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Energy efficient, facile and cost effective methodology for formation of inclusion complex of resveratrol with hp-β-CD

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Abstract: A distinctive, effortless, proficient and innovative process for preparation of inclusion complex (IC) of resveratrol (Res) with hp-β-CD was developed. The methodology overcame the hindrance of low solubility of Res which is a potential anticancer agent and makes its use more practical. The strategy involved utilization of a microwave meant for conventional use and the process of sonication in laboratory. It considerably saved time and labour besides cutting down on energy consumption. The prepared inclusion complexes were characterised by FTIR, UV-visible absorption spectroscopy, NMR, TGA, DSC, XRD and CHNS analysis. Phase solubility studies along with Gaussian studies proved the formation of 1:1 complex whose physical nature and stability was studied using XRD and UV-visible absorption spectroscopy respectively. This effortless and economical process could pave the way for the formation of inclusion complexes of other nutraceuticals and pharmaceuticals.

Keywords: effortless, anticancer, microwave, sonication process, inexpensive

1. Introduction

Nutraceuticals as defined in literature is a portmanteau of the words "nutrition" and "pharmaceutical".[1,2] These molecules possess a variety of physiological benefits and provide protection against the diseases and ailments like cardiovascular problems, obesity, diabetes, carcinoma, chronic inflammatory disorders and degenerative diseases.[3] In broader terms, they are defined as a food or a part of food that provides medical and health benefits. In conformity with their conjectured safety, prospective nutritional values and therapeutic effects, nutraceuticals have thus been of great interest to scientists.

These naturally occurring bioactive compounds are now being considered as the single most successful discovery of modern medicine.[4-6] The polyphenolic nature of the compounds like quercetin, curcumin, resveratrol etc. explain their antioxidant activities and enables them to quench the free radicals produced in diseased conditions. However, high hydrophobicity, low water solubility and sensitivity to external agents such as air, light and oxidative enzymes pose a serious threat to the stability and the bioavailability of these formulations.[7] These characteristics limit their use as food additives and therapeutics.

Resveratrol i.e. (trans-3,5,4'-Trihydroxystilbene) (Res) is a potential anticancer agent found free and in conjugated form in grapes, peanuts and other plant. It modulates lipid metabolism, inhibits platelet activation and their aggregation. It also protects lipoproteins from oxidative and free radical damage besides inhibiting tumour initiation, their promotion and progression. However, the problems concerning the physicochemical properties, such as poor solubility and photosensitivity have meant that no novel food has actually been fortified with this antioxidant.[8]

Concerted efforts are now being made to overcome physicochemical challenges and instability of natural bio-actives in order to improve their commercial viability and efficacy vis-a-vis their conventional counterparts. Nutraceutical formulation strategies reported in literature include: Liposomal carrier systems, electrospun fiber mats, microsponges and nanosponges, cyclodextrin complexation, biodegradable hydrogels, nanoemulsions, nanostructured lipid carriers, nanomicelles, nanoparticles, nanocapsules and nano-encapsulation, solid dispersions, self-emulsifying drug delivery systems (SEDDS) and microparticulate systems like microparticles, microspheres and microcapsules.[9] Cyclodextrin and modified cyclodextrin based nutraceutical carriers have an exceptional ability to enhance the therapeutic index of the bioactive agents by improving their inherent solubility, stability, permeation and bioavailabililty.[10-13] Pinho et al. reported cyclodextrins as cyclic oligomers capable of enhancing the biological, physical and chemical properties of bioactive molecules

like stilbenes.[14] The advantage of using these "cage like" molecules include their high biocompatibility and versatility to form complexes with a wide range of hydrophobic naturally occurring drugs. A number of complexation studies with cyclodextrins (CD) are available in literature[15-18] demonstrating the formation of 1:1 β CD complex with Res. Zou et al.[16] proved that hp-β CD was a better complexing agent for Res than β CD. Nu´n˜ez-Delicado[17] and his co-workers investigated complexation of Res with β CD and G_2- β CD. They confirmed that in addition to increasing Res hydrosolubility, entrapment in internal CD cavity delayed Res oxidation by lipoxygenase. The authors concluded that CD could be used as Res carrier systems since they acted as substrate reservoir in a dosage controlled manner. In another report, Venuti et al.[18] prepared res/sulfobutyl ether-cyclodextrin inclusion complexes (IC) by 30 min of sonication followed by stirring the mixture for 3 days. The prepared complex was found to be a potential drug for treatment of breast cancer. Apart from this, IC of various other bioactive molecules was also prepared. These studies clearly illustrated the merits of forming IC with cyclodextrin derivatives for enhancing the solubility and stability of these hydrophobic wonder molecules and lay their pathway to being capable, productive and an economical remedy for numerous infirmities.

For efficient incorporation in food or to be administered as an effective pharmacological drug various other IC have also been reported in literature (table 1)[18-29] *(only a few of them have been listed)* but all the reported methods are highly time engrossing. In the present day contemporary world time minimization and energy efficiency have become fundamental conditions for any new discovery. New methods of providing energy are being explored for the quick formation of the products without adversely affecting their desired healing properties.

In the present report, an inexpensive, quick and facile methodology has been developed for the formation of IC of Res which can find potential applications if loaded in daily diet. It does not require laborious and endless stirring and the IC is formed only in 100 seconds using the microwave methodology and within 15 minutes with sonication. This simple and quick approach not only produces instant results similar to the traditional stirring methods but also avoids the use of toxic solvents.

Table 1 List of IC's and their procedure as reported in literature

Targeting drug/Nutraceutical	Time taken	Reference
A characterization study of resveratrol/sulfobutyl ether-β-cyclodextrin inclusion complex and *in vitro* anticancer activity	30 min sonication followed by stirring for 3 days	Colloids and Surfaces B: Biointerfaces, 115 (2014) 22-28
Synthesis and spectroscopy studies of the inclusion complex of 3-amino-5-methyl pyrazole with beta-cyclodextrin	72 hrs of stirring	Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 134 (2015) 276–282
Preparation, characterisation and bioactivity evaluation of the inclusion complex formed between picoplatin and γ-cyclodextrin	5 days stirring	Carbohydrate Research, 396 (2014) 54–61
Inclusion complexes of poly(4-vinylpyridine)-dodecylbenzenesulfonic acid complex and cyclodextrins	2 days of stirring	Macromolecules, 35, (2002) 3997-4002
Fluorometric determination of curcumin in yogurt and mustard	Agitation of mixture at 70^0C for 4hrs	J. Agric. Food Chem., 50, (2002) 1355−1361
2,6-Dinitroaniline and β-cyclodextrin inclusion complex properties studied by different analytical methods	48 hrs of stirring	Carbohydrate Polymers, 2014 (113) 577–587
N-phenyl-1-naphthylamine/β-cyclodextrin inclusion complex as a new fluorescent probe for rapid and visual detection of Pd^{2+}	Stirred for 48 hrs at room temperature	Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 133 (2014) 73–79
Functional electrospun polymeric nanofibers incorporating geraniol–cyclodextrin inclusion complexes: High thermal stability and enhanced durability of geraniol	Stirring for 2 h at 80 °C + additional 5 h at room temperature	Food Research International, 62 (2014) 424–431
Inclusion complexes of β-cyclodextrin-dinitrocompounds as UV absorber for ballpoint pen ink	48 h at room temperature	Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 129 (2014) 551-564
Spectral investigations of Host–guest IC of 4,4'-methylene-bis (2-chloroaniline) with beta-cyclodextrin	stirring at room temperature for 24 h and then freezed for 24 h.	Carbohydrate Polymers 114 (2014) 558-566
Preparation and characterizations of solid/aqueous phases inclusion complex of 2,4-dinitroaniline with β-cyclodextrin	48 h stirring at room temperature	Carbohydrate Polymers, 107 (2014) 72–84
Transdermal delivery of the in situ hydrogels of curcumin and its inclusion complexes of hydroxypropyl-β-cyclodextrin for melanoma treatment	0.5 hr stirring using grinding method	International Journal of Pharmaceutics, 469 (2014) 31–39

