

# Crystal engineering of fisetin: a step towards improved biopharmaceutical parameters

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DOI: 10.25177/JFST.4.2.RA.461

Research

Received Date: 26<sup>th</sup> Jan 2019

Accepted Date: 20<sup>th</sup> Feb 2019

Published Date: 25<sup>th</sup> Feb 2019

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## CITATION

Renu Chadha et al., Crystal engineering of fisetin: a step towards improved biopharmaceutical parameters(2019) SDRP Journal of Food Science & Technology 4(2)

## ABSTRACT

**Background:** In the present study, crystal engineering strategy is employed to facilitate the supramolecular synthesis of three new crystalline forms of a potential flavonoidal molecule, Fisetin whose efficacy is hampered due to its low solubility. Fisetin cocrystals with GRAS status cofomers including glutaric acid, malic acid and theophylline are described herein. All the cofomers complement the phytoen with respect to hydrogen bonding.

**Method:** Mechanochemical grinding was utilized to prepare the cocrystals i.e., FGLU, FMAL and FTHY which were analyzed using DSC, FT-IR, PXRD and solid state NMR. The crystal structures determined using the PXRD pattern validated the existence of FGLU (triclinic crystal system ( $P_1$ )), FMAL (monoclinic  $P2_1/c$ ) and FTHY(triclinic crystal system ( $P_1$ )). In each of the crystal structures, intermolecular hydrogen-bonding motif involving the hydroxyl group(OH) of fisetin with the carbonyl group and the phenolic group of the cofomers was observed. The prepared cocrystals were further evaluated for their solubility, intrinsic dissolution and *in vivo/in vitro* profile.

**Results:** Solubility and dissolution studies of fisetin cocrystals were measured in aqueous buffer and demonstrated solubility improvement to be approximately 1.8-3.0 times higher as compared to the parent flavonoidal molecule, which subsequently led to improved pharmacokinetic parameters. Moreover, the antioxidant and antihemolytic effect of the cocrystals were found to be even high at low concentration when compared to fisetin molecule.

**Conclusion:** This report suggests cocrystallization as a viable approach to resolve the solubility and bioavailability issues that circumvent the use of a therapeutically potential isoflavone, fisetin.

**Keywords:** Fisetin, Cocrystals, DSC, FTIR, PXRD, Solid State NMR, Pharmacokinetics, Antioxidant, Antihemolytic and accelerated stability studies.

## 1.0 INTRODUCTION

The key objective of a novel drug development process is to synthesis a drug molecule with optimal physiochemical and biopharmaceutical parameters [1]. Solubility alarmingly influences the therapeutic utility of an API as it governs the dissolution rate and pharmacokinetic profile following better absorption in the gastrointestinal tract (GIT).

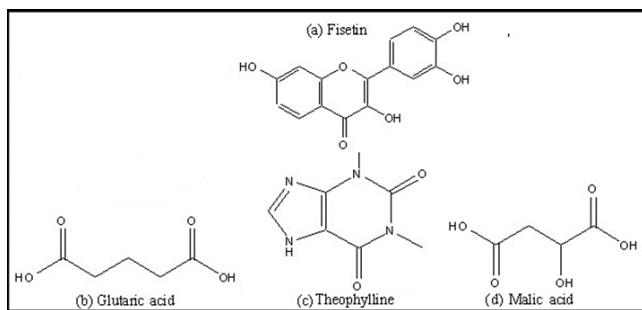
In the recent years the field of crystal engineering related to pharmaceutical industry has poised to address these two key issues and has matured into a paradigm for the supramolecular synthesis of new compounds with desired properties. The possible ways in which crystal engineering can modulate the physiochemical properties of molecular solids are by generation of various polymorphs, cocrystals, solvates and eutectics [2].

Cocrystals is a lucrative technique which has sparked the interest of many researchers in the crystal engineering field and is based on a concept of the supramolecular chemistry. Pharmaceutical cocrystals can be rationally designed to produce supermolecules with target structures and predetermined properties and has garnered the attention of pharmaceutical industry. The cocrystal comprises of a API and a GRAS status cofomer which aims to bring two different components into one crystal lattice via intermolecular force: hydrogen bonding,  $\pi$ - $\pi$  stacking, van der Waals forces, etc. [3]. Incorporation of a second constituents i.e., a cofomer in cocrystals is being utilized for more than a decade for manipulating the physiochemical properties of an API and has been a humdrum within the pharmaceutical industry.

Many patents have been registeted in the name of pharmaceutical cocrystals owing to their unique design and synthesis which involves various elements of non-obviousness. The increased patent portfolio of cocrystallization has made its way into the regulatory guidelines laid by US FDA which marks as a great success in the field of crystal engineering [4]. This manuscript reports the cocrystals of potential nutraceutical molecule, fisetin with various pharmaceutically acceptable cofomers.

Fisetin (FIS), 3,3',4',7-Tetrahydroxyflavone, is a flavonol that is present in many fruits, vegetables i.e., grapes, cucumbers, strawberries, onion, persimmons [5]. Recently, FIS has been reported to exhibit antiallergic [6], cardioprotective [7], antioxidant [8], anticancer [9], antihydroid [10], anti-inflammatory activity [11] and antimutagenic [12]. However, its extremely low aqueous solubility (<1 mg/mL) [13] has hampered its therapeutic benefits. FIS is a rather weak acid with a pKa of 7.42 and an unstable constituent in a basic environment [14], an appropriate salt former (a strong base) would exaggerate FIS' s instability whereas its cocrystals will improve the stability. So, cocrystallization will be a suitable method for improving the physicochemical properties of this bioactive molecule as it is concerned with the formation of a complex between neutral molecules [15]. Moreover, FIS has various competitive sites for hydrogen bonding i.e., donors and acceptors (four hydroxyls and one carbonyl), in its molecular skeleton, offering the possibility of cocrystal formation with the suitable cofomers with complementary functional groups. In this study, several pharmaceutically acceptable cofomers have been selected to prepare FIS cocrystals with an intension to improve its biopharmaceutical parameters.

Previous approaches for screening of solubilized forms of fisetin were based on empirical method like development of a nanoemulsion formation [16], liposomal formulation [17], complexation with a cyclophosphorus dimer [18]. As far as cocrystals are concerned three cocrystals of fisetin with nicotinamide, caffeine and isonicotinamide are reported however, the results are not supported by any biological evaluation [19,20]. In the present manuscript, an endeavor has been made to shed light on the development of few new cocrystals of fisetin (figure 1a) along with their pharmacokinetic analysis and evaluation for the improvement in biological activities including antioxidant activity and antihemolytic activity have also been performed. The three nontoxic cofomers, i.e., glutaric acid, theophylline, malic acid, utilized in this study have favorable aqueous solubility with intrinsic biological profile.



**Figure 1.** Chemical structures of FIS (a) and CCFs: THY (b), GLU (c), Mal (d).

Glutamic acid (figure 1b) is an FDA-approved API and is commonly used as a cocrystal coformer with high solubility[21]. Theophylline (figure 1c) (acts as a phosphodiesterase inhibitor, adenosine receptor blocker, and histone deacetylase activator) is a methylxanthine derivative obtained from tea possess diuretic, smooth muscle relaxant, bronchial dilation, cardiac and central nervous system stimulant activities[22]. The third coformer, Malic acid (figure 1d) is generally taken as a supplement and is an organic compound which is found in fruits (apple) and has been known for its therapeutic use to treat fibromyalgia and chronic fatigue.[23]

## 2.0 EXPERIMENTAL

### 2.1 Materials

The procurement of Fisetin ( $\geq 97\%$ , Alfa Aesar, England), glutamic acid ( $\geq 99\%$ , Sigma Aldrich, USA), Malic acid ( $\geq 98\%$ , HI-MEDIA, Mumbai, India), theophylline ( $\geq 98\%$ , HI-MEDIA, Mumbai, India) and ethanol ( $\geq 99\%$ , E.Merk Ltd, New Delhi, India) was done.

