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Enhancing biopharmaceutical parameters of bioflavonoid quercetin by cocrystallization.

Karan Vasisht*, Kunal Chadha, Maninder Karan, , Yashika Bhalla, Ashish Jena, Renu Chadha

University Insitute of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, INDIA

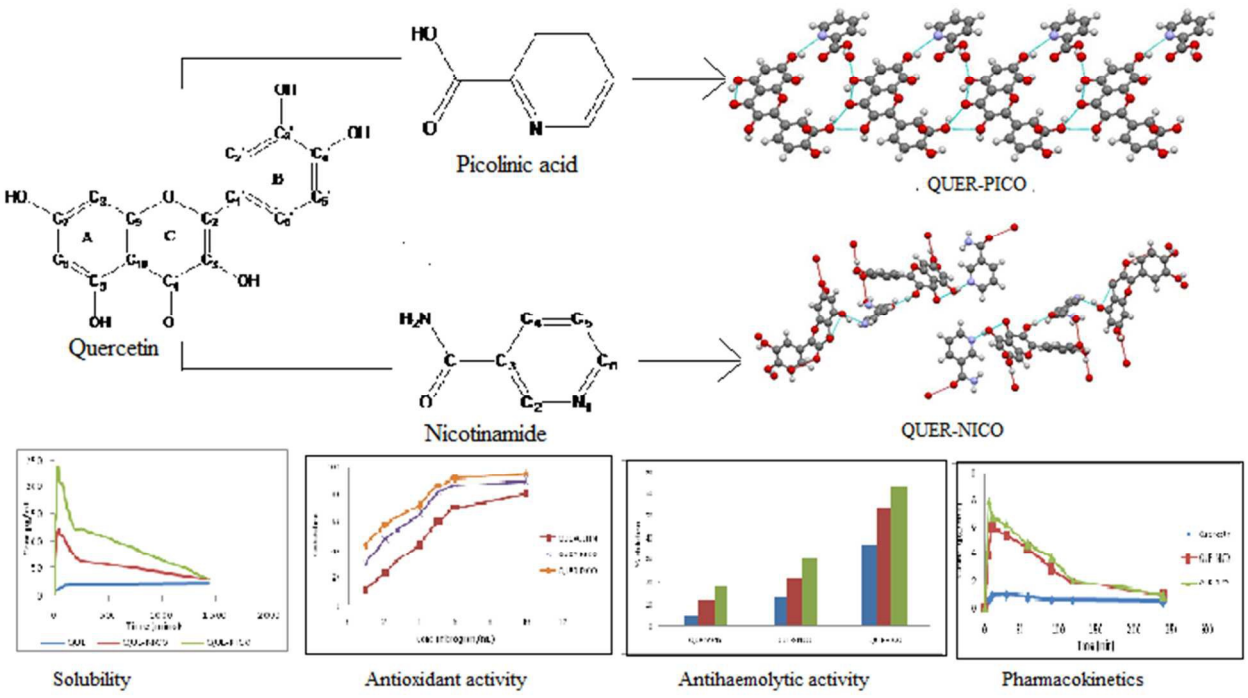
Corresponding Author:

Professor Karan Vasisht,

Email: kvasisht@hotmail.com

M: +91 9876067171

Graphical Abstract



Highlights

The study highlights the improved biopharmaceutical properties of quercetin with therapeutically active coformers: picolinic acid and nicotinamide using cocrystallization. The study is well supported by antioxidant, antihaemolytic and pharmacokinetic activities.

Abstract:

Quercetin, a polyphenolic nutraceutical belonging to flavonoid category of natural compounds, has been extensively studied as an antioxidant, anti-inflammatory, anticancer, glycoprotein P 450 enzyme inhibitor, cardioprotective and antihaemolytic substance. Despite an increased awareness of the efficacy and utility of this flavonoid, its use has been severely held back owing to its poor aqueous solubility and consequently reduced bioavailability. The present study reports two cocrystals of this valuable natural compound with therapeutically active coformers of GRAS status, nicotinamide and picolinic acid in an endeavour to overcome its limitations and exploit its full potential. The characterization of the cocrystalline phases prepared involved thermoanalytical methods (differential scanning calorimetery) and spectroscopic methods (FT-IR, and Powder X-Ray diffractometry). The confirmation was provided by ^{13}C solid state NMR analysis. The structures of the prepared cocrystalline phases were finally determined by utilizing the PXRD pattern in Materials Studio® software. A phenomenal improvement in biopharmaceutical parameters of cocrystals vs quercetin was evaluated by carrying out dissolution, equilibrium solubilty, pharmacokinetic, antihaemolytic and antioxidant studies.

keywords: nutraceutical, antioxidant, anti-inflammatory, antihaemolytic, pharmacokinetics, DSC, PXRD, Material Studio®.

1. INTRODUCTION:

Quercetin is one of the most abundantly found and widely studied nutraceutical belonging to subclass flavonol of flavonoids.[1] Quercetin is typically found in plants in different glycosidic forms of which rutin (quercetin-3-rutinoside) is most common, followed by thujin (quercitrin or quercetin-3-L-rhamnoside or 3-rhamnosyl quercetin).[2] However, the biological effects of the flavonoids are mainly due to the aglycone part with studies showing that the sugar moiety only affects the absorption of the flavonoid molecule.[3-5]

Quercetin like other natural antioxidants[6-8] exhibits wide range of biological effects including antibacterial,[9] antiviral,[9] antiallergic,[7] antihepatotoxic,[10,11] antiulcer,[12] and anti-inflammatory[12] activities. It is found to be one of the most effective inhibitor of oxidation of LDL (bad) cholesterol,[11] thereby reducing the risk of developing neurodegenerative diseases,[11-14] renal disorders[15] and atherosclerosis.[16] The cardioprotective role is also brought about by antithrombotic and vasodilatory properties in addition to inhibiting platelet aggregation and maintaining capillary integrity, permeability and fragility.[16-19] The research in therapy against cancer has also focused on the antitumor effects of quercetin to evaluate its potential as an anticancer agent.[19-24] Furthermore, quercetin also shows inhibition of numerous enzyme systems including glycoprotein cytochrome P-450 enzyme, tyrosine protein kinase, phospholipase A2, phosphodiesterases, mitochondrial ATPase, PI 3-kinase, protein kinase C aldose reductase and xanthine oxidase apart from the ability to activate Ca^{2+} and K^{+} channels.[25-27] The special attraction of this natural compound lies in the extremely low mammalian toxicity in addition to many beneficial effects.[28-30]

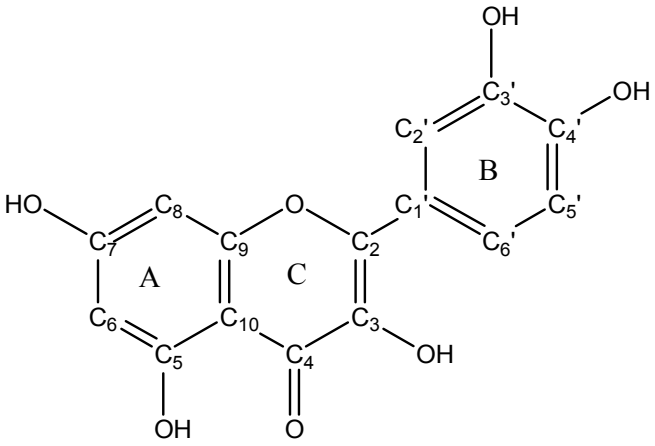
An evaluation of the role of dietary quercetin in clinical setting is hampered by the sparse aqueous solubility leading to insignificant absorption and low bioavailability along with limited solubility in pharmaceutically acceptable solvents.[31] Moreover, quercetin is also highly susceptible to metabolic conjugation in liver following oral administration and exists mostly as conjugated form in systemic circulation which further reduces its bioavailability.[32-36]

Thus, development of strategies to increase the solubility and absorption of quercetin with an aim to optimize its metabolism and pharmacokinetics are the need of the hour. Various groups of researchers have explored different strategies like preparation of β-cyclodextrin inclusion complexes,[37] nanoparticles,[38] salts,[39] hydrates,[39] solvates,[39-40] apart from applying crystal engineering approach to produce polymorphs and cocrystals.[40,41] The crystal engineering is the

concept of forming new solids with desired properties, using non covalent interactions, such as hydrogen bonds, electrostatic interactions, halogen bonds, π-π stacking and Van der Waals interactions.[41,42]

The cocrystal is a supramolecular single phase crystalline solid that incorporates two neutral molecules generally in a stoichiometric ratio,[43] where one is a nutraceutical in this case and the other is a coformer with desired properties.[44,45] The cocrystals of pharmaceuticals and recently of nutraceuticals with GRAS status compliant pharmaceutically acceptable cocrystal formers including carboxylic acids, amides, carbohydrates, alcohols, and amino acids have added a new dimension to technologies being developed to improve the biopharmaceutical properties of poorly absorbed molecules.[46,47]

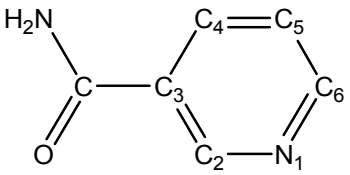
Quercetin is a polyphenolic molecule with five hydroxyl groups at different positions which act as both hydrogen acceptor and hydrogen donor centers along with two other hydrogen bond acceptors i.e. aromatic cyclic oxygen and keto group on the c ring. The molecule on account of its structure is highly susceptible to hydrogen bonding with complementary functionalities like hydroxyl groups, keto groups and aromatic nitrogen and thus holds a lot of promise for cocrystallization, [48-51] and has been picked up by few other groups of workers as well.



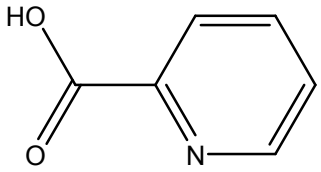
[$C_{15}H_{10}O_7$, (3,3',4',5,7-penthahydroxy-2-phenyl-chromen-4-one)]
Quercetin

Zaworotko et al[48,49] reported three novel cocrystals of quercetin with caffeine, isonicotinamide, and theobromine, with lucid details of crystal structure, dissolution and pharmacokinetic studies, but with no pharmacodynamic studies. Kruthiventi et al[25], describing two cocrystal of quercetin with antidiabetic agent metformin, did not have the pharmacokinetic and pharmacodynamic data. Interstingly, a recent report by Veverka et al [50] mentions twenty cocrystals of quercetin with as

many coformers but the characterization data is dismally inadequate, with no FTIR, PXRD or DSC scans to support the claims although some enzymatic inhibition studies have been done. The only basis of their claim is the mention of melting points, which, in the absence of any supporting data, can lead to a possibility of formation of even eutectics or salts as well.