2. Experimental

Materials and Methods

Resveratrol (Sigma-Aldrich, purity > 99%), hp-β-CD, (Sigma-Aldrich, purity > 99%), Ethanol, (Sigma-Aldrich, purity > 99%). Triple distilled water with conductance less than 3

μS cm^{-1} was used for the preparations. The 2-D structures of the materials used are reported in scheme 1

Method of Preparation

Preparation of Inclusion complex (IC)

Firstly, (Med I) IC was prepared by standard procedure reported in literature.[30] The second method (Med II) involved mixing of $4.38x10^{-3}$ mol hp-β-CD and $4.38x10^{-3}$ mol Res in a glass container with minimum amount of solvent mixture (ethanol:water::1:9). The mixture was microwave (IFB 20 PG 2S) processed for 100 sec at 800 W to get the product. The water was evaporated under vacuum. The data obtained after 25, 50 sec at 200, 400, 600 W revealed incomplete complexation. The third method (Med III) involved the same compositions of reactants as in Med II. However, the sample was sonicated for 15 min at 180 W using Hielsher UP200St ultrasonic device instead of microwave. The end product was obtained by lyophilization.

Preparation of Physical Mixture (PM)

The physical mixture of Res and hp-β-CD in 1:1 molar ratio was prepared by grinding in a pestle mortar.

Phase Solubility studies

Solubility studies were carried out by using the method reported by Higuchi and Connors[31] with some modifications. Excess Res was added to aqueous solution with increasing concentrations of hp-β-CD (0.5- 4 mmol L^{-1}) at 25, 30 and 37 ˚C. The system was left on stirring for 24 h at 850 rpm. The samples were centrifuged for 15 min and the supernatants were filtered through 0.45 μm nylon filters in order to separate excess Res. Further, the concentration of Res in each aqueous solution or "apparent solubility" was determined spectrophotometrically. The stability constant, K_c, was calculated from the straight line portion of the phase solubility diagram according to Higuchi-Connors equation (1).

$$Kc = \frac{\text{Slope}}{S_0 \times (1 - \text{slope})} \quad (1)$$

Where S_0 is the Res solubility in the absence of β-CD.

The experimental data obtained from phase solubility was used to calculate thermodynamic properties of the complex formation using the experimentally determined K_c values and the integrated form of the Van't Hoff equation

$$\ln K_c = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (2)$$

where ΔH is the enthalpy change for the reaction (kJ/mol), R is the universal gas constant (J/mol K), T is the absolute temperature (K), and ΔS is the entropy change for the reaction (J/mol K).

The Gibbs free energy (ΔG) was calculated using the above-mentioned parameters and equation (3) at standard conditions

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

Characterization of Inclusion complex

FTIR Spectra

FTIR spectra were recorded in the spectral region of 4000-500 cm^{-1} using Perkin-Elmer (RX1) spectrophotometer (Perkin-Elmer, Beaconsfield, Buck, U.K.) equipped with AgCl windows. The samples were scanned with a resolution of 4 cm^{-1} over the range.

UV-visible absorption Spectra

UV-visible absorption spectra were obtained in the spectral range of 200-380 nm using JASCO V 530 (4-21, Sennin-cho 2-chome, Hachioji, Tokyo 193-0835, Japan) model spectrophotometer with a precision of ± 0.2 nm using quartz cells with a path length of 1 cm.

NMR

^1H NMR spectra were recorded at 400 MHz on a Brucker FT-NMR spectrometer (Bruker, Switzerland) at 400 MHz in D_2O using TMS as internal standard. Chemical shifts of ^1H NMR spectra were measured in parts per million with respect to TMS.

Stability studies

Comparative studies of the stability of free Res, IC were carried out in solution form (ethanol:water =1:9) at room temperature under high energy UV exposure. The solutions were prepared dissolving 2 millimoles of Res and IC in 20 ml of solvent. At specified time intervals, samples were taken and the concentration of trans- Res was determined by UV-visible absorption spectroscopy. The results were expressed as percentage of the remaining trans- Res and percentage of Res converted to its non-functional cis form in IC by UV-visible absorption spectra. The results were obtained by a curve-fitting algorithm with a Gaussian-Lorenzian function.[32,33] The best curve fitting procedure was performed by iterative fits toward a minimum standard error. The relative composition of the component was computed to be the fractional area of the corresponding peak, divided by the sum of the area for all the peaks.

Powder X-ray diffraction

The information on the crystallinity of the prepared complex was obtained through X-ray diffraction (XRD; PANalycital X'pert Pro.) with Cu-K radiation (= 1.5418°A) in the range of 15–70˚C with 8˚C/min scanning speed.

CHNS Analysis

The elemental analysis was carried out using CHNS-O ANALYZER thermo scientific flash 2000 organic elemental analyzer.

SEM

The morphological estimations were done using Scanning electron microscopy (SEM; JEOL-JSM-7600F)

TGA

Thermal behaviour was investigated using SDT-Q-600 (TA instruments New Castle, DE). Sample measurements were carried out using in alumina pans under nitrogen atmosphere. The flow rate of nitrogen was 100.0 mL min^{-1}. Alumina crucibles were used for recording the curves. The samples were heated (~10 mg) to the given temperature at fixed heating rate (β) which was 10° C min^{-1}.

DSC

DSC measurements were performed with a DSC TA Q20 instrument equipped with a refrigerated cooling system (TA Instruments, New Castle, DE). Indium standard (melting point = 156.66 C, DH melting = 28.41 J g^{-1}) was used for calibration. Solid samples (6–11 mg) were pipetted out to 40 µl DSC aluminium pans, which were hermetically sealed with the lid quickly to prevent evaporation. DSC tests for samples were run with the conditions (i) equilibrating at 25 ˚C for 1 min, (ii) cooling the sample at the ramp rate of 10˚C min^{-1} to −50 ˚C, isothermal for 0.5 min, (iii) heating the sample at the ramp rate of 10˚C $min^{-1}$to 300˚C. An empty hermetically sealed pan was used as reference.

