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Original Article

ENCAPSULATION OF EXEMESTANE WITH β-CYCLODEXTRIN AND TERNARY AGENT: FORMULATION, EVALUATION AND ANTICANCER ACTIVITY

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ABSTRACT

Objective: A potent aromatase inhibitor, Exemestane, is well known for its anticancer action in breast neoplasm. The vital problem of this medicament is its poor aqueous solubility, which hinders its dissolution in body fluids. Therefore, the present study targets to enhance the water solubility of Exemestane by means of its complexation with β-cyclodextrin and a suitable ternary agent.

Methods: Inclusion complexes of Exemestane with β-cyclodextrin and ternary agent were prepared by kneading and lyophilization technique. Different hydrophilic polymers and organic acids were screened for their influential ability as a co-complex with a carrier; β-cyclodextrin. The validation of complex formation was carried out by various solid-state techniques. The geometry of Exemestane in the β-cyclodextrin cavity was picturized in docking studies. The *in vitro* anticancer activity of prepared inclusion complex formulations carried out using MCF-7 cell lines

Results: Phase solubility analysis proved HPMC E5 as the best ternary agent for complexation of Exemestane with β-cyclodextrin as it improved the stability constant of the drug from 665.92 M^{-1} to 1238.38 M^{-1}. The synthesized binary and ternary inclusion complexes exhibited 2.74 and 4.62 times enhanced solubilization of Exemestane. Likewise, the dissolution characteristics of Exemestane were improved, and drug release was increased by 1.18 and 1.42 times with binary and ternary freeze-dried formulations. Differential scanning calorimetry (DSC) and powder X-ray diffractometry (PXRD) study results presented the formation of binary and ternary complexes with significantly drooped crystallinity. Docking studies predicted encapsulation of rings A, B, and C of Exemestane in the cavity of β-cyclodextrin. In-line results were obtained in Fourier transform infrared (FTIR) studies. The cell growth inhibition of 62.78 % was achieved with a ternary complex of Exemestane which was far superior than the pure active moiety that showed mere 44.56 % of inhibition.

Conclusion: Altogether, it can be concluded that the inclusion complex of Exemestane boosted its aqueous solubility, resulting in its increased dissolution and *in vitro* anticancer activity in a breast cancer MCF-7 cell line.

Keywords: Exemestane, β-cyclodextrin, Polymer, Organic acid, Complexation, Ternary complex

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INTRODUCTION

A large number of cases every year reported uncontrolled cell growth and are diagnosed with cancer. Among females, breast cancer is the most common type of malignancy and is one of the major causes of their death. The number of breast cancer cases is still increasing every year so, the disease has become a chief concern for health sciences [1]. Development in genomics, proteomics, and biomedical research has enabled scientists to unveil the receptors, mediators, and signaling pathways involved in breast cancer pathogenesis. Breast cancer is mainly divided in four major molecular classes depending on the expression of hormone receptor (HR) and human epidermal growth factor receptor (HER) i) luminal A (HR+/HER2−); ii) luminal B (HR+/HER2+); iii) HER2+ and (iv) triple-negative (TNBC). 60 to 80 % of cases represent the luminal type of breast cancer which overexpresses hormone receptors, namely estrogen receptor and progesterone receptor [2].

Exemestane (EME), a low molecular weight steroidal moiety, acts as a pseudo-substrate and covalently binds to enzyme aromatase; a key mediator in estrogen synthesis; inhibiting it irreversibly, thereby obstructing hormone synthesis. This aromatase inhibitor is approved for the treatment of luminal and advanced breast cancer [3]. Exemestane is available in the market under the trade name "Aromasin" with a dose of 25 mg. However, it is reported that only 42% of a dose is absorbed in systemic circulation after its oral administration. Though efficacious, Exemestane shows troublesome pharmacokinetic properties as it belongs to BCS class IV; representing its low solubility and low permeability. Since the chemical structure of Exemestane shows the presence of a steroidal ring; it poses an aqueous solubility problem with a water solubility of only 0.0391 mg/ml [4, 5]. Enhancement in aqueous solubility of drug solves its absorption troubles to some extent by augmenting dissolution of medicine.

A number of solid-state and lipid-based nanotechnology approaches have been utilized to improve the solubility of Exemestane, but they pose few limitations owing to stability, scale-up, and cost issues [6]. Solubility enhancement of pharmaceutical activity by using cyclodextrin is one of the most commonly utilized approaches for many years and several products are in the market by using this carrier [7]. Use of derived cyclodextrins, namely, Methyl-β-cyclodextrin (M-β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) have been carried out to achieve solubility boost of Exemestane and the drug has shown maximum solubility as well as permeability enhancement with Methyl-β-cyclodextrin (M-β-CD) [8].

The ternary complexation technique involves the use of an active pharmaceutical ingredient, cyclodextrin, and a co-complexing agent, which is usually a hydrophilic polymer, organic acid amino acid, or surfactant [9]. These multicomponent systems tend to enhance the API solubilization capacity of cyclodextrin, thereby requiring its lesser amount and, thus in turn, reducing the volume of the final dosage form [10].

The current research targets to improve the solubility, dissolution rate, and anticancer activity of Exemestane by the use of natural cyclodextrin, β-cyclodextrin, and a suitable co-complexing agent. The effect of pH on the solubility of Exemestane in the presence of trio will also be studied. The binary, as well as the ternary complex of Exemestane, was prepared by kneading and lyophilization technique. These formulations were studied for solubility and dissolution rate enhancement. The solid-state of analysis of freeze-dried formulations

was carried out by use of DSC, PXRD, and FTIR analysis. The surface morphology of these formulations was examined with scanning electron microscopy. The geometry of Exemestane in the cavity of β-cyclodextrin was predicted through of docking studies. Furthermore, *in vitro* anticancer activity of Exemestane and its developed formulations were carried out using an MCF-7 cell line.

