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The new 1:1 stoichiometry complex formation of curcumin- \(p\)-SC[4]R has been investigated with the aim to enhance the solubility, bioavailability, stability and anti-oxidant activity as well as decreased \textit{in vivo} acute oral toxicity of curcumin by inclusion complexation. Thermodynamic parameters ∆S and ∆H are a negative value indicates that the inclusion complex was an exothermic process which occurred spontaneously. The inclusion complex was characterised by different analytical methods including FT-IR, PXRD, \(^1\)H-NMR, SEM, DSC, ESI-Mass, UV-Vis spectroscopy and Elemental analysis.

Introduction
Curcumin (CUR), bis (4-hydroxy-3-methoxyphenyl)-1,6-diene-3,5-dione, is a natural, low molecular weight, hydrophobic yellow-orange polyphenolic compound that is isolated from the rhizome of the spice herb curcuma longa Linn., commonly known as turmeric, belonging to the ginger family (Zingiberaceae). Turmeric has been extensively cultivated in India and Asian countries for use in cooking, textiles, as cosmetic agent for skin care, therapeutic uses etc. It is approved as a food additive by the WHO and the Food & Agriculture Organization.

Research over the past decade showed that curcumin has been extensively investigated for its potential therapeutic benefits with nominal side effects. The pharmacologically higher efficacy and safety profile of curcumin makes it a potential compound for treatment and prevention of a wide variety of human diseases. In spite of the applicable qualities and biological activities of this bioactive molecule it has not yet been approved as a therapeutic agent because of its poor solubility, low bioavailability and stability (short half-life). The inferior bioavailability of curcumin is due to its low absorption, rapid metabolism and systematic elimination from the biological system. Thus, an enhancement in the solubility, stability and bioavailability of curcumin is essential.

Many researchers are involved in the development of new strategies to improve upon the solubility, stability and bioavailability of curcumin with several reported methods. But there were own disadvantages like the change in the activity of modified curcumin and dispersion of nanoparticles. So, T. Harada and co-workers have examined the encapsulation of curcumin with cyclodextrin. But cyclodextrin is present inside the cell, leads to deformation of the cell structure. Fascinatingly, recent studies show that cell transfecion of calixarenes do not affect the structure of the cell and has numbers of medicinal applications. Recently, using the encapsulation of curcumin with \textit{para}\-sulfonatocalix[4]arene, an attempt has been made to increase the solubility and bioavailability of curcumin which is investigated by UV and fluorescence spectroscopic method. However, no such study has been reported with \(p\)-SC[4]R and hence the present study was designed.

Calixarenes are one of the major classes of macro cyclic organic compounds in supramolecular chemistry along with cyclodextrins, crown-ethers, cryptands and curcurbiturils, which are described as macro cycles with almost unlimited possibilities due to their ease in modification on lower as well as upper rims. Calixresorcinarenes a member of the calixarenes are large cyclic tetraters obtained from the acid-catalysed condensation of resorcinol with appropriate aldehyde. But characteristic of calixarenes is their insolubility of water. So, Shinikai and co-worker reported water soluble calixarene derivatives bearing a sulfonic acid groups at the upper rims. Due to the modified calixarenes at upper/ lower rim to determine their inclusion behaviour. Moreover, sulfonatocalixarenes have become a particularly important class in host-guest supramolecular chemistry because of their high solubility in water, stability and less toxicity than cyclodextrins with number of potential biological activities.

Calixresorcinarenes are third generation macro cyclic host molecules composed of resorcinol units linked by methylene bridges in 2- and 6- position. The bowl shaped structure of calixresorcinarenes have a concave binding cavity and high affinity towards various guests such as cations, anions and molecules with different sizes and various hydrophobic/ hydrophilic characteristics. The formation of inclusion complexes with biological compounds is an interesting application of the functionalized calixarenes. The development of water soluble calixarenes as receptors for biomolecules has

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Electronic Supplementary Information (ESI) available: Figures S1-S6
See DOI: 10.1039/c000000x/
become a very important field of research work in view of their potential applications to increase the bioavailability and to decrease the systematic toxicity of the biologically active compounds. Recently, there is one report from our laboratory on the binding studies of p-SC[4]R with biologically important drug molecules Lamotrigine.

The aim of the present study was to explore the effect of water soluble p-SC[4]R on the dissolution behaviour of curcumin. We selected p-SC[4]R as a drug solubilizing agent due to the ease of synthesis, availability of 8 phenolic units and a simple accessibility. In addition, they possess open and rigid structure and also number of possible conformations and binding positions with a hydrophilic outer surface and an apolar cavity at their centre that provides a hydrophobic matrix. Since curcumin is a symmetric lipophilic molecule, it can be easily entrapped in the hydrophobic p-SC[4]R cavity to form host-guest complex. We have carried out investigations including UV-Vis spectroscopy, FT-IR spectroscopy, Powder x-ray diffractometry, $^1$H-NMR, SEM, DSC and ESI-mass spectroscopy of the host-guest interactions between p-SC[4]R and curcumin. The stoichiometry of the inclusion complex and the apparent formation constant have been estimated. We have performed the phase solubility study, in vivo toxicity study as well as the stability of the inclusion complex formed and also determined the anti-oxidant ability by DPPH radical scavenging activity of inclusion complex.

**Experimental Section**

**Animals**

The Swiss albino mice (Mus musculus) were procured from the Laboratory Animal Centre of Zydus Research Centre (ZRC), Ahmedabad under the Animal Maintenance and License No. 167/1999/ CPCSEA from the Ministry of Social Justice and Empowerment, Government of India. The animal caring, handling and the protocols were approved by the Institutional Animal Ethics Committee (IAEC) of ZRC. The animals were acclimatized under a well regulated 12h:12h light-dark schedule at 26 ± 2 °C and relative humidity of 45-60% for 1 week before experiments.

