility of the drug with improved anti-inflammatory, antiproliferative and anti-angiogenic activity [54]. Also, another study concluded a greater cellular uptake, enhanced delivery and improved therapeutic efficacy towards cancer cells for the same inclusion complex compared to free curcumin[55]. Jiang and *co*-workers[56] confirmed a significant enhancement in antitumor activity of 9-nitrocamptothecin when complexed with HP-β-CD. Furthermore, the complexation approved an improvement in the anticancer activity when methotrexate was complexed with β-CD and then entrapped into niosomes, or when paclitaxel was loaded into β-CD nanosponges[57,58]. The complexation of these anticancer agents increased the amount entering cancer cells and lowered their IC50, thereby enhancing their pharmacological effects.

TPGS and HP-β-CD showed a synergistic effect on the inhibition of cancer cell growth. The addition of TPGS into RES-KL complex led to a significant higher cytotoxicity effect (p<0.05), with a 1.5 fold decrease in IC50 compared to the inclusion complex alone denoting that TPGS enhanced drug cytotoxicity. In a nanoparticle formulation, Abbad *et al.* reported that TPGS further enhanced the toxicity of Morin Hydrate, a naturally occurring bioflavonoid, against A549 cells both *in-vitro* and *in-vivo*[59]. Also, a TPGS-mixed micelle system loaded with RES has been formulated. The mixed micelles were proven to increase RES uptake by MCF-7/ADR cells and induced higher rates of apoptosis, whereas blank mixed micelles did not affect the proliferation of MCF-7/ADR cells (<10% inhibition) [20]. Moreover, TPGS per se was found to inhibit the

growth of A549 human lung carcinoma cells *in-vivo* and, in an *in-vitro* cell culture by inducing apoptosis [60] without harming normal cells [59].

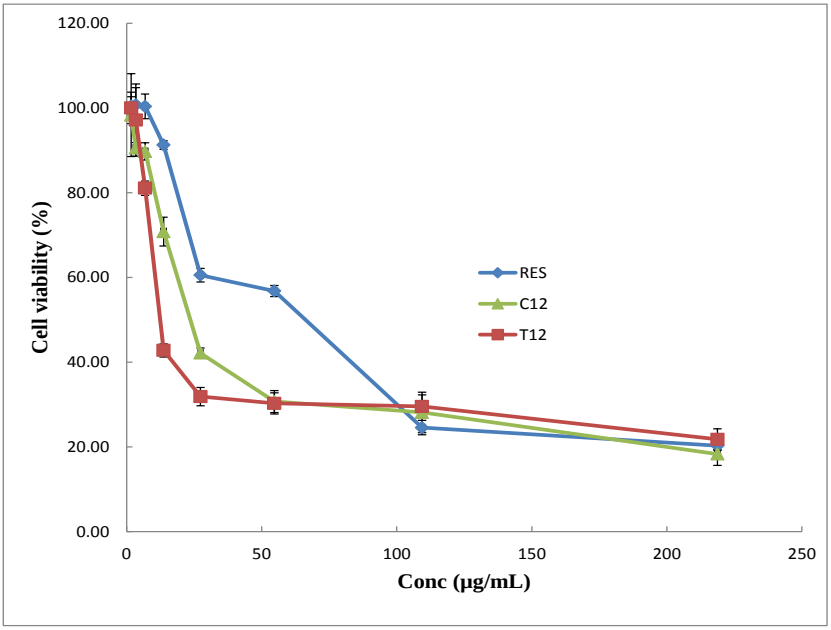


Figure 8: Cell viability of A549 lung cancer cells after exposure to RES, RES-CD complex (formula C12) and RES-CD/TPGS composite complex (formula T12) for 48 h at 37°C using MTT assay

Table 4: Calculated IC50 for RES, RES-CD complex (formula C12) and RES –CD/TPGS composite complex (formula T12)

Formula	RES	C12	T12
IC50 (µg/mL)	37	21	14

4. CONCLUSION

Among all tested hydrophilic carriers, KL and TPGS each used in a ratio of 2:1 with the drug, showed the highest enhancement in RES solubility (9-fold increase) using the solvent kneading method. Combining KL or KL/TPGS with RES, in different ratios, improved its solubility in a single step combined solvent evaporation-spray drying method. The enhancement of solubility depended on the concentration of KL used and was correlated with the decreased RES crystallinity as evidenced by XRD. The addition of TPGS to (1:12) RES/KL mixtures decreased

SDP particle size and modified SDP morphology. These effects led to improved drug amorphization and deposition using TSI achieving a respirable fraction exceeding 50%. Enhanced cytotoxicity was also achieved by using RES complexes where 1.8 and 2.6 fold decrease in IC50 values were seen following use of KL and KL/TPGS mixtures, respectively. This study reported the use of a simple single method for the combinatory effect of enhancement of solubility, efficacy and pulmonary deposition of RES using KL and TPGS as multifunctional excipients.

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