MATERIALS AND METHODS

Materials

Exemestane was purchased from Coral Drugs Pvt. Ltd., Murthal, Haryana, India. β-cyclodextrin, PVP K-30, PEG-4000, HPMC E5, tartaric acid, and citric acid were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Deionized water was used for all the experiments.

Phase solubility analysis

To determine the stability constant and complexation efficiency of EME with β-cyclodextrin (β-CD), a preliminary phase solubility analysis was carried out using Higuchi and Connors method. In 10 ml vials, excess EME was added to a 5 ml aqueous β-CD solution (0-10 mmol). The resulting suspension was vortexed for a minute before being placed in a shaker bath at 25 °C for 48 h. This suspension was then filtered using 0.22 µm pore size paper. For EME estimation, this filtrate was analyzed using UV-Visible spectroscopy (Schimadzu, UV-1800) at 252 nm. The phase solubility diagram was then constructed by plotting β-CD concentration versus EME concentration. Equations (1) and (2) were used to determine the values of the stability constant (Ks) and complexation efficiency (CE) respectively based on the slope of the resulting curve. Sw denotes the intrinsic aqueous solubility of EME (mM) in these equations [11].

$$Ks = \frac{Slope}{Sw\,(1-slope)} \ldots\ldots (1)$$

$$CE = \frac{Slope}{1-slope} \ldots\ldots (2)$$

Effect of hydrophilic polymers on complexation

Hydrophilic polymers namely, Polyethylene Glycol–4000 (PEG–4000), Polyvinyl Pyrrolidone (PVP K-30), and Hydroxypropyl Methyl Cellulose (HPMC E-5) were used as co-complexing agents in this investigation because they were found to considerably improve the solubility of visitor molecules with β-CD. Excess EME was added to a 5 ml aqueous β-CD solution (1-10 mmol) containing 0.5 % w/v of each polymer for this test. The suspensions were vortexed for one minute, followed by their filtration by use of 0.22 µm pore size filters [12]. UV-Visible spectroscopy at 252 nm was used to analyze these filtrates for EME estimation.

Effect of organic acids on complexation

Organic acids viz., Citric and tartaric acid were likewise investigated as ternary agents since they boosted the solubility of guest medicines when blended with β-CD. EME in excess was added in a 5 ml β-CD aqueous solution (1-10 mmol) that contained 0.5 % w/v organic acid [13]. Further processing of these suspensions was done as detailed earlier in section 2.2, with the results of phase solubility data guiding the final choice of co-complexing agent among hydrophilic polymers and organic acids for EME.

Effect of pH on complexation

Because EME is a weak basic molecule with a pKa of 5, pH changes have a significant impact on its solubility. As a result, the influence of pH, β-CD, and chosen ternary agent on solubilization of EME was investigated. The pH range of 1.5 to 11.2 was investigated as a useful pH range. Excess EME was added to buffered aqueous solutions containing 1 mmol β-CD alone or in combination with 0.5 %w/v HPMC, and the vials were maintained in a shaker water bath at 37 °C for 48 h. Aliquots from each vial were filtered and the EME concentration was determined using UV-Visible spectroscopy [10, 12].

Preparation of binary and ternary solid inclusion complexes

As A_L type of complex was formed during phase solubility studies, binary EME and β-CD complexes were synthesized in their equimolar ratio using kneading and freeze-drying methods. Also, by employing a lyophilization process a ternary complex of EME was formulated with an equimolar ratio of EME and β-CD added with 0.5 % w/v of HPMC and adjusting the pH to 1.5 [14].

Preparation of physical binary and ternary mixtures

Homogeneous mixing of sieved EME, β-CD and HPMC in a pestle mortar was carried out using the ingredient's same ratio as used for final complexation [13].

Kneaded binary complex

The binary kneaded complex of EME with β-CD was formulated in their 1:1 molar ratio. Weighed quantities of EME and β-CD were taken in a mortar and were homogenously mixed with a pestle, followed by their wetting with a small amount of ethanol-water mix (60:40 % v/v). This mixture was thoroughly kneaded with a pestle for nearly an hour until it became a paste. This paste was allowed to equilibrate for roughly 3 h before being vacuum dried at 40 °C and stored in a desiccator until further testing [15].

Preparation of freeze-dried binary and ternary complexes

EME and β-CD were mixed in water in a 1:1 mmol ratio, and the pH was adjusted to 1.5 with 0.1N HCl. This solution was then mixed for 48 h at room temperature on a magnetic stirrer before being freeze-dried. Lyophilization was carried out with a Martin Christ Alpha 2-4 LSC freeze drier. Lyophilization was carried out for 6 h at a vacuum of 0.3 mbar. Similarly, ternary complexes were prepared using a 1:1 mmol ratio of EME and β-CD with added 0.5 % w/v HPMC, and the pH was adjusted to 1.5. Further processing and freeze-drying was carried out in the same manner as that of binary complex [14].

Differential scanning calorimetry (DSC)

The produced cyclodextrin complexes were characterized using a number of techniques such as DSC, XRD, FTIR, and SEM. The thermograms of EME, β-CD, HPMC, EME-βCD-BC-FDC, and EME-βCD-HPMC-TC-FDC were recorded using a differential scanning calorimeter from Mettler-Toledo GmbH, Switzerland. At the beginning, indium was employed to calibrate the system's temperature axis and cell constant. The sample material was then weighed and studied at 10 °C/min heating rates over a temperature range of 100-300 °C with nitrogen purging at 50 ml/min in sealed pin-holed aluminum pans. The results were assessed using the STAR SW 12.10 software.

X-ray diffractometry

PXRD patterns of EME, β-CD, and synthesized complexes were recorded using Bruker's D8 advance diffract meter with Cu Kα radiation (1.54 A °) at 40 kV, 40 mA flowing through a filter of nickel. Data was collected in a continuous scan mode with a step size of 0.01 and a step time of 0.1 sec throughout an angular range of 5° to 85° 2Θ. The diffractograms were examined using DIFFRACplus EVA version 9.0 software.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of all of the aforementioned specimens were obtained using roughly 2 mg of sample each on a Perkin-Elmer Sp.2 spectrometer. IR wavelengths of 450 to 4000 cm^{-1}were used to scan the samples. The acquired spectra were analyzed using Spectrum 10 software.