Nicotinamide



Picolinic acid

In view of the available literature and the propensity of quercetin to form cocrystals, the compounds, nicotinamide and picolinic acid have been explored as suitable coformers in this study. Nicotinamide, being a vitamin with several uses in medicine has an added advantage of high aqueous solubility and is classified as safe for human consumption at doses below 3 g/day.[52,53] Picolinic acid is the body's prime natural chelator and an endogenous metabolite of l-tryptophan with wide range of beneficial pharmacological properties besides being safe and highly soluble. The established therapeutic efficacy and safety profile of these molecules being one part, the work carried out here has an added advantage in showing the complete characterization of cocrystals with techniques like DSC, FT-IR, PXRD, Solid State NMR and crystal structure determination by Material Studio software. Moreover, the complete pharmacokinetic profile as well as the dissolution profile together with the antioxidant and antihaemolytic activity have also been done showing a complete analysis of improvement in efficacy of cocrystals as compared to quercetin.

The method used for construction of supramolecular synthons essential for formation of cocrystals is liquid assisted grinding. The attractiveness of this technique lies in it being a low cost, efficient, and eco friendly method due to low amounts of solvent here.[54,55]

EXPERIMENTAL

Materials

The anhydrous crystalline form of quercetin, nicotinamide, piracetum, picolinic acid, succinimide and urea (all with >99% purity) were purchased from Sigma Aldrich. Solvents used for the study including ethanol, toluene, phosphoric acid, HPLC grade methanol and acetonitrile were purchased from Merck India Limited. The HPLC grade water was obtained from Millipore Water purification assembly installed on the premises of the institution.

Methods

Preparation:

The cocrystals of quercetin with nicotinamide (QUER-NICO) and picolinic acid (QUER-PICO) were prepared by using the 'solvent drop grinding' method.[54,55]

The QUER-NICO was prepared by constant trituration for 2 hours of stoichiometric amounts of 302 mg of quercetin and 122 mg of nicotinamide and QUER-PICO by trituration of quercetin (302 mg) and picolinic acid (123 mg) in a glass pestle mortar with the constant addition of a drop of ethanol at regular intervals. After the solvent addition was stopped, the grinding was continued till complete dryness. The dried cocrystals were stored in an airtight dessicator under controlled conditions of humidity and characterized.

Particle Size Determination:

All the powder samples prior to the studies were passed through mesh size number 44 giving a uniform particle size of about 8.5 μm as measured by the Mastersizer 2000 by Malvern Instruments Inc. (U.K.). (the figure showing the particle size distribution is given as supplementary information).

Differential Scanning Calorimetry

The thermal characterization of the prepared cocrystals was performed on the instrument DSC Q20 (TA Instruments, USA). The instrument was calibrated for temperature and heat flow accuracy using indium metal (mp.: 156.6 ^{o}C and ΔH: 25.45 Jg^{-1}). The samples were analyzed by placing a precise amount of sample (1-2 mg) in sealed aluminium pans which were heated at a rate of 5 ^{o}C/min from 50 to 350 ^{o}C under dry nitrogen, maintained at a flow rate of 50 cc/min.

Powder X-Ray Diffraction (PXRD)

The PXRD analysis was done on X'Pert PRO diffractometer system (PANalytical, Netherlands) with a Cu Kα radiation (1.54060 ^{o}A). The samples were analyzed with tube voltage set at 45 kV and current set at 40 mA. For this diffraction experiment on the 10 mm sample size, the divergence slit and anti-scattering slit settings were set at 0.48^{o}. The sample, placed in an aluminium sample holder, was measured by a continuous scan between 3^{o} and 50^{o} in 2θ with a step size of 0.017^{o} and a step time of 25 s/step.

Crystal Structure Determination

The crystal structure of QUER-NICO and QUER-PICO was determined by subjecting the powder XRD pattern of the cocrystal obtained to Powder Solve module of Materials Studio® (by Accelrys now BIOVIA Systems).

The process involved indexing, of the XRD pattern, obtained after removal of Kα2 peaks and smoothening of the experimentally obtained powder XRD pattern of the cocrystal. The indexing was done by utilizing the XCell module, which searched in orthorhombic, cubic, triclinic and monoclinic crystal systems giving us the suitable best unit cell. This was further optimized by Pawley Refinement. The structures of quercetin and coformer were sketched and energetically optimized using the Dreiding forcefeild in DMOL3 module. These structures were then imported into saved unit cell and structure solution was attempted by a direct space MonteCarlo simulated-annealing approach, and full-profile comparison method implemented in Powder Solve module with 10 simulated annealing cycles and 21100000 iterations in each cycle. Following the global optimization algorithm, the trial structures are continuously generated by modifying specified degrees of freedom in order to find the trial structure that yields the best agreement between calculated and experimental patterns (as denoted by a lowest possible value of Rwp). Finally Reitveld calculations involving hydrogen atoms was carried out on the structure solution obtained.

Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR patterns were obtained on a spectrum RX I FT-IR spectrometer (Perkin Elmer, UK) used in the diffuse-reflectance mode. The sample was prepared by mixing dry KBr (approx 20 mg) with the 2 mg of the sample and the mixture was finely ground in an agate pestle mortar. A manual press was used to form the pellet. The data was collected over the range of 4000 - 400 cm^{-1} and analyzed using Spectrum software.

Solid State NMR Analysis

The structure of the quercetin and the prepared cocrystals QUER-NICO and QUER-PICO were analyzed experimentally by solid state ^{13}C NMR analysis. This was carried out on Joel Resonance JNM-ECX400II Instrument at IISC, Bangalore, India. The solid state ^{13}C NMR spectra of the cocrystals are different from the spectra of the involved substances and therefore fulfil the last basic norm of cocrystal formation.

Dissolution Studies

The dissolution studies of quercetin, QUER-NICO and QUER-PICO were performed in phosphate buffer, pH 7.4 (I. P.) to mimic the conditions prevalent in the gastrointestinal tract. The excess of solid phase (50 mg) was added to 5 mL of the buffer pre-equilibrated at 37 ^{o}C in a stoppered flask. These flasks were then shaken on a water bath shaker at a constant rate of 120 rpm and temperature of 37 ^{o}C. The aliquots of 0.1 mL with replacement were withdrawn at time intervals of 5, 10, 15, 20, 25, 30, 40, 60, 80, 100, 120, 150, 180, 210 and 270 mins. The amount of sample withdrawn every time was, replaced with an equal amount of buffer. The samples were diluted with HPLC grade methanol to make final volume of 1 mL and filtered through 2 mm nylon filters before being assayed on the HPLC system for the content of quercetin. These samples were continued to be kept on the shaker under same conditions for 24 hours and analysed for quercetin to obtain the equilibrium solubility.

The remaining material in the flask after filtration was analyzed by DSC to check for the changes, if any, of the analyte. The cocrystal after 24 hours in phosphate buffer pH 7.4 breaks down in its constitutent materials.

The equimolar physical mixtures of quercetin with nicotinamide and picolinic acid in stoichiometric amounts were subjected to dissolution studies using phosphate buffer pH 7.4 (I. P). The sample withdrawal intervals, methodology, conditions and quercetin evaluation techniques were kept identical to that for cocrystals.

High Performance Liquid Chromatography

A LC-10A VP HPLC system (Shimadzu, Kyoto, Japan) consisting of a single pump, a photo diode-array detector and LC Solution for data collection was used. The chromatography was performed on a Sunfire C18 column (5 µ, 150 mm × 4.6 mm I.D.) supplied by Waters Corporation (Milford, MA, USA) with a Phenomenex RP-C18 guard column (California, U.S.A.). The mobile phase consisted of methanol (A): acetonitrile (B): water containing 0.1% (v/v) phosphoric acid (C) :: 40:10:50. The mobile phase was filtered through a 0.45 µ Millipore filter and degassed before use. The flow rate was kept at 0.8 ml/min and detector wavelength was set at 355 nm. The injection volume was 20 µL and the column temperature was set at 25 ^{o}C. Quercetin stock standard solutions were prepared by dissolving quercetin in methanol and used for solubility and dissolution studies. The calibration standards used were of 10, 20, 30, 50, 75 and 100 µg/ml concentrations.

Antioxidant Activity

The stable 1,1-diphenyl-2-picryl hydroxyl radical (DPPH) was used for determination of free radical scavenging activity of quercetin and the cocrystals according to the method described by Blois (1958) with suitable modifications.[56,57] Then 1 mL of 10mM methanol solution of DPPH was incubated with 1 mL of various concentrations (10, 6, 5, 4, 2 and 1 μg/mL) of each cocrystal in 30:70 mixture of methanol:water for 45 minutes at room temperature in a dark place. The final volume was made upto 5 ml with 50:50 methanol: distilled water. After 45 min, the absorbance of the resulting solution was recorded at 517 nm (UV–Visible EZ201, Perkin Elmer, USA). The control consisted of only 10 mM methanolic solution of DPPH and diluted to 5 mL with 30:70 :: methanol:water. Radical scavenging activity was expressed as the inhibition percentage and calculated by the formula:

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

This assay is based on the principle that stable organic nitrogen free radical DPPH accepts hydrogen from antioxidants like quercetin which act as hydrogen donors thus changing the deep violet color of solution from deep purple to yellow as the radical disappears and the yellow coloured 1,1-diphenyl-2-picrylhydrazine is produced. The degree of discolouration depends on the scavenging or the antioxidant potential of the antioxidant sample which in turn is affected by the solubilty of quercetin and its cocrystals.[56,57]

Antihaemolytic activity

The nutraceutical quercetin and its cocrystals were tested for antihaemolytic activity on rat red blood cells (RBC).[58-60] The protocol was duly approved by the Panjab University Animal Ethical Committee vide reference number PU/IAEC/S/14/85 dated 11/09/2013. About 2 mL blood of rats was withdrawn by retro-orbital route after anesthetization and collected in sterilized 5 mL centrifuge tubes containing equal amount of sterilized Alsevar's solution and centrifuged for 15 min at 3000 rpm at 4^{o}C. The supernatant was discarded and the packed RBC's were washed with pH 7 buffered isosaline solution and centrifuged for 15 min at 3000 rpm at 4^{o}C. This process was repeated 3 times. The final packed RBC obtained were then suspended as 10 % v/v (RBC solution in isosaline) in pH 7 buffered isosaline solution.

The inhibition of rat erythrocyte haemolysis by the quercetin, QUER-NICO and QUER-PICO was evaluated. To 500 μL of 10% (v/v) suspension of erythrocytes in isotonic phosphate

buffered saline (PBS), 4.5 mL of hypotonic phosphate buffer saline solutions with different concentrations of quercetin, QUER-NICO and QUER-PICO (50, 100, 150 µg/mL in hypotonic PBS pH 7) were added. The reaction mixtures were shaken gently while being incubated at 37°C for 30 min. Thereafter, the reaction mixtures were centrifuged at 3000 rpm for 15 min. The absorbance of the resulting supernatants was measured at 540 nm by spectrophotometer to determine the haemolysis. Likewise, the erythrocytes were treated with 4.5 mL of hypotonic PBS to obtain a complete haemolysis and used as standard. The inhibitory effect of the cocrystals was compared with quercetin. The percentage inhibition was calculated as the extent to which the haemolysis is prevented by the cocrystals as compared to the pure quercetin.

