ORIGINAL ARTICLE

Piperine-hydroxy acid-cyclodextrin inclusion complexes; antioxidant, anti-inflammatory, and stability studies: PART II

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Abstract

Introduction: Piperine (PIP) is a natural ingredient possessing important biological activities. However, its practical usefulness is limited due to its low water solubility. In our previous research article (PART A), we demonstrated inclusion complexation of PIP with cyclodextrins (CDs) in the influence of certain hydroxy acids resulted in tremendous improvement in physicochemical characteristics of PIP. The aim of current research work was to study biological properties and stability analysis of PIP and its lyophilized inclusion complexes. **Materials and Methods:** Initially, Job's plot experiment was carried out to assess the type of solubility and stoichiometry of complexes. The solid inclusion complexes were obtained by lyophilization and characterized by differential scanning calorimetry (DSC), X-ray powder diffractometry (XRPD), saturation solubility, *in-vitro* dissolution, *in-vitro* antioxidant activity, and *in-vivo* anti-inflammatory activity in carrageenan-induced rat paw edema model. The short-term stability studies were carried out as per the ICH guidelines. **Results:** During assessment, the complexes performed better in terms of *in-vitro* antioxidant and *in-vivo* anti-inflammatory activities than the native PIP. However, PIP: Hydroxypropyl β-CD (HPβCD): Ascorbic acid (AA) ternary complexes elicited immediate and maximum onset of anti-inflammatory action, as compared to other test samples. In the stability studies, no noteworthy changes were recorded concerning DSC, XRPD, and *in-vitro* dissolution studies over a period of 6 months except complexes with HPβCD. **Conclusion:** Taking everything into account, complexation of PIP with CDs, in the influence of AA, would be a successful way to improve its biological properties.

Key words: biological properties, complexation, cyclodextrin, hydroxy acids, piperine, stability studies

INTRODUCTION

Cyclodextrin (CD) complexation is the foremost interesting solubility enhancement method. CDs are oligosaccharides and are natural by-products of enzymatic starch degradation.[1-3] CDs have super molecular lattice structure with hydrophilic exterior and hydrophobic interior that reserve it to accommodate hydrophobic drug molecule in to its cavity.[4] Encapsulation with CDs promotes betterment in water solubility, dissolution rate, bioavailability, and stability of low aqueous soluble drugs without any change in their molecular structure and permeability properties.[5,6] Hydroxypropyl β-CD (HPβCD); the hydroxy derivative of βCD exhibits better water solubility with least toxicity, therefore, making it more preferable than fundamental one.[7] It has been published that efficiency of CD complexation could be improved by incorporation of certain ternary components such as low molecular weight hydroxy acids, hydrophilic polymers, and amino acids, to the complexation system.[8-10]

Piperine (PIP) [(2E,4E)-1-[5-(1,3-Benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine [Figure 1] is a pungent alkaloid observed in black pepper possessing antioxidant, anti-inflammatory, antihypertensive, antithyroid, and antitumor activities. The inadequate solubility of PIP results in poor dissolution rate in gastrointestinal tract after oral administration.[11-15] A few researchers reported inclusion

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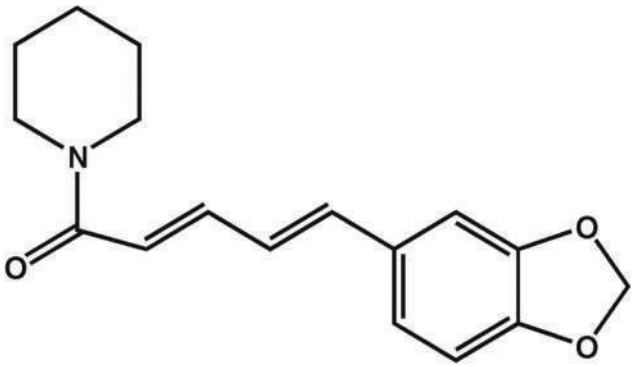

Figure 1: Chemical structure of piperine

complexation of PIP with CDs to improve aqueous solubility and bioavailability of PIP.[11,15] The nano-encapsulation of black pepper olio resin (PIP + Volatile oil) with HPβCD have been as a successful attempt to improve antimicrobial and antioxidant activity,[16] whereas Quilaqueo *et al*. in 2019[17] investigated improvement in bioaccessibility and antioxidant activity of PIP-βCD inclusion complexes.

In our previous research article (PART A), we demonstrated that inclusion complexation of PIP with CDs (βCD and HPβCD) in presence of ascorbic acid (AA) as a ternary component was the best approach toward enhancement in aqueous solubility and dissolution properties of PIP. In this present, investigation an attempt was made to prepare inclusion complexes of PIP with βCD and HPβCD in the presence of citric acid (CA) and AA as ternary components to improve the hydrophilicity in terms of *in-vitro* antioxidant and *in-vivo* anti-inflammatory activity of PIP. Initially, Job's plot data revealed the formation of 1:1 stoichiometry. The inclusion complexes of PIP with CDs were prepared by lyophilization technique and characterized by differential scanning calorimetry (DSC), X-ray powder diffractometry (XRPD), and further evaluated for saturation solubility, *in-vitro* antioxidant, and *in-vivo* anti-inflammatory activities of PIP. All the prepared inclusion complexes including pure PIP were subjected to short term stability studies as per ICH guideline (Q1A).

MATERIALS AND METHODS

Materials

PIP (Molecular weight: 285.34 g/mol, Purity 97%) was bought from Sigma Aldrich Chemicals Pvt. Ltd. (Mumbai, India). βCD (Molecular weight: 1135 g/mol, Purity 98%) was secured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). HPβCD (Molecular weight: 1400 g/mol, degree of substitution – 0.65, Purity 98%) was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). AA (Purity 98%) and CA (Purity 98%) were procured from Loba Chemie Pvt. Ltd. (Mumbai, India). Analytical grade reagents and glass distilled water were used throughout the experiment. All chemicals were used without further purification.

Job's plot experiment

The stoichiometry of the formation of complexes was confirmed by Job's plot experiment, where total molar concentrations of PIP and CDs were kept constant, but their mole fractions were different.[18-20] Variant molar ratios of PIP (10×10^{-6} M) in methanol:distilled water (50:50) and CDs (10×10^{-6} M) in distilled water were taken to a fixed volume of 10 mL such as 1:9, 2:8, 3:7, and so on. The prepared mixtures were mechanically shaken on an incubator shaker for 72 h at 298 ± 2 K. The maximum absorbance was measured for all solutions and the difference in absorbance of PIP in the presence and in absence of CDs was plotted against R.

$$R = \frac{[\text{Conc. PIP}]}{[\text{Conc. PIP}] + [\text{Conc. CD}]} \quad (1)$$

Where R = Mole fraction of solute.

Preparation of solid systems by lyophilization

Equimolar quantities of pure PIP (0.285 g) and both the CDs (βCD – 1.135 g and HPβCD – 1.4 g) with or without the addition of ternary components (0.25%w/v) were transferred to a beaker containing a mixture of 50 ml of methanol and 50 ml of distilled water. The prepared solutions were sonicated for 15 min and then allowed to stir for 72 h at 25±2°C with 150 rpm using a magnetic stirrer (REMI-CIS 24 plus Incubator Shaker, Mumbai, India). The resulting mixtures were filtered and placed in a deep freezer (ELCOLD, Denmark) at −80°C for 24 h. After deep freezing, the obtained solutions were lyophilized (DELVAC-Mini Lyodel, Chennai, India) at −80°C for 4 days. The received solid state complexes were kept in desiccators to avoid it from moisture absorption.