Molecular docking studies

Easy Dock Vina 2.2 and Autodock 4.2 were used to dock EME with β-CD. Structure File Generator and online SMILES Translator were used to create the ligand structure of EME and associated SMILES notations. For geometry optimization of molecules under interaction analysis, Open Babel 2.4.1 was used. To imitate the crystal structure of β-CD, the PDB co-crystal of β-amylase (PDB code: 1BFN, resolution-2.07 A °) was employed, and Easy Dock Vina 2.2 software was used to create binary complex docking findings. Autodock tools (ADT) version 1.5.6 was used to visualize the results [16].

Scanning electron microscopy

The surface morphology of binary and ternary EME complexes was examined using scanning electron microscopy, NovaNanoSEM.

Sample specimens were inspected by sputtering gold onto sample stubs with an ion sputter under a 0.000139-pascal vacuum and gluing the powder to a double-sided adhesive tape. The materials were scanned with an electron beam of 10 kV acceleration potential under the scanning electron microscope, and images were taken in secondary electron mode at several magnifications.

Assay and solubility determination of complexes

The content of EME in a freeze-dried complex was determined by dissolving a complex containing an equivalent of 3 mg EME in 5 ml of dichloromethane. The contents were vortexed until they formed a clear solution, then filtered through a 0.22 µm porous filter to determine the EME concentration using UV-Visible spectroscopy at 252 nm. To determine the solubilizing potential of β-CD and HPMC, excess binary and ternary complexes were added to 10 ml of water and placed in a 15 ml stoppered vial. The vials were stored in a shaker water bath (Lab Tech) at 37 °C for 48 h, filtered, and UV-Visible spectroscopy was used to determine the EME concentration. The experiment was repeated three times, with the average values taken into account [17].

Dissolution study

According to USFDA guidelines, dissolution tests were conducted for EME, PM of EME and β-CD, EME-βCD-BC-KC, EME-βCD-BC-FDC, and EME-βCD-HPMC-TC-FDC utilizing the USP-II, paddle-type dissolution test apparatus (Electrolab, India). The experiment was carried out at 37 °C±0.5 °C at 100 rpm with 900 ml of 0.5% sodium lauryl sulphate solution as a dissolution medium. After 10, 20, 30, and 45 min, aliquots from samples containing 25 mg of EME or its equivalent in PMs, binary, and ternary complexes were withdrawn, and the corresponding quantity of new dissolving medium was added to maintain sink conditions. The EME content of these aliquots was measured using UV-Visible spectroscopy after they were filtered (0.22 m pores size). The data was shown as a percentage of EME released versus time [6].

***In vitro* anticancer activity**

The MCF-7 cell line was utilized to test the influence of produced complexes on cell toxicity, as well as the effect of different sample concentrations on cell growth inhibition. The cell line was procured from National Center for Cell Science, Pune, India. Samples of β-CD, EME, EME-βCD-BC-FDC, and EME-βCD-HPMC-TC-FDC were investigated at doses of 10, 25, 50, 75, and 100 µg/ml. To dilute the samples, the culture medium was used (DMEM with high glucose, FBS, and Antimycotic 100X solution). In a 96-well plate, a suspension of MCF-7 cells was planted at a density of 1 X 10^4 cells per well. These cells were then exposed to all samples of different concentrations. The control group consisted of pure culture media, while the standard was 5-fluorouracil. All samples were incubated in triplicate for 24 h at 37 °C and 5% CO_2 in a CO_2 incubator (Thermo scientific BB150). The cells were incubated for an additional 4 h after the culture fluid was replaced with 20 liters of MTT (3-[4-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) and 80 liters of fresh medium. After that, each well received 200 µl of DMSO, which was incubated for 10 min until the formazan crystal reaction occurred. Because only living cells converted the yellowish MTT to the dark-colored formazan, each well was checked under a microscope to guarantee cell survival. The absorbance of each sample was measured at 550 nm using a microplate reader (Benesphera E21). The optical density and % cell inhibition parameters that were obtained were calculated [18].

RESULTS AND DISCUSSION

Phase solubility analysis

The aim of this study was to determine the strength of the interaction between Exemestane and β-CD molecules, as well as the stoichiometry of these two. Fig. 1 illustrates the results of the phase solubility analysis, which clearly shows a linear increase in EME concentration with an increase in β-CD concentration, validating the A_L curve type. As the slope of the curve is less than unity, a stoichiometric complex of 1:1 between the two was established. At 25 °C, the intrinsic solubility of EME in water was reported to be 0.132 mmol (Sw). The stability constant and complexation efficiency were found to be 665.92 M^{-1} and 0.0879, respectively, indicating that a stable complex had developed. A Ks value greater than 100 M^{-1} indicates the presence of stable complexes. As a result, β-CD was effective in enhancing EME solubility [19].

Earlier studies carried out with EME by the use of M-β-CD, HP-β-CD and HP-γ-CD also revealed similar results with the formation of a 1:1 complex between all types of cyclodextrin carriers and the API. The stability constants were reported to be approximately 3700 $M^{-1}$for all these carriers and EME [8]. These higher Ks values pointed to the significantly enhanced solubility as well as stability of EME with derived cyclodextrins as compared to natural β-CD.