Pharmacokinetic Studies

The pharmacokinetic studies were performed using plasma obtained from male Sprague Dawley rats.[49,58,59] The rats weighing 200-250 g (n = 3 per group) were deprived of only food for 18 hours prior to the start of the experiment. The gavage vehicle, an aqueous solution of 0.5 % sodium CMC was used to make suspensions of quercetin, QUER-NICO and QUER-PICO and delivered via oral gavage at a dosage equivalent to 100 mg of quercetin/kg body weight. Approximately 300 µL of blood was collected after 10, 30, 60, 90, 120 and 240 min in centrifuge tubes containing 10 µL of heparin solution. The sampling was done in duplicate. The samples were then centrifuged at 10000 rpm for 20 min, after which the plasma was transferred to sterile centrifuge tubes. The extraction of plasma was done three times with 100 µL of acetonitrile for each extraction.

Quantification of quercetin: The plasma samples were analyzed for quercetin content by HPLC using PDA detector. The calibration solutions for pharmacokinetic studies were made by spiking 100 µL of the rat plasma (extracted with acetonitrile) with appropriate amount of 1 mg/mL stock methanolic solution of quercetin to obtain concentrations of 0.1, 0.2, 0.4, 0.8, 1, 2, 4, 8 and 10 µg/mL.

Pharmacokinetic parameters were determined using a commercially available computer program, KINETICA® (Alfasoft Group Software Solutions Services for Science and Technology, UK & Ireland). The reported pharmacokinetic parameters included C_{MAX}, T_{MAX}, and mean retention time (MRT). The results of QUER-NICO and QUER-PICO were compared with quercetin control.

RESULTS AND DISCUSSION

The multicomponent crystals QUER-NICO and QUER-PICO, prepared by solvent drop grinding method were crystalline in nature but not suitable for single crystal analysis. Thus after characterization of the cocrystalline forms by techniques involving DSC, FT-IR, PXRD and SSNMR, the structure of cocrystals was determined by utilizing the PXRD pattern in Materials Studio® software. The characterized cocrystals were further studied for their enhanced solubility, dissolution and solubility profile apart from the measurement of improvement in antioxidant effect, antihaemolytic activity and pharmacokinetic parameters of the cocrystals.

Differential Scanning Calorimetry

The DSC curves depicting the thermal behaviour of quercetin, nicotinamide, picolinic acid, QUER-NICO and QUER-PICO are shown in Figure 1 and 2. The position of single sharp endotherms in Figure 1 (a), 2 (a), 1 (b) and 2 (b) show the melting points of quercetin, nicotinamide and picolinic acid to be 321.3, 130.6 and 138.1 ^{o}C respectively.

The melting endotherms of QUER-NICO and QUER-PICO, as shown in Figures 1 (c) and 2 (c) appear as single sharp peaks at 234.7 ^{o}C and 228.6 ^{o}C, respectively. The appearance of a single peak present between the melting points of quercetin and nicotinamide appears to convey the formation of a new crystalline form which could be a cocrystal.

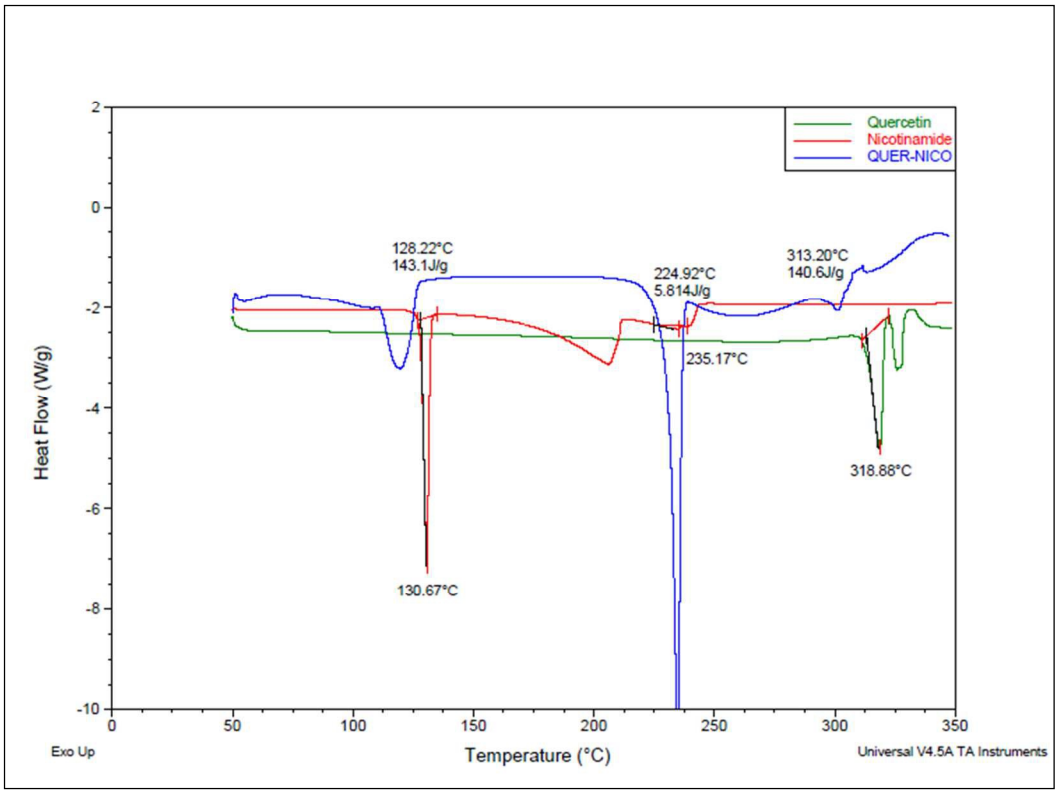


Figure I. Differential Scanning Calorimetry of quercetin and QUER-NICO.

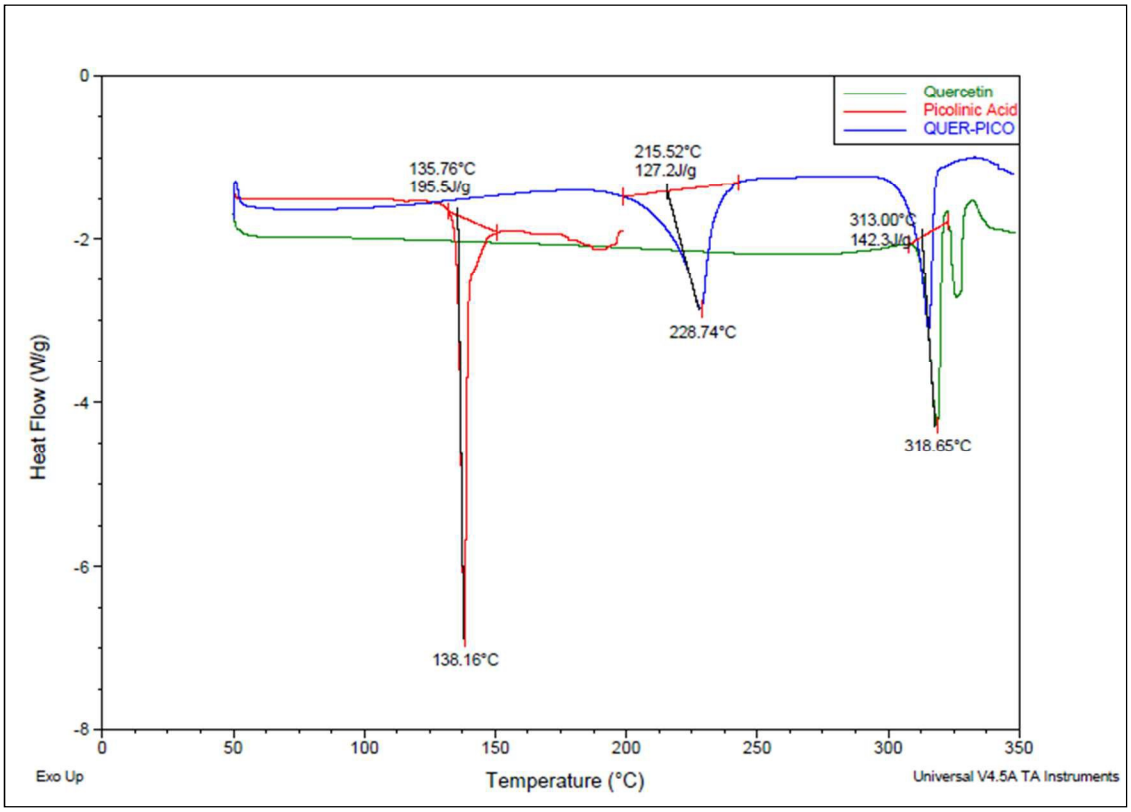


Figure 2. Differential Scanning Calorimetry of quercetin and QUER-PICO.

PXRD

The PXRD patterns for quercetin, nicotinamide, picolinic acid, QUER-NICO and QUER-PICO are shown in Figure 3. The Figure 3 (a), (b) and (c), show the powder XRD pattern of the constituent molecules, i.e. quercetin, nicotinamide and picolinic acid, respectively.

The PXRD pattern of quercetin nicotinamide cocrystal (QUER-NICO, Figure 3 d) shows new and distinct peaks at the 2θ values of 4.5 ^{o}, 8.8^{o}, 13.91^{o} and 16.25^{o} which are not originally present in the pattern of either quercetin or nicotinamide, while the peaks in quercetin at 9.79^{o} and 22.58^{o} and in nicotinamide at 22.80^{o} and 32.64^{o} have disappeared.

Moreover the peaks at 12.22^{o}, 14.44^{o} and 28.89^{o} in quercetin and at 11.43^{o}, 14.9^{o} and 28.56^{o} in nicotinamide have combined to give 12.48^{o}, 14.84^{o} and 29.42^{o} respectively.

Additionally, the peaks at 13.23^{o}, 17.23^{o}, 26.86^{o} in quercetin shifted to 13.17^{o}, 17.58^{o}, 26.67 ^{o} and the peaks in nicotinamide at 23.48^{o}, 25.49^{o}, 25.97^{o} showed a shift to 23.65 ^{o}, 24.97^{o}, 25.86^{o} respectively.

Similarly the powder XRD pattern of quercetin picolinic acid cocrystal (QUER-PICO, Figure 3 e), shows new peaks at 16.60^{o}, 16.89^{o} and 19.36^{o} of 2θ which are not present in the original pattern of either quercetin or picolinic acid.

The peaks at 9.79^{o}, 17.2^{o}, 26.8^{o}, 28.89^{o} in quercetin and at 8.7^{o}, 17.6^{o}, 26.5^{o}, 28.11^{o} in picolinic acid have combined to give 8.39^{o}, 17.53^{o}, 26.9^{o}, 28.23^{o} respectively in the cocrystal whereas the peaks in picolinic acid at 24.22^{o}, 26.83^{o}, 33.8^{o} have disappeared.