### 2.2 Designing of co-crystals

The first step for cocrystal synthesis is the design involving the analysis of the already existing associated crystal structures. This search is assisted by the Cambridge Structural Database (CSD) which is a reservoir of crystal structure. This search for already existing synthons of hydroxyl and carbonyl groups estimates the propensity of these groups present in fisetin molecule to form homo or hetero synthons with the functional complementary groups. The search was carried out using ConQuest software (version 1.7) which provides the data of pre-existing crystal struc-

tures in Cambridge Structural Database (CSD, version 5.36, 2014). A query for hydrogen bonding functional groups were prepared and explored for the hits. Based on the obtained result, suitable coformers were chosen to prepare the co-crystals.

### 2.3 Synthesis

The co-crystals of fisetin were prepared with the coformers: glutamic acid (GLU), theophylline (THY), malic acid (MAL) using solvent assisted grinding method. Fisetin (286 mg) and the corresponding coformers i.e., glutamic acid 132 mg, theophylline 180 mg and malic acid 134 mg, were combined in a 1:1 stoichiometric ratio in a mortar. The mixture was ground with a pestle for one hour with drop-wise addition of ethanol (6ml) with a micropipette, at room temperature, until dry. This procedure was continued till a dry mixture was obtained which were further stored in desiccators.

### 2.4 Identification and Characterization

#### 2.4.1 Differential scanning calorimetry (DSC)

DSC Q20 (TA Instruments, USA) was used to carry out DSC of all the samples. Samples were held in sealed aluminium pans under a dry nitrogen atmosphere (flow rate  $50 \text{ mL min}^{-1}$ ) which were further scanned at ramping rate of  $10 \text{ }^\circ\text{C min}^{-1}$ . The data was then collected using TA Q series Advantage software (Universal analysis 2000).

#### 2.4.2 Fourier Transform-Infra Red Spectroscopy (FTIR)

The FT-IR was performed in the KBr diffuse-reflectance mode (sample concentration 2 mg in 20 mg of KBr) using a Spectrum RX I FT-IR spectrometer (Perkin-Elmer, UK) in the range of  $4000\text{--}400 \text{ cm}^{-1}$ . The data was analyzed using spectrum software.

#### 2.4.3 Powder X-Ray Diffraction (PXRD)

X'Pert PRO diffractometer system (Panalytical, Netherlands) was utilized to analyze the PXRD patterns by means of a  $\text{Cu K}\alpha$  radiation ( $1.54060 \text{ \AA}$ ). The tube voltage and current was set at 45 kV and 40 mA respectively and the divergence slit and antiscattering slit were positioned at  $0.48^\circ$  while illumination on the 10-mm sample size. Analyzes of the PXRD pattern

was done at  $2\theta$  ranging from  $5^\circ$  to  $50^\circ$  (step size of  $0.017^\circ$ ). Pxd pattern obtained experimentally was refined by X'Pert High Score software.

#### 2.4.4 Solid State NMR (SSNMR)

Joel Resonance JNM-ECX400II Instrument from IISC, Bangalore, India was employed to obtain the solid state NMR of the prepared samples. The temperature was kept at 273 K with an acquisition time of 29.1s and polarization relaxation delay time was 5sec at 1024 complex data points.

#### 2.4.5 Crystal structure determination

Material Studio® software by BIOVIA system was employed to determine the structures of fisetin and its respective cocrystals. There were in all four steps starting with indexing of the peaks, Pawley fitting of the created cell, structure solution and lastly the Rietveld refinement. The indexing was done using X-cell for the peaks positioned at  $2\theta$  value ranging from  $5^\circ$ - $40^\circ$   $2\theta$  present in the PXRD pattern at position  $5^\circ$ - $40^\circ$   $2\theta$  and further a crystal unit cell was obtained. The unit cell was then refined using Pawley refinement to optimize cell parameters and lattice constants (minimum  $R_{wp}$ ). The molecular structures of FIS, GLU, THY and MAL were sketched using DMol3 module. The structures produced were geometrically optimized and were further imported inside the refined empty cell (having lowest  $R_{wp}$ ) and all of them were subjected to simulated annealing algorithm (10 cycles, 21000000 iterations in each cycle) using Powder Solve module.

#### 2.5 Equilibrium solubility studies

Equilibrium solubility study was performed at  $37^\circ\text{C}$  in water bath shaker (MSW-275) Macro Scientific Works, Delhi) at 200 rpm. An excess amount samples (approx. 50 mg) in 10 ml of phosphate buffer of pH 6.8 were kept for shaking for 24 hours. The obtained samples were then strained via a  $0.45\ \mu$  membrane filter which were further quantitatively analysed for fisetin using HPLC. At various time intervals (2 hrs and after 24 hrs) the samples were withdrawn. The FT-IR analysis was performed for the residual material.

#### 2.6 Intrinsic dissolution studies

Intrinsic dissolution study was carried out using rotating disk dissolution test apparatus, DS 8000 (Lab India Analyticals) at  $37^\circ\text{C}$  in phosphate buffer pH 6.8 with 150 rpm for 4 hours. Pellets of the pure drug and the respected cocrystals were prepared using hydraulic pellet press, and the pellet of the sample was held in the dissolution apparatus holder which was further immersed in dissolution media. The aliquot buffer (5 ml) was replaced with fresh buffer at multiple time points (15, 30, 45, 60, 90, 120, 180 and 240 min) and strained through  $0.45\ \mu\text{m}$  membrane filter. The quantitative analysis was carried out at 228 nm using Waters Alliance HPLC system (Photodiode Array Detector).

#### 2.7 High Performance Liquid Chromatography

Waters Alliance HPLC system which includes a Waters 2695 separation module, a Waters 2996 Photodiode Array Detector, and a  $4.6\ \text{mm} \times 150\ \text{mm}$  SunFire C18,  $5\ \mu\text{m}$  column (Waters Corporation, Milford, MA) was used for analysis. Stock solutions of the samples (fisetin and cocrystals) were prepared in phosphate buffer pH 6.8 to get various concentrations of calibration standards (10, 20, 30, 40, 50, 60, 70, 80, 90, 100  $\mu\text{g}/\text{ml}$ ).  $10\ \mu\text{l}$  of all the samples was injected in the column and analyzed by isocratic mobile phase a mixture of methanol and 0.1% ortho phosphoric acid (62:38) pumped at a flow rate of  $1.0\ \text{mL}/\text{min}$  through the column at a temperature of  $35^\circ\text{C}$ . The detector wavelength was set at 355 nm. Data acquisition and analysis were carried out using software Empower 2.0. The retention time of fisetin was determined to be 4.43 mins.

#### 2.8 Biological Studies.

##### 2.8.1 Antioxidant Activity

The scavenging activity for DPPH (2, 2' diphenyl-1-picrylhydrazyl) free radicals of fisetin and its cocrystals was performed as per the procedure demonstrated by Blis (1958) with appropriate modifications [24]. By dissolving 3.94mg (0.1 mM) DPPH in 100 ml of 50:50 methanol and water stock solution was obtained. The working solution was prepared by adding 1 ml stock solution in 1ml of cocrystal with different concentrations (1, 2, 3, 4, 5, 6, 7, 8, 10  $\mu\text{g}/\text{ml}$ ) and the

volume was made upto 5ml using methanol. Further, absorbance was recorded at 517 nm utilizing the spectrophotometer (UV-Visible EZ201, Perkin Elmer, USA). Fisetin and its cocrystals were kept aside in a dark place for 45 mins so that they react with DPPH solution. Standard control used in this experiment was Ascorbic acid due to its known antioxidant activity. The obtained absorbance was converted into the percentage antioxidant activity utilizing the following equation:

$$\% \text{Radical scavenging activity} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

### 2.8.2 Antihemolytic activity

The antihemolytic activity was performed on rat red blood cells (RBC). The RBC's were collected in centrifuge tubes having an equal volume of solution, this solution was further centrifuged at 3000 rpm at 4°C for 10 mins. The packed blood cells then were rinsed using isotonic (0.9% saline pH 7) buffer solution. These steps were repeated thrice. Ultimately the last cell volume (10% v/v) which was obtained was kept in isotonic buffer solution (pH 7). 500 µL of the final packed cells was added to 4.5 mL of hypotonic phosphate buffer saline solutions containing various concentrations of fisetin and its cocrystals (50, 100, 150 µg/mL in hypotonic PBS pH 7) incubated for 10 mins at room temperature and then centrifuged for 15 min at 3000 rpm at 4°C. The extent of haemolysis by fisetin and its cocrystals was evaluated by obtaining absorbance of the resulting supernatant at 540 nm by UV-Visible spectrophotometer (Perkin Elmer).

