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Studies on Inclusion Complexes of 1-Nicotinoyl-4-Aryl-3-Methyl 3a, 4-Dihydropyrazolo [3, 4c] pyrazoles with B-Cyclodextrin

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ABSTRACT
Some 1-Nicotinoyl-4-aryl-3-methyl 3a, 4-dihydropyrazole [3, 4c] pyrazoles were synthesised and their inclusion complexes were prepared with β-cyclodextrin. The compounds and their inclusion complexes were characterised by the study of their physical and spectral properties. The determination of stability constant of inclusion complexes and other thermodynamic parameters such as change in free energy, change in enthalpy and change in entropy, suggested the inclusion complex formation to be thermodynamically allowed. Further, the compounds and their inclusion complexes were screened for antibacterial activities against *Escherichia coli* (MTCC 40), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 87) and *Proteus vulgaris* (MTCC 426). It was found that that the formation of inclusion complex causes an increase in antibacterial activity.

Keywords: Nicotinic hydrazide, Fused pyrazoles, Inclusion complex, Thermodynamic stability, Antibacterial activity.

INTRODUCTION

Pyrazoles and fused pyrazoles exhibit a wide spectrum of biological and pharmacological activities. [1-4] Some important activities of these compounds include antifungal [5], antibacterial [6], antidepressant [7], anti-tubercular [8], insecticidal [9] etc. Secondly, there are also reports that fused pyrazoles coupled with a nicotinoyl unit, are showing excellent antimicrobial activities. [10] Since the bio accessibility of the compounds depends upon its solubility, one of the factors limiting the pharmacological activities of these compounds may be their poor solubility in polar medium. [11] The solubility and bio accessibility of these compounds may be enhanced by forming inclusion complex with β- cyclodextrin, nontoxic oligosaccharides. [12-13]

In the present work, an attempt has been made to synthesise some fused bispyrazoles with nicotinoyl unit such as of 1-Nicotinoyl-4-aryl-3-methyl 3a, 4-dihydropyrazolo [3, 4c] pyrazoles in their purest forms and to prepare their inclusion complexes with β- cyclodextrin. The formation of inclusion complexes was ascertained by the study of the physical and spectral characteristics. Thermodynamic properties were also studied to know the stability of inclusion complex and type of interaction in between the host and guest. Finally antibacterial activities of the compounds and their inclusion complexes were studied to know whether the inclusion complex formation had any impact on their biological activities.

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MATERIALS AND METHODS

Apparatus and Materials

All the chemicals of acceptable standards were procured from local market. Double distilled water was used as solvent. Electronic spectra were recorded on Shimadzu UV-1700 spectrophotometer. IR spectra were recorded in KBr pellets in Perkin-Elmer-1800 FT-IR spectrophotometer, and ^{1}H NMR spectra (DMSO-d_6) were scanned on a DRX-300 (300MHz) spectrophotometer using TMS as internal standard and chemical shifts were expressed in δ, ppm. Purity of synthesized compounds was checked by elemental analysis and homogeneity was checked by TLC using silica gel-G, as adsorbent. Melting points were recorded by open capillary method.

Synthesis of 1-Nicotinoyl-4-aryl-3-methyl 3a, 4-dihydropyrazolo [3, 4c] pyrazoles: The synthesis of the compounds was done in three steps as shown in scheme-I. [10]

i) ***Synthesis of 2-nicotinoyl-5-methyl-2,4-dihydro-3H-pyrazol-3-one;***

A mixture of nicotinic hydrazide (pyridine-3-carbohydrazide) (1.4g, 0.01mole) and ethyl acetoacetate (1.3g, 0.01mole) were taken in dry ethanol (10ml) and refluxed for 40 h. Excess of solvent was distilled off and the resultant residue was poured on crushed ice to obtain a pale white coloured residue (Compound-A).