Gaussian Studies

All the computational calculations for the synthesized complexes were performed using Gaussian 03 package. The structure of complexes was built using Gauss view 3.0 implemented in the Gaussian 03 package

3. Results and Discussion

The results obtained by FTIR, UV-visible absorption spectroscopy, TGA, XRD, ^{1}H NMR and CHNS for the three methods were found to be comparable and are discussed below.

The FTIR spectra of Res, hp-β-CD, physical mixture is shown in figure 1(A). Res showed peaks in the range of 1750-450 cm^{-1} with characteristic peaks at 1606, 1587, 1384,

965 cm^{-1} corresponding to C-C aromatic double bond stretching, C-C olefinic, C-O stretching and trans olefinic bond respectively.[30] The spectrum of hp-β-CD was characterized by the intense band at 3407 cm^{-1}, corresponding to vibration of the hydrogen-bonded OH groups, the band at 2926 cm^{-1} correspond to absorption of CH and CH_2 groups, and some other prominent peaks at 1654 cm^{-1}, 1156 cm^{-1}, and 1032 cm^{-1} were assigned to H-O-H bending, C-O and C-O-C stretching. In the spectra of PM, all characteristic bonds of Res and hp-β-CD were evident, indicating weaker or no interaction between Res and hp-β-CD when physically mixed. [34] However, in the spectra of IC, some characteristic bands of Res were found shifted or absent. Fig. 1(C) depicts the FTIR spectra of the IC prepared by different methods at different times in microwave. The results showed that in the IC formed by either of the methods, the band intensities decreased at 1384 and 965 cm^{-1} and disappeared at 1606 and 1587 cm^{-1}. This clearly evidenced the formation of IC.[35]

Clear spectral overlap indicated the compatibility between IC's formed by the three methods. Also, the shapes and intensities of the bands changed dramatically for the formation of IC as compared to pure Res or hp-β-CD. It further supported the formation of IC.[36]

Time and power optimization remained a major challenge while doing reactions with microwave methodology. For this, the reaction was carried out at different time intervals of 25, 50 and 100 sec. FTIR results indicated complete absence of characteristic peaks when the reaction was carried out for 25 sec. The band intensities at 1384 and 965 cm^{-1} decreased whereas they disappeared at 1606 and 1587 cm^{-1} indicating the formation of IC after 50 and 100 sec. Similar experiments were also carried out at different power modes of 150W, 300 W, 600 W, however, no product was obtained.

UV-visible absorption measurements have proved to be a very convenient method to explore the structural change and formation of complex.[37,38] The absorption spectra of Res, hp-β-CD, PM and IC were recorded according to the standard procedure. UV-visible absorption spectra of Res showed two bands, one centred at around 215 nm and the other one at about 300 nm.[39]After conversion from trans- to cis Res, the band at 300 nm disappears and a band at 285 nm was observed. The distinct and deformed peaks observed in PM indicates that Res and hp-β-CD are in their natives states and very weak interactions are occurring which leads to overlapping of peaks and thereby broadening of peak. The studies on IC further revealed not only simple superposition but also a slightly shifted peak at 310 nm (Fig. 2(C)). This may be taken as indirect proof of complex formation.[40]Results further indicated that the value of absorption (ΔA) at the peak wavelength of IC was almost 20 times the free Res. This increase in the peak absorption was attributed to enhanced solubility of the IC as

compared to free Res and hp-β-CD. Yet again the peaks obtained by the three methods completely overlapped indicating the formation of IC by the three methods.

It has been reported in literature[41] that Res exists in trans and cis isomeric forms of which trans form is more photo and thermo stable. Standard trans-res solutions also show photoisomerisation when exposed to daylight; they undergo changes in the trans stereoisomer ratio as the amount of the cis isomer increases to the detriment of the trans forms amount. The same reaction takes place when the solution is exposed to UV light. The longer the irradiation the higher the cis-res concentration, until about 2 h, where the ratio of the cis isomer reaches its maximum.[42] Bertacche et.al.[30] reported the stability of res and the corresponding IC both in the solid state and in ethanol solution at room temperature in the dark, at 4°C and under light exposure which proved the stability of Res and IC's in solid state. They concluded that the isolation rate of isomerization of trans to cis decreased with inclusion. The present study also revealed the decrease in conversion with inclusion when the system was nearly aqueous. The obtained % ages of cis and trans product with time as tabulated is given in Table 2.

Table 2 Stability of IC under high energy UV exposure

Time(min)	%age cis	%age trans
0	0	100
20	7.57	90.26
40	8.04	89.74
100	12.50	85.70
120	27.36	66.00
160	31.91	63.45
200	38.00	52.54
220	45.85	48.73

From the TG analysis of various moieties *viz.*, Res, hp-β-CD and the IC it was observed that the IC formed by three methods followed similar thermal behaviour. The results have been depicted in the TG curve (Fig. 3(a)). Comparing the decomposition behaviour it was observed that while Res decomposed in a single step, hp-β-CD, Res and the IC decomposed following two step mechanism (Fig. 3(b)); the first step being the removal of water in both hp-β-CD and the IC.

TG plots exhibited high thermal stability along with well-defined decomposition steps. It was observed that hp-β-CD was thermally most stable of all the given moieties and decomposed at 360 °C whereas Res was least stable. The higher decomposition temperature of IC (360 °C) vis-a-vis Res (340 °C) is an indication of superior thermal stability. The IC showed decomposition between 340 °C to 360 °C. This could be attributed to the fact that

pure Res showed decomposition at 340 ^{o}C. Therefore, Res present in the cavity of hp-β-CD also started decomposing at similar temperature. Similar decomposition behaviour was also observed by Macedo et al.[43] The activation energy for thermal decomposition was calculated using the Coats Redfern method.[44] The calculated activation energy came out to be nearly the same for all the three methods i.e. E_{MedI} = 74.94 kJ mol^{-1}, E_{MedII} = 74.90 kJ mol^{-1}, E_{MedIII} = 73.39 kJ mol^{-1}. From this it could be inferred that the product obtained by any of the methods followed the same decomposition behaviour. Also, the apparent activation energy and behaviour for thermal decomposition varied as: $E_{CD} > E_{IC} > E_{res}$ (Table 3) indicating that IC formed a stable complex compared to hp-β-CD and hence required less energy for decomposition.

Evidence for the formation of 1:1 IC was carried out by determining the stoichiometric ratio from TG curves. Bai et al.[45] had successfully devised a scheme to calculate stoichiometric ratio of the α-CD-based IC's in a solid-state form with four guests, 4-cresol, benzyl alcohol, ferrocene and decanoic acid, to verify the possibility and accuracy of a new method. According to the proposed method, if equation (1) as given below is satisfied by relative parameters then stoichiometric of Res: hp-β-CD can be defined as 1: n.

$$n\,M_{guest} - 2M\,H_2O \leq \frac{(aW_{pureCD} - kbW_{pureCD})M_{CD}}{W_{pureCD}} \leq n\,M_{guess} \qquad (1)$$

where M_{guest}, M_{H2O} and M_{CD} are the molar weight of guest (Res), water and hp-β-CD, respectively. $W_{pure\ CD}$, and $W'_{pure\ CD}$ represent the mass of total hp-β-CD with and without any guest, respectively. The parameters k, b and a were calculated by equations as already reported in literature. Substituting the values obtained from TG curves in the above equation with k = 0.917, b= 0.1279, a= 0.3442, the ratio 1:n was satisfied by the value 1:1, which confirmed the formation of complex in 1:1 stoichiometric ratio.