### 2.9 Pharmacokinetic studies in rat plasma

Male wistar rats were procured and used for the kinetic studies. Animals were accustomed with the regular feed/water ad libitum. The dose administered to the experimental rats was 50mg/kg. At various time intervals blood was withdrawn (approximately 0.2 ml) from the jugular vein cannula. The collected blood samples were kept in freezer until the HPLC analysis was performed.

Study protocol:

Overnight fasted rats were split in six groups (n=6) comprising of

Group I: Served as control (Received vehicle).

Group II: Rats were administered aqueous carboxymethylcellulose (CMC) (0.5%w/v)

Group III: Rats were given fisetin suspended in aqueous CMC (0.5%w/v)

Group IV: Rats received FTHY suspended in aqueous CMC (0.5%w/v)

Group V: Rats received FGLU suspended in aqueous CMC (0.5%w/v)

Group VI: Rats received FMAL suspended in aqueous CMC (0.5%w/v)

Sampling of plasma: Blood samples collected from the retro-orbital capillary plexus of rats (all groups) at specified time gap (15, 60, 120, 180, 240, 360, 420 min) was centrifuged for 20 mins at 5000 rpm to discrete plasma from blood sample. Subsequently, the volume was made upto 1 mL using solvent and further the samples were analysed by HPLC for fisetin content.

HPLC analysis of fisetin in plasma: The calibration standards were made by spiking 100 µL of the fresh pure plasma from untreated rat with an appropriate amount of 1 mg/mL methanolic stock solution of fisetin. The calibration standards made were of 5, 10, 15 and 20 µg/mL concentrations. Further, the samples were injected into the system at an injection volume of 20 µL, with mobile phase consisting of orthophosphoric acid: methanol (20:80) at a flow rate of 1.2 ml/min.

### 2.10 Accelerated stability conditions

The cocrystals were further subjected to accelerated stability conditions (40°C/75% RH for the period of 3 months) and the samples were analysed by DSC and PXRD pattern.

## 3.0 RESULT AND DISCUSSION

The solvent assisted grinding method had been employed to prepare the multicomponent crystals of fisetin, FIS-GLU, FIS-MAL and FIS-THY and were characterized thermally, spectroscopically and structurally via, DSC, FT-IR, PXRD and SSNMR. Further,

cocrystals were studied for their enhanced solubility and dissolution profile. Additionally the antioxidant activity and hemolytic activity was also measured for the prepared cocrystals in comparison to that of the pure fisetin molecule.

### 3.1 Design and Synthesis of Co-crystals

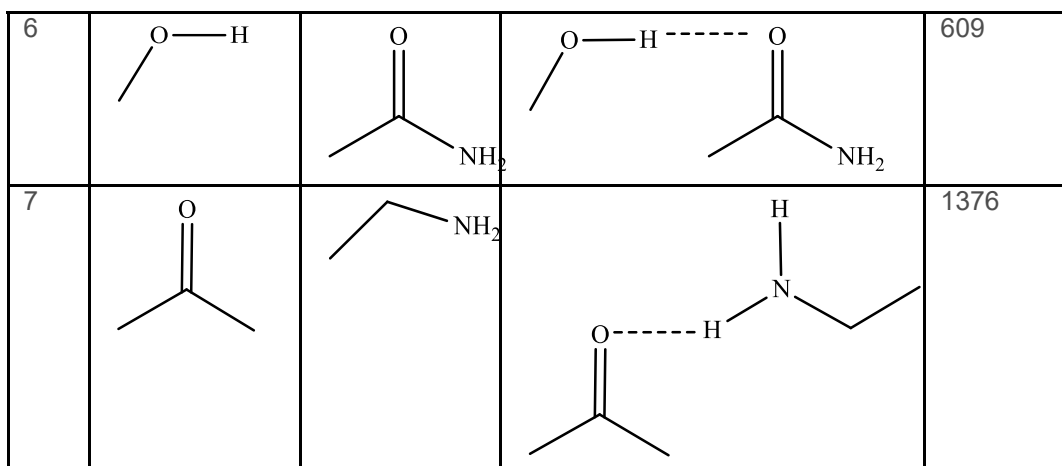
Firstly before the synthesis of cocrystal a CSD search was performed depending on the functional groups (hydroxyl (OH) and carbonyl (C=O)) present in FIS molecule. Search was carried out on each of the fragmented functional group following which the hits were obtained. The results obtained from the database showed high probability to prepare multicomponent

forms with specific functional groups.

Based upon CSD search (Table I), it was observed that ceratin functional groups (carboxylic acid group, aromatic nitrogen and carbonyl group) are more prone to form hydrogen bond with hydroxyl group and carbonyl group of FIS. henceforth, various cofomers including cytosine, piracetam, picolinic acid, nicotinic acid, histidine, theophylline, pyrogallol, theobromine, isonicotinic acid, urea, acetamide, benzamide, piperazine, picolinamide, 4-hydroxy benzamide, pyridoxine were tried. Cocrystals were obtained with GLU, MAL and THY, which were further characterized and evaluated.

**Table 1.** CSD statistics for potential functional groups

S.N	Function group present in drug	Potential functional groups	Supramolecular synthon	Hits
1				1181
2				4281
3				3413
4				2044
5				5954



### 3.2 Characterization of Co-crystals

#### 3.2.1 DSC

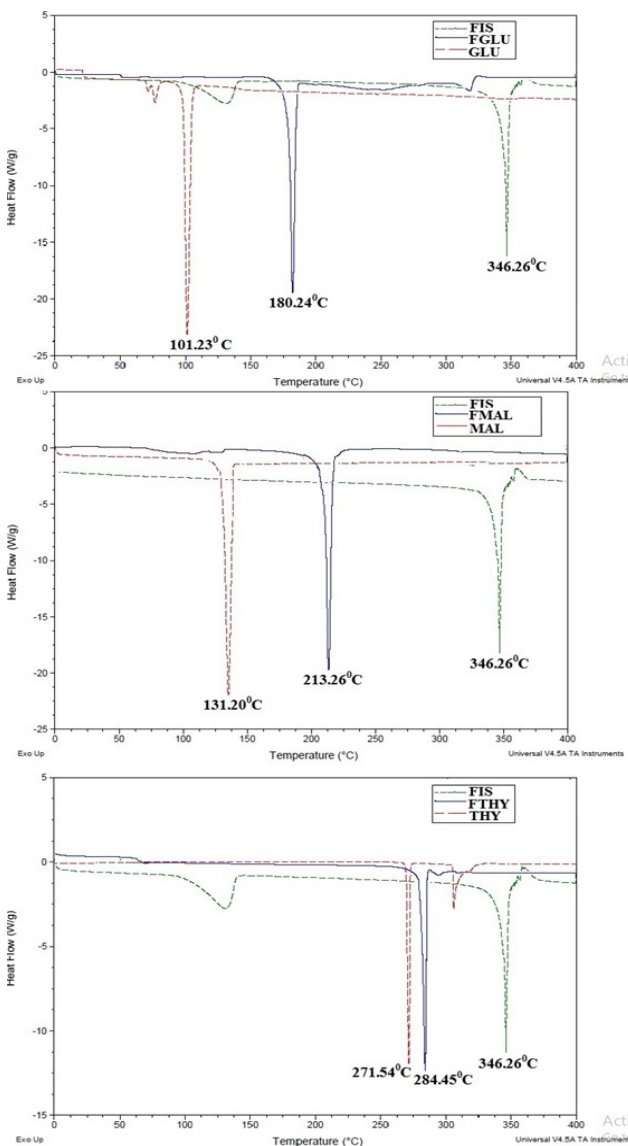
Single endothermic peaks were observed for FGLU (180.24°C), FMAL (213.26°C) and FTHY (284.45°C) respectively. They are different from their parent constituents i.e.,coformers, GLU(101.23°C), MAL (131.29°C), THY(271.54°C) and the pure drug FIS (346.26°C).The thermograms are are given in Figure 2.