IR (KBr):3101($CH_{str.}$ArH), 2948($CH_{str.}$CH3), 1687, 1654($C=O_{str.}$), 1600cm^{-1}($C=N_{str.}$);

^{1}HNMR (DMSO-d6):δ7.54-8.79(m, 4H, Ar-H), 4.89(s, 2H, CH_2), 2.26(s, 3H, CH_3)

ii) *Synthesis of 4-Benzylidene-2-nicotinoyl-5-methyl-2,4-dihydro-3H-pyrazol-3-one* *(A):*

Compound-1(0.20g, 0.001mole) was dissolved in a buffer solution of 10ml acetic acid and anhydrous sodium acetate (0 .082g, 0.001mole). Benzaldehyde (0.106g, 0.001mole) was added to it. The resultant reaction mixture was refluxed for 12hr, cooled, filtered and poured on crushed ice. Solid 4-(bezylidene)-2-nicotinoyl-5-methyl-2, 4-di-hydro-3H-pyrazol-3-one appeared gradually which was filtered and dried.

Characteristics of (A):

IR (KBr):3101(C-$H_{str.}$,Ar-H), 2922(C-$H_{str.}$,CH), 1709(C=$O_{str.}$), 1592cm-1(C=$N_{str.}$);

^{1}HNMR (DMSO-*d*6):d7.09-8.01(m, 9H, Ar-H), 6.22(s, 1H, =CH-Ar), 2.10(s, 3H, CH_3).

Similarly, compounds B (4-(4-bromobenzylidene)-2-nicotino-yl-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one) and C(4-(3-nitrobenzylidene)-2-nicotino-yl-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one) were prepared as per the above methodology. The spectral characteristic of B and C were given below:

Characteristics of (B):

IR (KBr) :3093 (C-$H_{str.}$,Ar-H),2913(C-$H_{str.}$,CH3),1711(C=$O_{str.}$), 1600(C=$N_{str.}$),738cm-1(C-$Br_{str.}$);

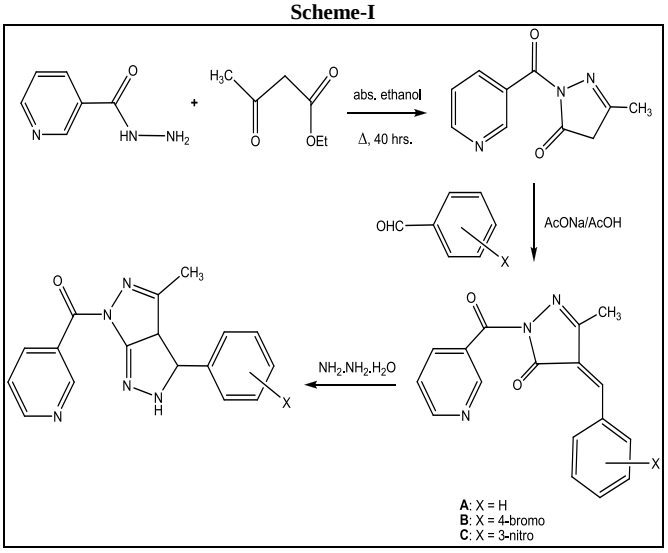
^{1}HNMR (DMSO-*d*6:d6.94-7.94 (m,8H,Ar-H), 6.26(s,1H,=CH-Ar), 2.12(s, 3H,CH_3).

Characteristics of (C):

IR (KBr):3091(C-$H_{str.}$,Ar-H), 2916(C-$H_{str.}$,CH), 1717(C=$O_{str.}$), 1589cm-1(C=$N_{str.}$);

$_1$HNMR (DMSO-*d*6):d7.07-7.99(m, 8H, Ar-H), 6.19(s, 1H, =CH-Ar), 2.17(s, 3H, CH_3).