Perusal of literature revealed that very few researchers have commented on the stoichiometry of the formed IC.[46,47] Bru *et al* [47] reported that 1:1 IC had the highest correlation coefficient, thus, specifying the complex formation in 1:1 ratio. The present study also evidenced the formation of 1:1 stoichiometric ratio for IC.

Molecular modelling calculations were further carried out to compare the experimental TG data with theoretical computations. Minimum energy optimized structures for the complexes were obtained using Gaussian software as given in Fig. 4. The minimum energy of the optimized structure and activation energy calculated using TG were in sync and followed a similar trend suggesting that the IC formed was more stable than hp-β-CD (Table 3).

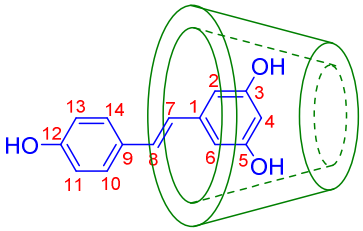
Table 3 Comparison of activation energy using TG analysis and minimum energy optimized structure using Gaussian.

Moieties	E_{act}	
	TGA (kJ mol^{-1})	Energy optimization (a.u.)
Res	63.30	-761.00
hp-β-CD	90.67	-5592.52
IC	74.90	-6353.96

XRD is a useful technique for the detection of CD encapsulation and is a useful method to assess the degree of crystallinity of the given sample. Supporting evidence for complex formation was obtained from this. Perusal of literature reveals that the formation of an IC between hp-β-CD and a crystalline guest meant that the latter would no longer exist in the crystalline state and, consequently, the diffraction pattern of the complex would not be a simple superposition of those of the two compounds. When a true complex is formed, the overall number of crystalline structures gets reduced and amorphous structures increase.[34] Hence IC exhibits less intense peaks. It was observed that pure Res was highly crystallized and exhibited intense crystalline peaks between 5° and 50°.[48] The XRD pattern of hp-β-CD showed no crystalline peak indicating amorphous nature.[49,50] The obtained XRD of PM confirmed the loss of identity of the individual compounds (Fig. 5) showing characteristic peak of mixture of Res or hp-β-CD but as it was not a true complex, the peaks retained their intensity and sharpness. This type of behaviour was found to be consistent with other studies in literature reported for the formation of IC's.[51,52,53] Meanwhile, the XRD pattern of the three IC's showed no characteristic peak of Res, which was distinct from the PM, hp-β-CD. The diffraction peaks relating to crystalline behaviour were no longer detectable in complex system and the reduction of the crystalline degree were further considered as an indirect proof of complex formation [52] The exact overlapping XRD pattern indicated the similarity of the IC's formed by any of the methods.

Nuclear Magnetic Resonance spectroscopy (NMR) is one of the most useful techniques to study the interactions of host-guest complexes. It provides the information about right orientation of the guest molecule inside the cavity of host molecules.[54] So, if IC of hp-β-CD with Res is synthesized, then the inside protons of hp-β-CD cavity shall show the shifting in hp-β-CD value due to their interaction with res molecule. Similarly, Res protons shall also be sensitive to this changed environment and as a result, they will also show the chemical shifting in ^1H NMR spectrum. The proton NMR spectra of Res, hp-β-CD and inclusion compounds (Med I, Med II and Med III), obtained from three different methods were recorded in D_2O solvent.

Table 4 Comparison of δ value for various protons of Res, Med I, Med II and Med III.



Entry	H-2	H-4	H-6	H-7	H-8	H-10	H-11	H-13	H-14
Res	6.643	6.306	6.643	7.139	6.954	7.490	6.897	6.897	7.490
PM	6.465	6.259	6.465	7.027	6.815	7.430	6.888	6.888	7.430
Med I(t)	6.491	6.286	6.491	7.018	6.826	7.439	6.903	6.903	7.439
Med II (M)	6.480	6.281	6.480	7.017	6.825	7.439	6.901	6.901	7.439
Med III(S)	6.484	6.281	6.484	7.019	6.828	7.439	6.902	6.902	7.439

The proton NMR spectra of pure Res, physical mixture (PM) and ICs are shown in Fig. 6 and their δ values in Table 4. The ^{1}H chemical shifts of pure Res and Med I were found to be in agreement with literature[55] and it confirmed the formation of IC's. Data in Table 4 showed that chemical shifting (Δδ) for Res protons in ICs were in range of 0.006 ppm to 0.163 ppm. As expected, the NMR signals of protons H-2 and H-6 showed maximum downfield chemical shifting Δδ, *i.e.* 0.163 ppm and confirmed the insertion of aromatic ring bearing two hydroxyl groups inside the hp-β-CD bucket. H-4 proton showed little chemical shift 0.027 ppm as it is positioned at the bottom of hollow hp-β-CD cavity and not affected much by the –OH group of hp-β-CD. Furthermore, Vinylic protons H-7 and H-8 in IC's shifted towards downfield and appeared at δ 7.018 and 6.826 ppm, with Δδ value of 0.120 ppm and 0.130 ppm, respectively. H-11 and H-13 showed the slightest chemical shifting of range 0.006 ppm, as they are away from the hp-β-CD cavity. It proved that the second aromatic ring with one –OH group should not be capped with the second molecule of hp-β-CD and confirmed the formation of 1:1 IC's. The other ICs Med II and Med III also showed similar type of downfield chemical shifting as in Med I and validated the formation of IC's from Microwave and sonication methods too. However, in Med II and Med III protons showed same Δδ value with ±0.01. This might be due to the different morphological structures obtained from using three different methodologies (discussed in next section).

Furthermore, when the proton NMR (δ 5.8 and 0.5 ppm region) of hp-β-CD, Med I, Med II and Med III were compared, hp-β-CD protons in IC Med I showed chemical shifting which matched with the literature value. Med II and Med III also showed similar type of shifting which proved the formation of similar IC.

PM is formed by simply mixing both the reactants Res and hp-β-CD without using any external energy, therefore Res molecule should not be inserted inside the hp-β-CD cavity. But still, protons showed downfield chemical shift, because there may be a little interaction between these two molecules due to the presence of –OH groups on both the compounds.

The IC of Res was obtained in the solid and solution phase. The stoichiometric ratio and stability constants were derived from the changes in the solubility of res in the presence of increasing concentrations of hp-β-CD. The obtained phase solubility plots at 25,30 and 37° C have been shown in Fig 7. The plots indicated that the solubility increased with the increase in temperature with a linear dependence. Consequently, according to Higuchi and Connors[53] the plots could be considered A_L type suggesting the formation of 1:1 IC. Further, for all the plots the slope of straight line was less than 1, thus it was inferred that the increase in solubility was due to the formation of 1:1 IC[30]. The results were in correlation with the results obtained from TGA studies and Gaussian calculations which also indicated the formation of 1:1 IC.

