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Original Article

Enhancement of the Antimicrobial Properties of SAP Using Beta-Cyclodextrin as an Encapsulation Agent

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ABSTRACT

Objectives: The objective of this endeavour is to prepare β-cyclodextrin (β-CD) inclusion complex with sulfonamide (**SAP**) using the co-evaporation technique and screened its effect on *in-vitro* antimicrobial property against some Gram-positive and Gram-negative bacteria.
Methods: A new sulfonamide SAP (4-amino-N-(1, 5-dimethyl-3-oxo-2-phenyl-2, 3-dihydro-1H-pyrazol-4-yl) benzene sulfonamide) **4** has been synthesized by using Ampyrone with N-Acetylsulfanilyl chloride and characterized by FT-IR, NMR, and LC-MS spectroscopy. The inclusion complex of **4** with β-CD is achieved in the semi aqueous medium by using Sonicator and is characterized with the help of UV–Vis, Fourier Transform Infrared spectrometer, Scanning Electron Microscope, Differential Scanning Calorimetry, Thermo gravimetric Analyzer. Finally, effect of in-*vitro* antimicrobial activity of the inclusion complex has been studied with the free SAP.
Results: Successful conversion of the new Sulfonamide to its inclusion complex with β-CD has been achieved by co evaporation method. *In- vitro* antimicrobial studies of both SAP and its inclusion complex with β-CD shows the better enhancement in Inclusion complex across the free Sulfonamide.
Conclusion: The above study shows that inclusion complex of sulfonamide with β-CD offers a simple and attractive solution to improve the bioactivity of pharmaceutical and pharmacokinetic properties of Sulfonamide.

Keywords: β-Cyclodextrin; Sulfonamide; Antimicrobial Property; Inclusion complex.

INTRODUCTION

Wounds permit the microorganism deposition and their growth, leading to skin and soft tissue infections which delay in the healing process. Hence, antibiotics have been indiscriminately used for the treatment of such infections and aggravated by extending therapies, leading to the development of microbial resistance[1]. Thus, antibiotics had been losing their capacity against pathogens and new therapies had to be adopted control multiresistant bacteria[2]. Therefore, natural and synthetic antibacterial agents are better alternative to overcome this issue.

Sulfonamides are a class of family of synthetic drugs used pharmacologically as antibacterial agents to treat or prevent the many kinds of bacterial inflammations as they have ability to inhibit the bacterial growth in wounds or infected organs without appreciable toxicity to normal tissues. They have some other interesting medicinal properties, such as hypoglycemic[3], antitumor, antiviral drugs, diuretics, antithyroid agents, anti-carbonic anhydrase[4-7]. Recently, it is reported that a host of structurally novel sulfonamide derivatives show substantial antitumor and anticancer activity[8-14]. Sulfonamides are described as antibiotics, but these bioactive molecules possesses with some issues regarding the solubility and stability to environmental stress.

Hence, for enhancing the physical, chemical and biological properties of these bioactive molecules, they are encapsulated within Cyclodextrins (CDs). Moreover, CDs have been described as safe for humans and approved by the Food and Drug Administration (FDA) [15]. These are cyclic oligosaccharides composed of glucose units linked by α-1, 4-glycosidic bonds[16] related to natural products formed during bacterial digestion of cellulose[17]. These are made-up of three types, i.e., α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, composed of 6, 7, and 8 α-1, 4 -glycosidic bonds respectively[18-19]. CDs can form inclusion complexes that enclose various organic compounds in their unique cyclical structure because of their toroidal shape with a hydrophobic surface inside and free hydroxyl groups[20]. Due to these specialties, they are widely used as additives for a variety of consumer items for enhancing the physical and chemical properties of products[21]. Even though long-term researches have been carried out, but very little information about bioactivity of CDs against microorganism has been published to date.

Among of these cyclic oligosaccharides, β- Cyclodextrin is most popular glucose unit for inclusion of various types of molecules like drugs, heterocyclic compounds, polymers, pesticides etc., because it can be sourced from a renewable natural material (i.e., starch), and possesses some great characteristics like cheapness, nontoxic, biodegradable, and non hazarders to environment[22].