Scheme-I



iii) ***Synthesis of 1-nicotinoyl-4-phenyl-3-methyl-3a,4-dihydropyrazolo[3,4-c] pyrazole*** (D):

Compound A (0.34g, 0.001mole) and hydrazine hydrate (0.01g, 0.002mole) were taken in dry ethanol (10ml) and a few drops of acetic acid (as catalyst) was added to it. It was then refluxed for 9h. Finally, the content was concentrated, cooled and poured on crushed ice. The solid product obtained was washed several times with water and then dried (D).

Similarly compounds E(1-Nicotinoyl-4-(4- bromo benzylidene)-3-methyl-3*a*,4-dihydropyrazolo[3,4-*c*]pyrazole) and F(1-Nicotinoyl-4-(3-nitrobenzylidene)-3-methyl-3*a*,4-dihydropyrazolo[3,4-*c*] pyrazole) were prepared as per the above methodology. The spectral characteristic of E and F were given in Table1 and 2.

Aqueous Phase Solubility Measurements

The aqueous phase solubility of the compound was studied by Higuchi-Corner method at various concentrations of β-cyclodextrin (0-10mM). [14] Accurately weighed sample of these compounds were shaken in rotary flash shaker at room temperature in a series of conical flask for a period of 48 hours till the attainment of equilibrium. The solutions were filtered through whatmann-42 filter paper and were analyzed in a UV-visible spectrophotometer. The various values of absorbance at λ-max were plotted against different concentrations of β-cyclodextrin.

Synthesis of inclusion complexes

The inclusion complexes of the compounds with β-cyclodextrin were prepared as per co-precipitation method. [15-18] Proper concentrations of the solutions of these compounds were added drop by drop to β-cyclodextrin solution of the required concentration. Stirring of the solutions was carried out for a period of 48 hours. The stirred solutions were filtered. The filtrates were cooled for 24 hours in refrigerators. The precipitates obtained are filtered, washed with water and dried in open atmosphere for 24 hours.

Study of thermodynamic properties

The stability constant of the complexes K was calculated with increasing temperature. From the slope and intercept of the linear plot of lnK vs. 1/T, ΔH and ΔS are calculated by using van't Hoff's equation [18-19]

$$\ln K = \Delta H/RT - \Delta S/R$$

Evaluation of Antibacterial activity

The antibacterial activity of compounds was studied as per cup-plate method. [18, 20] The solutions of the test compounds were prepared in dimethyl sulphoxide (DMSO) at 500µg/ml. The bacterial strains of *Escherichia coli* (MTCC 40), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 87) and *Proteus vulgaris* (MTCC 426) were inoculated into 100ml of the sterile nutrient broth and incubated at 37±1°C for 24 hours. The density of the bacterial suspension was standardized by McFarland method. Well of uniform diameter (6mm) were made on agar plates, after inoculating them separately with the test organisms aseptically. The drug (500µg/ml) and the test compounds (500µg/ml) were introduced with the help of micropipette and the plates were placed in the refrigerator at 8- 10°C for proper diffusion of drug into the media. After two hours of cold incubation, the Petri plates were transferred to incubator and maintained at 37±2°C for 18-24 hours. Then the Petri plates were observed for zone of inhibition by using venire scale. The results were reported by comparing the zone of inhibition shown by the test compounds with standard drug (Tetracycline). The results were the mean value of zone of inhibition of three sets measured in millimetre.

RESULTS AND DISCUSSION

The synthesis of compounds was confirmed from physical data (Table 1) and spectral data (Table 2). The elemental composition significant changes in colour, melting point (Table 1), a shift in UV-Visible absorption maximum and Infra-Red signals of was similar to theoretical data (Table 1).The Infra-Red and NMR data indicated the presence of expected bonds and groups in the newly synthesized compounds. The inclusion complex formation was ascertained from characteristic absorption peaks (Table-2).