**Chemicals and reagents**

Curcumin (MW, 368.38; purity ≥ 99.0 %), Resorcinol (purity ≥ 99.8 %), DPPH and other chemicals were purchased from Sigma Aldrich. All other reagents were analytical grade reagents and was used without purification. The host p-SC[4]R (MW, 864.84) was synthesized according to the literature procedure. The p-SC[4]R was characterized by various spectroscopic techniques and the data was compared with standard values. Ultra-pure water was used for the preparation of inclusion complexes from Millipore Synergy system (Millipore, Bedford, MA, USA).

**Instruments**

UV-Vis measurements were performed for curcumin, p-SC[4]R and the inclusion complex by using a Jasco V-570 spectrophotometer at ambient temperature (25 ± 1 °C). All the samples were dissolved in H$_2$O / EtOH (50%) mixture and scanned in the 200 to 600 nm wavelength range to obtain the absorption spectra. The FT-IR spectra were obtained on Bruker Tensor-27 FT-IR spectrometer with KBr pellets in the range of 4000–400 cm$^{-1}$. Elemental analyses were performed on GmbH Vario Micro cube elemental analyzer. $^1$H-NMR spectra were recorded on a Bruker Avance III 400 spectrometer at 400 MHz & 500 MHz and 125 MHz respectively, with TMS as an internal standard. The following abbreviations were used to indicate NMR-multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad). The ESI–MS experiments of all samples were performed on Applied Biosystems, API 2000 LC/MS/MS instrument (USA) equipped with electrospray ionization source operating in the positive ion mode. X-ray diffraction (XRD) patterns were performed on a SEIFERT–FPX (XR&D/7); using Cu Ka X-ray lines at 1.5406 Å as the radiation source at 40 kV and 30 mA power and also Make Philips XPERT MPD. The liquid chromatographic system used was an isocratic HPLC waters system (USA) consisting of Waters 515 HPLC pump. Waters variable wavelength UV-Vis detector equipped with Waters Empower 2 solution software and a sample injector fitted with a 20 µL sample loop using a C18 system column (150 × 3.9 mm I.D., 5 µm particle size, Waters, USA) at ambient temperature (25 ± 1 °C). The detection wavelength was set at 428 nm. The mobile phase was a mixture of methanol / H$_2$O (50:50%, v/v) (containing 3% glacial acetic acid) filtered with 0.45 µm membrane filter paper at 1.0 mL/min flow rate. DSC analysis of all samples were analyzed in a Shimadzu model DSC-60 (Japan) calibrated with indium. SEM microscopy of curcumin, p-SC[4]R, physical mixture of curcumin/p-SC[4]R and inclusion complex were performed with a Leo 440i, Leo Electron Microscopy Ltd, Cambridge CB1 3QH, England to visualize the surface morphology.

**Preparation of inclusion complex of curcumin and p-SC[4]R**

The inclusion complex was prepared by mixing curcumin and p-SC[4]R according to the previously reported method described by Menon. Briefly, a 1:1 molar ratio of curcumin (0.368 g, 1 mM) and p-SC[4]R (0.865 g, 1 mM) were dissolved in 100 mL of ultrapure water, stirred for 48 h at 37 °C by rotary shaker in the dark and filtered through 0.45 µm membrane filter to eliminate undissolved materials. The filtrate was dried at 60 °C under 2–5 torr vacuum to collected the inclusion complex of curcumin and p-SC[4]R (Scheme 1).

**Preparation of physical mixture of curcumin and p-SC[4]R**

A physical mixture of curcumin / p-SC[4]R having a 1:1 molar ratio were prepared by simple grinding using an agate mortar and pestle to obtain a uniformed physical mixture.

**In vitro evaluations of curcumin / p-SC[4]R inclusion complex**

Dissolution studies of pure curcumin, physical mixture of curcumin / p-SC[4]R and inclusion complex were conducted as per standard protocol using USP rotating paddle type-II apparatus (USP-II) filled with 900 mL of 0.2 M HCl aqueous solution of dissolution medium (pH 1.2), maintained at 37 ± 1°C and agitation speed at 100 rpm. All samples containing 30 mg of curcumin were used and sprinkled directly on the surface of the dissolution medium. An aliquots of dissolution samples were withdrawn at predetermined intervals, filtered through a 0.45 µm membrane filter and analyzed by HPLC with a UV detector. An equivalent amount of fresh dissolution medium was added to maintain constant volume and sink condition. The dissolution experiment were conducted in triplicate at 37 °C.

**Phase solubility analysis**

The phase solubility analysis of curcumin with p-SC[4]R were examined according to the method described by Higuchi and Connors with some minor modification. In brief, an excess amount of curcumin was added to each 10 mL sample of p-SC[4]R aqueous solutions with the different concentrations ranging from 0 to 0.0012 mM. Each flask was sonicated for 1 h in capped condition and agitated for 72 h at 25 ± 1°C in an orbit shaker incubator (Newtronics, India) at 100 rpm. After equilibrium, the suspensions were filtered through 0.45 µm membrane filters to remove undissolved compounds. The resulting clear filtrate was diluted and analysed by HPLC. All
protected from light. The phase solubility diagram was determined by UV-Vis spectroscopy at 428nm. The difference between the concentration of the equation (1); line portion of the phase solubility diagrams using the following equation:

\[ K_c = \frac{\text{Slope}}{S_0 (1-\text{Slope})} \] … (1)

Where, \( S_0 \) is the intrinsic solubility of curcumin in the absence of \( p-\text{SC}[4]\)R and slope means the corresponding slope of the phase solubility diagram.