However the peaks at 12.21^{o}, 14.39^{o}, 14.94^{o} in quercetin and at 24.22^{o}, 24.97^{o} and 27.9^{o} in picolinic acid showed shift to 11.75^{o}, 14.57^{o}, 14.89^{o}, 24.10^{o}, 25.54^{o} and 27.4^{o} respectively

The presence of new and distinct peaks apart from the absence of some peaks, lead us one step closer to confirming the formation of a new cocrystalline phase in each case.

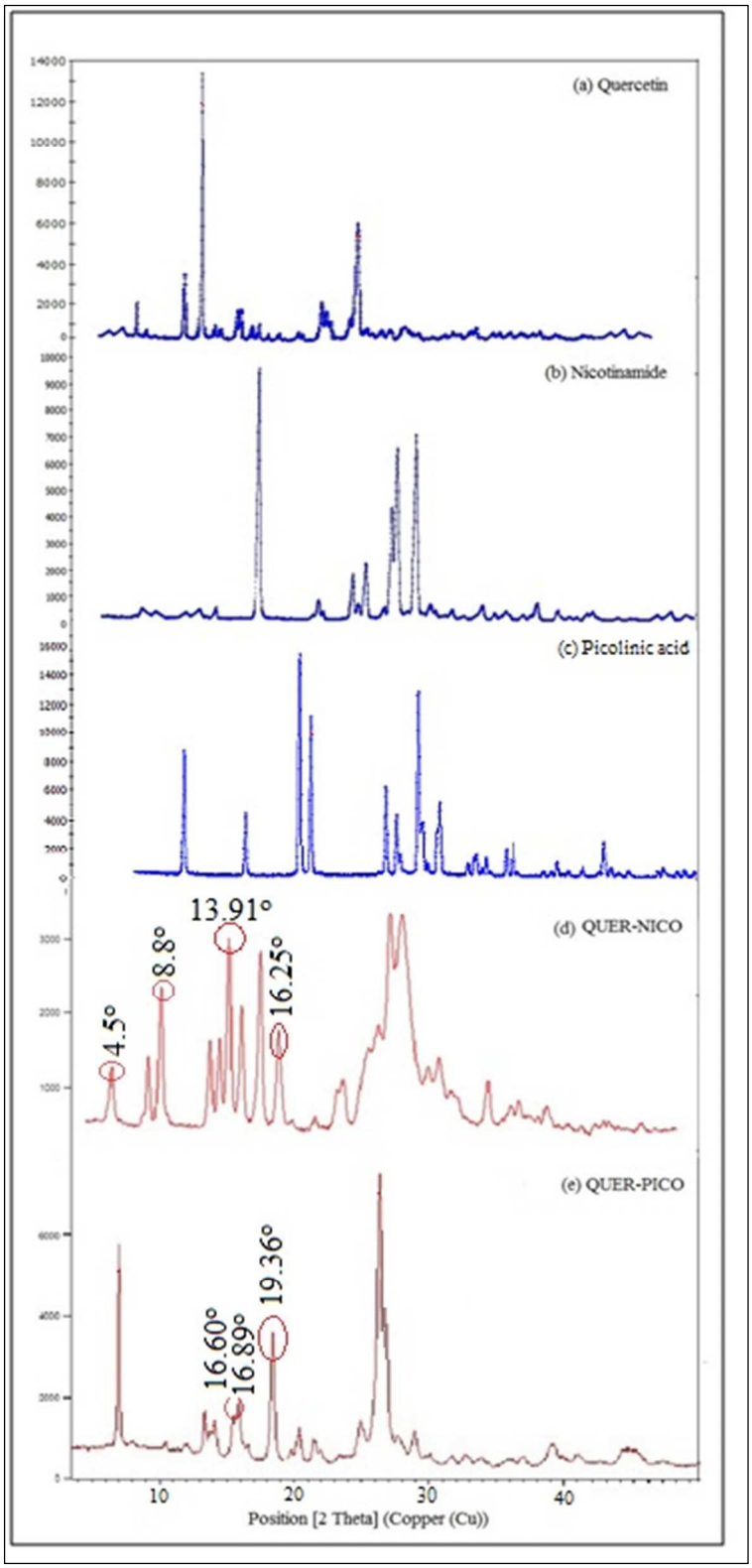


Figure 3. Powder X-Ray Diffraction Spectra

Crystal Structure Determination

The powder diffraction pattern of the quercetin, QUER-NICO and QUER-PICO cocrystal were subjected to computational calculations to obtain a crystal structure solution of the formed cocrystals.

The structure determination of quercetin was carried out using its powder X-Ray diffraction pattern as a single crystal data for anhydrous quercetin was not available. The structure obtained (Figure 4) was in orthorhombic unit cell with Pn21a space group. The structure showed hydrogen bonding of one quercetin molecule with 4 other quercetin molceules. The hydroxyl group at C3 is bifurcated to form hydrogen bond with -OH group on C5 and C7 of adjacent quercetin molecules forming an OH....OH homosynthon.

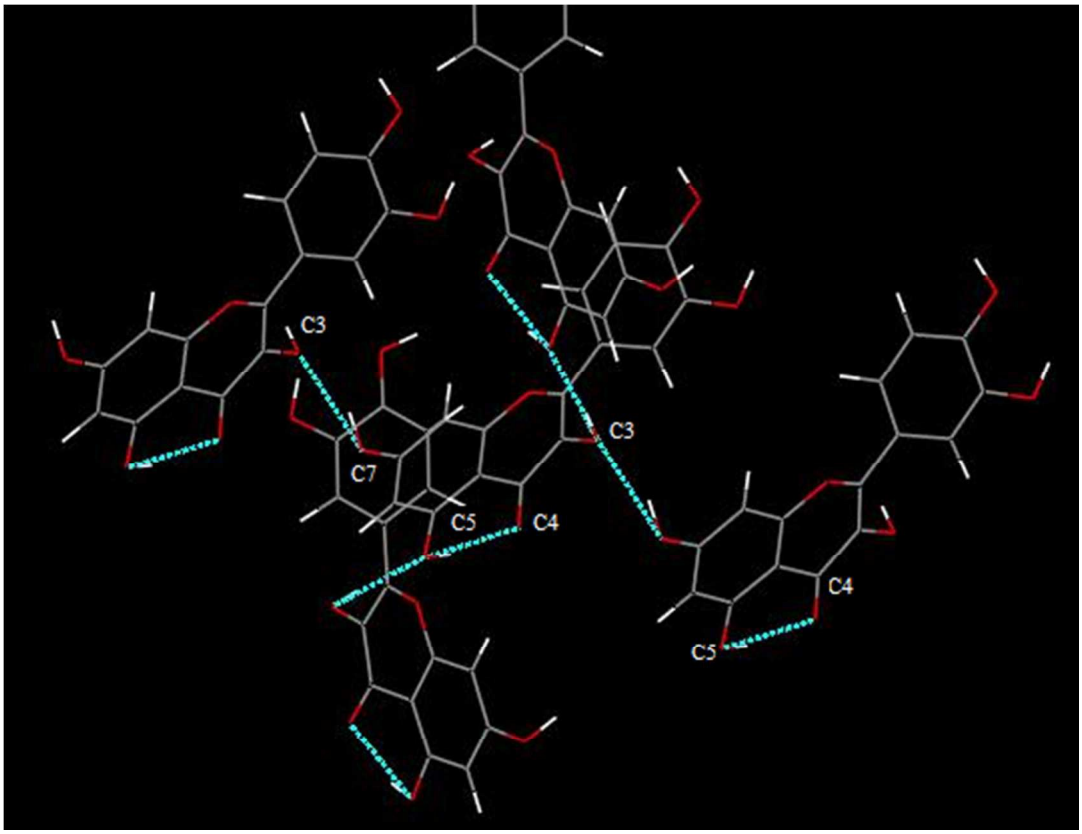


Figure 4. Crystal structure of quercetin. (Structure showing hydrogen bonding between different quercetin molecules in pure quercetin structure analysis

The structure elucidation of QUER-NICO was initiated with the removal of Kα-2 peaks and followed by the stripping and smoothening of the experimental powder XRD pattern in the software.

The comparative pattern, in Figure 5 (a), depicting the powder XRD pattern obtained (i) experimentally, (ii) after removal of Kα-2 peaks and (iii) simulated from the determined crystal structure, does not indicate any shift in the position of the peaks except for some expected changes in their intensities.

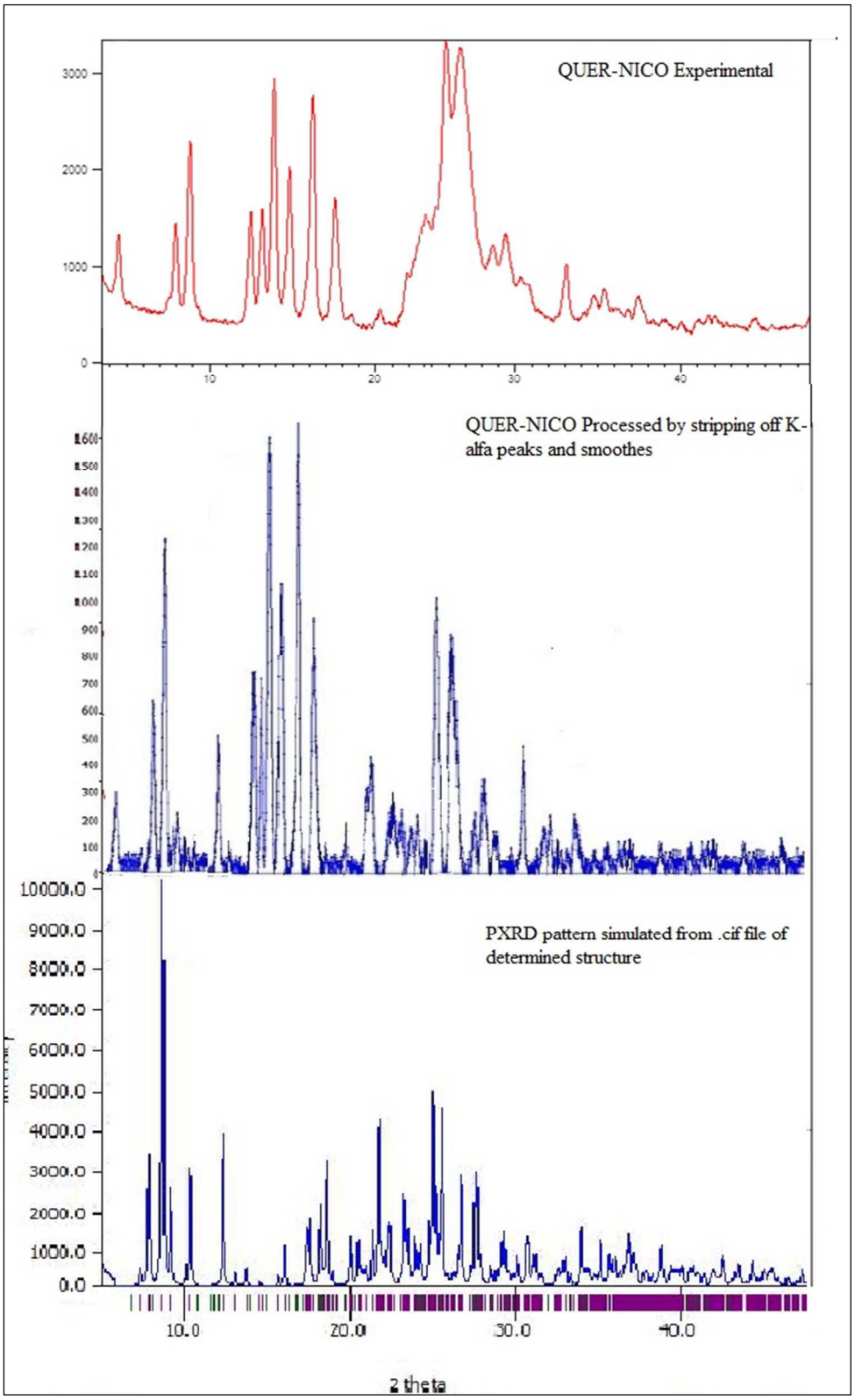


Figure 5 (a).Comparison of QUER-NICO PXRD pattern, (i) obtained experimentally, (ii) pattern obtained by processing of experimental pattern by stripping off Kα peaks and smoothening and (iii) pattern obtained from the .cif file of determined structure of cocrystal.