Table 1: Physical properties of the compounds with and without inclusion complex

S. No.	Compound/complex	Colour	Melting Point (°C)	Elemental Analysis (%) Theoretical (Experimental)			
				C	H	N	O
1	Compound- D	Bright white	221	67.1 (67.2)	4.6 (4.7)	23.1(23.0)	5.2 (5.1)
2	Compound- D with β-CD	Dull White	273				
3	Compound- E	Pale yellow	206	52.9 (53.0)	3.6 (3.7)	18.2 (18.2)	4.2 (4.1)
4	Compound- E with β-CD	Pale white	281				
5	Compound- F	yellow	220	58.2 (58.3)	4.1 (4.0)	24.0 (23.9)	13.7 (13.8)
6	Compound- F with β-CD	Pale yellow	288				

Compound- D: 1-Nicotinoyl-4-phenyl-3-methyl-3a,4-dihydropyrazolo[3,4-c]pyrazole
Compound- E:1-Nicotinoyl-4-(4-bromobenzylidene)-3-methyl-3a,4-dihydropyrazolo[3,4-c]pyrazole
Compound- F: 1-Nicotinoyl-4-(3-nitrobenzylidene)-3-methyl-3a,4-dihydropyrazolo[3,4-c]pyrazole

Table 2: Spectral data of the compounds with and without inclusion complex

S. No.	Compound/complex	UV λ_{max} (nm)	IR(KBr) cm^{-1}	NMR
1	Compound- D	262	3392(N-H str), 3019(C-H str, Ar),1650(C=O str), 1541(C=N str)	δ 8.78(s,1H, NH) 7.04-7.86(m,9H,Ar-H), 4.88-4.89(dd,2H,CH-CH),2.14(s,3H,CH_3),1.612(s), 1.427(s), 0.880(t)
2	Compound- D with β-CD	264	3401(N-H str), 1651(C=O str), 1403(C=N str),	δ 7.264(s,1H, NH), 2.3(d),2.329(s),1.576 (s), 0.859(t)
3	Compound- E	261	3617(?), 3385(N-H str),3020(C-H str, Ar),1650(C=O str), 1534(C=N str), 762(C-Br str)	δ 8.601(s,1H, NH) 7.729-7.263(m,8H,Ar-H), 2.352(s,3H,CH_3),1.612(s), 1.427(s), 0.880(t)
4	Compound- E with β-CD	263	3396(N-H str),3021(C-H str, AR),1648(C=O str), 1534(C=N str), 761(C-Brstr	δ 7.263(s,1H, NH) 7.729-7.263(m,8H,Ar-H), 2.355(s,3H,CH_3),2.329(s),1.571-0.832(s), 0.880(t)
5	Compound- F	261	3411(N-H str),3021(C-H str, Ar),1646(C=O str), 1530(C=N str)	δ 8.85(s,1H, NH) 7.09-7.87(m,8H,Ar-H),4.80-4.81(dd,2H,CH-CH), 2.05(s,3H,CH_3)
6	Compound- F with β-CD	262	3434(N-H str),3019(C-H str, AR),1650(C=O str), 1529(C=N str)	δ 7.264(s,1H, NH) 7.09-7.87(m,8H,Ar-H),4.181-4.159(dd,2H,CH-CH), 1.574(s,3H,CH_3)

Compound- D: 1-Nicotinoyl-4-phenyl-3-methyl-3a,4-dihydropyrazolo[3,4-c]pyrazole
Compound- E:1-Nicotinoyl-4-(4-bromobenzylidene)-3-methyl-3a,4-dihydropyrazolo[3,4-c]pyrazole
Compound- F: 1-Nicotinoyl-4-(3-nitrobenzylidene)-3-methyl-3a,4-dihydropyrazolo[3,4-c]pyrazole

Table 3: Thermodynamic data of inclusion complexes at 298 K

S. No.	Compound/ complex	K M^{-1}	ΔG KJ/mol	ΔH KJ/mol	ΔS KJ/mol
1	Compound- D with β-CD	150	-12.41	-2.25	0.034
2	Compound- E with β-CD	375	-14.68	-2.05	0.042
3	Compound- F with β-CD	174	-12.78	-3.59	0.030