DSC

DSC examination of all samples was assessed using a DSC analyzer (TA Instruments, SDT Q600 USA). Five milligram of sample was taken within a closed aluminum crucible and placed into a temperature controlled DSC cell. The temperature range was measured from 0 to 200°C under a nitrogen atmosphere (flow rate 100 ml/min) at a heating rate of 10/min.[21]

XRPD

XRPD investigation of pure PIP, both the CDs and lyophilized complexes were carried out using X-ray diffractometer (BRUKER – D2 PHA-SER, Germany) with tube anode Cu over the interval 10–90°/2θ. The operational data were as: Generator tension (voltage) 30 kV, generator current 10 mA, and scanning speed 2°/min.

Percentage of drug content and saturation solubility studies

The percentage drug content was determined by dissolving binary and/or ternary lyophilized complexes equivalent to 5 mg of PIP in 50 ml of distilled water. The solutions were filtered through Whatman filter paper no. 41, appropriately diluted if necessary and examined spectrophotometrically at 341 nm (Shimadzu ultraviolet (UV)-visible Spectrophotometer 1800, Japan).

The saturation solubility studies were performed in triplicate. The excess amount of PIP and lyophilized inclusion complexes was placed in solubility tubes containing 10 ml of distilled water and allowed to shake for 24 h at 25°C in an incubator shaker (REMI-CIS 24 plus Incubator Shaker, Mumbai, India). After equilibration, samples were withdrawn, filtered through Whatman filter paper no. 41, and analyzed by a UV spectrophotometer (Shimadzu UV-visible Spectrophotometer 1800, Japan) at 341 nm. The results of saturation solubility studies were statistically analyzed by ANOVA (Instate GraphPad software Inc. Version 3.05).

In-vitro dissolution studies

In-vitro dissolution studies of samples were performed in 0.1N HCl (ELECTROLAB–TST-06L/LX, New Mumbai, India) by paddle method.[22,23] Ten milligrams of PIP or its equivalent amount of the complexes were placed in a dissolution vessel containing 900 mL of 0.1N HCl maintained at 37 ± 0.5°C at 50 rpm. Five milliliters of samples were withdrawn at 2, 5, 10, 15, 30, 45, and 60 min time intervals. The volume of dissolution media was adjusted to 900 ml by replacing each 5 ml aliquot withdrawn with 5 ml of fresh 0.1 N HCl. The filtered solutions were analyzed spectrophotometrically at 342 nm.

In-vitro antioxidant activity

1,1-diphenyl-2–picryl–hydrazyl/2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was performed to study *in-vitro* antioxidant activity.[24] The DPPH solution (0.1 mM) in methanol and inclusion complex solutions (100 µg/ml) in distilled water were prepared. Both the solutions were mixed in various concentrations of inclusion complexes and kept in darkness. Thirty minutes later, the absorbance was measured spectrophotometrically (Shimadzu 1800, Japan) at 517 nm. Blank was prepared without the addition of the drug. AA was used as standard and the capability to scavenge the DPPH radical was calculated by the following equation,

$$\text{DPPH Scavenged}\ (\%) = \frac{A_{control} - A_{Test}}{A_{control}} \times 10 \qquad (2)$$

$A_{Control}$ is the absorbance of the control reaction and A_{Test} is the absorbance of the complex. The IC_{50} value was also determined to indicate antioxidant activity. This is the concentration of fractions that inhibits the formation of DPPH radicals by 50%. The results of antioxidant activities were statistically analyzed by ANOVA (Instate GraphPad software Inc. Version 3.05).

In-vivo anti-inflammatory studies

In-vivo anti-inflammatory studies were approved by the Institutional Animal Ethical Committee (the Protocol No. APTRF/RP-29/1718 dated 08/08/2017) for APT Testing and Research Pvt. Ltd. Pune, India and executed as per CPCSEA (Committee for the purpose of control and supervision of experiments on animals) guidelines. Healthy Wistar male rats weighing 150–180 g were used in the present study. Three rats per cage were housed in polypropylene cages with a stainless steel grill top for the facilities of food, water bottle, and bending of clean paddy husk. The pelleted feed and potable water passed through the "Aquaguard" water filter were provided to the animals. The room temperature maintained between 22 ± 3°C, relative humidity 50–60%, and illumination cycle set to 12 h light and 12 h dark.

The test samples and standard drug (indomethacin 10 mg/kg) were administered orally to the respective group of rats. After 15 min of dosing, animals were given a subplantar injection of 0.1 ml of 1% carrageenan in the right paw. The paw volume of all rats was measured at 0, 1, 2, 4, and 6 h by Vernier Caliper. The % inhibition of inflammation was calculated using the following formula,

$$\%\ \text{Inhibition} = \frac{C - T}{C} \times 100 \qquad (3)$$

Where "C" represents mean edema in control and "T" represents mean edema in the group treated with the standard drug and test drug.[25] The statistical analysis as performed using ANOVA followed by Dunnett's *t*-test and expressed as mean±standard error mean.

Stability studies

The pure PIP, βCD, HPβCD, and lyophilized complexes (PBA: PIP+βCD+AA and PHA: PIP+HPβCD+AA) were subjected to the accelerated stability study for 6 months as per ICH guidelines (Q1A) at 40±2°/75±5% RH for up to 6 months.[26] Samples were put in glass bottles and kept in a stability chamber (Remi SC-19 *plus* Stability Chamber, India). Periodically (initial, after 3, and 6 months) samples were removed and characterized for DSC, XRD, and dissolution analysis as per the procedure described in DSC, XRD, and *in-vitro* dissolution studies sections.

RESULTS AND DISCUSSION

Job's plot

The Job's plot obtained by plotting absorbance of the drug against the mole fraction of the drug interacting with CDs [Figure 2] which displayed the maximum peak at 0.5 mole fraction. The drug absorbance increased with increase in PIP concentration up to the molar ratio 1:1 and then decreased, indicating the formation of inclusion complexation at 1:1 stoichiometric ratio, this is the strongest evidence for the previous phase solubility results.[27]

DSC

DSC investigation technique has stood out to examine the connections among drugs and CDs during the complex formation. When drug molecules are embedded into the CD cavity, their melting points usually shift to a different temperature or disappear.[28,29]

The DSC curves of PIP [Figure 3A], CA [Figure 3D] and AA [Figure 3E], exhibited sharp melting endotherms at 134°, 162°, and 195°, respectively, indicating their crystalline nature. The appearance of broad endotherm at 120° in the DSC curve of βCD [Figure 3B] was attributed to the dehydration process from the βCD cavity. The binary [Figure 3F] and ternary (PBC – Figure 3H, PBA – Figure 3J) complexes showed the appearance of broad endothermic peaks at 81°, 85°, and 88°, respectively, revealing water loss and disappearance of sharp melting endotherm of PIP ensuring entrapment of PIP in to βCD cavity with replacement of water molecule.