The appearance of sharp, peak with definite melting point in the DSC indicate that the new solid form prepared is crystalline in nature and is phase pure. Moreover, the appearance of a single endothermic event present between the melting point of fisetin and respective coformers further point towards the existence of cocrystals over eutectics.

#### 3.2.2 PXRD

The formation of new crystalline phases as conferred by DSC was further confirmed by the PXRD pattern of the cocrystal (Figure 3) when compared to the parent molecule and the corresponding coformer.

PXRD is a reliable technique to obtain qualitative information regarding the formation of cocrystals. The existence of a distinct PXRD pattern of the resultant products conveys the generation of a new phase. The events pertaining to new peak appearance, disappearance of characteristic reflections and distortion in the original peaks provides information about certain interactions taking place within the fisetin and the respective coformers resulting in the cocrystals.



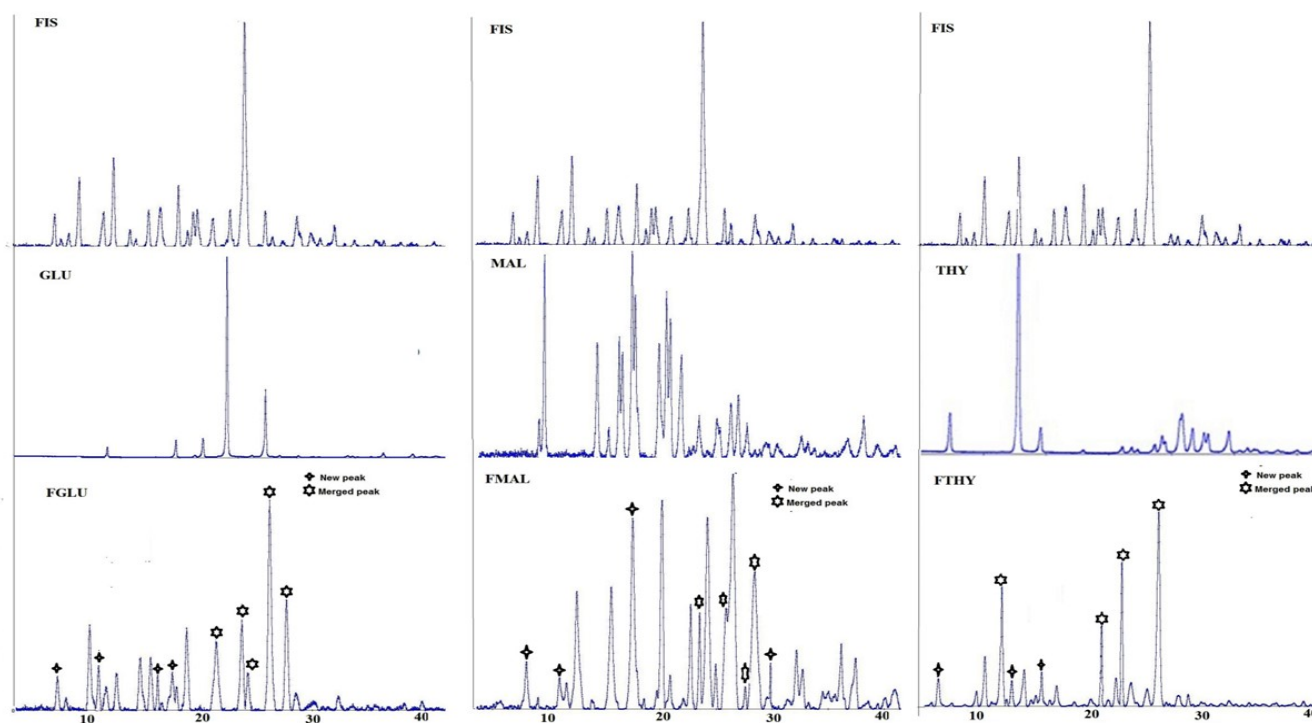
**Figure 2.** DSC of FIS, coformers, FGLU,FMAL and FTHY



Some new peaks in FGLU, at  $7.3^\circ, 11.0^\circ, 16.2^\circ$  and  $17.5^\circ$  have appeared. A few characteristic peaks at  $9.1^\circ, 9.8^\circ, 15.3^\circ, 19.6^\circ, 20.4^\circ, 20.9^\circ, 22.7^\circ$  in PXRD of FIS and  $21.9^\circ$  in PXRD of GLU disappeared. The peaks at  $21.2^\circ$  in PXRD of GLU and  $21.3^\circ$  in PXRD of FIS have merged to  $21.4^\circ$  in PXRD of FGLU, peaks positioned at  $23.3^\circ$  in PXRD of GLU and  $23.8^\circ$  in PXRD of FIS have produced a peak at  $23.7^\circ$  in PXRD of FGLU whereas, peak at  $24.04^\circ$  in PXRD of GLU and  $24.1^\circ$  in PXRD of FIS have merged to  $24.2^\circ$  in PXRD of FGLU. Besides, this peak at  $26.0^\circ$  in PXRD of GLU and peak at  $25.5^\circ$  in the PXRD of FIS has merged to give a peak at  $26.2^\circ$ . Some new peaks at  $5.6^\circ, 11.03^\circ, 17.5^\circ, 29.6^\circ$  were observed in FMAL whereas few peaks at

$9.8^\circ, 19.6^\circ, 20.4^\circ, 21.3^\circ, 31.4^\circ$  of FIS disappeared. Besides this, peaks at  $23.5^\circ$  ( $23.6^\circ$  in MAL merged with  $23.8^\circ$  in FIS),  $25.9^\circ$  ( $25.8^\circ$  in MAL merged with  $25.5^\circ$  in FIS),  $27.6^\circ$  ( $27.4^\circ$  in MAL merged with  $27.3^\circ$  in FIS) and  $28.3^\circ$  ( $28.15^\circ$  in MAL merged with  $28.0^\circ$  in FIS) were observed in FMAL. Many peaks in the PXRD of both FIS and MAL were found to be shifted significantly from their initial positions.

Few new peaks have appeared at  $6.3^\circ, 14.4^\circ$  and  $16.06^\circ$   $2\theta$  in FTHY. Besides this, peaks positioned at  $12.9^\circ, 21.3^\circ, 23.8^\circ$  and  $25.5^\circ$  in FIS fused with  $12.8^\circ, 20.1^\circ, 23.6^\circ$  and  $26.7^\circ$  in THY and resulted in peaks with  $2\theta$   $12.7^\circ, 21.03^\circ, 23.5^\circ$  and  $26.8^\circ$  respectively in FTHY.



**Figure 3.** Diffraction patterns of FIS, all the CCF, FGLU, FMAL and FTHY

### 3.2.3 FT-IR

Infrared spectroscopic technique has been extensively used to identify the intermolecular interaction and hydrogen bond directed associations in the field of crystal engineering [25].

The shift in vibrational bands of Fisetin as well as the respective cocrystals has been observed in the cocrystals FGLU, FMAL and FTHY (Figure 4).