Table 4: Anti-microbial studies of the compounds with and without inclusion complex

Comp. Code (500µg/ml)	*Escherichia coli* (MTCC 40)	*Bacillus subtilis* (MTCC 441)	*Staphylococcus aureus* (MTCC 87)	*Proteus vulgaris* (MTCC 426)
	Diameter of zone of inhibition (mm)			
Compound-D	10±0.005	08±0.007	09±0.004	07±0.004
Compound-D With β-CD	11±0.004	09±0.006	10±0.005	09±0.003
Compound-E	08±0.006	07±0.004	10±0.006	09±0.006
Compound-E With β-CD	10±0.006	09±0.003	11±0.004	10±0.003
Compound-F	08±0.006	12±0.004	08±0.003	06±0.004
Compound-F With β-CD	10±0.005	14±0.002	09±0.007	07±0.003
Control (DMSO)	-	-	-	-
Standard (Trtracycline-10mg/disc)	31±0.007	26±0.003	21±0.003	24±0.006

The mean value of zone of inhibition of three sets

The higher melting point of inclusion complexes than the compounds may be attributed to the fact that extra amount of thermal energy is required for the latter to bring it out of β-cyclodextrin cavity. The shift in UV-Visible absorption maximum and Infra-Red signals of characteristic absorption peaks (Table 2) may be attributed to the transference of the compound from a more protic environment to a less protic environment within the cavity of β-cyclodextrin. Such changes in spectral characteristics due to inclusion complex formation may be due to the weak interactions like hydrogen bonding, vander Waal's forces, hydrophobic interactions etc. between the guest compound and the host as proposed earlier. [21-23] The aqueous phase solubility plots of the compounds within β-cyclodextrin solution (Fig. 1) exhibited a linear increase in solubility of these compounds with increasing concentration of β-cyclodextrin. Since the slopes of all the plots were less than unity, the stoichiometry of these complexes may be 1:1. The thermodynamic stability constants (K_T) of inclusion complexes were determined by using Benesi-Hilderband relation. [21] Good linear correlations were obtained for a plot of $1/\Delta A$ verses $[\beta\text{- CD}]_o$ for compounds (Fig. 2). The values of K_T for all the complexes were calculated using the relation.

$$K_T = \text{Intercept/Slope}$$

The K_T values of the inclusion complexes of compounds with β- Cyclodextrin were found to be 150, 375, 174 M^{-1} respectively (Table 3). The data obtained were within 100 to 1000 M^{-1}(ideal values) indicating appreciable stabilities for the inclusion complexes through host-guest interaction like vander Waal's force, hydrophobic interaction etc. [22-23] The thermodynamic parameters associated with the interaction of the compound with β-cyclodextrin for 1:1 stoichiometry were calculated by determining stability constant (K_T - values) at different temperatures. The K_T - values were found to decrease with rise in temperature as expected for an exothermic process (de encapsulation). The graph of ln K verses inverse absolute temperature (Fig. 3) produced linear plots from which the value of ΔH, ΔS and ΔG were calculated using van't-Hoff's equation (Table 3). In Table 3, it is found that ΔG values were negative for all the inclusion complexes. These data clearly demonstrated that formation of inclusion complexes of compounds D, E and F with β-cyclodextrin was a spontaneous process.