The DSC graph of HPβCD [Figure 3C] represented broad endotherm at 61.88° due to the dehydration process and absence of melting endotherm indicated the amorphous phase of HPβCD. In case of HPβCD binary (PH-Figure 3G) and ternary (PHC-Figure 3I, PHA-Figure 3K) systems, sharp PIP endotherm was disappeared, indicating a strong physical

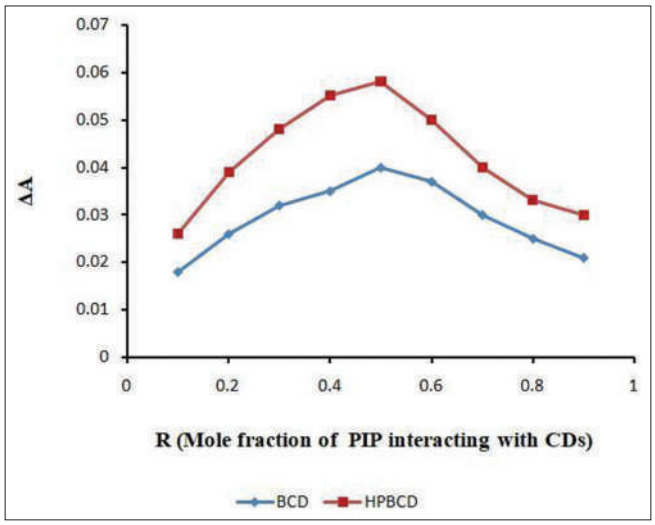

Figure 2: Job's plot for confirmation of stoichiometry of inclusion complexation

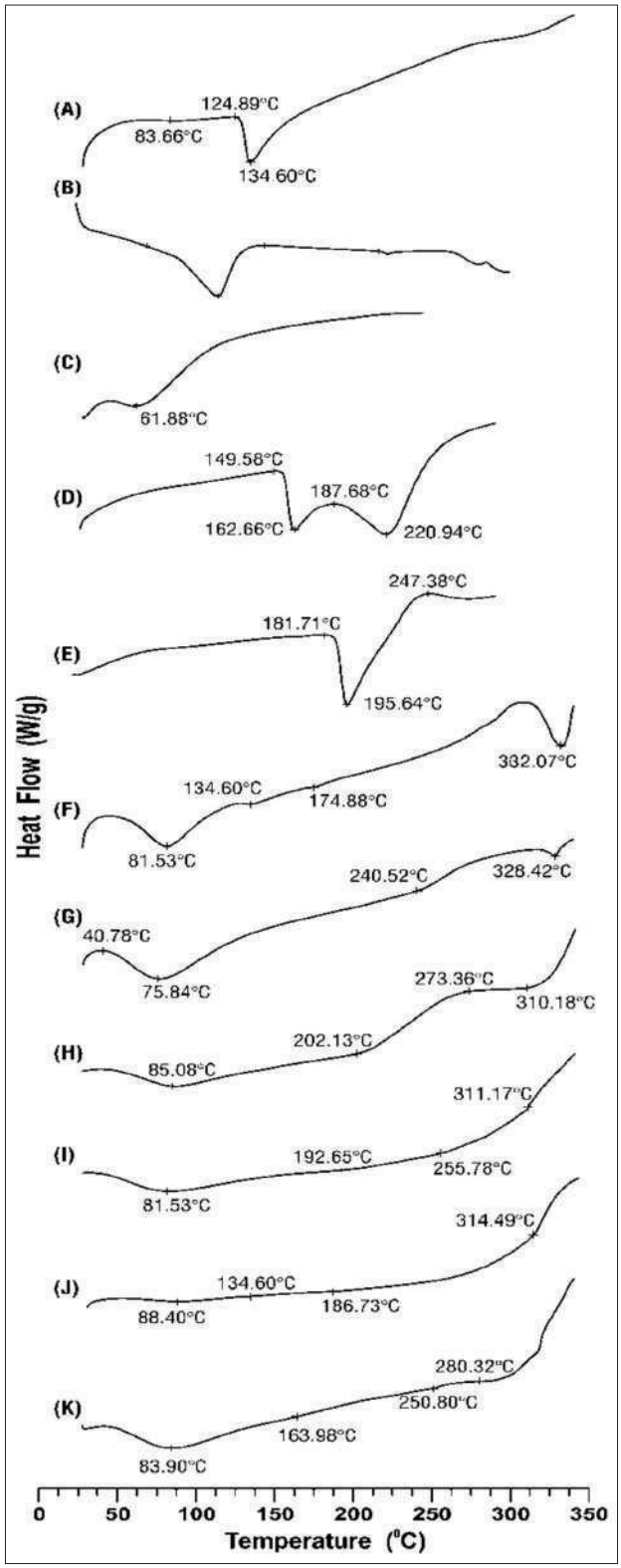

Figure 3: Differential scanning calorimetry thermogram of piperine (PIP) (A), β-cyclodextrins (CD) (B), hydroxypropyl β-CD (HPβCD) (C), citric acid (CA) (D), AA (E), PB (F), PH (G), PBC (H), PHC (I), PBA (J), PHA (K); PB: PIP + βCD; PH: PIP + HPβCD; PBC: PIP + βCD + CA; PHC: PIP + HPβCD + CA; PBA: PIP + βCD + AA; PHA: PIP + HPβCD + AA

interaction between PIP and HPβCD which resulted in stable inclusion complexation in solid state.[30] Similar findings have been reported by Teixeira *et al*. in 2013,[16] where HPβCD has been utilized to form inclusion complexes with black pepper oleoresin.

XRPD

XRPD is an extremely helpful strategy to relegate the physical condition of the drug and complexes by examining their respective diffractograms. XRPD diffractograms of all samples are displayed in Figure 3. The diffractogram of pure PIP [Figure 4A] indicated its crystalline nature at 14.61, 14.63, 14.65, 14.67, and 14.69 (2θ) with peak intensities 1102, 1077, 1110, 1108, and 1213, respectively. The sharp peak intensities were also observed for βCD [Figure 4B], CA [Figure 4D], and AA [Figure 4E] revealing their crystalline nature, where a typical halo pattern was recorded for HPβCD [Figure 4C] with its amorphous nature.

In the case of βCD binary PB [Figure 4F] and ternary PBC [Figure 4H], PBA [Figure 4J], systems these peaks were observed to be suppressed indicating the loss of crystallinity of the drug. However, some crystalline traces were still observable which might be due to crystalline natured βCD, CA, and AA. Whereas, for HPβCD binary PH [Figure 4G] and ternary PHC [Figure 4I], PHA [Figure 4K] systems, these peaks were observed to be completely diffused or disappeared, endorsing amorphization of PIP achieved by lyophilization technique[31] and formation of inclusion complexation with CDs, which was further contributed for improvement in physicochemical properties of PIP.[21,32]

Percentage of drug content and saturation solubility studies

The percentage drug content for PB (PIP+βCD) was found to be 24±1% (w/w), while for PBC (PIP+βCD+CA), PBA (PIP+βCD+AA), PH (PIP+HPβCD), PHC (PIP+HPβCD+CA), and PHA (PIP+HPβCD+AA), complexes were found to be 36±1% (w/w), 38±2% (w/w), 34±2% (w/w), 51±3% (w/w), and 51±1% (w/w), respectively, which indicated significant improvement in complexation efficiency of ternary systems as compared to that of binary systems in case of both CDs due to incorporation of CA and AA as a ternary components.