During the last few years, the researcher has found that the ability of β-cyclodextrin to achieved successful molecular inclusion complexes with a various drug molecules in the pharmaceutical area, as it increased solubility, enhanced bioavailability, improved stability, masking of bad taste or odor, reduced volatility and contribution to drug release systems[23-26]. Hence, mostly the inclusion complexes of drugs with β -CD have been widely used in recent years in the pharmaceutical region.

MATERIALS AND METHODS

Materials

(4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)

benzenesulfonamide) is synthesized by using N-Acetylsulfanilyl chloride and 4-amino-1,2-dihydro-2,3-dimethyl-1-phenylpyrazol-5-one, N-Acetylsulfanilyl chloride was purchased from Sigma-Aldrich chemicals (Bengaluru, India), 4-amino-1,2-dihydro-2,3-dimethyl-1-phenylpyrazol-5-one & β-cyclodextrin from S.D. Fine Chem. Ltd.(Mumbai) and were used without further purification.

Methods of preparation[27]

Synthesis of N-(4-(N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)sulfamoyl)phenyl)acetamide (3)

In scheme 1 Compound **3** is synthesized by dissolving 4-amino-1,2-dihydro-2,3-dimethyl-1-phenylpyrazol-5-one **1** (0.01 mmol) in a mixture of anhydrous acetone and pyridine (10 ml). This mixture was then cooled to 0-5°C while stirred and then 4-acetamidobenzenesulfonyl chloride **2** (0.01 mmol) was added portion wise over a period of 10 min and stirred for overnight at RT. The sticky heterogeneous mass thus obtained was then filtered, washed with water (30 ml) and dried to get crude product. The compound was purified by recrystallization from a mixture of ethyl acetate: methanol (9:1) to give a white solid.

Synthesis of 4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzenesulfonamide (4)

N-(4-(N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl) sulfamoyl) phenyl)acetamide **3** (3.0 g) was dissolved in 50 ml ethanol containing conc. HCl. The solution was heated at constant temperature of 100°C for 4-5 hours under stirring. It was then cooled to RT and adjusted the pH to 6 by using 10% NaOH. The white solid formed thus was filtered, washed free from acid and dried.

M.P. 265 ^{O}C

Yield: 62%.

Solubility: DMSO, Acetonitrile and DMF solvents.

IR of KBr: υmax/cm^{-1} = 3464 cm^{-1} (NH_2 stretching), 3329 cm^{-1} (NH stretching), 1633 cm^{-1} (C=C aromatic, stretching) 1149 cm^{-1} (SO_2 stretching).

^{1}H NMR (400MHz, DMSO, TMS, s = singlet, d = doublet, t = triplet, h = heptet, m = multiplet): (δ, ppm) = 8.64 (s, 1H, -NH), 5.67 (s, 2H, NH_2), 2.01 (s, 3H, CH_3), 3.02 (s, 3H, CH_3), 6.54- 7.46 (m, 7 H, Ar-H).

LC–MS (TOF MS ES +ve) for $C_{17}H_{18}N_4O_3S$ (m/z), 359 $[M+1]^+$.

Preparation of Sulfonamide/β-Cyclodextrin Inclusion Complex[28]

The inclusion complex was formulated (scheme 2) followed by the Co evaporation technique. Briefly, 1gm of β-CD was dissolved in 50 ml of distilled water and a solution containing 1 gm weight of newly synthesized (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzenesulfonamide) of 50 ml 60 % ethanol was added under stirring, This mixture was then sonicated for 15 min to dissolve the SAP and β- CD. The resulting solution was stirred at 50 ^{O}C for 1 - 2 h. After equilibrate to room temperature, the solution was filtered through 0.45 mm membrane filter. The resultant clear solution, then dried by subjecting it to evaporation at 35 ^{O}C for three days to get the desired product.

RESULTS AND DISCUSSION

Identification of inclusion complex formation

In the present investigation, inclusion complex formation is identified by a number of parameters like UV-Visible, FT-IR, DSC, TGA, SEM, and finally evaluate for antimicrobial study.