**Determination of Job’s plot**

Job’s method involves determination of binding stoichiometry of curcumin and \( p-\text{SC}[4]\)R in aqueous solution according to the reported method with minor variation. Briefly, equimolar (0.01mM) EtOH-water (50:50; v/v) solutions of curcumin and \( p-\text{SC}[4]\)R were mixed to a fixed volume by varying the molar ratio from 0.1 to 1, keeping the total concentration of solutes constant. After 1h stirring, the absorbance of each solution was measured by UV-Vis spectroscopy at 428nm. The difference between absorbance of curcumin with and without \( p-\text{SC}[4]\)R was plotted against \( R \), where \( R \) is obtained by following equation (2);

\[ R = \frac{[\text{Curcumin}]}{[\text{Curcumin}]+[p-\text{SC}[4]\)R]} \] .... (2)

**Effect of temperature on the curcumin / \( p-\text{SC}[4]\)R complexation**

The effect of temperature on the solubility of curcumin with \( p-\text{SC}[4]\)R was determined similar to the phase solubility study described above; the only variation was the temperature of the medium. The complexation study was investigated at different temperatures of 25, 35 and 45°C. The phase solubility diagrams were plotted as defined above and the thermodynamic parameters of the inclusion complex between curcumin and \( p-\text{SC}[4]\)R were obtained by plotting the logarithm of the stability constants against the correlative of temperature. The values of enthalpy and entropy deviations were calculated following the Van’t Hoff equation (3) and free energy changes for the complexation was calculated by the Gibbs equation (4).

\[ \ln K_c = - \frac{\Delta H}{RT} + \frac{\Delta S}{R} \] .... (3)

\[ \Delta G = -RT \ln K_c \] .... (4)

**Stability studies of curcumin / \( p-\text{SC}[4]\)R complex**

**Storage stability**

The colorant curcumin and the inclusion complex of curcumin / \( p-\text{SC}[4]\)R solutions were prepared by dissolving 1 gm each in 100mL of Milli-Q water and divided into four groups at room temperature (̴ 25°C). One group was placed in the dark condition, another group was placed in natural light condition, third group was placed in an incubator set at 45°C temperature to investigate the effect of heat and the last group was exposed to UV light to investigate the effect of UV radiation on the stability of curcumin for 40 days. Quantitative analysis were performed at weekly intervals and the concentration of appropriate dilutions of curcumin was analyzed by spectrophotometer. The results were expressed as percentages of the remaining curcumin. The colorant concentration was monitored during the experimental period and the calculated degradation rate constant was analyzed by linear regression analysis of the logarithm of the percentages of remaining curcumin against time. Each sample analysis was repeated twice and the mean value is considered.

**pH stability**

The concentrations of pure curcumin and curcumin / \( p-\text{SC}[4]\)R inclusion complex, 0.001mM of colorants were diluted in water: ethanol solution with a 50%: 50% (v/v). The solutions were prepared just before taking the measurements. The solutions, 5 mL, were adjusted to pH values in the range of 2–12 using buffer solutions and the wave length of absorbance was determined at 428nm by using a Jasco V-570 spectrophotometer.

**Assay of Anti-oxidant activities**

The anti-oxidant activity of the inclusion complex of curcumin/\( p-\text{SC}[4]\)R was compared to curcumin by DPPH radical scavenging assay method. In brief, a series of concentrations (1 mg/mL) of pure curcumin, \( p-\text{SC}[4]\)R and its inclusion complex were dissolved in methanol: water (50:50 %; v/v) and appropriately diluted. A 0.3 mM (0.013 gm/mL) of DPPH solution was prepared in methanol: water (50:50%; v/v). Then, 2 mL of DPPH solution was added to 2 mL of sample solutions at different concentration and the mixture were incubated at room temperature for 30 min. The free radical scavenging activity of inclusion complex as well as pure curcumin and \( p-\text{SC}[4]\)R were determined by spectrophotometer.
measured by monitoring the decay of absorbance of DPPH solution at 518 nm in the presence of the inclusion complex as well as p-SC[4]R and curcumin alone using a Jasco V-570 UV-Vis spectrophotometer. The anti-oxidant activity of the solutions as well as that of ascorbic acid was assayed by same procedures for the purpose of comparison. The experiment was performed in triplicate. DPPH scavenging activity was calculated as per the following equation (5):

\[
\text{% of scavenging activity} = \frac{Ac - As}{Ac} \times 100 \quad \ldots (5)
\]

Where \(Ac\) is the absorbance of the DPPH solution and \(As\) is the absorbance of the DPPH solution with the inclusion complex as well as pure curcumin and p-SC[4]R.

**In vivo evaluations of the curcumin / p-SC[4]R complexation**

**Acute oral toxicity (LD\(50\))**

The acute oral toxicity of the inclusion complex of curcumin/p-SC[4]R was evaluated in Swiss albino mice using the Organization for Economic Co-operation and Development (OECD) guidelines 425 (OECD, 3 October, 2008) (Acute Oral Toxicity-Modified Up and Down Procedure). The procedure followed was according to the earlier reported method described by Menon et al.\(^2\). Swiss albino mice were used to assess the toxicity level. The mice were fasted overnight prior to the dosing. During the period of fasting, mice were weighed and the inclusion complex of curcumin with p-SC[4]R were administered. The inclusion complex of curcumin with p-SC[4]R at dose of 2750 mg/kg was administered to four Swiss albino mice in a single dose by oral gavage. All mice were observed individually after dosing at least once during the first 30 min, periodically during the first 24 h with special attention given during the first 4 h and daily thereafter, for a total of 14 days for clinical signs as well as morbidity and mortality. The fasted body weight of each mice is determined and the dose is calculated according to the body weight. The acute oral toxicity studies were calculated by AOT425 software prepared by US Environmental Protection Agency.

**Statistical Analysis**

All of the experiments in the study were performed using the origin 6.1 software. All the samples were prepared and analyzed in triplicate. The data expressed as the mean ± standard deviation (SD). The differences were detected by one-way analysis of variance (ANOVA) with a statistical significance for \(p\) value < 0.05.

**Results and discussion**

In this study, we prepared the solid inclusion complex of curcumin / p-SC[4]R which was confirmed by various spectroscopic and physicochemical methods. We also investigated in vitro as well as in vivo assessments, which are discussed below.