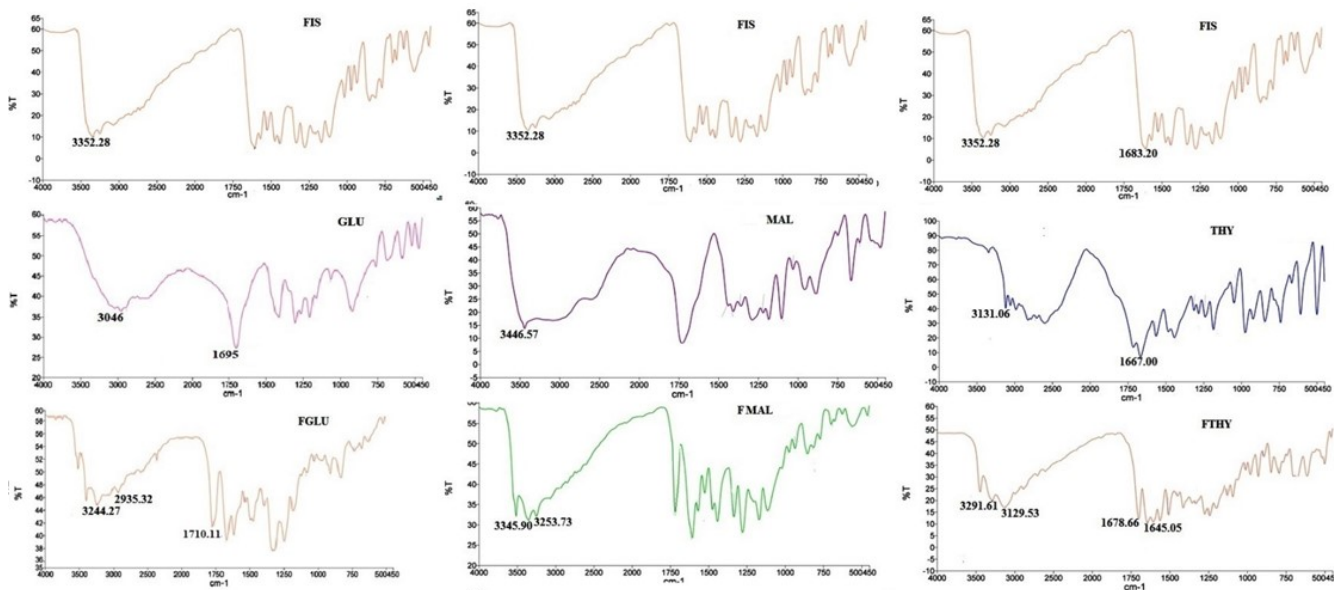
There is a shift in the position of hydroxyl (-OH) group of fisetin at  $3352\text{ cm}^{-1}$  as well as hydroxyl stretch (-OH) and carbonyl (C=O) stretch at  $3046\text{ cm}^{-1}$  and  $1695\text{ cm}^{-1}$  of glutaric acid to  $3244\text{ cm}^{-1}$ ,  $2935\text{ cm}^{-1}$  and  $1710\text{ cm}^{-1}$  respectively in FGLU.

Whereas in the case of FMAL, the hydroxyl (-OH) stretch at  $3352\text{ cm}^{-1}$  of fisetin and (-OH) stretch at  $3446\text{ cm}^{-1}$  of MAL have appeared at  $3345\text{ cm}^{-1}$  and  $3253\text{ cm}^{-1}$ . The absence of peaks in the region of



C=O stretch corresponding to the carboxylate salts (1750-1700  $\text{cm}^{-1}$ ) shows that in FMAL and FGLU both the constituents i.e., the drug and the coformer are in the neutral state neglecting the salt formation. The hydrogen bond existence in FTHY can be manifested by the shift in the hydroxyl group (-OH) from

3352  $\text{cm}^{-1}$  and carbonyl (C=O) group from 1683  $\text{cm}^{-1}$  of FIS, and N-H stretching from 3131  $\text{cm}^{-1}$  and carbonyl (C=O) stretch from 1667  $\text{cm}^{-1}$  of THY to 3291  $\text{cm}^{-1}$ , 1678  $\text{cm}^{-1}$ , 3129  $\text{cm}^{-1}$  and 1645  $\text{cm}^{-1}$  respectively.



**Figure 4.** FTIR spectra of FIS, coformers, FGLU, FMAL and FTHY

### 3.2.4 SSNMR

SSNMR offers insight into the structural information which in collaboration with the vibrational spectroscopy, powder X-ray diffraction and crystal structural data can act as definite evidence for existence of cocrystals. The sensitivity of this analytical tool provides valuable information regarding the perturbations in the chemical environment attributing to the variation in the supramolecular interactions within the cocrystals. Significant differences were observed in the chemical shift of the carbon attached to the functional groups participating in the hydrogen bond between fisetin molecule and the respective coformers (Figure 5).

#### FGLU

The spectra of FGLU indicates the existence of a specific non-covalent interaction between the glutaric acid molecule and fisetin molecule. There is a shift in the peak position at C3, C4 and C7 of fisetin from 137.1, 170.8 and 169.7 ppm to 136.4, 172.9 and 165.8 ppm in the cocrystal alongwith, shift in peak

position corresponding to C1 and C2 of glutaric acid from 181.9, 34.1 ppm to 180.9 and 33.8 ppm. These chemical shifts are due to the involvement of the hydroxyl groups (C7 and C3) and carbonyl group (C4) of fisetin and the hydroxyl and carboxylic group (C1 and C4) of glutaric acid in hydrogen bonding forming O...HO heterosynthon.

#### FMAL

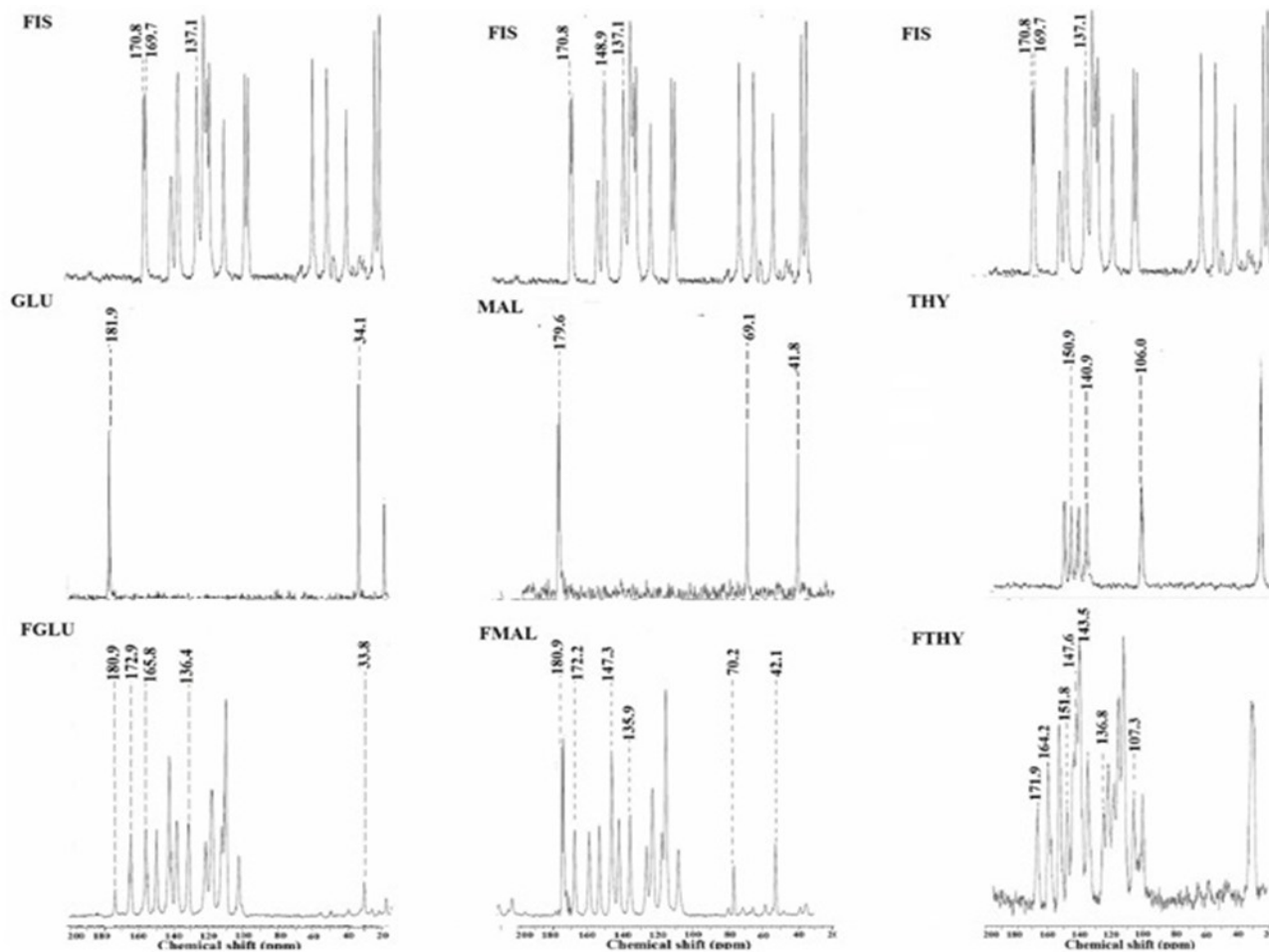
Similarly, the peak positions at C3 (137.1), C4 (170.8) and C4' (148.9) of fisetin have shifted to 135.9, 172.2, 147.3 alongwith shift in the peak position of malic acid at 179.6, 69 and 41.8 ppm corresponding to C2, C3 and C4 to 180.4, 70.2, 42.1 ppm respectively. These variations infer that there is involvement of hydroxyl groups positioned at C7 and C4' of fisetin and carbonyl group (C1 and C4) and hydroxyl group (C2) of glutaric acid.