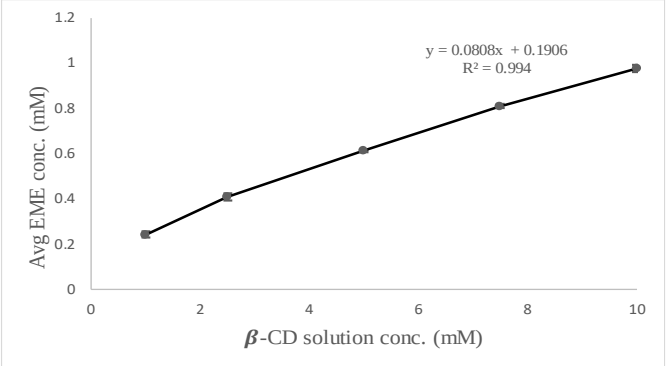


Fig. 1: Phase solubility study of EME in the presence of different concentrations (1-10 mmol) of β-CD (n=3, mean+SD)

Effect of hydrophilic polymers on complexation

Hydrophilic polymers, Polyethylene Glycol-4000 (PEG-4000), Polyvinyl Pyrrolidone (PVP K-30), and Hydroxypropyl Methyl Cellulose (HPMC E-5) were used to investigate the solubility, stability constant, and complexation efficiency of β-CD with EME. Every polymer screened had a collaborative impact with β-CD, as seen in fig. 2, with HPMC E5 being the most effective, followed by PVP K-30 and PEG-4000. At 10 mmol concentration of β-CD, the solubility improvement was 7.44 times better than pure EME, and with 0.5 %w/v polymer added, the solubility was 14.24, 11.51, and 8.33 times greater with HPMC E5, PVP K-30, and PEG-4000, respectively. Polymers improved Ks and CE values, implying that the EME, β-CD, and polymer produced a ternary complex. These findings are summarized in table 1. Enhanced values of Ks and CE in ternary mixtures pointed to the increased strength of interactions of β-CD with EME in the presence of ternary polymer, which could be ascribed to the liberation of high-energy water molecules present in the cavity of β-CD, and raised Van der Waals interactions as well as hydrogen bonds [11]. HPMC E5 was chosen for further studies since it had a higher potential for solubilization enhancement, Ks, and CE.

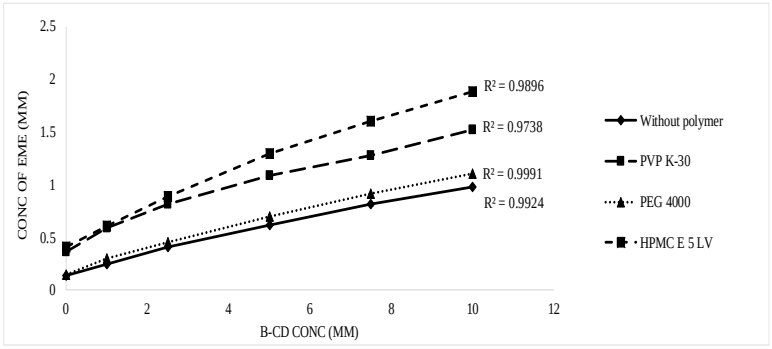


Fig. 2: The comparative solubility profile of EME in the presence of hydrophilic polymer (n=3, mean+SD)

Table 1: The complexation efficiency of β-CD and EME in the presence of 0.5%w/v of polymers

Polymer	Ks (M^{-1})	CE
β-CD	665.92	0.087
β-CD-HPMC E5	1238.38	0.163
β-CD-PVP K30	842.68	0.111
β-CD-PEG 4000	745.59	0.098
β-CD–Citric acid	816.57	0.107
β-CD–Tartaric acid	763.91	0.100

Effect of organic acids on complexation

Both organic acids worked along with β-CD to increase the solubility of EME. As shown in fig. 3, 0.5 %w/v citric acid increased the solubility of EME by 9.36 times, while tartaric acid increased it by 8.60 times when mixed with a 10 mmol β-CD solution. These organic acids also increased the values of Ks and CE, implying the formation of a ternary β-CD complex. The enhanced stabilization of complex in the presence of weak organic acids could be attributed to increased electrostatic interactions and hydrogen bonding [10]. Table 1 illustrates these values and it is evident from the table that the chosen hydrophilic polymers except PEG 4000 formed a more stable complex with EME when combined with β-CD than the organic acids. They also increased the solubility of EME by a greater percentage than organic acids. The lesser CE of organic acids as compared to hydrophilic polymers with β-CD could be due to their lesser affinity towards the cavity of β-CD, which hinders them from the inclusion process in contrast to hydrophilic polymers, which actively participated in the inclusion phenomenon [10]. As a result, HPMC E5 was chosen for further complexation experiments as it was proven to be the best ternary agent for EME along with β-CD.

Effect of pH on complexation

Since EME is a weak basic molecule with a pKa of 5.0, the pH of the media has a significant impact on its solubility. Hence, the combined influence of pH, β-CD, and polymer (HPMC E5) on EME solubility was investigated. It is a well-established fact that the ionisation status of a molecule is governed by the pH of the media, which in turn impacts its complexation efficiency with the chosen carrier [20]. Fig. 4 shows the influence of pH on EME solubility in the presence of β-CD and HPMC E5. The experimental study found that the solubility of EME was highest at a pH of 1.5 when present with HPMC E5 and β-CD. Therefore, while formulating binary and ternary complex of EME by freeze-drying technique, pH of the final solution was adjusted to 1.5 with 0.1 N HCl.

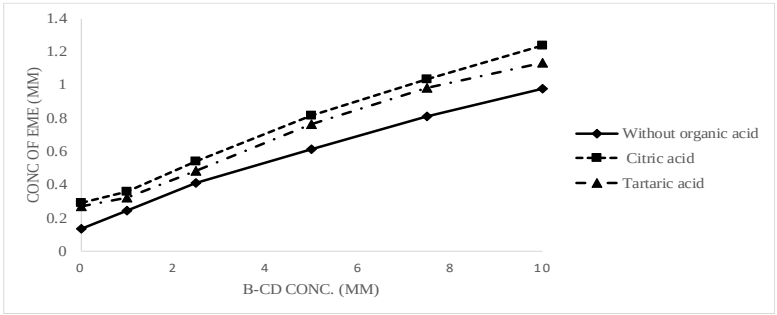


Fig. 3: The comparative solubility profile of EME in the presence of organic acid (n=3,+SD)