**Characterization of solid complex of curcumin / p-SC[4]R**

Yield 0.77 g. (89%), Elemental analysis for C\(_{53}\)H\(_{23}\)O\(_{22}\)S\(_{4}\) 1232.16 C, 51.62; H, 4.25; O, 33.73; S, 10.40 %, Found C, 51.65; H, 4.23; O, 33.75; S, 10.37 %. FT-IR (KBr) \(\nu\), cm\(^{-1}\) 3510 (-OH stretching, broadened), 1629 (strong –C=O), 1605 (–C=C stretching vibration), 1459 (-C-O), 1191 (-SO\(_3\)-H), 1026 (-C-O-C-stretching). \(^1\)H NMR (400 MHz, DMSO-d\(_6\)) \(\delta\) = 3.813 (S, 6H, J= 11.5 Hz, -OCH\(_3\)), 5.232 (S, 2H, J=12.40, -CH methylidine), 4.104 (m, 4H, J=6.4 Hz, -CH), 6.627 (d, 2H, -CH), 6.751 (d, 2H, -CH), 6.784 (S, 2H, -CH), 7.090 (S, 4H, Ar), 7.431 (d, 2H, -CH), 7.562 (S, 2H, -OH), 9.891 (S, 4H, -SO\(_3\)H), 8.451 (S, 8H, -OH, p-SC[4]R), 1.247 (d, 12H, J= 6.8 Hz, -CH\(_3\)). ESI-MS m/z 1232.1 (Curcumin + p-SC[4]R).

**UV-Vis analysis**

The UV-Vis spectra of curcumin, p-SC[4]R and inclusion complex of curcumin / p-SC[4]R with visual colour changes are shown in Fig. 1. The results showed three various peaks at 428, 458 and 585 nm of pure curcumin, p-SC[4]R and inclusion complex respectively. The wavelength of maximum absorption (\(\lambda_{max}\)) of curcumin was remarkably altered by the addition of p-SC[4]R. Apparent absorption changes of curcumin have been observed with and without p-SC[4]R. On addition of p-SC[4]R, the absorption maximum of free curcumin showed a considerable redshift from 428 nm to 585 nm with a concomitant increase in the absorption intensity. Thus, this UV-Vis spectra indicates the successful formation of the inclusion complex between curcumin and p-SC[4]R.

![Fig. 1 UV-Vis absorption spectra of pure curcumin, p-SC[4]R and inclusion complex in H\(_2\)O/EtOH (50%) with visual colour changes.](image-url)

**FT-IR analysis**

The FT-IR spectra of curcumin, p-SC[4]R, physical mixture of curcumin / p-SC[4]R and inclusion complex are presented in Fig. 2. The FT-IR spectra of pure curcumin showed sharp absorption band at 3510 cm\(^{-1}\) indicating the presence of phenolic –OH stretching vibration. The sharp peaks at 1628 cm\(^{-1}\) has a predominantly mixed C=O & C=C groups and 1603 cm\(^{-1}\) is indicative of symmetric aromatic stretching vibration. The sharp peak at 1455 cm\(^{-1}\) is attributed to the phenolic C-O while enolic C-O peak was obtained at 1275 cm\(^{-1}\). Absorption at 1025 cm\(^{-1}\) is indicative of C=O-C asymmetric stretching vibration; In peak at 958 cm\(^{-1}\) is due to benzoate trans C-H vibration while at 712 cm\(^{-1}\) is C-H vibration of aromatic ring. The FT-IR spectra of p-SC[4]R consisted of broad absorption bands of –OH stretching at 3456 cm\(^{-1}\) while three characteristic absorption peaks at 1187, 1115 and 1057 cm\(^{-1}\) indicative of –SO\(_3\) group. The FT-IR spectra of the physical mixture is found to have merged with lower intense broad OH peak due to the interactions with supramolecule during complex formation and also all sharp peaks of curcumin as well as p-SC[4]R were observed along with few peaks and reveal reduced sharpness rather than the
physical mixture was essentially a combination of the spectra of the two molecules of curcumin with p-SC[4]R. However, the curcumin / p-SC[4]R inclusion complex shows no features similar to pure curcumin. Several insignificant absorption peaks of curcumin from 500-1200 cm\(^{-1}\) almost disappeared and sharp – OH stretching band of curcumin at 3510 cm\(^{-1}\) broadened, which indicates the inclusion of curcumin into the p-SC[4]R cavity.

PXRD analysis

The powder X-ray diffractometry is a useful method, which has been proven to provide insightful information for studying complexation between p-SC[4]R and curcumin. Fig. 3 shows the XRD pattern of curcumin, p-SC[4]R, physical mixture of curcumin / p-SC[4]R and the inclusion complex. The XRD pattern of curcumin showed intense and sharp peaks indicating its crystalline nature. The XRD pattern of curcumin has major diffraction peaks at 20 ≈ 12.14\(^\circ\), 14.84\(^\circ\), 18.75\(^\circ\), 21.72\(^\circ\), 24.09\(^\circ\), 25.22\(^\circ\)and 27.77\(^\circ\). whereas, the XRD pattern of p-SC[4]R revealed three broad peaks at 20 ≈ 11.08\(^\circ\), 18.61\(^\circ\) and 23.22\(^\circ\), consistent with its amorphous character. The XRD pattern of physical mixture displayed essentially a superposition of the pattern of the two compounds of curcumin and p-SC[4]R, confirming the absence of complex formation and both retained their original physical characteristic. However, the XRD pattern of as received curcumin with p-SC[4]R inclusion complex showed no diffraction peaks except halo diffraction pattern, indicating that the material was typically amorphous, having some amount of channel type structure and exhibited none of the characteristic peaks of curcumin indicating that complete complexation was achieved between curcumin and p-SC[4]R.