Fourier transform-infrared (FTIR) spectra

FTIR is a useful technique to prove the existence of both guest and host molecules in their inclusion complexes[29]. Figure **1** shows the FTIR spectra for the (a) (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzenesulfonamide), (b) β-Cyclodextrin and (c) Inclusion complex. The spectrum of the inclusion complex looks almost similar to the pure β-Cyclodextrin which proves the evidences of formation of the inclusive complex. Besides that, a broad hydroxyl band of pure β-Cyclodextrin at 3303.21 cm^{-1} was found to be narrowed in the FTIR spectrum of the inclusion complex which is a good indication of the formation of it. This is a most common phenomenon observed by many researchers in synthesizing the inclusion complex between β-Cyclodextrin (host) and a guest molecule.

The IR spectrum of the non complex and complex exhibited the identical peaks in comparison of the IR of the previous compound supporting our conclusion that there is no chemical reaction has been taking place. The frequencies for β-Cyclodextrin observed at 3303.21 cm^{-1}, 2921.29 cm^{-1}, 1139 cm^{-1}, and 1059.92 cm^{-1} which corresponds to the symmetric and antisymmetric stretching of γ[OH], γ[CH2], γ[C–C] and bending vibration of γ[O–H] respectively. Meanwhile the frequencies for SAP (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzene sulfonamide) were recorded at 3464.27 cm^{-1}, 3329.25 cm^{-1} and 1149.61 cm^{-1} for the respective functional groups such as γ[NH_2], γ[N-H] and vibrational stretching of γ[SO_2].

Differential Scanning Calorimetry (DSC)[30]

Sulfonamide, β-Cyclodextrin, and its complex were subjected to Differential Scanning Calorimetry (DSC) analysis. The measurements were carried out at a heating rate of 10 ^{O}C/min. In order to provide the same thermal history, each sample (1.2 to 1.8 mg) was heated from room temperature to 200 ^{O}C and rapidly cooled down to room temperature, then the DSC scan was recorded by heating from 0 to 340 ^{O}C. The thermal curves are given in Figure **2**. The DSC curve of Sulfonamide shows one characteristic sharp peak at around 265 ^{O}C, indicating the melting point of the drug. The DSC curve shows that the sharp peak at around 265 ^{O}C, which is observed for Sulfonamide, increases in the inclusion complex at around 280 ^{O}C. Furthermore, the wide peak at 115 ^{o}C, which is observed for β- Cyclodextrin, shifts to 125 ^{o}C in the inclusion complex, indicating that the inclusion complex does not contain much residue of Sulfonamide or β-Cyclodextrin, thus suggesting that the SAP is well dispersed in the β-Cyclodextrin cavity.

Thermo gravimetric analysis

Thermo gravimetric analysis (TGA) of IC is done to identify the changes in weight percent with respect to temperature change[31]. TGA was performed on pure β-Cyclodextrin, SAP and inclusion complex. Fig.**3** shows TGA results plotted in the temperature range of 50 ^{O}C to 800 °C. β-Cyclodextrin exhibits weight losses due to decomposition of macrocycles at 345 ^{O}C. Meanwhile Sulfonamide exhibited weight losses at 280 ^{O}C and 600 ^{O}C which were due to the degradation of benzyl part and -SO_2-NH- respectively. Then the inclusion complex undergoes weight losses in two stages and lost 50% of its original weight at 320 ^{O}C and remaining at 500 ^{O}C. The first stage is due to the dehydration of water molecules and the second stage is due to the decomposition of β-Cyclodextrin and Sulfonamide. It means that the formation of inclusion complex has changed the thermal degradation properties of β-Cyclodextrin and Sulfonamide. This phenomenon

suggests that formation of an inclusion complex decrease the thermal stability of β-Cyclodextrin.

Absorption Spectra

Absorption spectrum is also used to confirm the formation of inclusion complex. In this study, absorption spectra of β-Cyclodextrin, (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzenesulfonamide) and inclusion complex were taken into consideration. Fig **4** shows that β-Cyclodextrin has low absorption throughout the wavelength; hence, its absorbance can be neglected. Absorption spectra for sulfonamide is slightly upper than β-Cyclodextrin whereas, inclusion complex had an increased intensity at all points of wavelength due to the formation inclusion phenomena between β-Cyclodextrin and sulfonamide.