#### FTHY

Perturbations due to hydrogen bonding at C3, C4 and C7 of fisetin corresponding to 137.1, 170.8 and

169.7 ppm have are responsible for shift in their position to 136.8, 171.9, 164.2 ppm respectively in FTHY. Whereas, the peak positioned at 150.9, 140.9 and 106.0 ppm corresponding to C2, C9 and C8 of theophylline have shifted to 151.8, 143.5, 107.3 ppm

in FTHY. These variations in chemical shifts provide evidence for the existence of hydrogen bond between the hydroxyl group positioned at C3 of fisetin and carbonyl oxygen at C2 position of theophylline resulting in the formation of OH...O=C heterosynthon.

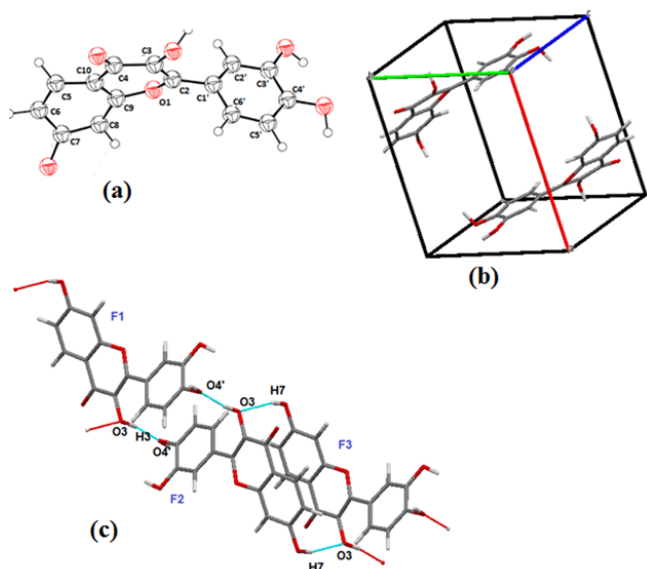


**Figure 5:** ssNMR patterns of FIS,coformers, FGLU,FMAL and FTHY

### 3.2.4 STRUCTURE DETERMINATION

As discussed in experimental section the crystal structure was determined from PXRD pattern utilizing BIOVIA software. Firstly the crystal structure of fisetin was determined as no report is available on its crystal structure.

Fisetin molecule crystallizes in P-1 space group of triclinic system. The fisetin molecules are linked via homosynthons between the hydroxylic group at C3 of F1 which donates its hydrogen to the oxygen of the hydroxylic group at C4' of the adjacent F2 molecule forming a homosynthon (OH3...OH4'). Also, the hydrogen of the phenolic group at C7 of F3 donates its hydrogen to the oxygen of hydroxylic group at C3 of F1 (homosynthon OH7...OH3) (Figure 6).



**Figure 6.** FIS (a) asymmetric unit, (b) Crystal packing pattern along b axis, (c) Hydrogen bonded interactions in FIS

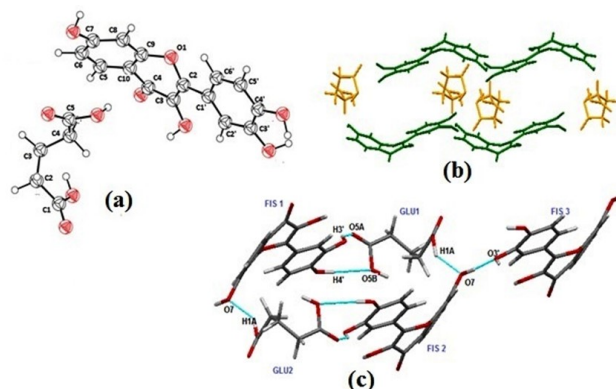
The cocrystals are prepared from heteromeric interactions by breaking the homomeric interactions amongst the FIS molecule.

Crystallographic data are summarized in Table 2 for the FIS cocrystals. The cocrystal (Fis-GLU) crystallizes in the  $P1$ — space group of triclinic system, with one FIS and one GLU molecule in the asymmetric unit (Figure 7 a). The FIS-MAL cocrystal crystallized in the  $P2_1$  space group of the monoclinic system. The asymmetric unit contains one molecule each of FIS and MAL (Figure 8a). The FIS-THY cocrystal crystallized in the  $P1$ — space group of the triclinic system, with one FIS molecule and one THY molecule in the asymmetric unit. (Figure 9a).

#### FGLU

Structural analysis of FGLU reveals the presence of centrosymmetric tetramers assembled by GLU and FIS molecules, as illustrated in Figure 7c. The homosynthon ( $H3' \dots O4$ ) of fisetin breaks to form cocrystal with glutaric acid via heterosynthon ( $H3' \dots O5A$ ). In these assemblies, a ring motif is formed consisting of heteromolecular synthons in FIS1-GLU1 i.e.,  $O4' - H4' \dots O5B$  (1.78 Å, 178u), wherein the hydroxyl group present at  $C4'$  position of ring B of fisetin (FIS1) donates hydrogen to the oxygen of hydroxyl

group positioned at  $C5$  of glutaric acid (GLU1),  $O1A - H1A \dots O7$  (2.20 Å, 166u) in which the hydrogen of the hydroxyl group positioned at  $C1$  of glutaric acid (GLU 1) donates its hydrogen to the oxygen of the hydroxyl group positioned at  $C7$  of fisetin molecule (FIS 2). Besides this, heterosynthon  $O3' - H3' \dots O5A$  (2.04 Å, 147u) exists between hydrogen of phenolic group at position  $C3'$  of ring B (FIS 1) and carbonyl oxygen positioned at  $C5$  of glutaric acid (GLU 1). Additionally homomeric interactions also exist within fisetin molecules FIS2-FIS3 connected to each other via  $O7 - H7 \dots O3'$  hydrogen bond. Glutaric acid being a small aliphatic diacid molecule fits in the voids of the fisetin molecule. The packing pattern of FGLU is such that GLU molecules are oriented approximately perpendicular to the chain of FIS molecules (figure 7b).



**Figure 7.** FGLU (a) asymmetric unit, (b) Crystal packing pattern where green and orange colour represent FIS and GLU molecules respectively, along the b axis, (c) Hydrogen bonded interactions in FGLU cocrystal.

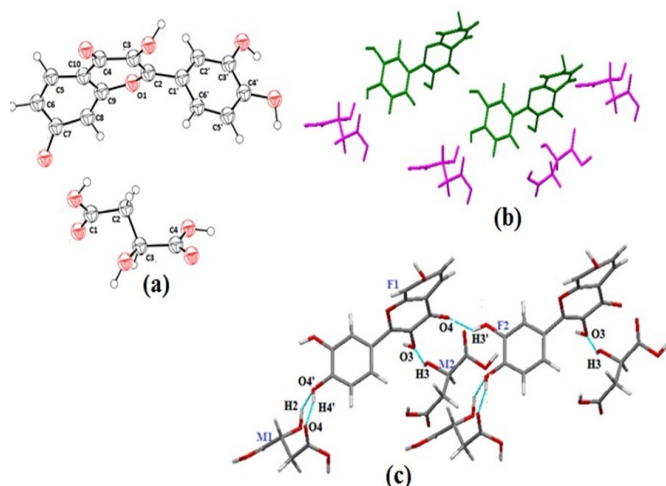
#### FMAL cocrystal.