The stability constants (K_C) for the IC were calculated from the straight line plots of Fig. 7 considering 1:1 stoichiometry. The values have been listed in Table 5. The K_C values obtained at different temperatures were plotted in a van't Hoff plot. IC showed a linear function between K_c and the inverse of absolute temperature (1/T). The stability constant decreased with the increase in temperature indicating an exothermic process. The results were in comparison with the other reports reported in literature on the IC formation of Res.[56,57] Also the obtained high K_C values indicated that the equilibrium was displaced towards the formation of IC.

The phase solubility data was further used to calculate the thermodynamic parameters using equations (2) and (3). The calculated thermodynamic parameters have been tabulated in Table 4.The negative value of enthalpy change (-ΔH) indicated that the interaction process of Res with hp-β-CD was exothermic in nature. The negative value of ΔG further indicated that the interaction process was spontaneous in nature. The obtained negative value for entropy changes could be explained considering that inclusion moderately hinders the free rotation of the included molecule around the symmetry axis [57]

Table 5 Aqueous solubility of res in the absence of hp-β-CD (S_o) solubility, stability constants (K_c) and thermodynamic parameters

Temperature(°C)	S_o(mmol^{-1})	K_c (M^{-1})	-ΔG (kJmol^{-1})	-ΔH (kJ mol^{-1})	-ΔS(J K^{-1}mol^{-1})
25	0.00090	719.42	15.74		5.33
30	0.00125	652.55	16.32	17.33	3.33

37	0.00139	575.00	16.84	1.59

Elemental analysis is an important tool for characterization of newly synthesized complexes. CHNS analysis of pure Res, hp-β-CD and IC are recorded in Table 6. The C, H % observed was found to be well in agreement with calculated values and was a good measure to assess the purity of the complexes. It was observed that complex formed was the same irrespective of the employed methodology. It also supported the evidence given by Gaussian studies that 1:1 (Res:hp-β-CD) IC formation took place.

Table 6 Analytical data depicting obtained % ages of C, H, N in Res, hp-β-CD and Med (I), (II) and (III)

Compounds	C	H
Res	72.95 (73.61)	5.32 (5.25)
hp-β-CD.12H_2O	42.76 (42.96)	6.87 (7.72)
Med(I)	46.76(46.48)	6.61 (7.44)
Med (II)	46.33 (46.48)	6.84 (7.44)
Med(III)	48.85 (46.48)	6.43(7.44)

The DSC data also provides evidence for the formation of IC. It has been reported by Marques et al.[58] that the inclusion of a guest into the host molecules such as CD's causes changes in the crystal lattice, melting, boiling, and sublimation points get shifted at different temperatures or disappears. The thermograms of Res, hp-β-CD, PM and IC have been shown in Fig. 8. The obtained DSC of Res was typical of a crystalline substance showing a sharp endothermic peak at 269.9°C corresponding to the melting point of pure Res. The thermogram of hp-β-CD showed a wide endothermic peak at about 110°C, which referred to the release of water. In the physical mixture the characteristic thermal profile of Res was shifted to a lower temperature in the region of 152.8°C. Further the thermogram of PM indicated a superimposition of thermogram of Res and hp-β-CD indicating no or very weak interaction in the PM. The thermogram of the IC showed a shifted peak at 105°C, the shift of peak to the lower temperature could be attributed to the change in the properties followed by complete complexation indicating an interaction between Res and hp-β-CD. This also indicated an increase in the amorphous character in the complex which was also supported from the XRD data. All these results showed that Res was wrapped into the cavity of hp-β-CD.[59]

SEM (Fig. 9) is a reliable technique for studying the structural aspects of materials and it was employed to investigate the microstructures of IC in solid state.[60-62] The obtained shapes were very different from pure Res which had been reported to be bar shaped by Le et

al.[60] and pure hp-β-CD which were reported as spherical particles with cavity structures by Chen et al.[36]

The PM indicated the presence of both the bars and open spherical structures indicating the presence of both the compounds and maintaining their individual identity. SEM images of the IC prepared by all the three methods clearly indicated the formation of irregular complex in which the original morphology of hp-β-CD and Res disappeared. Tiny aggregates of amorphous and irregular pieces were seen. The observed shape was in accordance with the literature [60-62] Liu *et al*.[51] reported for the formation of IC's by long stirring conditions.

Conclusion

In the light of our findings it can be concluded, that the complex was prepared under solvent free conditions with new innovative simple microwave methodology involving only 100 sec of reaction time and the powerful sonication laboratory method. The obtained product was characterized with FTIR, ^{1}H NMR, TGA, DSC, XRD and CHNS analysis. The product was found to be exactly similar to the one synthesized in literature by 120 hrs of long refluxing conditions. This inexpensive and quick methodology will find potential applications in food industry and may also provide a gateway for cancer treatment for patients using quick microwave methodology at home. Sonication method can also be applied for the synthesis of IC of other nutraceuticals. Work in this direction is in progress in our laboratory.

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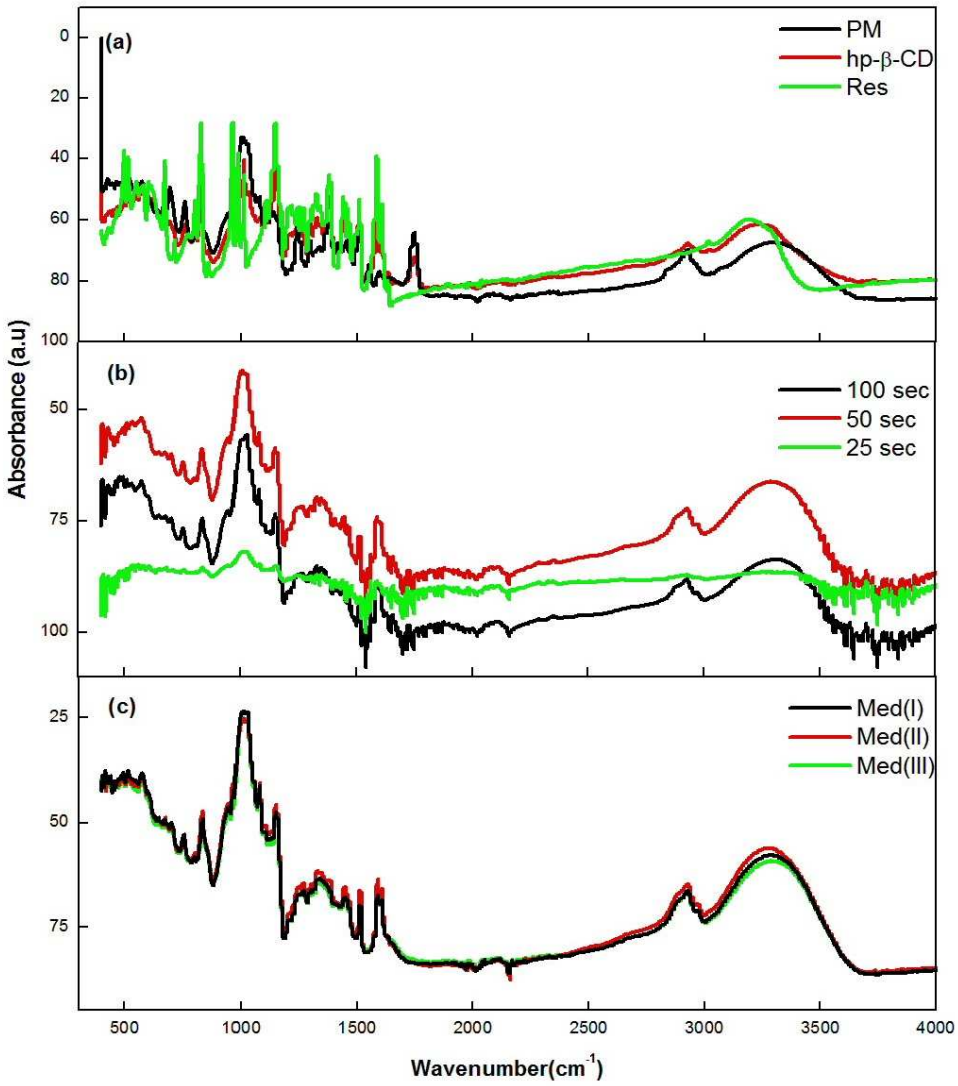