Scanning Electron Microscope

Scanning electron microscopy (SEM) was used in the morphological analysis of Sulfonamide, β-Cyclodextrin, and the prepared Sulfonamide/β-Cyclodextrin complexes. Fig. 5 shows that sulfonamide particles in the physical mixture method were small in size with reduced effective surface area, due to agglomeration. They remained dispersed and physically adsorbed on the surface of b-cyclodextrin.

Biological Evaluation

Antibacterial activity

In vitro antibacterial screening of the newly synthesized (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzenesulfonamide) compound and IC has been screened against two Gram-positive bacteria *viz.*, *Staphylococcus aureus* and *Bacillus mycoides*, two Gram-negative bacteria *viz.*, *Escherichia coli* and *Proteus vulgaris*, by agar diffusion method[32]. *Streptomycin* was used as standard drug for comparison.

The antibacterial potentialities of the both compounds were estimated by placing pre-sterilized filter paper disks (5 mm in diameter) impregnated with 100 µg/disk using dimethylsulfoxide (DMSO) as solvent, after 24-28 hour incubation at 37 °C. The minimum inhibitory concentration (MIC) method applied using different concentrations per disk against Gram-negative and Gram-positive bacteria for determining the activity (**Table 1**).

Screening results of the both compounds established that inclusion complex showed excellent activity and they are more active at low MIC compared to the standard drug, while compounds newly synthesized (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzenesulfonamide) also shows good activity against all the four bacterial strains.

Antifungal activity

In vitro antifungal activities of the newly synthesized compounds were evaluated against two fungal strains *viz.*, *Aspergillus flavus* and *Trichoderma viridae*. Both compounds were tested *in vitro* using the agar disk diffusion method[33], taking Nystatin as a reference drug. The two fungal *A. flavus* and *T. viridae* were isolated on Sabouraud dextrose agar (oxoid). They were isolated from clinical samples and identified to the species level according to different API systems (biomerilux). The antifungal potentialities of the tested compounds were estimated by placing pre-sterilized filter paper disks (5 mm in diameter) impregnated with 100 µg/disk using dimethylsulfoxide (DMSO) as solvent at 28^{O}C after 5 days incubation for fungi. The minimum inhibitory concentration (MIC) method was applied using different concentrations per

disk against fungi for determining the activity. (**Table 2**)

The screening results of the compound data reveal that Inclusion complex showed better activity as compared to sulfonamide against tested micro organisms with the reference drug, while sulfonamide shows good activity against two fungal strains. The overall activity profile of the both compounds was found to be good and they inhibited the growth of fungal organisms to a remarkable extent and they were found to be lethal even at MIC as low as that of reference drug.

CONCLUSION

In the present study, an formation of inclusion complex of SAP with β- CD has been achieved by using co evaporation technique to enhance its antimicrobial activity. Formation of the inclusion complex was identified by FT-IR, DSC, TGA, SEM and UV Absorbance . *In vitro* study evaluated against four bacterial stains - *S.aureus, B.mycoides, E.coli, P.vulgris,* and fungi *A.flavus, T.viridae* with respect to *Streptomycin* and *Nystatin* as reference drugs used. Although the encapsulation of SAP in β-CD appears to be the best option to enhanced the applicability of sulfa group as antimicrobial agent.

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Conflict of interest

The author(s) declare that there is no conflict of interest regarding the publication of this paper.