HPLC analysis

The HPLC analysis results of pure curcumin, p-SC[4]R and inclusion complex of curcumin with p-SC[4]R, by Isocratic reverse phase HPLC method, is shown in Fig. S1 (ESI†). The chromatograms of pure curcumin and p-SC[4]R are showing a peak at retention time of 6.75 min and 12.90 min respectively. The chromatogram of the inclusion complex also shows the peaks of both curcumin and p-SC[4]R at the same retention times, indicating the existence of both curcumin as well as p-SC[4]R in the inclusion complex.

\(^1\)H-NMR Analysis

NMR spectroscopy is a widely used and indeed the most powerful technique for the study of inclusion of guest curcumin into the hydrophobic p-SC[4]R cavity in solution. Inclusion complex of curcumin in p-SC[4]R cavity is confirmed by the change in chemical shifts of the guest and host protons, in
comparison with the chemical shifts of the protons in the free molecules. The $^1$H-NMR spectra of pure curcumin, \textit{p-SC[4]R} and curcumin / \textit{p-SC[4]R} inclusion complex are shown in Fig. 4 in DMSO-$d_6$ as a solvent. The chemical shift (\(\delta\)) values of curcumin, \textit{p-SC[4]R} and inclusion complex are given in Table 1.

Table 1 Chemical shift values (\(\delta\)) of the $^1$H NMR corresponding to curcumin in the presence and absence of \textit{p-SC[4]R} in DMSO-$d_6$.

<table>
<thead>
<tr>
<th>Proton assignment</th>
<th>(\delta) Curcumin (ppm)</th>
<th>(\delta) \textit{p-SC[4]R} (ppm)</th>
<th>(\delta) Curcumin / \textit{p-SC[4]R} complex (ppm)</th>
<th>(\Delta\delta = \delta - \delta_0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>3.819, 3.838</td>
<td></td>
<td>3.813</td>
<td>-0.006, -0.025</td>
</tr>
<tr>
<td>b</td>
<td>4.528</td>
<td></td>
<td>4.523</td>
<td>-0.005</td>
</tr>
<tr>
<td>c</td>
<td>7.567, 7.589</td>
<td></td>
<td>7.562</td>
<td>-0.005, -0.027</td>
</tr>
<tr>
<td>d</td>
<td></td>
<td>9.885</td>
<td>9.891</td>
<td>0.006</td>
</tr>
<tr>
<td>e</td>
<td></td>
<td>8.439</td>
<td>8.451</td>
<td>0.012</td>
</tr>
<tr>
<td>f</td>
<td></td>
<td>1.243</td>
<td>1.247</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Generally, it was observed that the aromatic part of curcumin and methylene bridged (\(\text{a, b & c-protons}\)) are shifted to the up field (lower chemical shift in ppm) region in the complex as compared to pure curcumin protons. Neither appearance of new peak nor splitting was observed in this complex. These chemical shifts resulted owing to the binding of the aromatic segment of curcumin within the hydrophobic cavity of \textit{p-SC[4]R} and the ring current effect resulted in shielding of protons. On the other hand, the peaks (\(\text{d, e & f-protons}\)) corresponding to \textit{p-SC[4]R} are shifted to the downfield (higher chemical shift in ppm) region due to the binding of curcumin with \textit{p-SC[4]R} inside the cavity via $\pi-\pi$ interactions as well as the wide rim with stronger interactions with its hydroxyl groups. This data indicates that the differences in chemical shifts (ppm) for curcumin peaks in curcumin / \textit{p-SC[4]R} complex compared to pure curcumin, is due to the presence of strong complexation. Thus the $^1$H-NMR spectra strongly confirms that the guest molecule curcumin penetrate deeply into the hydrophobic cavity of \textit{p-SC[4]R}.

**DSC Analysis**

Fig. 5 shows the DSC curves of pure curcumin, \textit{p-SC[4]R}, their physical mixture and inclusion complex of curcumin with \textit{p-SC[4]R}. The DSC curves of curcumin presents a single sharp endothermic peak at 183 °C, corresponding to the melting point of crystalline curcumin followed by an exothermic influence because of the thermal degradation of the substance at higher temperatures. The DSC thermogram of \textit{p-SC[4]R} displayed a relatively broad peak appearing at 235 °C, which could have been formed owing to its amorphous nature. The DSC thermogram of physical mixture exhibited a combination of the

![Fig. 5 DSC thermo grams of (A) Pure curcumin, (B) \textit{p-SC[4]R}, (C) Physical mixture and (D) Inclusion complex.](image-url)
curcumin as well as p-SC[4]R molecules indicating the absence of close association between these molecules, apart from this the variance of curcumin thermogram intensity has reduced, while the two molecules are simply mixed together. In contrast, the DSC thermogram of the inclusion complex of curcumin with p-SC[4]R showed disappearance of the melting endothermic peak of curcumin. The appearance of a new small peak at 173 °C together with the shifting of one representative band is suggestive of a change in the substance structure and of a compact interaction between curcumin and p-SC[4]R.

SEM Analysis
The SEM analysis is the most ideal qualitative technique for measuring the surface roughness and for imaging the surface texture of the substance. Not only there is amorphisation of curcumin/p-SC[4]R (halo pattern in Fig.3) but uniform at the tiny level the inclusion solid has aragonite of irregular shaped of 1 μm width (Fig.6D). The morphology of the curcumin and curcumin / p-SC[4]R inclusion complex were evaluated by SEM as shown in Fig. 6. The curcumin existed in extremely rounded spherical and uniform particles while p-SC[4]R appeared as rice-seed like or long rod like morphology, which is observed for most of the precipitated particles. However, the electron micrograph of the physical mixture of the two powders of curcumin / p-SC[4]R exhibited some similarities with the rod like shape of p-SC[4]R and the rounded spheres of curcumin found in combination of large and small agglomerates. In contrast, the curcumin / p-SC[4]R inclusion complex shows a change in particle’s morphological appearance, to contain neither rounded spherical structures nor rice-seed like patterns but exhibited aggregation into irregularly shaped amorphous particles. This micrograph expose the development of aragonite mineral aggregates from a middle pseudo-hexagonally basal platter. This growth process is the middle prime pattern nucleus particles. This micrograph expose the development of aragonite indicative of the presence of an apparent interaction between curcumin and p-SC[4]R.