This powder XRD pattern after removal of Kα-2 peaks was subjected to indexing by X-Cell module which gave among other solutions, a monoclinic unit cell (P21/A) which had highest figure of merit and incorporated all the peaks of the powder XRD pattern. The Pawley refinement of this P21/A cell was done three times to obtain the Rwp of 4.49. The crystal structure (Figure 5 (b-d)) of QUER-NICO reveals that the aromatic nitrogen and the carbonyl moiety of the amide group of nicotinamide as well as carbonyl group in quercetin acts as hydrogen bond acceptors. All the hydroxyl groups of quercetin, although can act as hydrogen bond acceptors or donors, but here only act as donors. Two nicotinamide molecules interact with one quercetin molecule at two positions, one at OH on C3 forming a OH….O=C (of amide moiety of nicotinamide) heterosynthon and another at hydroxyl group on C5 of quercetin forming a OH…N_{arom} supramolecular heterosynthon. Besides this, the quercetin interacts with three adjacent quercetin molecules by forming hydrogen bonds (OH on C3' of first with OH on C4' of second molceule and OH on C4' of first with OH on C3' of third molecule. Also seen are hydrogen bonds between OH on C7 of first with OH on C7 of second and third molecule.

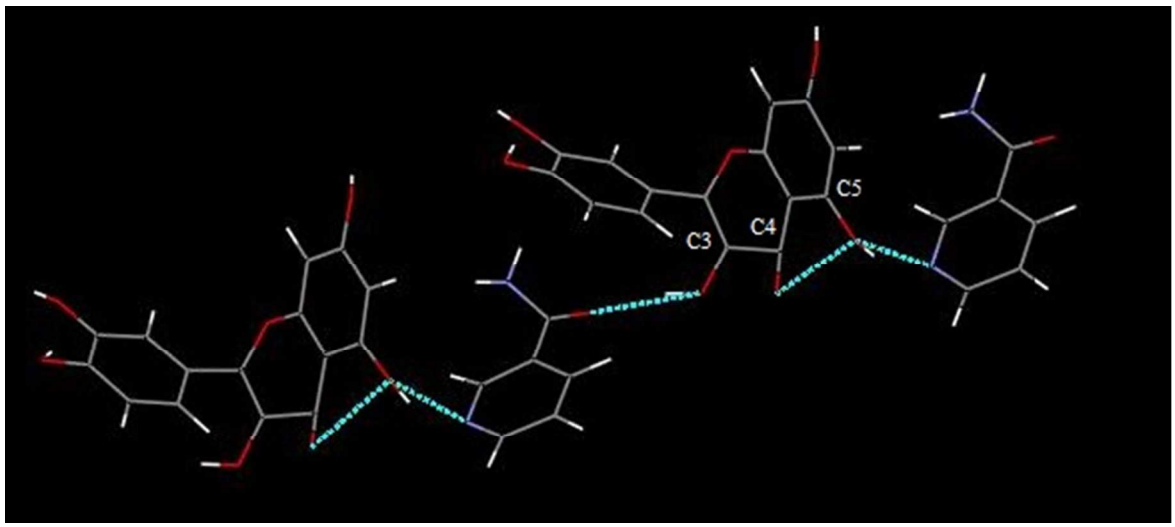


Figure 5 (b).Hydrogen bonding between quercetin and nicotinamide

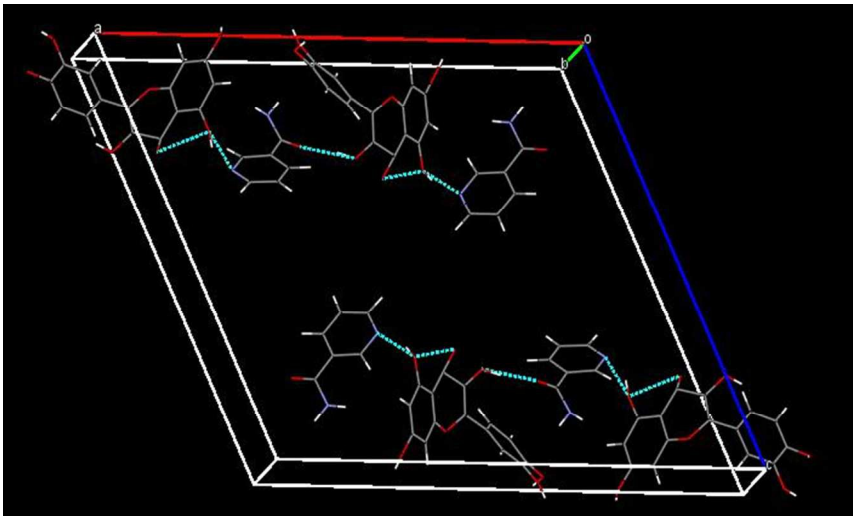


Figure 5(c). Quercetin and nicotinamide molecules in the QUER-NICO Monoclinic unit cell

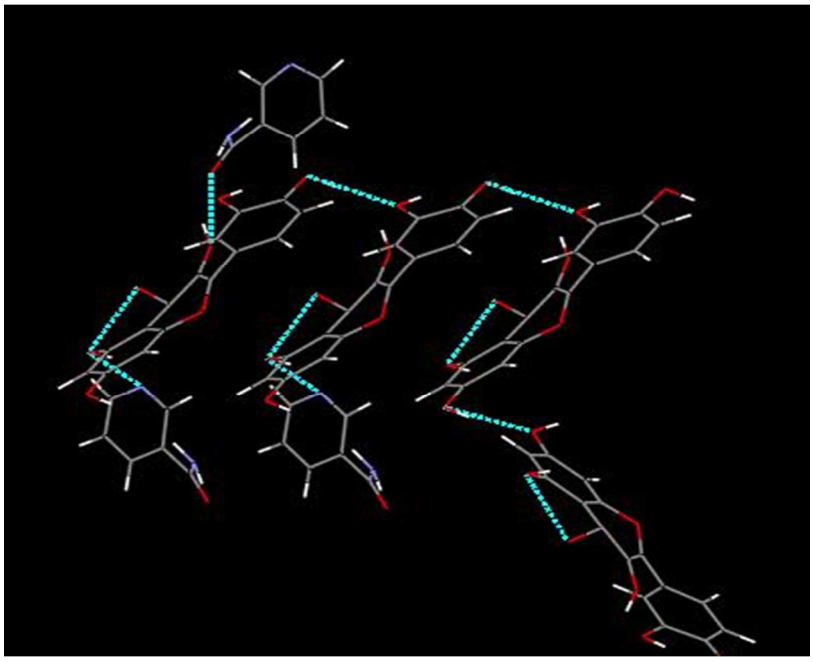


Figure 5 (d). Hydrogen bonding between different quercetin molecules in the QUER-NICO

Similarly, the powder XRD pattern of cocrystal QUER-PICO was also processed by removal of Kα-2 peaks followed by stripping and smoothening. Figure 6 (a) shows the comparison of the (1) experimental pattern, (ii) the processed pattern and (iii) the similated pattern from the .cif file of determined structure. The comparison of these three patterns also shows similarity in position of the peaks although there is expected difference in the intensities of the peaks.