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Table 1. Antibacterial activity

Minimum inhibitory conc. (MIC)

Comp.	Gram positive		Gram negative	
	S.aureus	*B.mycoides*	*E.coli*	*P.vulgris*
Sulfonamide	20	22	17	21
Inclusion complex	17	19	14	20
Streptomycin	23	25	20	22

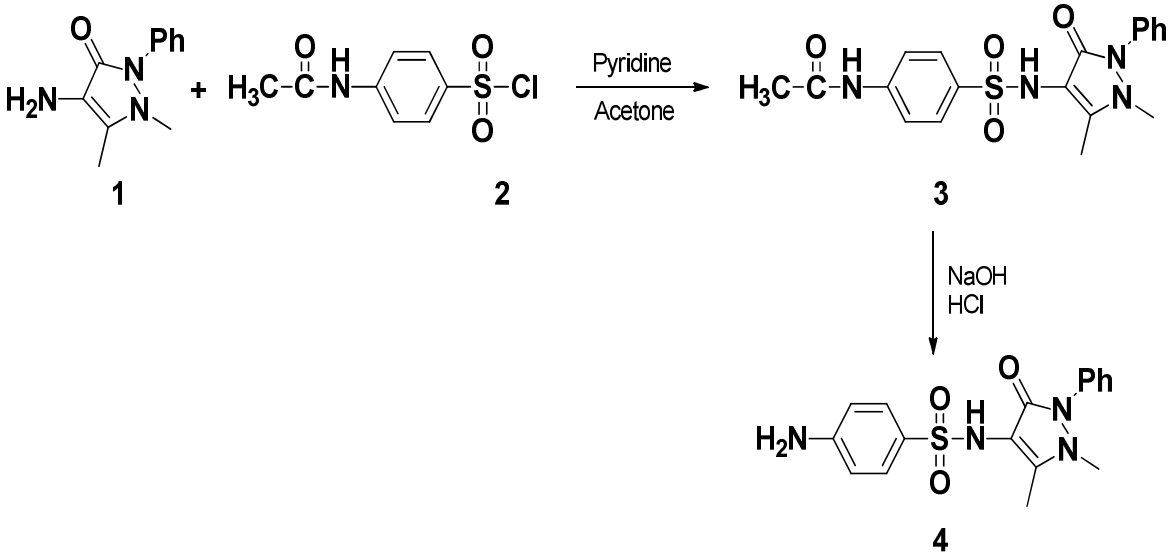
Negative control (DMSO) - No activity
Concentration 100 µg/disk

Table 2. Antifungal activity

Minimum inhibitory conc. (MIC)

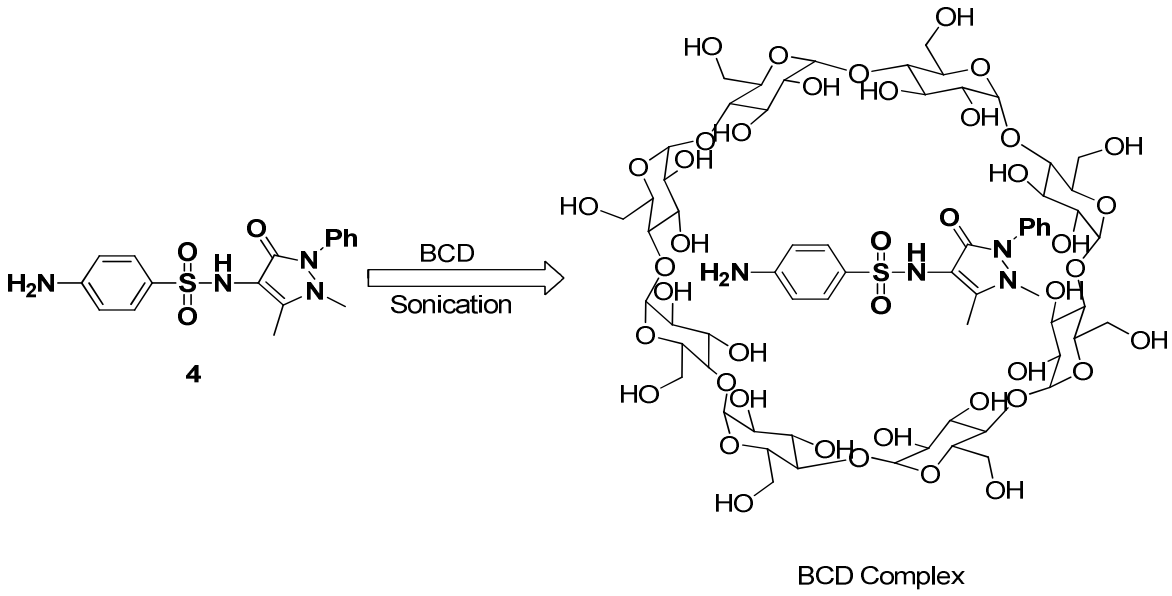
Comp.	*A.flavus*	*T.viridae*
Sulfonamide	18	24
Inclusion complex	15	20
Nystatin	23	27