The saturation solubility data of prepared lyophilized inclusion complexes are displayed in Table 1 which illustrates remarkable improvement in solubility as compared to pure PIP ($P < 0.001$). From the observed findings, it could be concluded that the addition of hydroxy acids as ternary components resulted in improvement in complexation efficiency as compared to binary complexes which further gave betterment in hydrophilicity of PIP.[33]

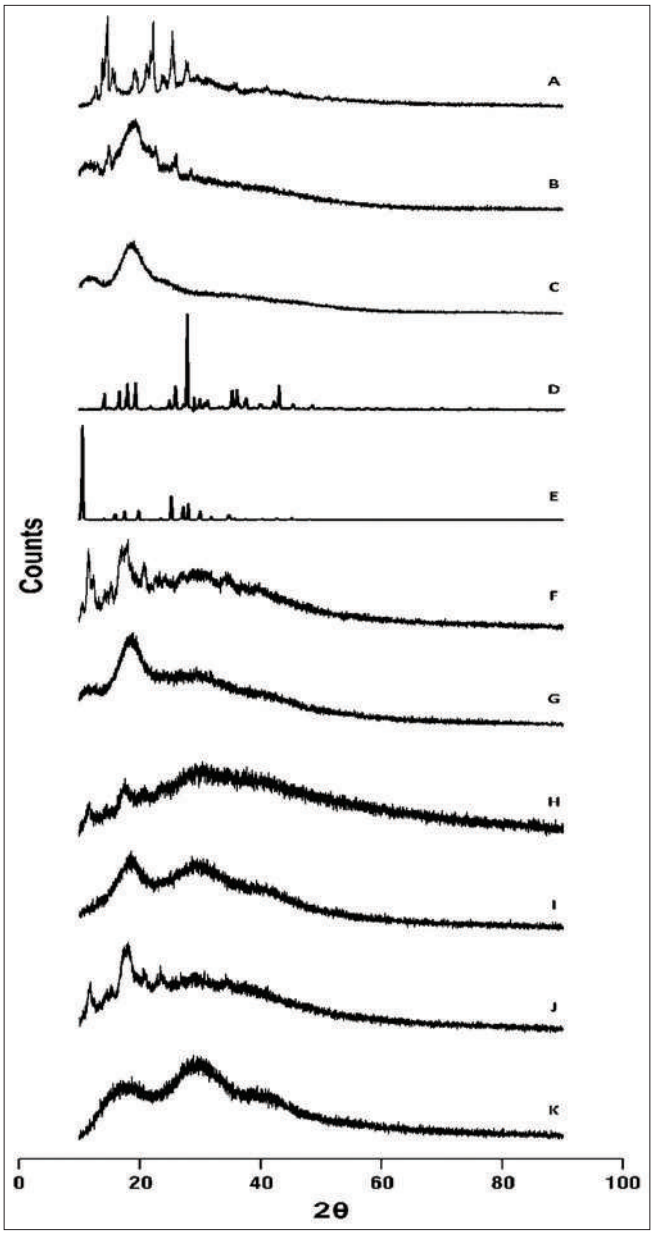

Figure 4: X-ray powder diffractometry diffractogram of piperine (PIP) (A), β-cyclodextrins (CD) (B), hydroxypropyl β-CD (HPβCD) (C), citric acid (CA) (D), AA (E), PB (F), PH (G), PBC (H), PHC (I), PBA (J), PHA (K); PB: PIP + βCD; PH: PIP + HPβCD; PBC: PIP + βCD + CA; PHC: PIP + HPβCD + CA; PBA: PIP + βCD + AA; PHA: PIP + HPβCD + AA

In-Vitro dissolution studies in 0.1N HCl at pH 1.1

The *in-vitro* drug release profile in 0.1N HCl at pH 1.1 is illustrated in Figure 5, where the percentage of drug dissolved is plotted against time. The % drug release at 10 min (DR_{10}) for PIP, PB, PH, PBC, PHC, PBA, and PHA was found to be 7 ± 1, 22 ± 3, 13 ± 2, 18 ± 2, 12 ± 2, 22 ± 2, and 100 ± 5, respectively, and dissolution efficiencies of the same at 10 min (DE_{10}) were calculated as 3 ± 0.8, 8 ± 1, 7 ± 1, 9 ± 2, 6 ± 0.3, 9 ± 1, and 38 ± 4, respectively [Table 2]. A significant difference between the dissolution efficiencies of PIP and

complexes ($P < 0.001$) was observed. From the findings, it was noticed that the drug release rate was rapid and complete from HPBCD binary (PH) as well as ternary systems (PHC, PHA) indicating HPβCD performed well as complexing and solubilizing agent.[32] The PHA complexes have shown 100% drug release at 10 min time interval suggesting addition of AA as a ternary component resulted in improved stability (phase solubility studies) and complexing property.

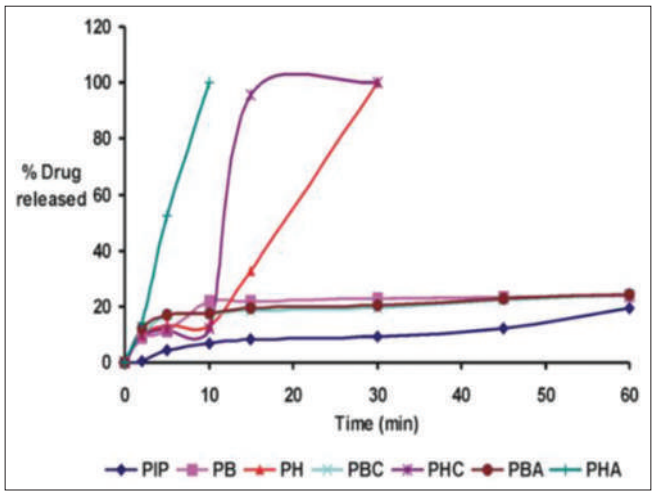

Figure 5: Dissolution profile of piperine (PIP) and all inclusion complexes in 0.1N HCl at pH 1.1; PB: PIP + β-cyclodextrins (CD); PH: PIP + hydroxypropyl β-CD (HPβCD); PBC: PIP + βCD + citric acid (CA); PHC: PIP + HPβCD + CA; PBA: PIP + βCD + AA; PHA: PIP + HPβCD + AA

Table 1: Saturation solubility of all systems

System	Saturation solubility* (μg/ml)	Fold increase in solubility
PIP	0.0079±1	-
PB	0.0523±1[a]	6.5
PBC	0.075±1.2[a]	9.4
PBA	0.117±1.2[a]	14.8
PH	3.3±1[a]	417
PHC	4.24±2.2[a]	537
PHA	6.44±2.4[a]	815

PB: PIP + βCD; PH: PIP + HPβCD; PBC: PIP + βCD + CA; PHC: PIP + HPβCD + CA; PBA: PIP + βCD + AA; PHA: PIP + HPβCD + AA; * mean±D (n=3); Standard deviation; [a]P value compared to pure PIP (P<0.0001). PIP: Piperine, CD: Cyclodextrin, HPβCD: Hydroxypropyl β-CD, CA: Citric acid

In-vitro antioxidant studies

In-vitro antioxidant activities of pure PIP and all lyophilized complexes are shown in Table 2. Accordingly, PBA, PHC, and PHA complexes exhibited better antioxidant activity than pure drug and other complexes [34]. Similar results have been observed by Jullian *et al*. (2007) and Teixeira *et al*., where formation of inclusion complexes improved antioxidant activity of encapsulated moiety.[35,16] The IC_{50} value is a guide to antioxidant values, explicating the addition of AA as a ternary component gave synergistic contribution for insightful effect on antioxidant activity (P <0.001). It could be reported that the inclusion complexation of PIP with CDs in the presence of AA as a ternary component might have reduced the chances of its auto-oxidation.