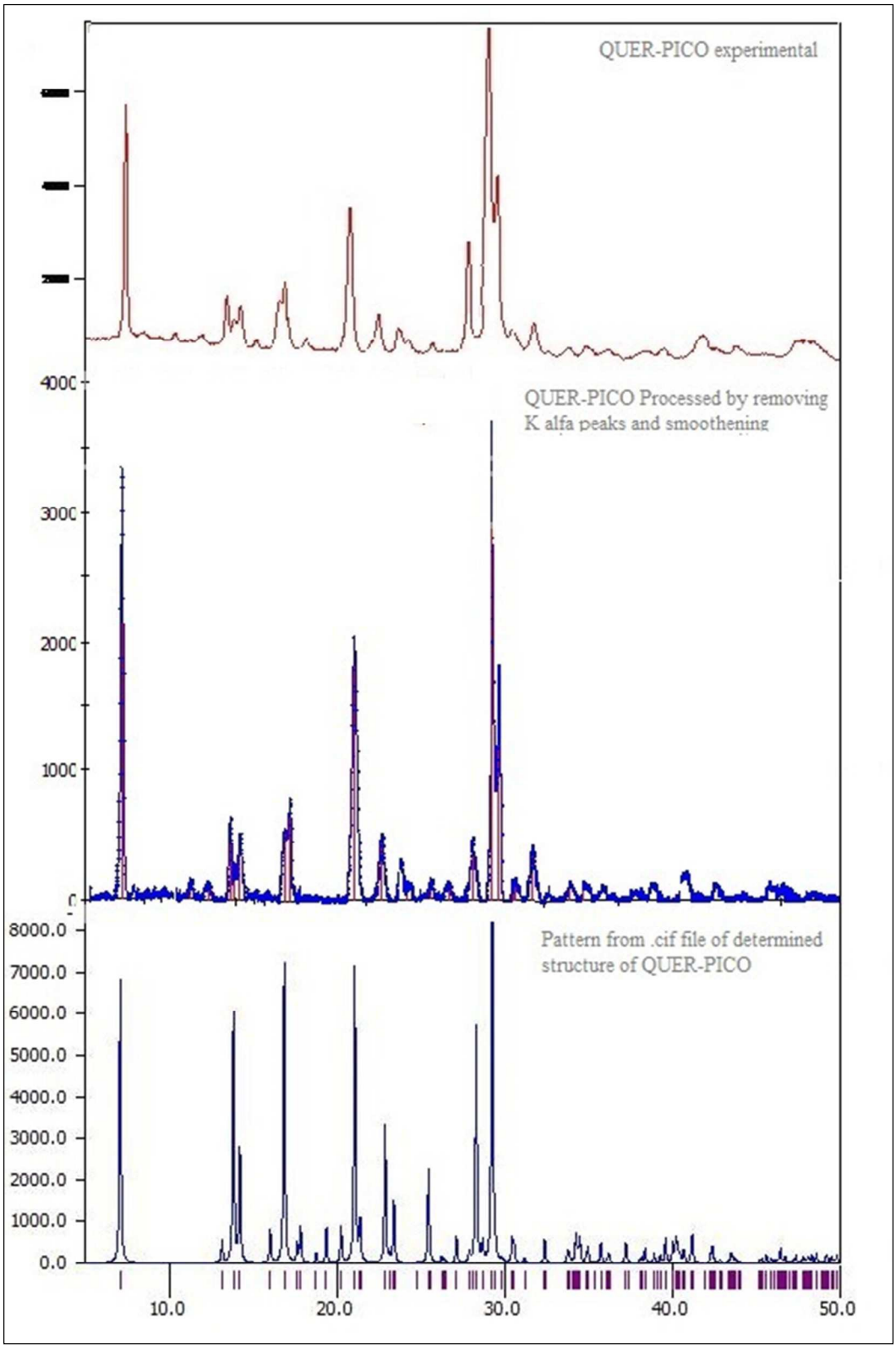


Figure 6 (a).Comparison of QUER-PICO PXRD pattern, (i) obtained experimentally, (ii) pattern obtained by processing of experimental pattern by stripping off Kα peaks and smoothening and (iii) pattern obtained from the .cif file of determined structure of cocrystal.

The powder XRD pattern of QUER-PICO after being subject to removal of ks and smoothening is shown to possess the crystal structure as shown in Figure 6 (b and c). The indexing of PXRD pattern in XCell threw up P1 space group in triclinic unit cell. The solution structure obtained after refinement using Reitveld had Rwp = 9.45%. The structure solution shows that the hydrogen bonds between quercetin molecules at hydroxyl groups on C7, seen in the crystal structure of quercetin are replaced by hydrogen bonds with picolinc acid. The OH group on C7 of quercetin molecule is bifurcated to form hydrogen bonds with hydroxyl group of carboxylic acid moiety of one picolinic acid (OH....OH homosynthon) and with aromatic ring N of second molecule of picolinic acid (N_{arom}...OH heterosynthon). Another picolinic acid molecule is hydrogen bonded by its OH group on carboxylic acid side chain with the OH group on C5 of quercetin. Besides this, one quercetin molecule is also attached to four other quercetin molecules at various points. These points are seen at hydroxyl groups on C5, C3 and also on the substituent ring at C3' and C4'. The crystallographic parameters are shown in Table 1.

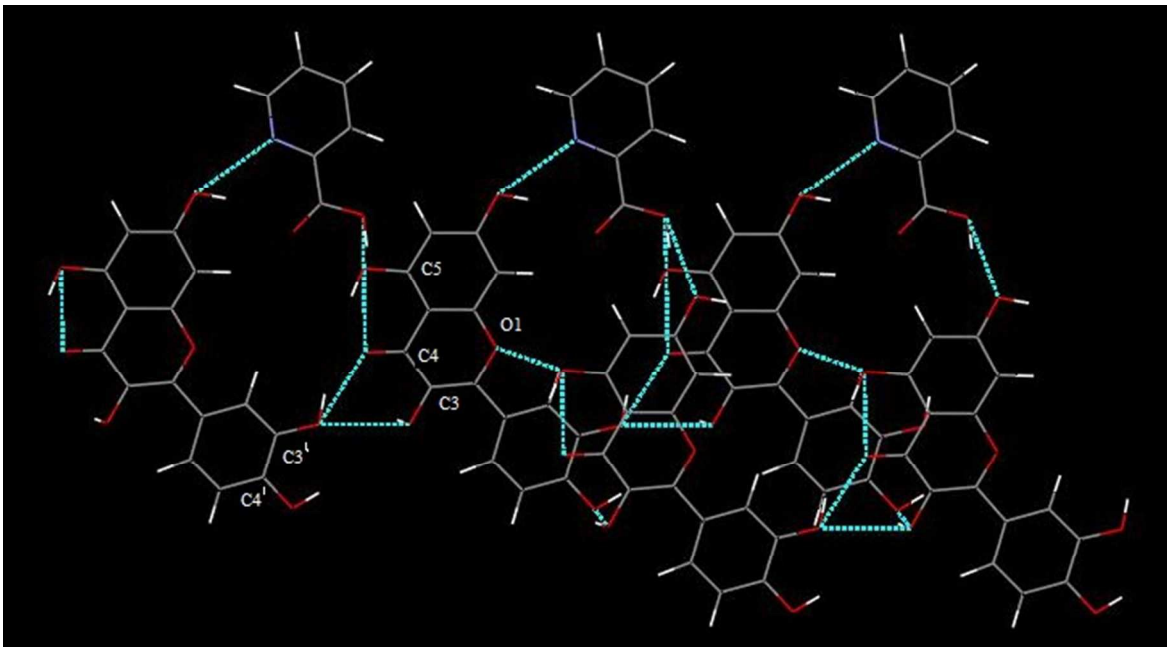


Figure 6 (b). Hydrogen bonding between quercetin and picolinic acid.

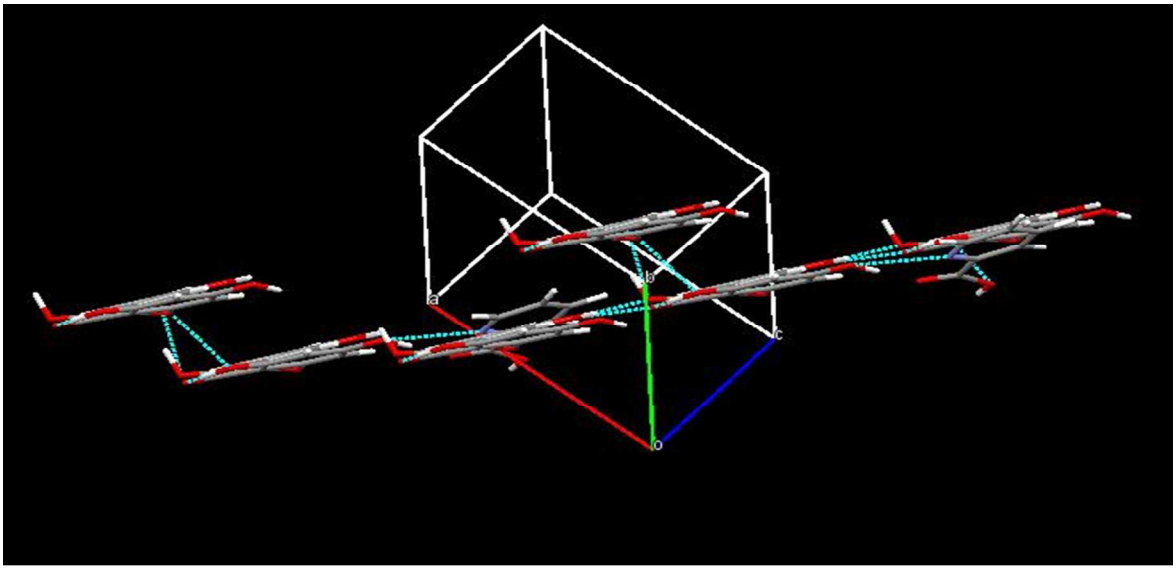


Figure 6 (c) Stacking diagram of QUER-PICO in unit cell.

Table 1.Crystallographic parameters.

	Quercetin	QUER-NICO	QUER-PICO
Formula	C15 H10 O7	C15 H10 O7, C6 H6 N2 O	C15 H10 O7, C6 H5 N O2
sum formula	C15 H10 O7	C21 H16 N2 O8	C21 H15 N O9
MW	302.23	424.36	425.34
crystal system	Orthorhombic	Monoclinic	triclinic
spacegroup	P n 21 a	P 21/a	P 1
a (Å)	14.7998	24.5332	12.6465
b (Å)	11.2379	5.2575	7.9602
c (Å)	10.3512	22.0935	5.3536
α (deg)	90	90	58.9404
β (deg)	90	112.6259	92.1712
γ (deg)	90	90	99.689
Cell Volume (V)	1721.598	2630.37	454.322
D g/cm3	1.166	1.072	1.555
Z	4	4	1
2θ range	3.5084 - 49.9864	3.5084-49.9864	5.084-49.9904

FT-IR

The vibrational spectroscopy provides complimentary molecular level information depending upon the shifts in the absorption bands which are relative to the input components and help to determine the functional groups involved in molecular interaction. The Figure 7 (a), (b) and (c) describe the FT-IR pattern of quercetin, nicotinamide and picolinic acid respectively.

The Figure 7 (d) shows FT-IR spectrum of QUER-NICO. The peak at 3370.4 cm^{-1} in quercetin, corresponding to phenolic –OH stretch shifts to 3422.2 cm^{-1} and the peak at 1681.7 cm-^{1} due to –C=O stretch of amide moiety of nicotinamide shifts to 1705.2 cm^{-1} in cocrystal confirming their involvement in hydrogen bonding in cocrystal. There are also seen corresponding shifts from 3160.8 cm^{-1} in the spectrum of nictotinamide to 3175.2 in cocrystal spectrum confirming the involvement of amide group of nicotinamide in hydrogen bonding.

The Figure 7 (e) depicting QUER-PICO shows substantial shifts confirming hydrogen bond formation in cocrystal. The phenolic -OH stretch in quercetin at 3370.4 cm^{-1} shifts to 3492.7 cm^{-1} and the carboxylic acid –OH stretch in picolinic acid at 3110.5 cm^{-1} shifts to 3265.7 cm^{-1}. Furthermore the absence of peaks around 1650.1 cm^{-1} and 1400.3 cm^{-1} shows that picolinic acid is not present in its anionic form but as a neutral molecule.