ESI-MS analysis
The ESI-MS spectra has shown itself to be a very convenient method in describing the non-covalent interaction between p-SC[4]R and curcumin guest, owing to its high sensitivity, rapid and low sample consumption. ESI-MS spectra of the solid inclusion complex of curcumin / p-SC[4]R, pure curcumin and p-SC[4]R shows in Fig. 7. The ESI-MS spectra of pure curcumin shows three molecular ion peaks with m/z at 368.1, 369.1 and 391.4 corresponding to curcumin mass (M), M+1 and M+ Na respectively, while p-SC[4]R shows peak at 865.3. The positive mode ESI MS spectra of the inclusion complex shows two peaks at 1232.1 and 1255.4, which were due to p-SC[4]R + curcumin and p-SC[4]R + Na-curcumin, which indeed suggest the presence of binding of curcumin with p-SC[4]R. Also negative mode ESI MS spectra of inclusion complex shows two peaks at 1230.8 and 1230.0, due to p-SC[4]R + curcumin – H (M-1) and p-SC[4]R + curcumin – 2 (M-2), which also suggest the presence of superficial interaction between curcumin and p-SC[4]R. These results clearly indicates that the binding ratio is 1:1 between curcumin and p-SC[4]R during complexation.

Elemental analysis
The analytical calculation of inclusion complex for C, H, O and S% was found to be 51.62, 4.25, 33.73 and 10.40% respectively, while practical values of C%, H%, O% and S% is 51.65, 4.23, 33.75 and 10.37% in that orders. The values of curcumin / p-SC[4]R inclusion complex were confirmed mostly based on the data from carbon and oxygen elements, elucidating that the curcumin was completely complexes with the p-SC[4]R in 1:1 stoichiometry.

In vitro evaluations
Dissolution analysis
In vitro dissolution profiles of curcumin / p-SC[4]R inclusion complex, pure curcumin and the physical mixture of curcumin / p-SC[4]R at the equivalent ratio are shown in Fig. S2 (ESI†). As seen in the Fig. S2, curcumin / p-SC[4]R inclusion complex exhibited an initially rapid and manifestly superior dissolution release than the corresponding physical mixture and pure curcumin with an accumulative dissolution of over 90%. The dissolution process attained a constant value within 30 min and dissolved completely in 40 min. At the end of 3 h, pure curcumin, physical mixture and inclusion complex released were 9.89%, 5.49% and 0.83% respectively. These results indicate that the rise in solubility, reduced curcumin crystallite size and an enhanced curcumin wettability lead to the conversion of curcumin to the amorphous state. The pure curcumin showed the sluggish dissolution rate owing to its hydrophobicity that produced the powder to levitate on the surface of the dissolution medium. The physical mixture also resulted in higher dissolution compared to pure curcumin and the dissolution is attributable to the in situ formation of readily soluble inclusion complex. In short, the results concluded that the curcumin / p-SC[4]R inclusion complex showed much faster dissolution than the pure curcumin and this enhanced dissolution leads to an improved oral bioavailability of curcumin.

Phase solubility diagram
The phase solubility diagram of curcumin / p-SC[4]R complex investigated at 25 °C is shown in Fig. 8. The phase solubility diagram is a widely useful technique to studies curcumin/p-SC[4]R complexation since it not only provides the solubilizing capacity of the p-SC[4]R but also allow the apparent stability constant (K_a) to be calculated by analyzing the solubility curves according to the reported method described by Higuchi and Connors. The diagram showed that a linear relationship exists among the quantity of curcumin solubilized and the molar
Fig. 7 ESI-MS spectra of (A) curcumin, (B) p-SC[4]R and (C) curcumin/p-SC[4]R inclusion complex (ES+ mode) as well as ES- mode spectra of inclusion complex (D).
concentration of \( p\text{-SC}[4]\text{R} \) in solution, which in turn define the \( A_L \) type phase solubility diagrams according to Higuchi and Connors. The phase solubility diagram studies revealed a correlation coefficient square value (\( R^2 \)) of 0.9997 and slope calculated was 0.9844 observed in linear regression equation \( y = 0.9844x + 0.000015 \), which is less than 1, indicating the complexation to be first order and suggesting a 1:1 curcumin / \( p\text{-SC}[4]\text{R} \) stoichiometry. The apparent stability constant (\( K_c \)) of inclusion complex was calculated from the slope of the linear plot of the curves according to equation (1). The apparent stability constant value of \( K_c \) (1:1) was found to be 985 mM\(^{-1}\) indicating that the complex formed between curcumin / \( p\text{-SC}[4]\text{R} \) was quite stable. In fact, always apparent stability constant values are within the range of 100 to 1000 mM\(^{-1}\). These results indicates the formation of a stable 1:1 inclusion complex between curcumin and the polar cavity of \( p\text{-SC}[4]\text{R} \).

Further, stoichiometry of curcumin/\( p\text{-SC}[4]\text{R} \) complexes was confirmed by Job’s plot method shown in Fig. 9. In Job’s plot, the maximum value was found at \( R=0.5 \) and a highly symmetrical outline demonstrated the presence of an inclusion complex with 1:1 stoichiometry. These results are in agreement with the phase solubility studies.

Fig. 8 The phase solubility diagram of curcumin / \( p\text{-SC}[4]\text{R} \) host-guest system at 25 °C as per Higuchi Connors method.

Fig. 9 Job’s plot obtained for the complexation of curcumin with \( p\text{-SC}[4]\text{R} \) from absorption measurement at \( \lambda=428 \text{ nm} \).