Fig. 1 FTIR spectra (a) Comparison of Res, PM and hp-β-CD (b) Med II at different times (c) Comparision of Med (I,II,III)

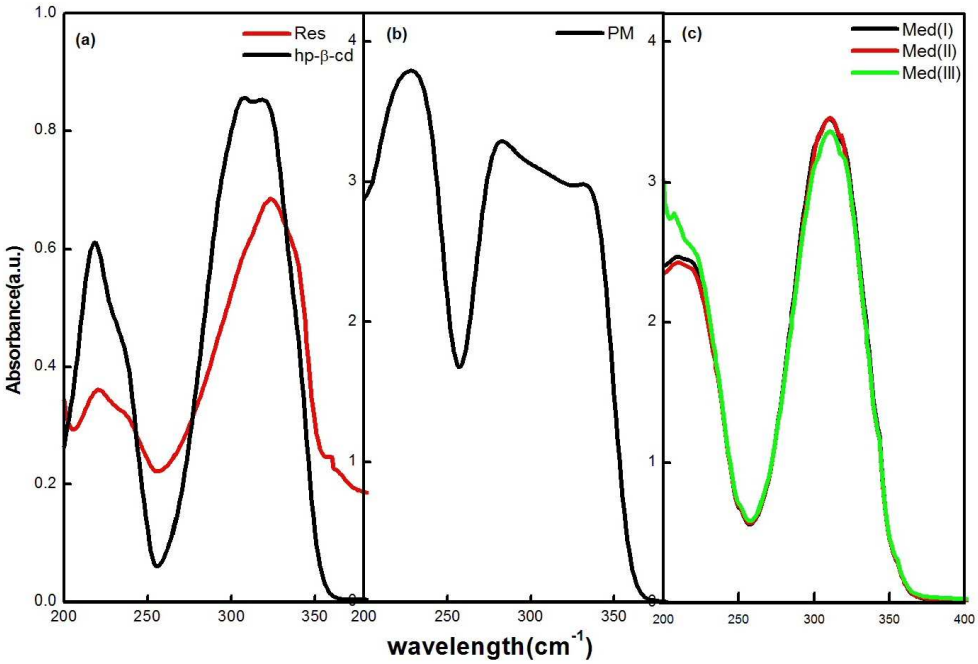


Fig 2. UV-visible absorption spectra of (a) Res (H_2O+C_2H_5OH) and hp-β-CD(H_2O) (b) PM (H_2O) (c) Comparison of Med (I,II,II) (H_2O)

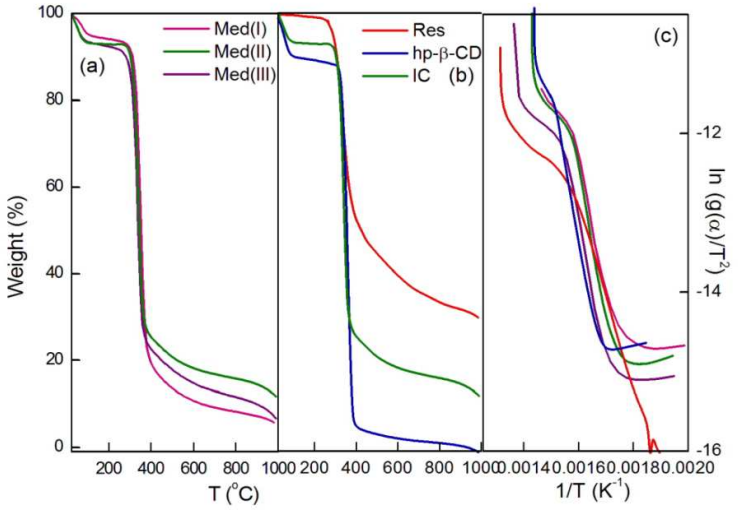


Fig. 3 TG curves for (a) the IC formed by three methods, (b) res, hp-β-CD and IC; (c) Coats Redfern plot