Negative control-DMSO-no activity
Concentration 100 µg/disk



1 2 3 4

Scheme- 1



4

BCD Complex

Scheme- 1

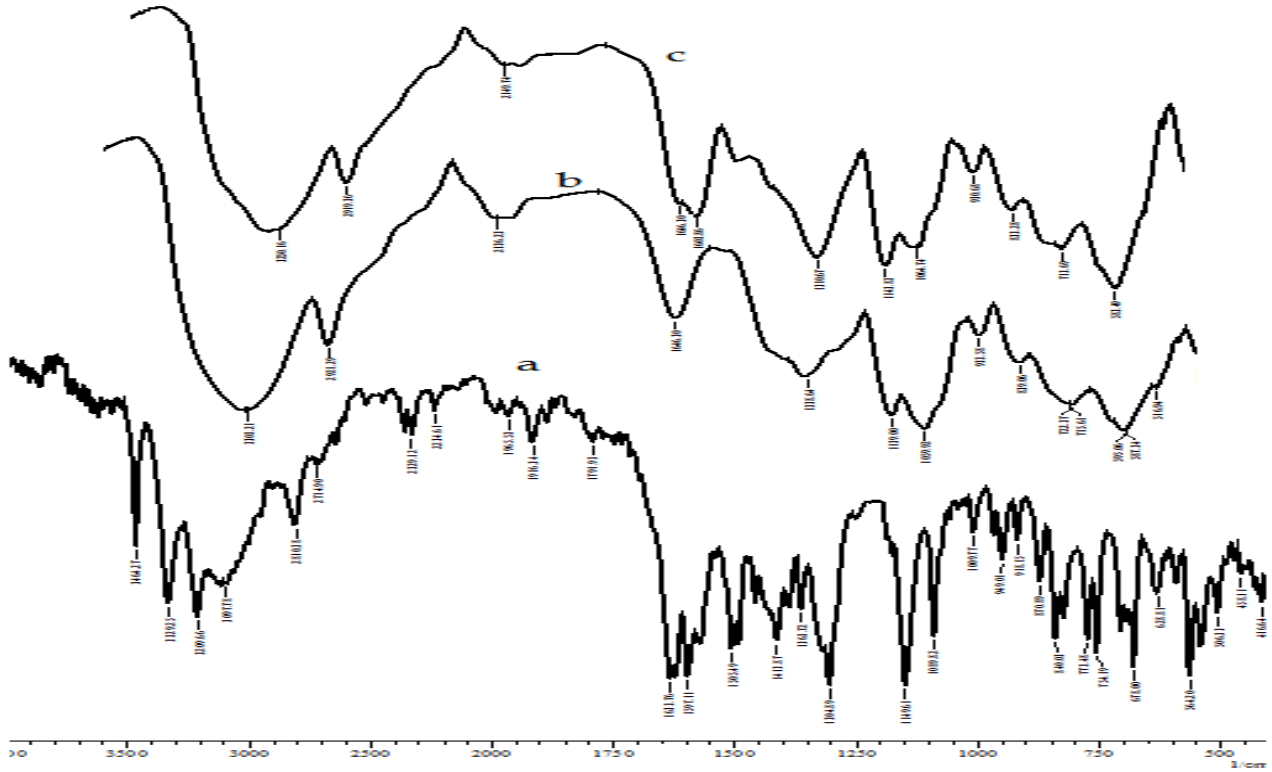


Figure 1. The Fourier transform-infrared (FTIR) spectra of (a) SAP- (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzenesulfonamide) (b) β-Cyclodextrin (c) inclusion complex of SAP/β-Cyclodextrin