In-vivo anti-inflammatory activities

All data relevant to *in-vivo* anti-inflammatory activities are displayed in Table 3 and Figure 6. Pure PIP has shown delayed anti-inflammatory action which was at 4 h due to poor absorption. However, PBA complexes indicated positive % inhibition at 2 h due to betterment in hydrophilicity by inclusion with βCD. The PHA complexes have shown immediate and maximum onset of action at 0.5 h revealing faster absorption of PIP, due to wetting property of CDs, increased complexation efficiency by addition of AA as ternary component and formation of inclusion complexes.

In general, the PIP shows anti-inflammatory activity by inhibiting the expression of interleukin–6, matrix metallopeptidase-13, and reducing the production of

Table 2: *In-vitro* antioxidant activities of all systems

System	% antioxidant activity of all systems at various concentrations (μg/ml)[a]						IC_{50} (μg/ml)
	1	2	4	8	16	20	
PIP	18±1	29±1	34±2	37±3	40±1	43±2	24±1
Standard	19±1	25±2	34±2	45±2	58±3	76 ±2	10 ±1[b]
PB	35±2	36±2	38±1	39±1	41±2	43 ±1	37 ±1[b]
PH	28±2	34±2	38±3	41±3	41±1	41 ±2	31 ±2[b]
PBC	35±3	36±2	38±3	39±2	41±2	43 ±2	37±2[b]
PHC	37±2	40±1	40±2	49±3	49±3	52 ±2	15±1[b]
PBA	35±3	37±1	40±2	44±2	45±2	50 ±2	15±2[b]
PHA	38±3	42±1	48±2	51±2	51±2	60 ±3	9±1[b]

PB: PIP + βCD; PH: PIP + HPβCD; PBC: PIP + βCD + CA; PHC: PIP + HPβCD + CA; PBA: PIP + βCD + AA; PHA: PIP + HPβCD + AA; [a]Indicates mean±S.D. (n=3); [b]Significant difference compared to IC_{50} value of PIP (P<0.001). PIP: Piperine, CD: Cyclodextrin, HPβCD: Hydroxypropyl β-CD, CA: Citric acid

Table 3: *In-vivo* anti-inflammatory activity in carrageenan-induced rat paw edema model

Time (h)	% inhibition*				
	DC	STD	PIP	PBA**	PHA**
0	−62±12	−49±12	−58±9	−57±16	−64±9
0.5	−68±3	−49±7	−60±3	−61±5	68±1
1	−67±4	47±10	−62±4	−60±2	68±2
2	−67±2	49±3	−56±2	58±4	72±8
4	−61±4	45±5	57±7	55±4	70±4
6	−56±3	44±4	55±2	55±2	70±4

*Mean±SD (n=6); (**) sign indicates non-significant difference compared to pure PIP (P>0.05); (−) sign indicates increase in paw edema; (+) sign indicates decrease in paw edema; SD: Standard deviation; DC: Rats received carrageenan; STD: Rats received carrageenan and indomethacin; PIP: Rats received carrageenan and PIP; PBA: Rats received carrageenan and βCD ternary complexes of piperine with AA; PHA: Rats received carrageenan and HPβCD ternary complexes of piperine with ascorbic acid. PIP: Piperine, CD: Cyclodextrin, HPβCD: Hydroxypropyl β-CD, AA: Ascorbic acid

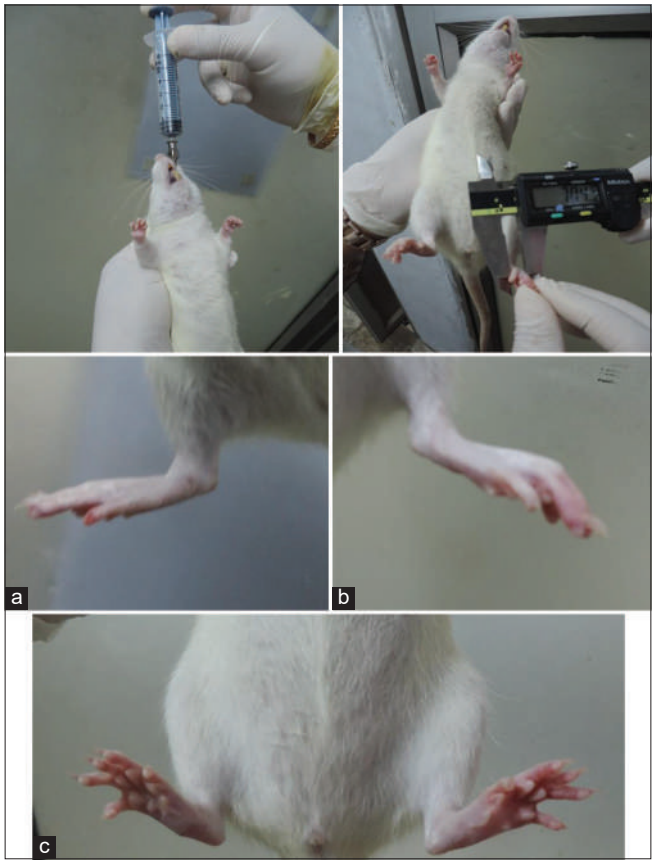

Figure 6: Visual representation of *in-vivo* anti-inflammatory study (a) normal paw (b) inflamed paw (c) difference in paw

Prostaglandin E2.[25,36] Furthermore, it inhibits LPS-induced expression and production of inflammatory factors.[37] The inclusion complexation of PIP increases its aqueous solubility and might be useful for enhancing the stability through the gastrointestinal tract which increases the drug concentration at the site of action, further resulted in the enhancement of anti-inflammatory activity.

Stability studies

Stability studies – DSC

DSC assessment was performed to have a look at the thermal behavior of natural PIP in addition to lyophilized inclusion complexes at distinct time durations (initial, 3, and 6 months). The thermograms of all samples subjected to accelerated stability study are displayed in Figure 7.

The shape of the melting endotherm of PIP [Figure 7I] was observed to be intact all throughout the ageing period. The DSC thermogram of βCD [Figure 7aII] demonstrated broad endotherm at 120°C at initial stage indicating presence of crystalline phase which continued as it was during the entire ageing period [Figure 7bII and cII].

At the time of aging, the form of HPβCD thermogram [Figure 7III] was found to be modified. During beginning stage [Figure 7a.III], the wide endotherm of HPβCD (due to loss of water molecule) was seen to be at 61°C. During aging period, it was shifted to at higher temperatures, that is, 88°C [Figure 7b.III] and 77°C [Figure 7c.III], respectively, due to moisture absorption.