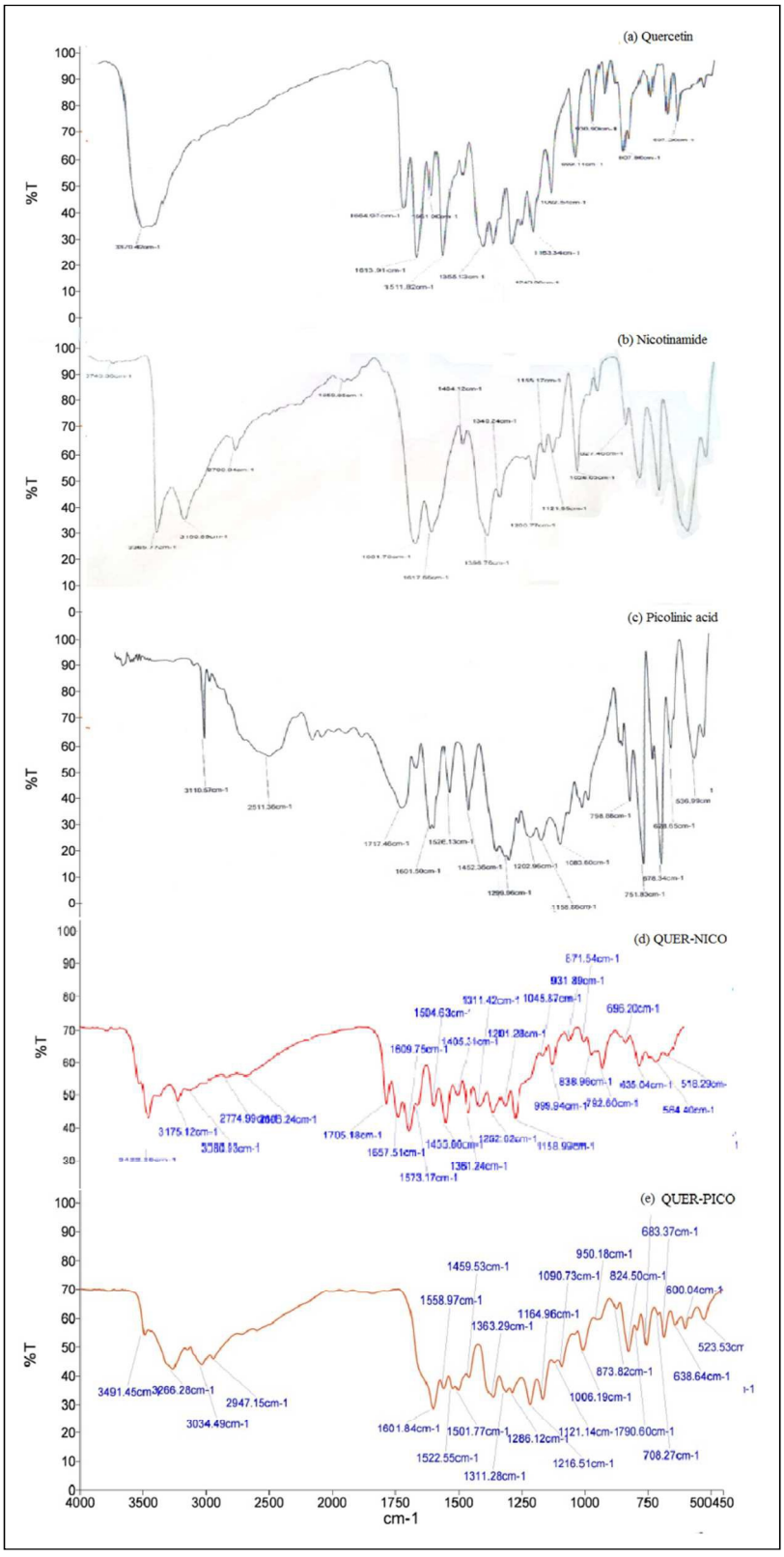


Figure 7. FT-IR spectra

^{13}C Solid State NMR Analysis

The analysis of the ^{13}C solid state NMR data shows significant difference in position of some peaks as highlighted in the Figures 8(a) for QUER-NICO and 8(b) for QUER-PICO.

The Figure 8(a) representing QUER-NICO, shows major perturbations at C-3 and C-5 atoms of quercetin to which OH groups involved in hydrogen bonding are attached. The peaks at 138.3 (C-3) and 160.54 (C-4) shift to 139.3 and 162.46 respectively in the cocrystal. Furthermore, the peak corresponding to the carbon of amide group in side chain of nicotinamide appearing at 168.7 shifts to 166.4 along with the shifts in the peaks of carbons adjacent to the aromatic nitrogen in pyridine ring from 153.7 and 148.2 to 154.8 and 147.9 respectively. The high intensity peak at 147.9 in the cocrystal pattern is likely to be the result of superimposition of peak due to the C-2 in quercetin and the carbon present adjacent to aromatic N in nicotinamide.

The Figure 8(b) representing QUER-PICO, depicts the shift in peaks corresponding to C-4, C-5 and C-7 of quercetin from 177.0, 160.5 and 165.5 to 176.14, 158.89 and 164.5 respectively in the cocrystal. The peak corresponding to carbon of side chain carboxylic acid of picolinic acid shifts from 166.5 to 164.5 and the peaks due to carbons adjacent to the aromatic nitrogen of pyridine shows shifts from 147.8 and 145.84 to 149.05 and 146.6 respectively.

These shifts are irrefutable proof of the involvement of these atoms in hydrogen bonding in our cocrystals.

The comparative study of solid state ^{13}C NMR spectrum of quercetin, nicotinamide and QUER-NICO in Figure 8(a), and that of quercetin, picolinic acid and QUER-PICO in Figure 8 (b), compliments the information regarding the formation of hydrogen bonding as elucidated by PXRD and FT-IR analysis.

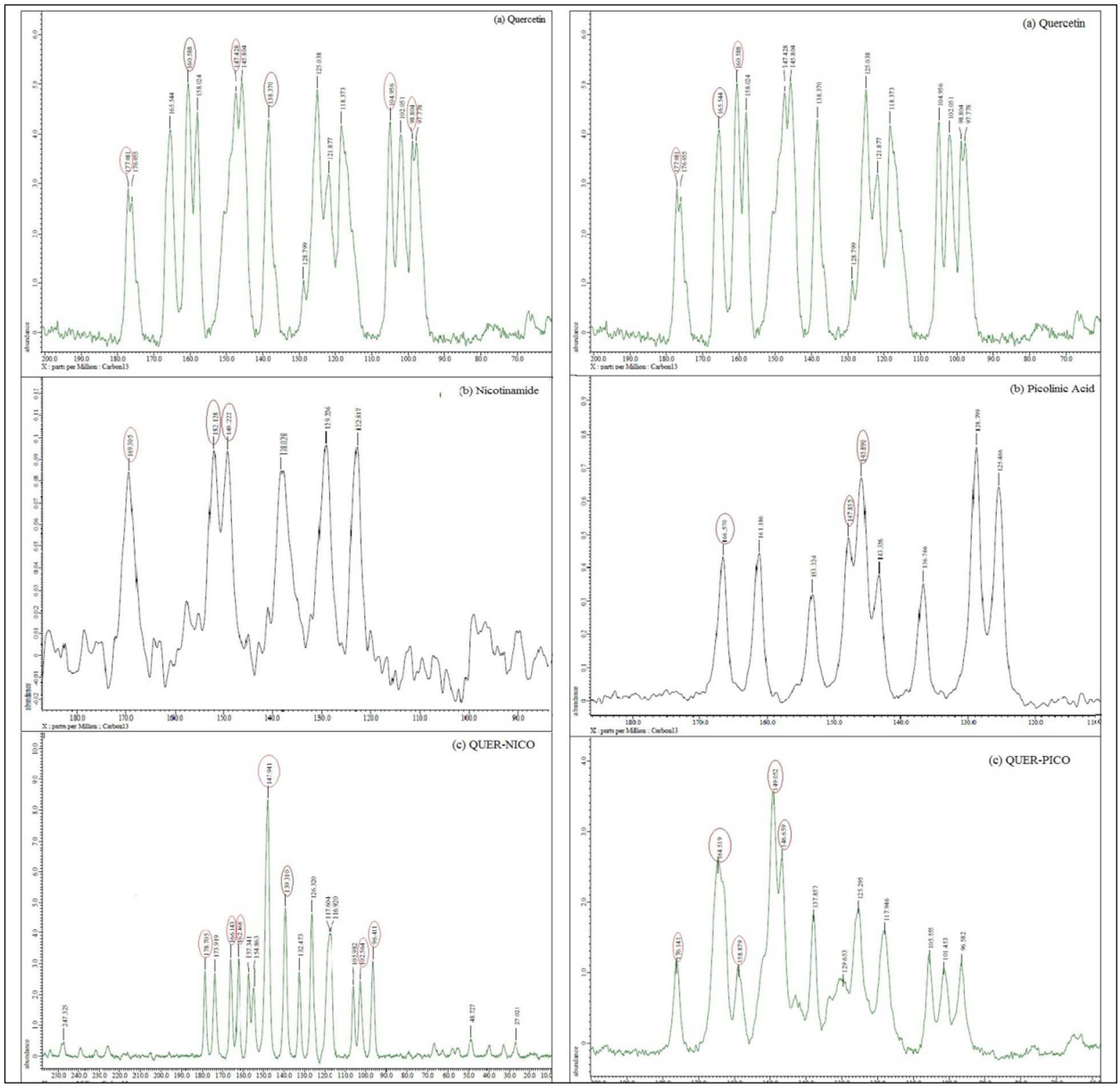


Figure 8 (a) Solid state ^{13}C NMR spectra of quercetin, nicotinamide and QUER-NICO

Figure 8 (b) Solid state ^{13}C NMR spectra of quercetin, picolinic acid and QUER-PICO

Figure 8. Solid State ^{13}C-NMR spectra

Dissolution and Equilibrium Solubility Studies

The results of dissolution and solubility studies are shown in Figure 9 (a and b). The results depict a significant improvement in the dissolution rate and the S_{max} (maximum solubility concentration) of the cocrystals QUER-NICO and QUER-PICO as compared to quercetin.

In cocrystals, the S_{max} was found to be 115.15 µg/mL and 237.54 µg/mL for QUER-NICO (at 50 mins) and QUER-PICO (40 mins), respectively as compared to the S_{max} of 22.6 µg/mL achieved by quercetin alone. However, subsequently there was seen a decline in enhancement of the

solubility of cocrystals after S_{max} was achieved. This was probably due to the breakdown of cocrystal into quercetin and its coformer and the subsequent rapid conversion of quercetin to quercetin dihydrate in aqueous medium at the end of the equilibrium solubility experiment (24 h). However, the improvement in solubility during 1- 3 h period is high enough to give increased drug exposure in pharmacokinetic studies.

To fully comprehend the upshot of cocrystallization of quercetin, the physical mixtures of the constitutents were also subjected to on dissolution and solubility studies.

These studies clearly show no improvement in the solubility and dissolution profile of quercetin in its physical mixtures as showcased in Figure 9 (c). This essentially confirms that the improved solubility of quercetin is due to the formation of cocrystals.