Assay and solubility determination of complexes

According to phase solubility experiments, EME formed a 1:1 combination with β-cyclodextrin. Hydrophilic polymers were added to boost the complexation propensity, with HPMC E5 exhibiting the largest improvement. So, 1 mmol of each EME and β-CD were mixed for the binary complex, and 0.5 %w/v of HPMC E5 was added for the ternary complex, followed by a pH adjustment to 1.5. After kneading or freeze-drying, the EME content of the complexes was evaluated. According to UV analysis, the assay of EME was 79.63 % in the binary complex, EME-βCD-BC-FDC, and 83.89 % in the ternary complex, EME-βCD-HPMC-TC-FDC. As shown in fig. 5, saturation solubility tests revealed a significant improvement in complex ternary solubility over complex binary solubility. The notable rise in the aqueous solubility of the ternary complex could be the result of the interaction of HPMC E5 with the surface of β-CD, which caused increased hydrophilicity of the carrier system [10]. Freeze-dried binary and ternary complexes increased EME solubility in water by 2.74 and 4.62 times, respectively. API solubility was raised by 2.10 times in the kneaded binary complex of EME with β-CD. This study showed that using the lyophilization procedure; enhanced solubility of EME can be achieved as compared to the kneading technique.

Another attempt to enhance the solubility of EME was carried out by Sangamwar *et al.* using phospholipid sodium deoxycholate by means of a solid dispersion technique. Their developed formulation also enhanced the solubility of EME by 11.7-folds; however, the aqueous media chosen has the pH of 1.2. The advantage of the current investigation is the achievement of significantly enhanced aqueous solubility of EME even with the use of simple excipients and scalable formulation technique [4].

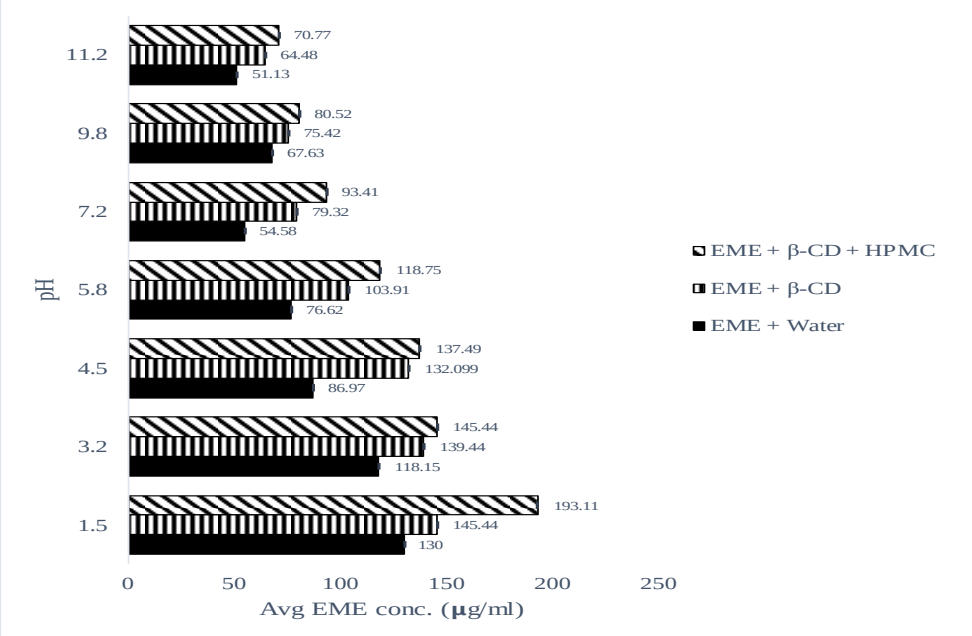


Fig. 4: Effect of pH on solubility of EME in the presence/absence of polymer and co-polymer (n=3, mean+SD)

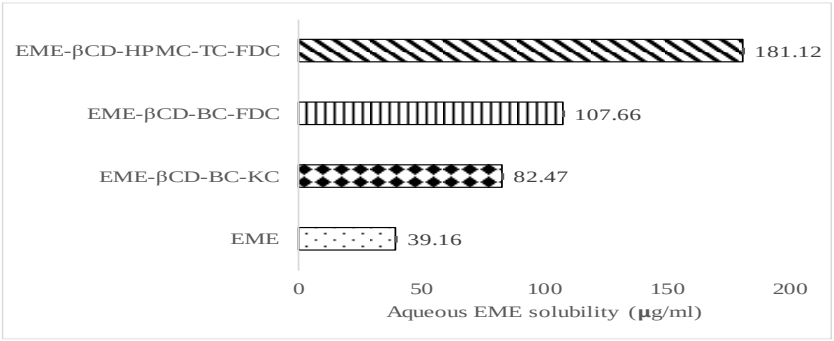


Fig. 5: The saturation solubility data of EME, kneaded binary complex (EME-βCD-BC-KC), freeze-dried binary complex (EME-βCD-BC-FDC), and freeze-dried ternary complex (EME-βCD-HPMC-TC-FDC) (n=3, mean+SD)

Dissolution study

The proportion of drugs dissolved against time is used to understand the results of dissolution studies. The dissolution characteristics of the EME and its carriers are shown in fig. 6. In designated media, the dissolution profile of EME revealed drug release of 15.76 % at the first time point, 10 min, and only 43.02 % at 45 min. This percent drug release was found to improve even with a simple physical mixture of EME with β-CD that released 31.36 % EME after 10 min and 50.97 % EME after 45 min. This result could be attributed to the wettability influence of β-CD on EME release. The kneading and freeze-drying processes resulted in EME complexation in the cavity of the β-CD, resulting in increased drug release in the EME-βCD-BC-KC, EME-βCD-BC-FDC, and EME-βCD-HPMC-TC-FDC formulations. After 10 min, a complex binary formulation created by kneading obtained a 36.79 % drug release, and after 45 min, it reached 61.34 %. Similarly, with the EME-βCD-BC-FDC formulation, 38.59 % EME release was seen in the first 10 min, increasing to 63.90 % at the 45-minute mark. At the last time point of 45 min, the ternary complex demonstrated a maximum drug release of 74.08 %, which is 1.72 times more than pure EME.