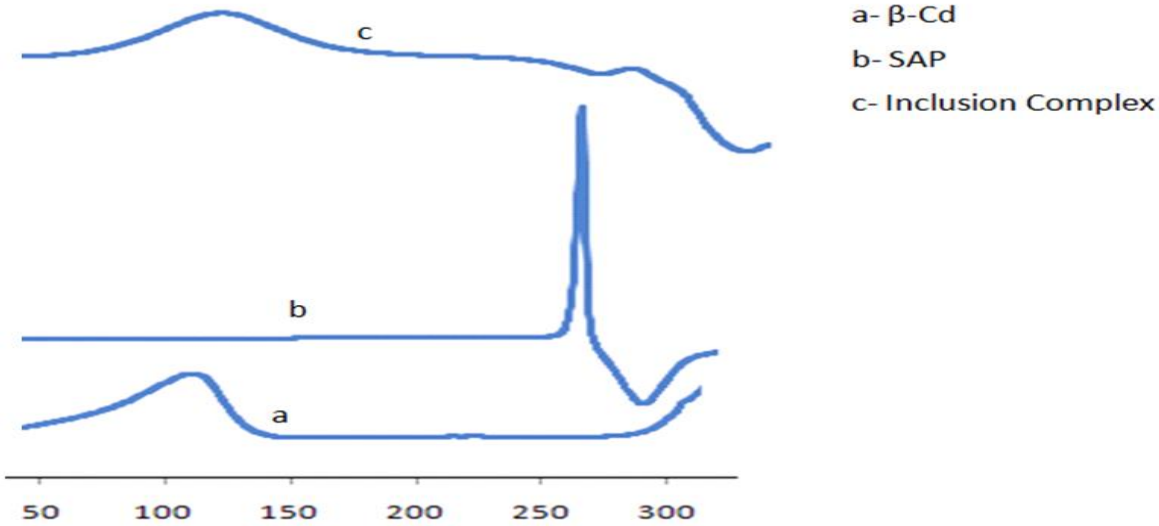


Figure 2. DSC spectra of (a) SAP- (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzenesulfonamide) (b) β-Cyclodextrin (c) inclusion complex of SAP- β-CD

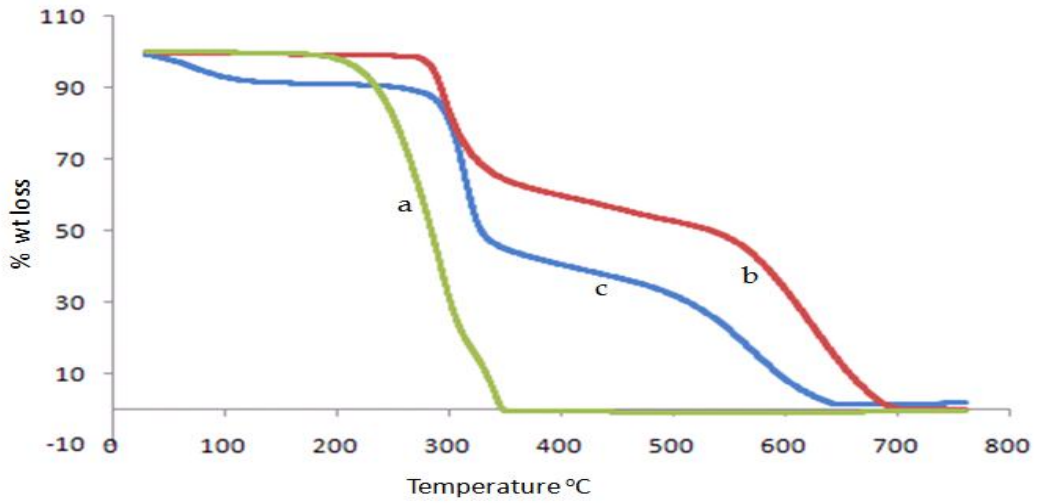


Figure 3. TGA spectra of (a) SAP- (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4- yl)benzenesulfonamide) (b) β-Cyclodextrin (c) inclusion complex of SAP- β-Cyclodextrin.