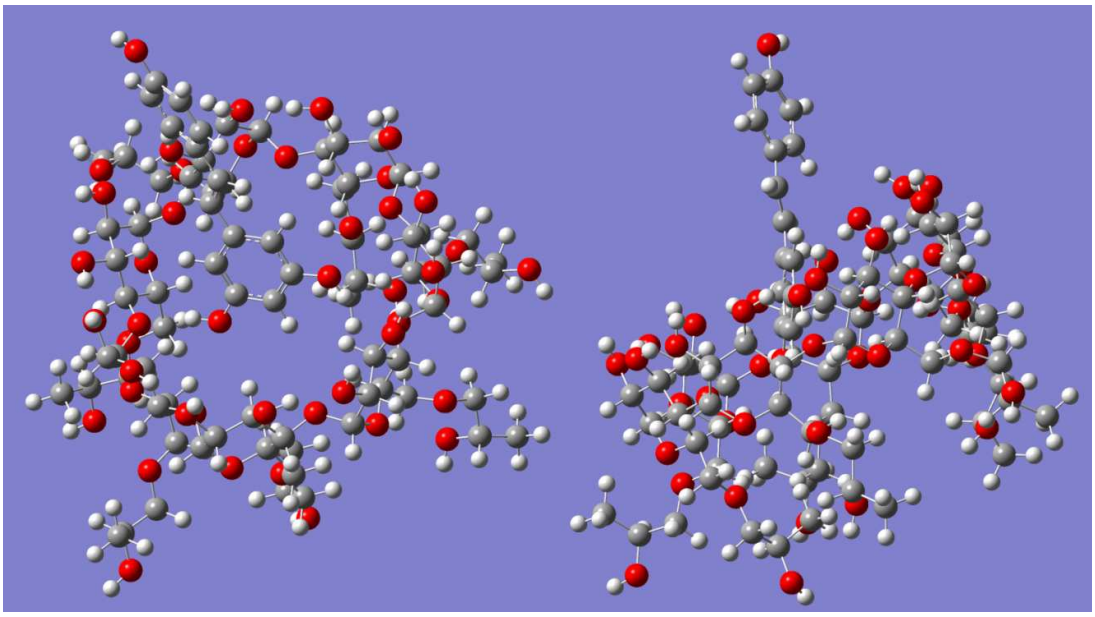


Fig. 4 Minimum energy optimized structure for IC (a) Top view (b) Side view

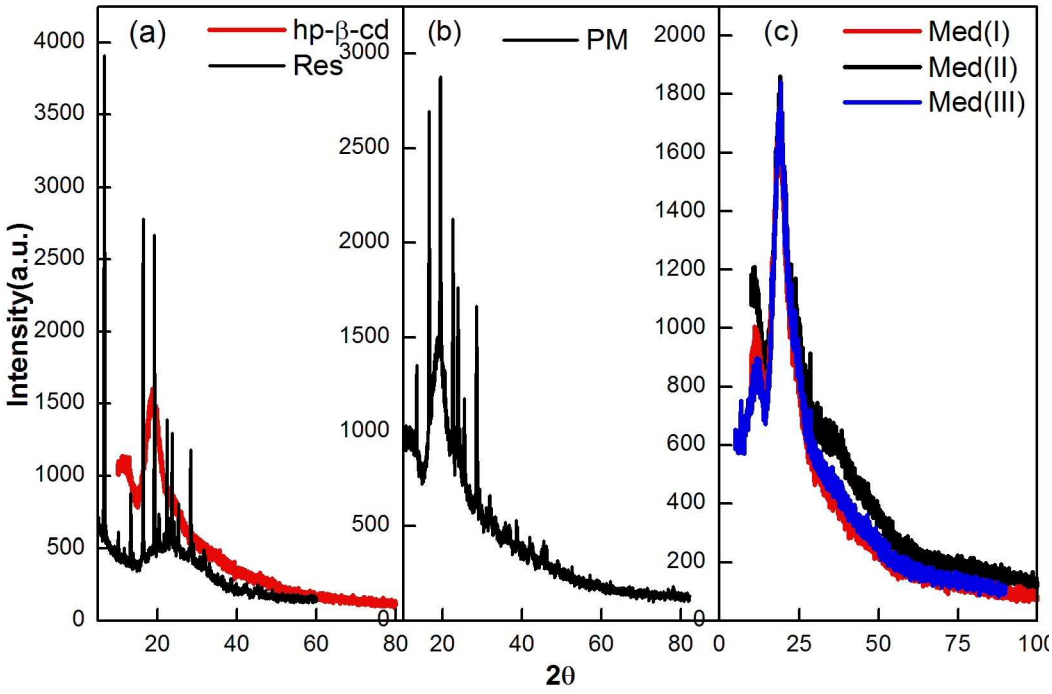


Fig. 5 XRD pattern of (a) Res and hp-β-CD (b) PM (c) Comparison of IC

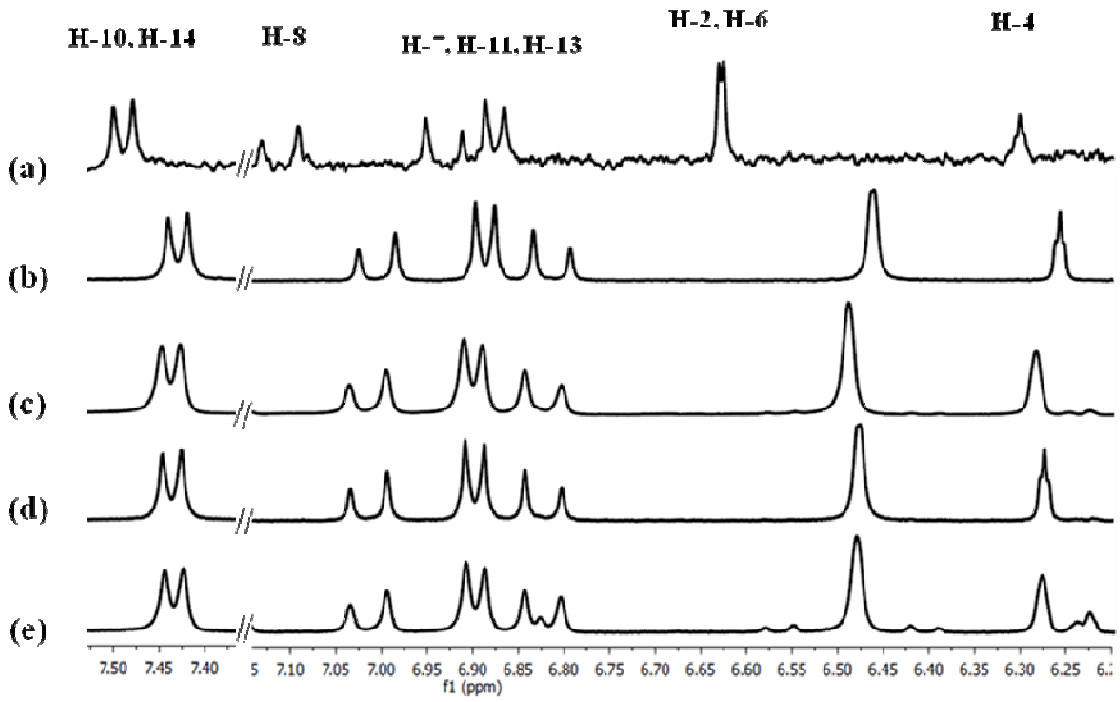


Fig. 6 ^{1}H NMR (400 MHz, D_2O) spectra (expanded between δ 7.4 and 6.0 ppm) of **(a)** Res; **(b)** PM; **(c)** Med I; **(d)** Med II and (e) Med III.

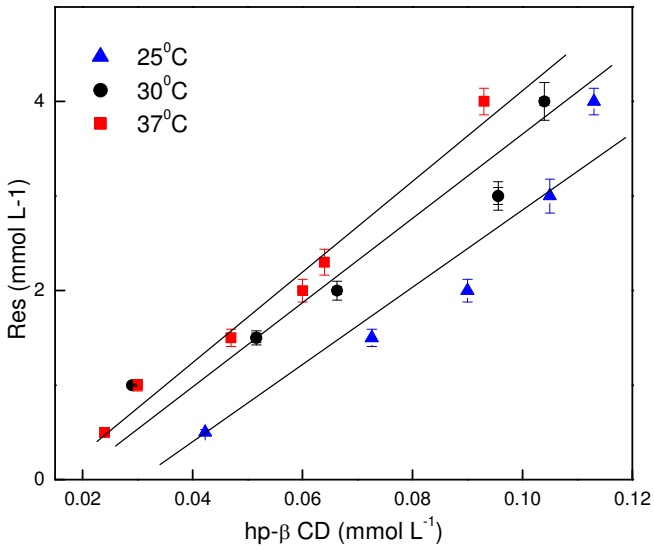


Fig. 7. Straight line portion of the phase solubility diagrams for Res at different concentrations of hp-β-CD