The βCD ternary complexes, PBA [Figure 7a.IV], showed broad endotherm during initial time. This indicated entrapment of PIP by loss of water molecules, which continued up to 6 months [Figure 7b.IV and c.IV]. Ternary component (AA) gave stability to the system and had performed its role consummately.

The PIP complexes with HPβCD demonstrated lower endotherm at all time periods [Figure 7v] as compared to natural PIP and other complexes. This was due to dehydration and entrapment of drug molecules into the CD cavity, resulting in the formation of inclusion complexes. The presence of dehydration endotherm and disappearance of PIP melting endotherm clearly ascribed for amorphous nature of PHA complexes. After 6 months, we could not collect PHA sample due to glassy nature of the complexes, which might be because of moisture absorption.[38]

Stability studies – XRPD

XRPD diffractograms of all systems which were subjected to stability studies are displayed in Figure 8. Pure PIP [Figure 8.I] and βCD [Figure 8.II] with sharp peak intensities recommended their crystalline nature, whereas halo pattern of diffractogram of HPβCD indicated its amorphous nature [Figure 8.III]. The βCD ternary complexes [Figure 8. IV] indicated diffused peak intensities and traces of some crystallinity of βCD and AA throughout the 6 months period. In the case of HPβCD ternary [Figure 8.V] system, a

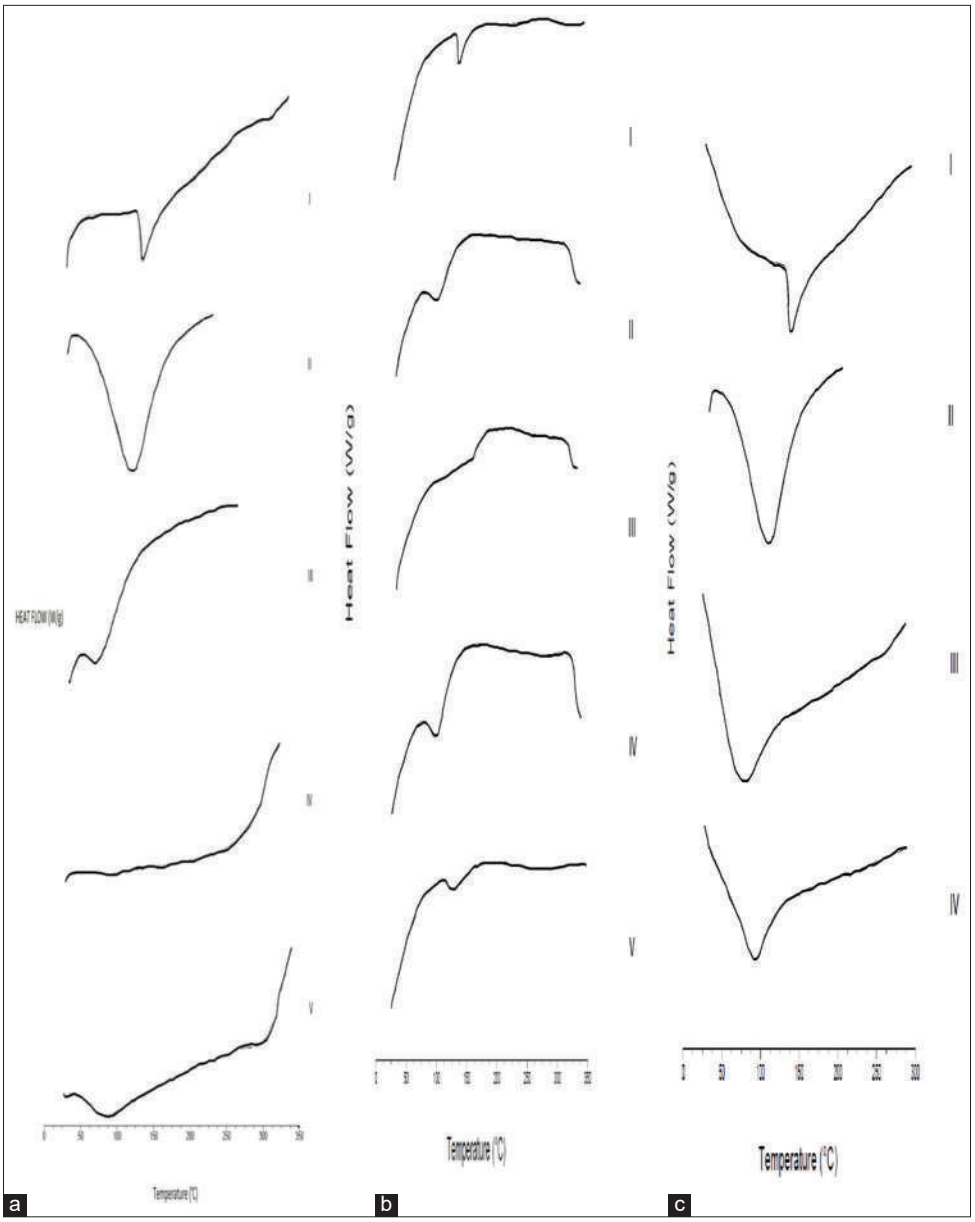

Figure 7: Differential scanning calorimetry (DSC) thermograms of all aged samples. (a) DSC thermograms of all systems at initial stage. (b) DSC thermograms of all systems after 3 months. (c) DSC thermograms of all systems after 6 months. Where, piperine (I), β-cyclodextrins (CD) (II), hydroxypropyl β-CD (III), PBA (IV), PHA (V)

similar halo pattern was observed up to 3 months as of initial characterization [Figure 8a.V]. These halo patterns endorsed the transformation of crystalline PIP to its amorphous form.

Stability studies – In-vitro dissolution Studies

Although PIP has shown up to 25% release at 60 min after 6 month, it illustrated that pure drug has not undergone any change in the content of API, as far as its stability concerned it remained intact throughout the 6 months period [Figure 9a-c]. In case of PBA complexes, no drastic change in the dissolution profile was to be observed. For HPβCD ternary complexes (PHA), up to 3 months, similar dissolution profiles were observed as that of initial time [Figure 9a].