**Effect of temperature on the complexation between curcumin and \( p\text{-SC}[4]\text{R} \)**

The phase solubility diagrams of curcumin / \( p\text{-SC}[4]\text{R} \) inclusion complex shown in Fig. S3 (ESI†) at different temperatures of 25, 35 & 45°C are to calculate the stability constants (\( K_c \)) and the thermodynamic values for the formation of the curcumin / \( p\text{-SC}[4]\text{R} \) complexes. All the diagrams showed that the solubility of curcumin increased linearly along with the concentration of \( p\text{-SC}[4]\text{R} \), confirming earlier observations of \( A_L \) type diagram suggestive of the formation of 1:1 stoichiometry of the complex. The intercept, slope and stability constant obtained from the curcumin/\( p\text{-SC}[4]\text{R} \) phase solubility diagrams is given in Table 2, according to linear regression equation and stability constant (\( K_c \)) equation (1). Increase in the intercept value indicates that the aqueous solubility of curcumin raised with temperature as well as the total molar concentration of curcumin in aqueous media containing \( p\text{-SC}[4]\text{R} \) increase at higher temperature. However, the stability constant \( K_c \) decreases with increasing temperature, representing that the inclusion of curcumin into \( p\text{-SC}[4]\text{R} \) was an exothermic process. Furthermore, some thermodynamic parameters of inclusion complex could also be obtained from the phase solubility studies (Fig. 10) at different temperatures of 25, 35 and 45 °C. The values of enthalpy (\( \Delta H \)) and entropy (\( \Delta S \)) changes were calculated from stability constant (\( K_c \)) using the integrated form of the Van’t Hoff equation (3) at different temperatures. The inclusion complex of curcumin / \( p\text{-SC}[4]\text{R} \) showed in Fig. 10, exhibited a linear association (\( R^2 = 0.998 \)) between logarithm of stability constant (\( K_c \)) and the inverse of the absolute temperature (1/T). Thermodynamic parameters \( \Delta H = -24.08 \text{ kJ/mol} \) and \( \Delta S = -6.29 \text{ J/mol} \) can be calculated from the slope and intercept respectively. Also, the free energy changes (\( \Delta G \)) for the inclusion complex formation was calculated by the Gibbs free energy equation (4). The value of \( \Delta G \) was found to be -17.79 kJ/mol. The negative value of enthalpy changes (\( \Delta H \)) shows that the interaction processes between curcumin and \( p\text{-SC}[4]\text{R} \) was exothermic. These interaction processes may consist of the displacement of water

![Van’t Hoff plot for curcumin / \( p\text{-SC}[4]\text{R} \) inclusion complex.](image)

**Table 2** The intercept, slope, stability constant and Gibbs free energy obtained from curcumin / \( p\text{-SC}[4]\text{R} \) phase solubility diagrams at different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Intercept ( (\times 10^4 \text{ M}) )</th>
<th>Slope</th>
<th>( R^2 )</th>
<th>Stability Constant ( (K_c, \text{M}^{-1}) )</th>
<th>( \Delta G ) (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.15</td>
<td>0.985</td>
<td>0.99</td>
<td>985.00</td>
<td>-17.09</td>
</tr>
<tr>
<td>35</td>
<td>0.91</td>
<td>0.993</td>
<td>0.99</td>
<td>772.33</td>
<td>-17.04</td>
</tr>
<tr>
<td>45</td>
<td>1.71</td>
<td>0.999</td>
<td>0.99</td>
<td>587.65</td>
<td>-16.87</td>
</tr>
</tbody>
</table>
molecules from the cavity of the p-SC[4]R by more hydrophobic curcumin, with the formation of hydrogen bond or other low energy interaction and an increase in van der Waals interaction between molecules. The entropy changes (\(\Delta S\)) is negative indicating that complexation resulted in the system environment becoming more stable and orderly, owing to the decrease in the translational and rotational degrees of the curcumin compared to p-SC[4]R in the complexed state. Overall, the \(\Delta G\) negative value indicates that the formation of curcumin / p-SC[4]R inclusion complex is a spontaneous processes.

**Stability of free curcumin and curcumin / p-SC[4]R complex**

**Storage stability**

In the present study, the potential of p-SC[4]R to protect curcumin from light, heat and UV loss was investigated. The degradation of curcumin and its inclusion complex with p-SC[4]R complex when exposed to light, heat and UV radiation over 40 days period is shown in Fig. S4 (ESI†). The stability of curcumin has been reported earlier by light exposure. For curcumin exposed to light, heat and UV, all groups of curcumin free and complex forms were gradually degraded, while the degradation occurred at a slower rate for curcumin in the complex form. Thus, light, heat and UV appears to interfere significantly with the integrity of colorant curcumin. After 40 days, the amount of curcumin remaining was 57% and 78% for free and complex form respectively. Measurement of curcumin colour intensity after stored under dark condition showed that the difference between complex and without complexation was smaller suggesting that a protective action by p-SC[4]R on curcumin against light, heat and UV irradiation. As a result, the improvement in the stability of curcumin by complexing with p-SC[4]R is exceedingly important in the food and pharmaceutical field.

**pH stability**

Fig. S5 (ESI†) shows the stability results of the pure and complexed curcumin between pH ranges from 2-12. The curcumin / p-SC[4]R inclusion complex displayed better stability compared to the pure colorant for the pH range of 1-7. However, at pH values 8-12, degradation occurred for pure curcumin as well as curcumin complex and both the solutions visually changed the colour from yellow to red. Curcumin is exposed to hydrolytic degradative reactions in basic media and is therefore unstable in an alkaline medium, so it is more suitable to distinguish curcumin in the media whose pH value is maintained below 7. For curcumin, p-SC[4]R and its complex with p-SC[4]R as well as ascorbic acid, the degradation occurred at a slower rate for curcumin in the complex form. Thus, light, heat and UV appears to interfere significantly with the integrity of colorant curcumin. After 40 days, the amount of curcumin remaining was 57% and 78% for free and complex form respectively. Measurement of curcumin colour intensity after stored under dark condition showed that the difference between complex and without complexation was smaller suggesting that a protective action by p-SC[4]R on curcumin against light, heat and UV irradiation. As a result, the improvement in the stability of curcumin by complexing with p-SC[4]R is exceedingly important in the food and pharmaceutical field.