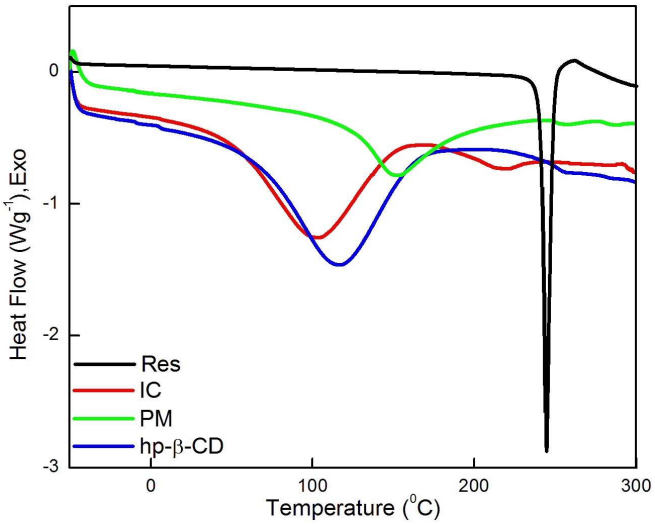


Fig 8. DSC curves Res, IC, PM, hp-β-CD

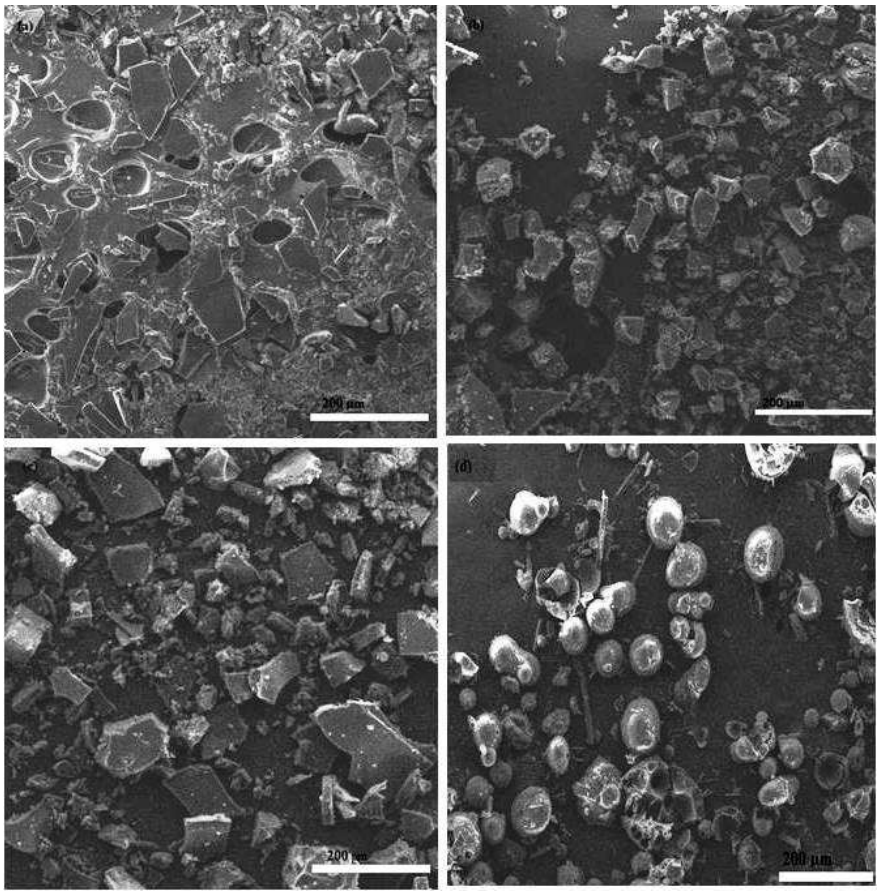
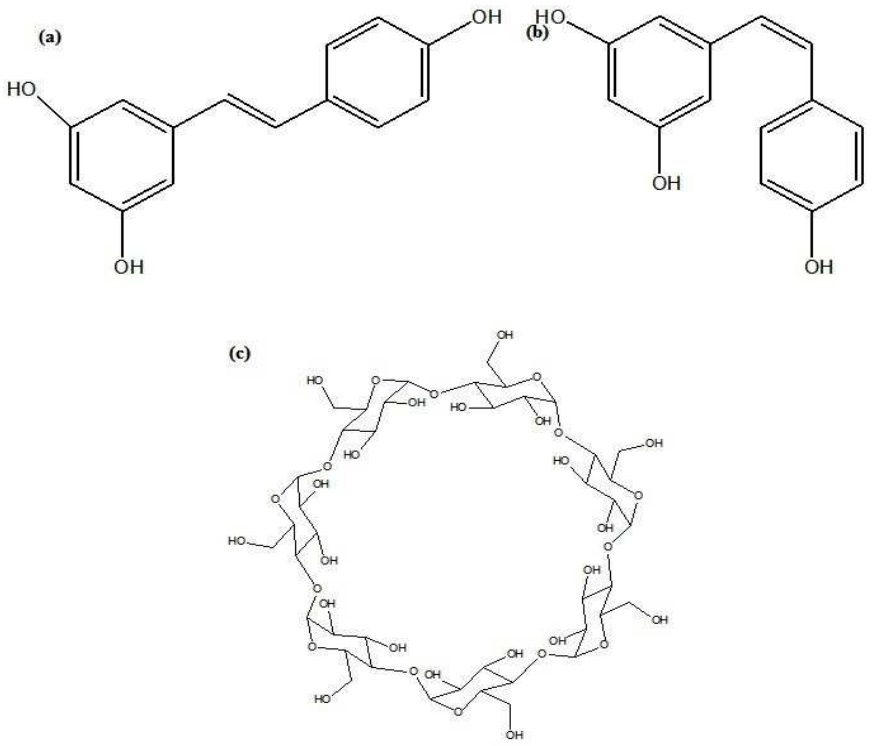


Fig. 9 SEM images of the IC prepared by three methods (a) Med I (b) Med II (c) Med III (d) PM



Scheme 1 Representative structures of (a) Trans Res (b) Cis Res (c) hp-β-CD

Graphical Abstract

Energy efficient, facile and cost effective methodology for formation of inclusion complex of resveratrol

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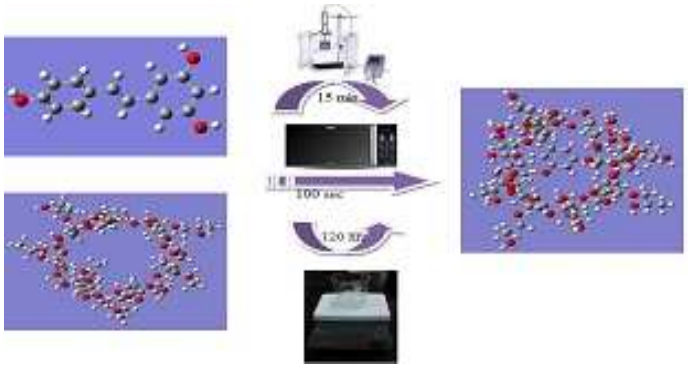

Stable inclusion complex was obtained by using a greener, energy proficient approach via ultrasonic method.