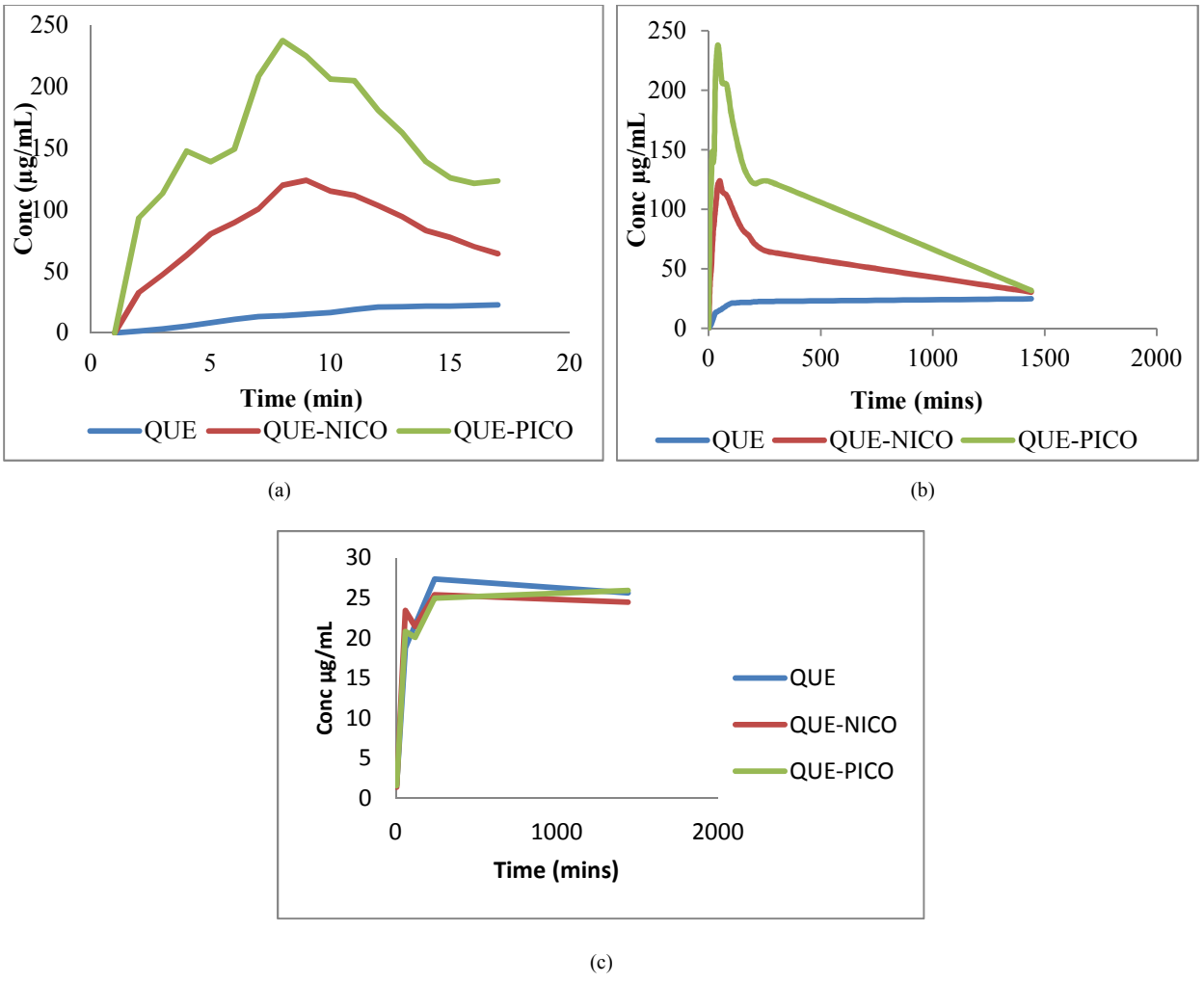


Figure 9: Dissolution studies of : (a) Cocrystals for first 270 mins, (b) Cocrystals upto 24 hours (c) Physical mixtures of quercetin and equimolar quantities of coformers upto 24 hours

Antioxidant Activity

The results of the DPPH free radical scavenging activity done for quercetin, QUER-NICO and QUER-PICO are presented in the Figure 10. There is seen enhanced activity in case of QUER-PICO and in case of the cocrystals as compared to quercetin itself. However, the increase in activity is much more in case of QUER-PICO as against QUER-NICO.

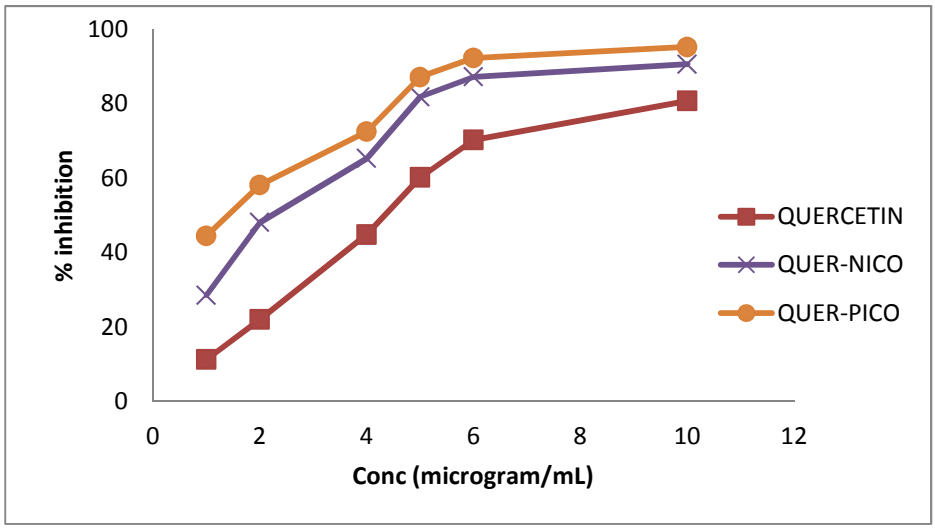


Figure 10. DPPH radical scavenging activity.

Antihaemolytic Activity

The antihaemolytic activity measured the percentage inhibition of haemolysis of rat red blood cells by quercetin. The results presented in Figure 11 clearly show the inhibition of haemolysis by QUER-PICO to be almost three times and by QUER-NICO to be almost double that of quercetin. These results are in agreement with the dissolution and the solubility profile which have shown the cocrystals to be more soluble as compared to quercetin, and out of these, further, the form QUER-PICO is much more soluble than QUER-NICO.

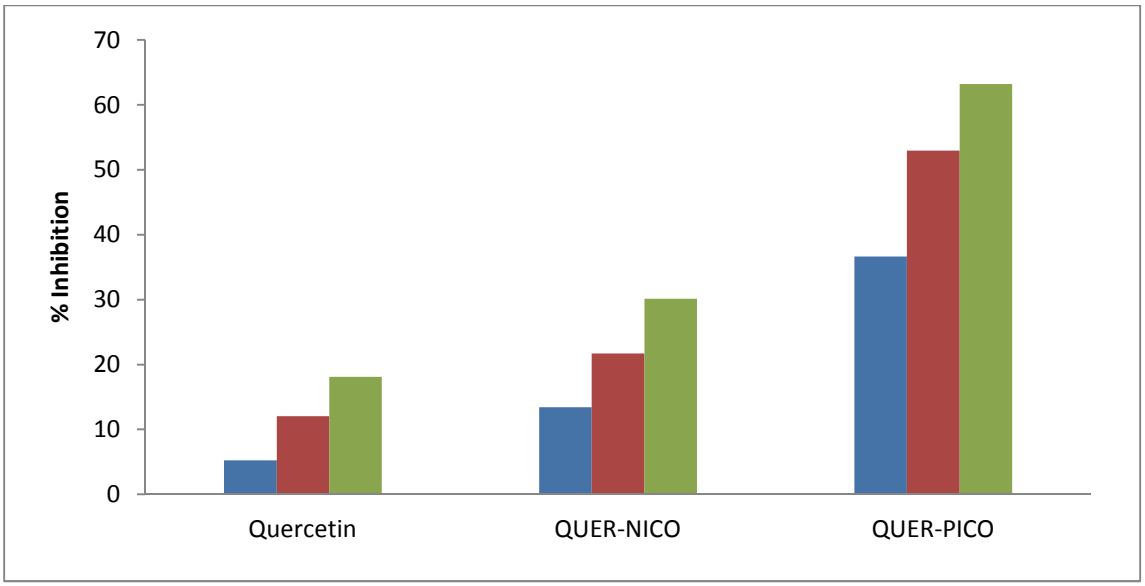


Figure 11. Antihaemolytic activity

Pharmacokinetic studies

The pharmacokinetic profile of quercetin and its cocrystals as shown in Figure 12 with the parameters tabulated in Table 2 shows that the quercetin in the form of its cocrystals has a much higher absorption leading to shorter T_{max} and higher C_{max} . The relative bioavailability (calculated as AUC_{tot} of the cocrystal divided by AUC_{tot} of quercetin) is the availability of quercetin in biological systems for pharmacological action and calculated to be 1.5 and 1.75 for QUER-NICO and QUER-PICO respectively. The higher plasma concentration of quercetin from its cocrystals justifies its increased solubility.

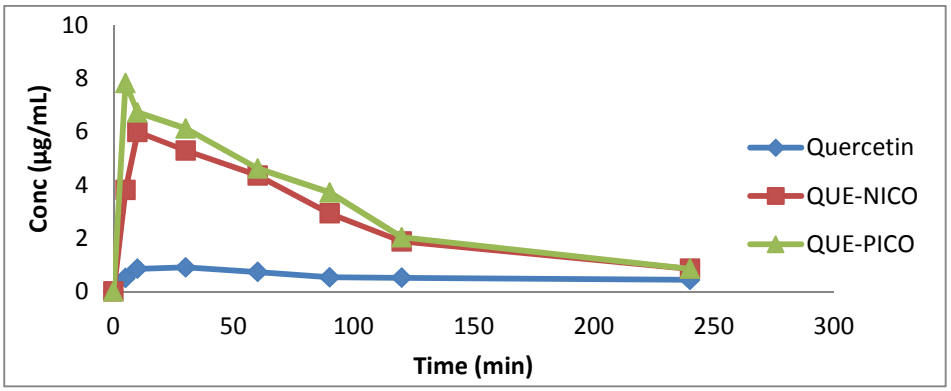


Figure 12. The pharmacokinetic profile

Table 2. Pharmacokinetic parameters.

Pharmacokinetic parameters	Quercetin	QUER-NICO	QUER-PICO
T max (min)	30	10	5

C max (µg/mL)	0.9117± 0.001	5.9972+ 0.001	7.832 + 0.005
Mean retention time (min)	80.17	112.32	103
AUC tot (µg/mL * min)	467.26	728.4	817.664
Relative Bioavailability		1.558858	1.749894

Conclusion

The cocrystallization has been successful as an effective means to improve upon the biopharmaceutical parameters of quercetin. The cocrystals, QUER-NICO, and QUER-PICO, prepared by an effective and an eco friendly technique confirms to the standards of green chemistry and have been characterized by a variety of techniques involving thermal and spectroscopic methods. The crystal structure analysis for pure quercetin and its cocrystals clearly shows the breaking of the -OH...OH- homosynthon between the adjacent quercetin molecules to make way for the -OH...O=C heterosynthon in QUER-NICO and N_{arom}...OH heterosynthon in QUER-PICO. The increased dissolution rate and higher solubility leads to improved pharmacokinetic profile apart from imparting increased efficacy as an antioxidant and an antihaemolytic agent.

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Conflict of Interest

None

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