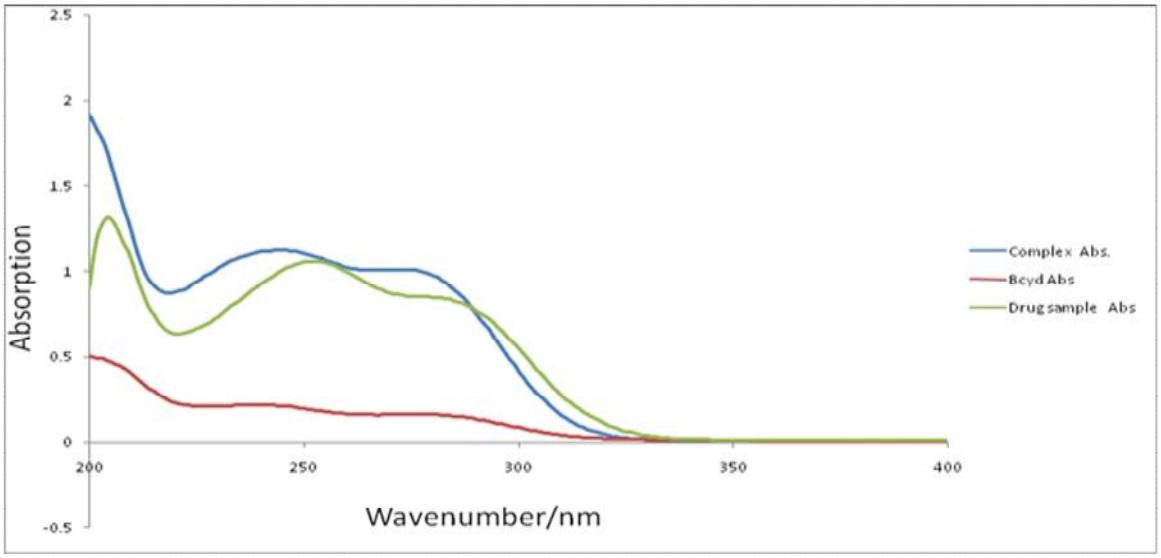


Figure 4. Absorption spectra of (a) β-Cyclodextrin (b) (4-amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)benzenesulfonamide) (c) inclusion complex.

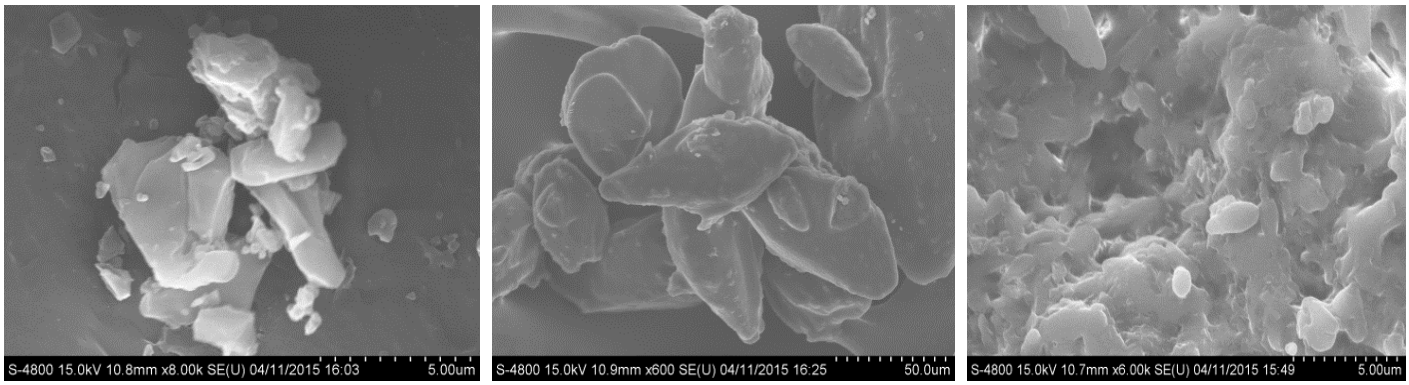


Figure 5. Absorption spectra of (**a**) β-Cyclodextrin (**b**) (4-amino-N-(1, 5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4- yl)benzenesulfonamide) (**c**) inclusion complex.