Thus, it can be stated that HPMC E5 improved the inclusion of EME in the ternary complex, resulting in increased drug release. Altogether, the inclusion, complexation, solubility, and dissolution characteristics of the EME were significantly improved with its β-CD complexes. These parameters were further enhanced considerably with the addition of HPMC E5, a ternary hydrophilic agent. The formulated ternary complex of EME showed an almost similar dissolution profile as that of the one obtained with methyl-β-cyclodextrin reported by Yavuz *et al.* [8]. However, methyl-β-cyclodextrin is the preferred choice of carriers when the formulation is to be delivered via the ophthalmic and nasal route, but not the oral route. Hence, the advantage of the current research lies with the selection of suitable carriers that can be given via the oral route; furthermore, these excipients produced optimal results in terms of drug release and hence has the potential to direct further research for the development of an oral formulation that can have market value [21].

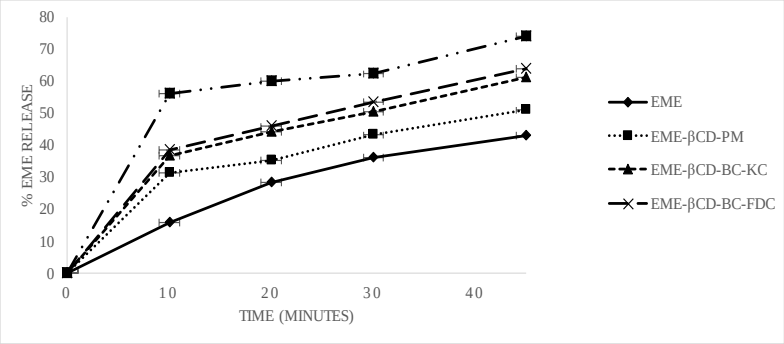


Fig. 6: The dissolution profiles of EME, physical mixture, binary and ternary complexes (n=6, mean+SD)

Differential scanning calorimetry (DSC)

Fig. 7 depicts DSC patterns of EME, β-CD, HPMC E5, and complexes prepared by freeze-drying. β-CD exhibited a broad endotherm in the temperature range of 107-139 °C due to dehydration. HPMC E5 showed a tiny exothermic event at the temperature range of 196.25 °C, which could be attributed to its oxidation. The absence of any other thermal event in the studied temperature range directs its amorphous nature. EME showcased a sharp melting endotherm at 197.64 °C due to its crystalline nature [22]. The enthalpy of this melting endotherm was 88.36 J/g.

EME-βCD-BC-FDC, the binary lyophilized complex, produced a small melting event at 196.16 °C, indicating retention of the crystalline nature of EME in the complex; however, the crystallinity of the complex was turned poor. The enthalpy associated with this thermal event was 5.94 J/g and this reduced enthalpy value further confirmed a significant reduction in crystallinity of EME in the complex. Similarly, freeze-dried ternary complex, EME-βCD-HPMC-TC-FDC, also displayed a slight shift of M. P. to 196.56 °C with an enthalpy of 19.02 J/g. These results again directed to the noteworthy dropping of EME crystallinity in the ternary complex, indicating its successful synthesis and major interaction at the molecular level. A remarkable reduction in peak height and enthalpy in the melting event of EME in complexes indicated structural engulfment of EME in the β-CD cavity due to complexation. However, the appearance of small intensity melting thermal events in DSC thermograms indicated pinches of EME crystals in both binary and ternary complexes. All these samples were further investigated by PXRD to conclude alterations at the solid-state level of EME due to complexation.

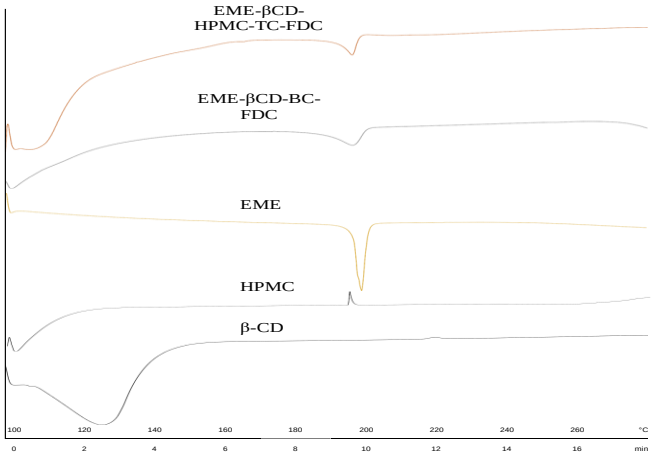


Fig. 7: DSC thermograms of β-CD, HPMC, EME, binary complex (EME-βCD-BC-FDC), and ternary complex (EME-βCD-HPMC-TC-FDC)

Powder X-ray diffraction (PXRD)

The PXRD patterns of β-CD, HPMC E5, EME, and their lyophilized binary and ternary formulations are shown in fig. 8. The partly crystalline structure of the cross-linked polysaccharide, β-CD, was demonstrated by a few strong peaks at 2θ values of 5.1, 7.3, 17.6, and 23.2. The diffused X-ray diffraction pattern of HPMC E5 indicated its amorphous nature. Because of its strong crystalline nature, the diffractogram of EME showed distinct characteristic peaks at 2θ values of 6.2, 14.6, 16.9, 18.1, and 24.5 [23]. The X-ray diffractogram of the lyophilized binary complex, EME-βCD-BC-FDC, revealed weak intensity diffraction peaks at 2θ values of 6.2 and 14.5 which can be attributed to the reduced crystallinity of EME in a carrier, β-CD. Also, the ternary lyophilized formulation, EME-βCD-HPMC-TC-FDC, presented a nearly diffused diffraction pattern with a complete absence of distinct diffraction peak that confirmed a profound reduction in crystallinity of EME due to its engulfment in void β-CD. Thus, the PXRD data indicated that the prepared ternary formulation had a higher amorphous character than the binary one. The results of PXRD are in agreement with the DSC data, suggesting that the crystallinity of the freeze-dried formulations was significantly lower than that of the pure active ingredient, EME, and the drug was on the verge of transition to a completely amorphous state.