The crystal packing (Figure 8b) and hydrogen bonding in the FIS-MAL cocrystal are depicted in Figure 8c. In this cocrystal the homosynthon of fisetin ( $O3' \dots H7$ ) breaks to form heterosynthon ( $O3' \dots H3$ ) with MAL.

The structural analysis shows that the fisetin (F1) molecule interact with two adjacent malic acid (M1 and M2) molecules. Oxygen of the hydroxyl group ( $O4'$ ) of F1 is bonded to the hydrogen of hydroxyl group (H2) positioned at  $C2$  of M2 forming a motif

(H4'O4'...H2O2). Whereas, the hydrogen (H4') of the hydroxyl group at C4' of F1 is bond with the oxygen of carbonyl group at C4 of malic acid (M1) (O4'H4'....O4=C4).

Additionally, F1 is also linked with M2 via hydrogen bonding that exists between the oxygen of the hydroxyl group at C3 position of F1 and the hydroxy hydrogen (O3H3) at C3 of M2. Besides, this the oxygen of the carbonyl group at C4 of fisetin (F1) accepts hydrogen from the hydroxyl group positioned at C3' of another fisetin molecule (F2) leading to intermolecular interactions amongst the fisetin molecules.

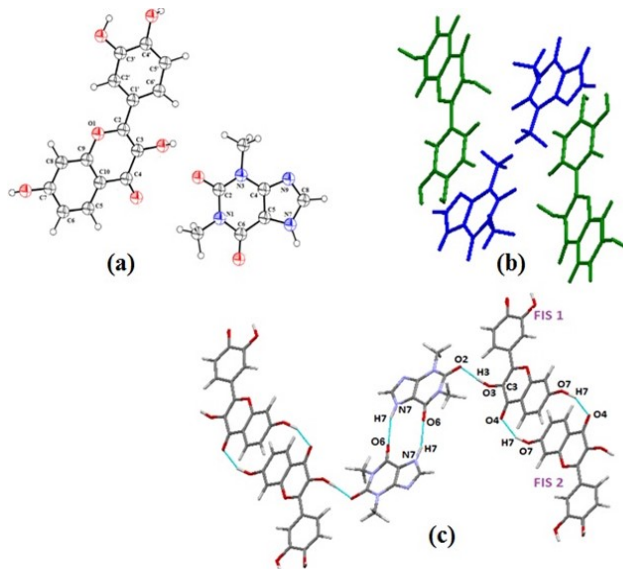


**Figure 8.** FMAL (a) asymmetric unit, (b) Crystal packing pattern where green and pink color represent FIS and MAL molecules respectively, along b axis, (c) Hydrogen bonded interactions in FMAL cocrystal

#### FTHY

The hydrogen bond between fisetin (FIS) and theophylline (THY) is formed between the hydroxyl hydrogen at C3 of FIS and the carbonyl oxygen atom at C2 of THY. Also the two theophylline molecules are held together in a cyclic motif (NH...O=C) by hydrogen bonding leading to  $aR^2_2(8)$  homodimer. The

homodimeric unit is linked with adjacent FIS molecules in sheets by O...H hydrogen bond between hydroxyl group located at C3 of FIS which acts as hydrogen bond donor and carbonyl oxygen at C2 (hydrogen bond acceptor) of THY via O3H3...O2 hydrogen bonds. The homodimers of THY are capped in centre with two sheets of flavanoid molecule on each end (Figure 9c). These are further connected via O...H hydrogen bond existing between oxygen of the carbonyl carbon (hydrogen bond acceptor) at C4 of F1 with hydrogen of hydroxyl group at C4 of F2 i.e., O4...H7O7 (F2). The oxygen atoms of phenol groups on the adjacent FIS molecules face each other in a close packed arrangement (Figure 9b), which stabilizes the 3-dimensional structure. The synthon (O4...H3') originally present in fisetin lattice breaks to form heterosynthon (O3H3...O2) with THY.



**Figure 9.** FTHY (a) asymmetric unit, (b) Crystal packing pattern where green and blue colour represent FIS and THY molecules respectively, along b axis, (c) Hydrogen bonded interactions in FTHY cocrystal

Table 2. Crystallographic parameters

Crystallography Parameters	FIS	FGLU	FMAL	FTHY
Chemical formula	C15 H10 O6	C20 H18 O10	C19 H16 O11	C22 H18 N4 O8
Stoichiometry		1:1	1:1	1:1
Temperature	Room Temperature as specified 25°C	Room Temperature as specified 25°C	Room Temperature as specified 25°C	Room Temperature as specified 25°C
Crystal system	Triclinic	Triclinic	Monoclinic	Triclinic
Cell volumes(A°3)	847.503	731.639	1279.62	853.524
Space group	P-1	P-1	P2/n	P-1
Cell lengths	a 10.8694 b 9.3598 c 8.5290	a 11.6887 b 8.9868 c 7.4230	a 12.0193 b 10.4781 c 10.3186	a 12.4461 b 8.5843 c 8.0286
Cell angles(deg)	$\alpha$ 94.7742 $\beta$ 95.2038 $\gamma$ 99.6883	$\alpha$ 86.9352 $\beta$ 104.1696 $\gamma$ 104.5818	$\alpha$ 90.0000 $\beta$ 100.0403 $\gamma$ 90.0000	$\alpha$ 94.1500 $\beta$ 93.8458 $\gamma$ 90.5247
Z	2	2	4	2
2 $\theta$ range	5° 45°	5° 45°	5° 45°	5° 45°
Rwp(%)	12.8	15.1	18.5	17.2
CCDC No.	1884089	1884086	1884087	1884088

#### 4.0 Equilibrium solubility and dissolution studies

The ability to solubilize a defined therapeutic dose of the API plays a very critical role in the context of drug development. The solubility profile of an API also influences the absorption process. Moreover, solubility together with permeability governs the bio-availability[26]. The thermodynamic solubility and kinetic solubility profile herein has been determined by performing equilibrium solubility and intrinsic dissolution. Equilibrium solubility indicates the solubility values whereas the intrinsic dissolution values reveal the dynamics of the whole solubility process and the difference in concentration of API during different time periods. Dissolution study represents a key link between *in-vitro* and *in-vivo* utilization of the API.[27]

The prepared cocrystals of fisetin display a spring and parachute phenomenon (Figure 10) since the two molecules (i.e., fisetin and respective cofomer) dissociate into the individual constituents within the short span of time, owing to weak hydrogen bond holding the molecules together. The spring and parachute effect provides sufficient period of time for absorption of the cocrystals before their dissocia-

tion. The breakdown of the cocrystal into individual constituent was confirmed by the FTIR analysis of the residue. The residual material was analyzed after 2 and 24 hours of the solubility study to examine any change in the prepared co-crystals. The FT-IR analysis showed that all the three co-crystals existed as a single unit upto 2 hours. However, FT-IR spectra after 24 hours resembled that of the parent flavonoidal molecule. Thus, suggesting the breakdown of co-crystals.