**Anti-oxidant activity of curcumin in free and complex form**

The anti-oxidant activity of curcumin and inclusion complex of curcumin / p-SC[4]R measured by the DPPH radical scavenging activity was compared to standard ascorbic acid (Fig. 11). DPPH is an efficient antioxidant which is a stable free radical and proton donating substance, generating a deep violet colour in organic solvent. Its progressive discoloration in the presence of curcumin indicated that it is acting as an anti-oxidant. The reduction capability was determined by monitoring the decrease in absorption by DPPH radical at 516 nm. The effect of curcumin, p-SC[4]R and curcumin / p-SC[4]R inclusion complex on DPPH staining was measured quantitatively from 0.5 to 10 µg/mL. The % of DPPH radical scavenging was increased by increasing the concentration of pure curcumin, p-SC[4]R and its complex with p-SC[4]R as well as ascorbic acid (Fig. 11). The calculated inhibitory concentration (IC\(_{50}\)) value of curcumin and inclusion complex of curcumin / p-SC[4]R were 3.93 ± 0.03 µg/mL and 3.56 ± 0.07 µg/mL respectively, as compared to IC\(_{50}\) value of ascorbic acid for DPPH radical which is 4.31 ± 0.08 µg/mL. However, the calculated inhibitory concentration of p-SC[4]R is 3.69 ± 0.05, due to Calix[4]resorcinarene which can transfer of electron or hydrogen atoms to the non-radical form of DPPH is highly efficient antioxidant agents. This means curcumin in complex form with a concentration of only 3.56 ± 0.07 µg/mL was

<table>
<thead>
<tr>
<th>Animal</th>
<th>Time (h)</th>
<th>Dose (mg/kg)</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>500</td>
<td>(\checkmark)</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>2500</td>
<td>(\checkmark)</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
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<td>(\checkmark)</td>
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<td>(\checkmark)</td>
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<td>12</td>
<td>48</td>
<td>(\checkmark)</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>3000</td>
<td>(\checkmark)</td>
</tr>
</tbody>
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required to scavenge 50% of DPPH radicals while the concentration of curcumin free and standard ascorbic acid needed to scavenge the same amount of the radicals were 3.93 μg/mL and 4.31 μg/mL correspondingly. These results clearly indicates that curcumin in the presence of p-SC[4]R is a powerful free radical inhibitor. These results also indicate that complex formation could be useful to maintain and even enhance the anti-oxidant activity of curcumin.

**In vivo acute toxicity study**

The in vivo acute toxicity studies, LD50 of the solid curcumin / p-SC[4]R inclusion complex was found to be greater than 2500 mg/kg of body weight in Swiss albino mice. In mice, a single dose of free para-sulfonato-calixarene at doses equivalent to 2–5 g/kg in humans shows no acute toxicity.[9] At the end of the 14 days period, all the survived animals were weighed, no substantial changes were observed as compared to the initial weight on the 1st day. The animals appeared active and healthy during the study. During the acute toxicity study and body weight measurements the animals did not show any toxic effects. As a part from the toxicity observations, there were no signs of gross toxicity in mice. An autopsy at the end of study did not reveal any gross pathological abnormalities and adverse pharmacological effects in all mice. Therefore, above study indicates that curcumin / p-SC[4]R was non-toxic to an oral dose LD50 of about 2750 mg/kg (Table 3) of body weight in Swiss albino mice. The solid lipid curcumin particle earlier reported was found to have an oral LD50 in rats as well as in mice which was found to be 2000 mg/kg of body weight for a dose of 720 mg/kg28.

**Conclusion**

In summary, we have successfully prepared the inclusion complex of curcumin/p-SC[4]R at 1:1 complexation stoichiometry with 985 mM−1 stability constant and also confirmed by Job’s plot method (R=0.5). The evidences obtained by various spectroscopic methods using UV, FT-IR, HPLC, DSC, SEM, PXRD, 1H NMR and ESI-Mass analysis. From the temperature dependent studies, the thermodynamic parameters ∆S & ∆H changes mean a negative value indicates that the inclusion complex of curcumin/p-SC[4]R was an exothermic process which occurred spontaneously. The formation of such an inclusion complex covered moderate degrees of protection to curcumin from light, thermal & UV radiation degradation during storage and the pH stability of inclusion complex is better in the acidic media in comparison of curcumin alone. The DPPH scavenging capacity of curcumin on complexation with p-SC[4]R was enhanced compared to pure curcumin. Moreover, in vivo oral acute toxicity showed that there is a remarkable changes in the toxicity of curcumin alone indicating that the pure curcumin becomes less toxic as it increases the LD50 value (2750 mg/kg) as compared to the previously reported LD50 value (2000 mg/kg) of curcumin solid lipid particle after complexation. All these results concluded that p-SC[4]R complexation of curcumin takes place in the hydrophobic cavity of p-SC[4]R Further research could examine the chemical, biological and processing properties & their application in pharmaceutical and functional foods.

**Acknowledgements**

Nikunj N. Valand gratefully acknowledge DST, New Delhi for INSPIRE Junior Research Fellowship. Prof. N K Jain, Flora Shah, Namrata Bhagia (Dept. of Life Science) and Kalpesh Solanki (Dept. of Forensic Science) are acknowledged for their help in HPLC study.

**References**

Table of Content:


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The target to enhance the solubility, bioavailability, stability and anti-oxidant activity as well as decreased in acute oral toxicity of curcumin by 1:1 curcumin/\( p \)-SC[4]R inclusion complexation.