From observed findings, it could be concluded that no significant changes were seen in DSC, XRD and dissolution patterns of PIP and βCD ternary complexes (PBA) up to 6 months. As like βCD complexes, HPβCD complexes of PIP (PHA) were also stable up to 3 months. After 6 months, it was difficult to collect the sample of PHA as it was found in a glassy state, which might be because of moisture absorption by complexes (plasticizer effect by water vapors) and enthalpy relaxation process (material encounters gradual loss of energy in terms of enthalpy)[39] of amorphous HPβCD. These complexes have stable up to 3 months and plausibly all up to 6 months by addition of some antiplastisizers (polyvinyl pyrrolidone, hydroxypropyl methylcellulose, polyethylene glycol, etc.) to the complexes and by suppressing the rate of enthalpy relaxation.[38,39]

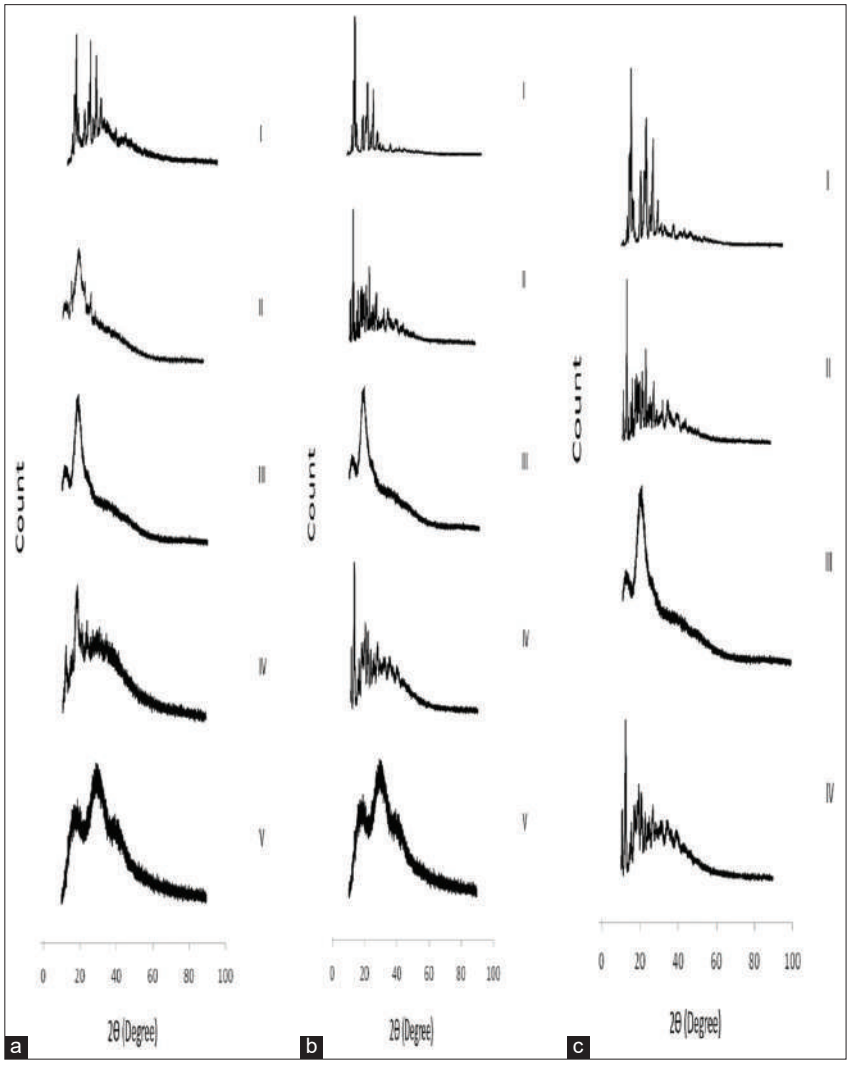

Figure 8: X-ray powder diffractometry (XRPD) diffractograms of aged samples. (a) XRPD diffractograms of all systems at initial time. (b) XRPD diffractograms of all systems after 3 month. (c) XRPD diffractograms of all systems after 6 months. Where, piperine (I), β- cyclodextrins (CD) (II), hydroxypropyl β-CD (III), PBA (IV), PHA (V)

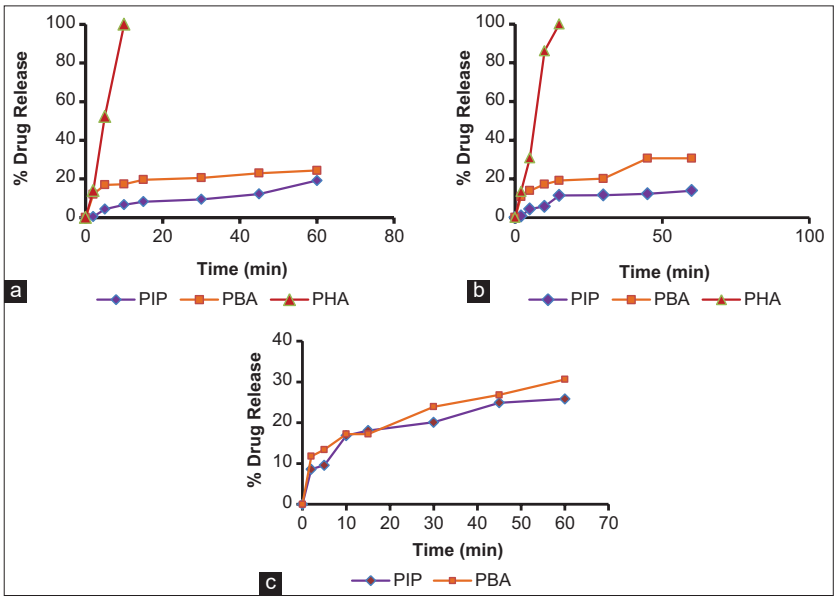

Figure 9: Drug release profiles of aged samples. (a) Drug release profile of all systems at initial stage. (b) Drug release profile of all systems after 3 months. (c) Drug release profile of all systems after 6 months

CONCLUSION

The present work exhibited successful formation of inclusion complexes of PIP with βCD and HPβCD in presence and/or absence of CA and AA by lyophilization technique, resulting in remarkable improvement in biological properties of complexed PIP than native one. The characterization studies confirmed the formation of inclusion complexes. HPβCD complexes prepared with AA gave incredible improvement in *in-vitro* antioxidant and *in-vivo* anti-inflammatory activities. Stability studies concluded that lyophilized inclusion complexes of PIP with both CDs were stable up to 3 months. Nonetheless, *in vivo* pharmacokinetic studies of supramolecular complexes of PIP with CDs may be necessarily conducted in future for further formulation development.