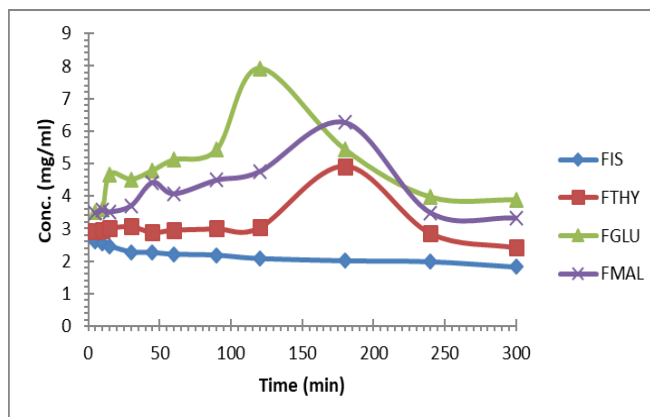


Figure 10. Dissolution profile of fisetin and its cocrystals



The improvement in the equilibrium solubility of fisetin was observed in its cocrystals which is found to be 3.03 times more for FGLU(7.93 mg/ml), 2.40 times more FMAL(6.27 mg/ml) and 1.88 times more for FTHY (4.92 mg/ml). The maximum concentration was attained for FGLU and FMAL at 120 min and for FTHY at 180 mins which started decreasing steadily to a value comparable to the pure fisetin molecule (2.61 mg/ml). The superior solubility and IDR pattern of fisetin cocrystals can be justified on the basis of the solubility profile and melting point of the coformers, the orientation of molecules in the crystal lattice and strength of supramolecular interactions. FGLU showed the maximum solubility which may be attributed to the fact that the molecules in FGLU are packed in bilayer pattern in crystal lattice while in FMAL the molecules are packed in multilayers. Whereas, in FTHY the intra and intermolecular interactions suggest strong crystal lattice resulting in less solubility in comparison to other cocrystals. Additionally, coformer glutaric acid has lowest melting point as compared to MAL and THY.

The better physicochemical properties of the new engineered cocrystals (FGLU, FMAL and FTHY) encouraged its further development, and thus further pharmacokinetic and pharmacodynamic studies were performed.

### 5.0 Accelerated Stability Study

The cocrystals of FIS were further subjected to accelerated stability conditions (40°C/75% RH for the period of 3 months) and the samples were analysed by DSC. The cocrystals were found to be stable also, no significant changes were seen in DSC thermogram (Table no. 3)

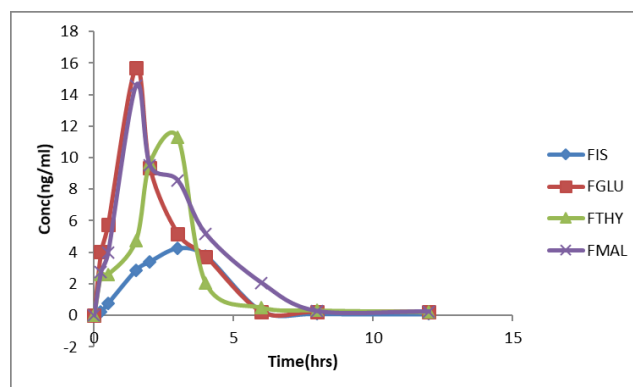
**Table 3.** Melting point of samples after 3 months

Samples	Melting point	Melting point after 3
FGLU	180.24°C	179.63°C
FMAL	213.26°C	214.31° C
FTHY	284.45°C	283.25° C

### 6.0 Bioavailability

Bioavailability of the prepared cocrystals was determined by measuring of “how much” and “how quickly” the active component of the cocrystal gets absorbed in the biological system. It has been evident from the literature that if upon cocrystallization there is an enhanced in vitro solubility then there is a probability of an increased  $C_{max}$  as well. Therefore, bioavailability is a combination of solubility, dissolution and permeability profile of an API.[28]

In order to elucidate the pharmacokinetic characteristics the prepared fisetin cocrystals were administered in rats. The results showed that all the cocrystals had higher  $C_{max}$  and AUC, and a shorter  $T_{max}$  as compared to that of pure fisetin (figure 11). The highest concentration was achieved by FGLU which is approximately 3.7 times in 1.5 hours followed by FMAL 3.4 times in 1.5 mins and FTHY 2.6 times in 3 hours when compared to pure FIS (Table No.4).



**Figure 11.** Pharmacokinetic profile

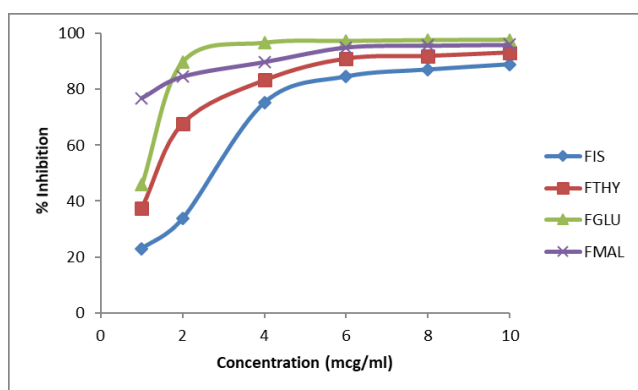
**Table 4.** Biopharmaceutical parameters of fisetin cocrystals

	$C_{max}$ (ng/ml)	$T_{max}$ (min)	AUC(ng/ml min)
FIS	4.2	30	48840
FGLU	15.7	15	181489
FMAL	14.5	15	168447
FTHY	11.2	30	130179

## 7.0 Biological evaluation

### 7.1 Antioxidant activity

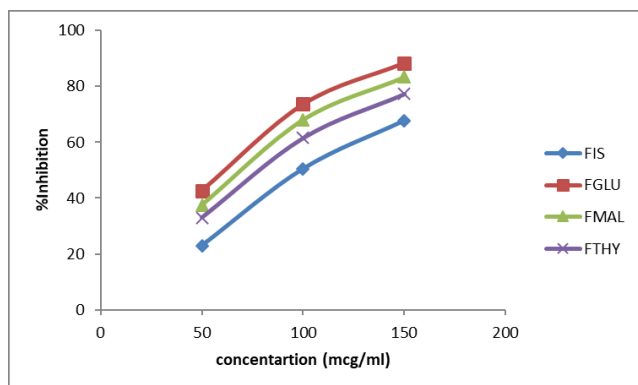
The cocrystals were further evaluated for their antioxidant effect by determining the hydrogen donating capability of the fisetin in its cocrystals by performing the DPPH assay. The cocrystals showed a substantial melioration in scavenging the free radical (DPPH<sup>•</sup> radical). The free radical-scavenging activity was found to be maximum for FGLU > FMAL > FTHY (Figure 12). Improvement in scavenging activity was also found to be concentration dependent where the maximum activity was observed at 10 mcg/ml.



**Figure 12.** Antioxidant activity

### 7.2 Anti-hemolytic

The effect of cocrystallization on antihemolytic activity of fisetin was studied on rat RBC's. Maximum inhibition of hemolysis was depicted by FGLU(88%) followed by FMAL(83%) whereas FTHY showed 77% antihemolytic activity as compared to the parent molecule which is 67% (Figure13).



**Figure 13:** Antihemolytic activity

## 8.0 Conclusion:

The present study demonstrates the design, synthesis and evaluation of pharmaceutical cocrystals of a poorly water soluble bioflavonoidal molecule, fisetin via crystal engineering approach. The article illustrates that the improvement in solubility, pharmacokinetic parameters and biological activity is dependent on the presence of hydrogen bond interactions amongst the coformers and the parent phytochemical molecule. Finally, the study showcases the successful application of crystal engineering technique towards improving the clinical performance of fisetin.

## Acknowledgements

The authors wish to thank Department of Science and Technology (DST), New Delhi (File number: SR/SI/OC-90/2012) and University Grants Commission (UGC-RFMS), New Delhi (F.5-94/2007(BSR)).

## ABBREVIATIONS

CSD	Crystal structure database
FIS	Fisetin
FGLU	Fisetin-glutaric acid
FMAL	Fisetin-malic acid
FTHY	Fisetin-theophylline
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared
KBr	Potassium bromide
PXRD	Powder X-ray diffraction
SSNMR	Solid-state nuclear magnetic resonance
HPLC	High performance liquid chromatography
DPPH	2, 2' diphenyl-1-picrylhydrazyl
GLU	Glutaric acid
MAL	Malic acid
THY	Theophylline
GRAS	Generally regarded as safe
IDR	Intrinsic dissolution rate
SAG	Solvent assisted grinding
API	Active pharmaceutical ingredient
RBC	Red blood cells
SCXRD	Single crystal X-ray diffraction
AUC	Area under the curve



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