Scanning electron microscopy

SEM microphotographs of binary and ternary complexes of EME produced by freeze-drying are shown in fig. 9(a) and (b). The surface morphology of the binary formulation, EME-βCD-BC-FDC, displayed small, irregularly shaped particles of β-CD that have engulfed EME. Few EME crystals that have not been complexed were also visible. EME-βCD-HPMC-TC-FDC, a lyophilized ternary complex, revealed freeze-dried β-CD particles that obscured EME and HPMC. EME crystals were barely visible in ternary formulations, indicating that it was better entrapped in the ternary inclusion complex.

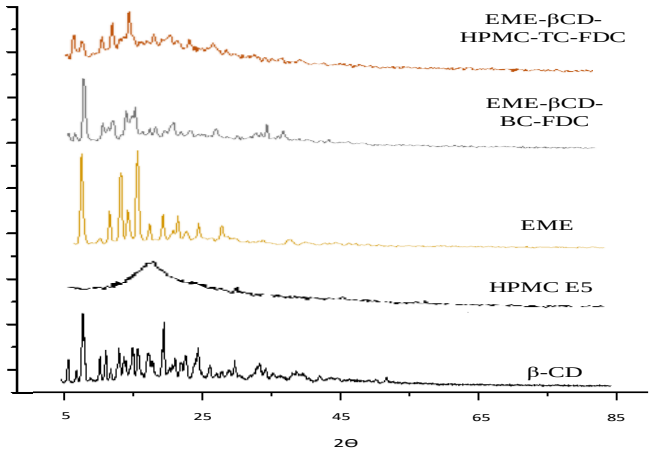
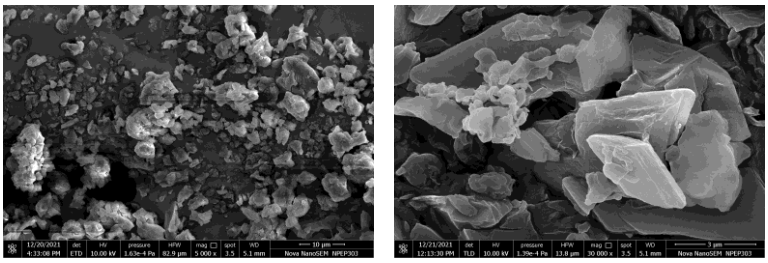


Fig. 8: The X-ray diffractograms of β-CD, HPMC E5, EME, binary complex (EME-βCD-BC-FDC), and ternary complex (EME-βCD-HPMC-TC-FDC)



(a) (b)

Fig. 9: SEM Photomicrographs of (a) binary formulation EME-βCD-BC-FDC and (b) ternary formulation EME-βCD-HPMC-TC-FDC

FTIR spectroscopy

The FTIR spectrum of β-CD reveals the vibration of free–OH groups between 3300 and 3500 cm^{-1}. At 2926.23 cm^{-1}, the vibrations corresponding to–CH stretching may also be recognized. IR absorption peaks for EME were found at 2943 cm^{-1}, 1731 cm^{-1}, and 902-622 cm^{-1}which were attributed to-CH stretch, C=O, and-CH bend, respectively. The classic aliphatic C=C stretch peaks at 1656 and 1458 cm^{-1} were also evident in its FTIR spectrum [24]. The peaks corresponding to the C=C stretch have been masked in the binary lyophilized complex, EME-βCD-BC-FDC, showing entrapment of steroidal rings A, B, and C of EME in the cavity of β-CD. Furthermore, a reduction in peak intensity was seen in the C=O stretch, indicating the weak interaction of the carbonyl group with β-CD. Also, other characteristic peaks of EME were visible in the spectrum though are of reduced intensity, which indicated weaker dynamic interaction in duo and partial entrapment of EME in βCD cavity.

All the characteristic EME peaks disappeared in the FTIR spectrum of the freeze-dried ternary complex, EME-βCD-HPMC-TC-FDC, indicating efficient entrapment of the steroidal ring of API in a bucket of β-CD. Only the peak corresponding to C=O stretch was seen though were of significantly reduced intensity indicating minimal involvement of this functional moiety in chemical interaction with β-CD. By simply comparing the FTIR spectra of EME with its binary and ternary complexes, it can be stated that the interaction of EME with β-CD was markedly improved in a ternary complex in the presence of HPMC E5 that favored interaction which led to an efficient concealing of typical peaks of EME.

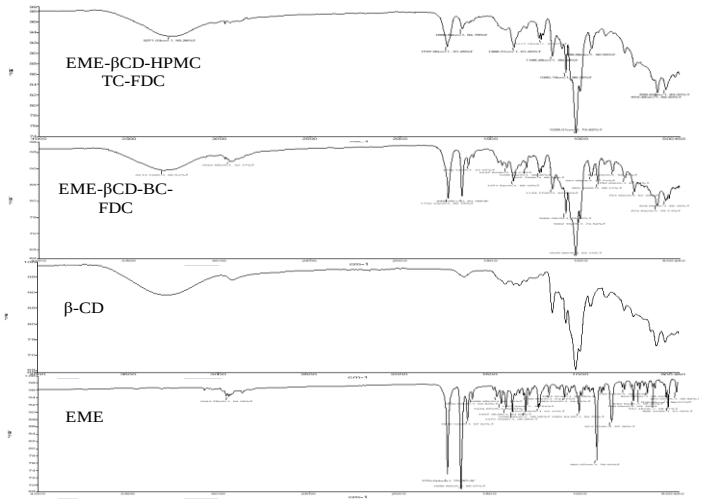
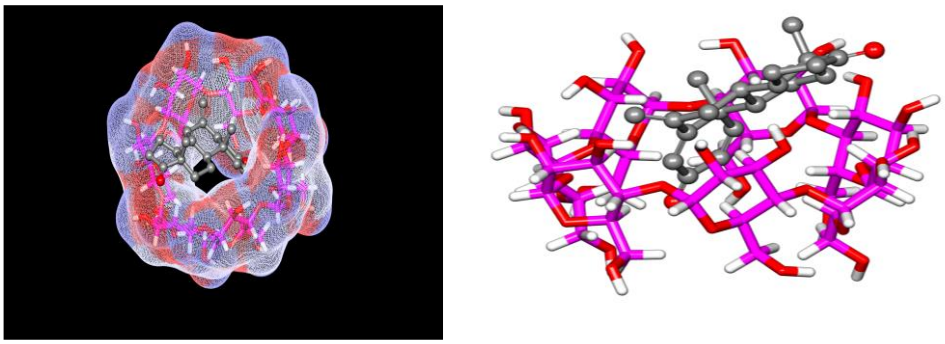