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REFERENCES

1. Ficarra R, Ficarra P, Bella MR, Raneri D, Tommasini S, Calabrò ML, *et al*. Study of the inclusion complex of atenolol with β-cyclodextrins. J Pharm Biomed Anal 2000;23:231-6.
2. Zingone G, Rubessa F. Preformulation study of the inclusion complex warfarin-β-cyclodextrin. Int J Pharm 2005;291:3-10.
3. Hebeish A, Aly A, Shafei A, Zaghloul S. Innovative starch derivatives as textile auxiliaries for application in sizing, finishing and flocculation. Starch-Starke 2008;60:97-109.
4. Praveena A, Prabu S, Rajamohan R. Encapsulation of quercetin in β-cyclodextrin and (2-hydroxypropyl)-β-cyclodextrin cavity: *In-vitro* cytotoxic evaluation. J Macromol Sci A 2017;54:894-907.
5. Aree T. β-Cyclodextrin inclusion complexation with tricyclic antidepressants desipramine and imipramine: A structural chemistry perspective. J Pharm Sci 2020;109:2086-3094.
6. Jo G, Lee BY, Kim EJ, Park MH, Hyun H. Indocyanine green and methyl-β-cyclodextrin complex for enhanced photothermal cancer therapy. Biomedicines 2020;8:476-84.
7. Young J. Citric acid chemical laboratory information profile. J Chem Ed 2003;80:480.
8. Pokharkar V, Khanna V, Venkatpurwar V, Dhar S, Mandpe L. Ternary complexation of carvedilol, β-cyclodextrin and citric acid for mouth dissolving tablet formulation. Acta Pharm 2009;59:121-32.
9. Higuchi T, Connor K. Phase-solubility techniques. Adv Anal Chem Instr 1965;4:117-212.
10. Brewster M, Loftsson T. Cyclodextrins as pharmaceutical solubilizer. Adv Drug Deliv Rev 2007;59:645-66
11. Ezawa T, Inoue Y, Tunvichien S, Suzuki R, Kanamoto I. Changes in the physicochemical properties of piperine/*β*--Cyclodextrin due to the formation of inclusion complexes. Int J Med Chem 2016;2016:8723139.
12. Acharya S, Momin A, Gajjar A. Review of piperine as a bio-enhancer. Am J PharmTech Res 2012;2:32-44.
13. Vijayakumar R, Nalini N. Piperine, an active principle from *Piper nigrum*, modulates hormonal and apolipoprotein profiles in hyperlipidemic rats. J Basic Clin Physiol Pharmacol 2006;17:71-86.
14. Kumar S, Singhal V, Roshan R, Sharma A, Rembhotkar G, Ghosh B. Piperine inhibits TNF-α induced adhesion of neutrophils to endothelial monolayer through suppression of NF-κB and IκB kinase activation. Eur J Pharmacol 2007;575:177-86.
15. Ezawa T, Inoue Y, Murata I, Takao K, Sugita Y, Kanamoto I. Characterization of the dissolution behavior of piperine/cyclodextrins inclusion complexes. AAPS Pharm Sci Tech 2018;19:923-33.
16. Teixeira B, Ozdemir N, Hill L, Gomes C. Synthesis and characterization of nano- encapsulated black pepper oleoresin using hydroxypropyl beta-cyclodextrin for antioxidant and antimicrobial applications. J Food Sci 2013;78:1913-20.
17. Quilaqueo M, Millao S, Luzardo-Ocampo I, Campos-Vega R, Acevedo F, Shene C, *et al.* Inclusion of piperine in β-cyclodextrin complexes improves their bioaccessibility and *in vitro* antioxidant capacity. Food Hydrocolloids 2019;91:143-52.
18. Ol'khovich M, Sharapova A, Lavrenow S, Blokhina S, Perlovich G. Inclusion complexes of hydroxyl propyl β-cyclodextrin with novel cytotoxic compounds; solubility and thermodynamic properties. Fluid Phase Equilib 2014;384:68-72.
19. Desai S, Poddar A, Sawant K. Formulation of inclusion complex-based orally disintegrating tablet of eslicarbazepine acetate for improved oral bioavailability. Mater Sci Eng C 2016;58:826-34.
20. Patro N, Sultana A, Terao K, Nakata D, Jo A, Urano A, *et al*. Comparison and correlation of *in vitro*, *in vivo* and *in silico* evaluations of alpha, beta and gamma cyclodextrin complexes of curcumin. J Incl Phenom Macro Chem 2014;78:471-83.
21. Jadhav P, Petkar B, Pore Y, Kulkarni A, Burade K. Physicochemical and molecular modeling studies of cefixime-l-arginine-cyclodextrin ternary inclusion compounds. Carbohydr Polym 2013;98:1317-25.

22. Shao B, Cui C, Ji H, Tang J, Wang Z, Liu H, *et al*. Enhanced oral bioavailability of piperine by self emulsifying drug delivery systems: *In vitro*, *in vivo* and *in situ* intestinal permeability studies. Drug Deliv 2015;22:240-7.
23. Singh D, Garg M, Sharma H. Development and evaluation of standardized solid dosage formulations of Trikatu. Int J Adv Pharm Biol Chem 2014;3:250-5.
24. Killedar S, Mali S, More H, Nadaf S, Salunkhe S. Phytochemical screening and in vitro antioxidant potential of *Memecylon umbellatum* Burm leaf extracts. J Drug Deliv Ther 2014;4:30-5.
25. Mujumdar A, Dhuley J, Deshmukh V, Raman P, Naik S. Anti-inflammatory activity of piperine. Jpn J Med Sci Biol 1990;43:95-100.
26. ICH, Q1A, Stability Testing of New Drug Substances and Products Q1A(R2), International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Dated 6 February; 2003.
27. Talegaonkar S, Khan A, Khar R, Ahmad F, Khan Z. Development and characterization of paracetamol complexes with hydroxypropyl-β-cyclodextrin. Iran J Pharm Res 2007;6:95-9.
28. EI-Maradny H, Mortada S, Kamel O, Hikal A. Characterization of ternary complexes of meloxicam-HPβCD and PVP or l-arginine prepared by the spray drying technique. Acta Pharm 2008;58:455-66.
29. Liu X, Lin H, Thenmozhiyal J, Yung CS, Paul CH. Inclusion of acitretin in to cyclodextrins; Phase solubility, photostability, physicochemical characterization. J Pharm Sci 2003;92:2449-52.
30. Shinde V, Shelake M, Shetty S, Cahvan-Patil A, Pore Y, Late S. Enhanced solubility and dissolution rate of lamotrigine by inclusion complexation and solid dispersion technique. J Pharm Phamacol 2008;60:1121-9.
31. Shah M, Karekar P, Sancheti P, Vyas V, Pore Y. Effect of PVP K30 and/or l-arginine on stability constant of etoricoxib-HPβCD inclusion complex; preparation and characterization of etoricoxib–HPβCD binary system. Drug Dev Ind Pharm 2008;35:118-29.
32. Shah M, Pore Y, Dhawale S, Burade K, Kuchekar B. Physicochemical characterization of spray dried ternary micro-complexes of cefuroxime axetil with hydroxypropyl- β-cyclodextrin. J Incl Phenom Macrocycl Chem 2013;76:391-401.
33. Sapte S, Pore Y. Inclusion complexes of cefuroxime axetil with β-cyclodextrin: physicochemical characterization, molecular modeling and effect of l-arginine on complexation. J Pharm Anal 2016;6:300-9.
34. Swaroop B, Mishra I, Priyadarsini I. β-Cyclodextrin inclusion complexes of curcumin. BARC Newslett 2007;285:208-12.
35. Jullian C, Moyano L, Yanez C, Olea-Azar C. Complexation of quercetin with three kinds of cyclodextrins: An antioxidant study. Spectrochim Acta A Mol Biomol Spectrosc 2007;67:230-4.
36. Bang J, Oh D, Choi H, Sur B, Lim S, Kim J, *et al*. Anti inflammatory and anti-arthritic effect of piperine in human interleukin 1β-stimulated fibroblast-like synoviocytes and in rat arthritis models. Arthritis Res Ther 2009;11:1-9.
37. Wang B, Zhag Y, Huang J, Dong L, Tingting L, Xueyan F. Anti-inflammatory activity and chemical composition of dichloromethane extract from *Piper nigram* and *P. longum* on permanent focal cerebral ischemia injury in rats. Rev Bras Farmacogn 2017;27:369-74.
38. Ambike K, Mahadik KR, Paradkar A. Stability study of amorphous valdecoxib. Int J Pharm 2004;282:151-62.
39. Jondhale S, Bhise S, Pore Y. Physicochemical investigations and stability studies of amorphous gliclazide. AAPS Pharm Sci Tech 2012;13:448-59.

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