Fig. 10: FTIR spectra of EME, β-CD, binary and ternary systems, EME-βCD-BC-FDC, EME-βCD-HPMC-TC-FDC

Molecular docking studies



(a) (b)

Fig. 11: **(a) Binding mode of the exemestane in binary inclusion complex from the side [3D Ball and stick model of EME (grey color) and wire-mesh model of β-CD] and (b) Binding pose of EME with β-CD. [Ball and stick model of EME (grey colour) β-CD (pink colour)]**

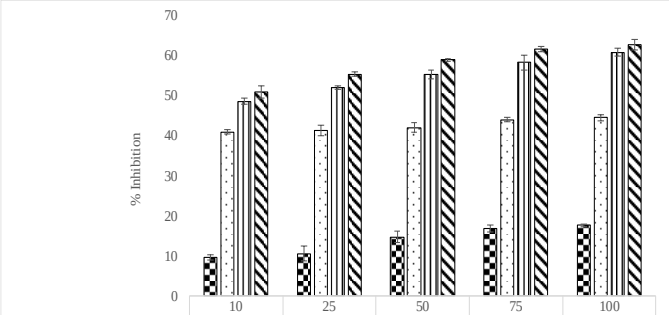


Fig. 12: Percentage inhibition values of β-CD, EME, EME-βCD-BC-FDC, and EME-βCD-HPMC-TC-FDC with respect to concentration (n=3, mean+SD)

In order to investigate the molecular interaction between EME and β-CD, *in silico* analysis was conducted, and the conformation of EME in the cavity of β-CD was studied. Fig. 11(a) and 11(b) illustrate these docked conformations. Steroidal rings A, B, and C of EME were found to be in the inner circle of β-CD, while ring D, along with its carbonyl group, was revealed to be on the perimeter. The results of docking studies on the structural orientation of EME in the β-CD cavity were in-line with the results of FTIR spectra confirming protrusion of the carbonyl group of ring D from the β-CD cavity. Hydrogen bonding connections and pi-pi stacking interactions were found to be involved between the steroid ring of EME and a the-OH group of β-CD. Docking tests also revealed that EME had a binding affinity of-6.054 kcal/mol with β-CD. No stearic impediment was found when EME interacted with β-CD, confirming the formation of a stable complex in the duo.

***In vitro* anti-cancer activity**

The percentage inhibition of β-CD, EME, EME-βCD-BC-FDC, and EME-βCD-HPMC-TC-FDC on MCF-7 cells was examined at concentrations of 10-100 µg/ml, and the results are shown in fig. 12. β-CD demonstrated a poor anticancer effect. When compared to the pure drug; EME, both binary and ternary inclusion complexes showed significantly higher percentage inhibition levels, 60.84 %, and 62.78 %, respectively at a concentration of 100 µg/ml. The higher cell growth inhibition with inclusion complexes compared to the pure drug was owing to improved drug availability due to API's complexation with β-CD, which resulted in its amplified solubility. Among the inclusion complexes, the ternary cyclodextrin complex, EME-βCD-HPMC-TC-FDC, had a larger percentage of cell inhibition than the binary complex over the entire concentration range, which could be attributed to the formulation's improved solubilization. The ternary complex, therefore, established its superiority in terms of *in vitro* anticancer activity.

CONCLUSION

The binary and ternary inclusion complexes of Exemestane were prepared by kneading and freeze-drying method as EME-βCD-BC-KC, EME-βCD-BC-FDC, and EME-βCD-HPMC-TC-FDC. HPMC E5 was chosen as a ternary co-complexing agent among the examined hydrophilic polymers and organic acids based on phase solubility research findings. The medicine's higher water solubility as a result of ternary complexation resulted in a significant increase in drug dissolution with a drug release of 74.08% after 45 min.

A range of solid-state characterization techniques, including DSC, PXRD, FTIR, and SEM, were used to confirm the complex formation. FTIR research revealed that the steroid rings A, B, and C of EME interacted with β-CD in binary and ternary complexes. Similar results were predicted in docking investigation, where the entanglement of these three rings of EME in the inner vacuum of β-CD was seen, whereas the ring D was, along with its carbonyl functionality, was seen to thrust outwards.

The MCF-7 cell line was used to test anticancer efficacy *in vitro*, and both binary and ternary inclusion complexes suppressed cell growth better than pure EME. In terms of altered solid-state features of EME, the ternary inclusion complex of EME with β-CD and HPMC exhibited superior results, resulting in a significant increase in water solubility, drug release, and *in vitro* anticancer activity.

ABBREVIATIONS

EME: Exemestane, DSC: Differential scanning calorimetry, HPMC E5: Hydroxyl propyl methyl cellulose E5, PEG 4000: Polyethylene glycol

4000, PVP K30: Polyvinyl pyrrolidone K30, PXRD: Powder X-ray diffraction, SEM: Scanning electron microscopy, β-CD: β-cyclodextrin.

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Nil

AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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