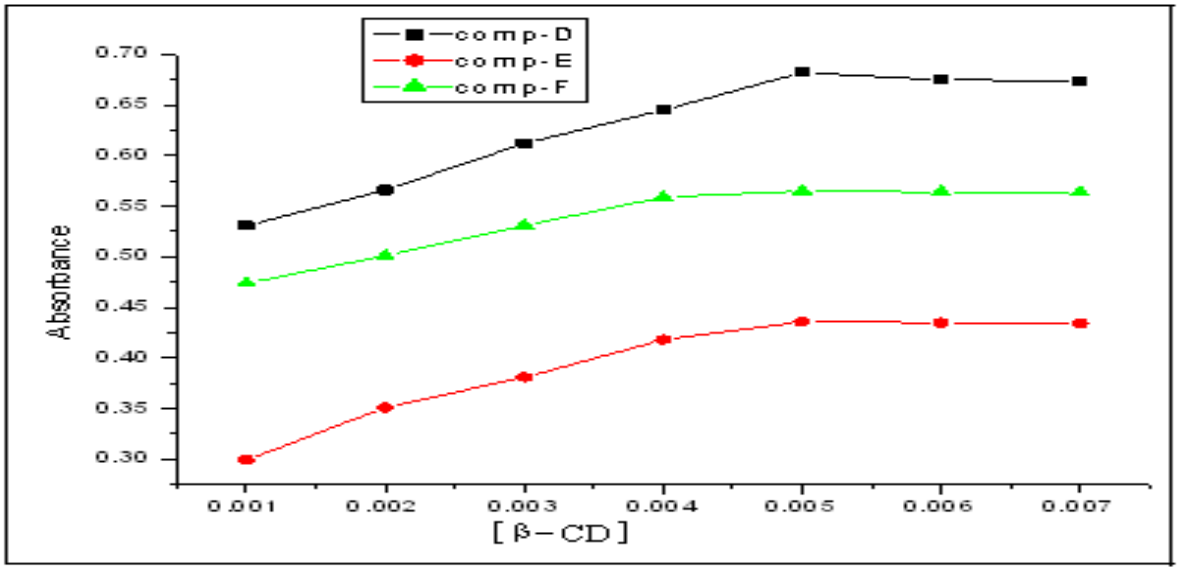


Fig. 1: Aqueous Phase Solubility of the compounds

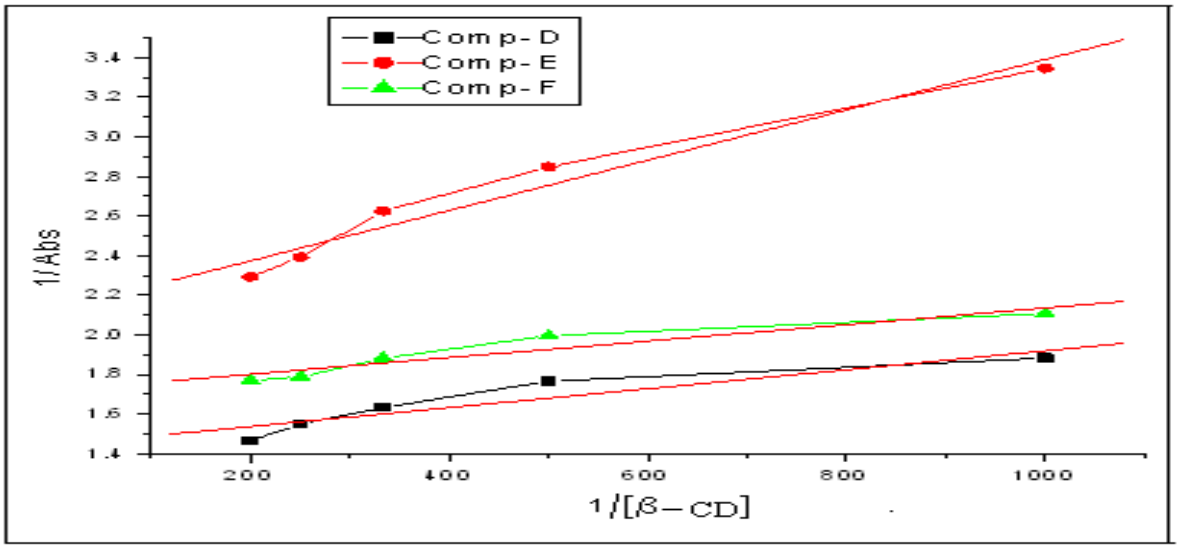


Fig. 2: Plot of 1/Absorbance Vs. 1/ [β-CD]

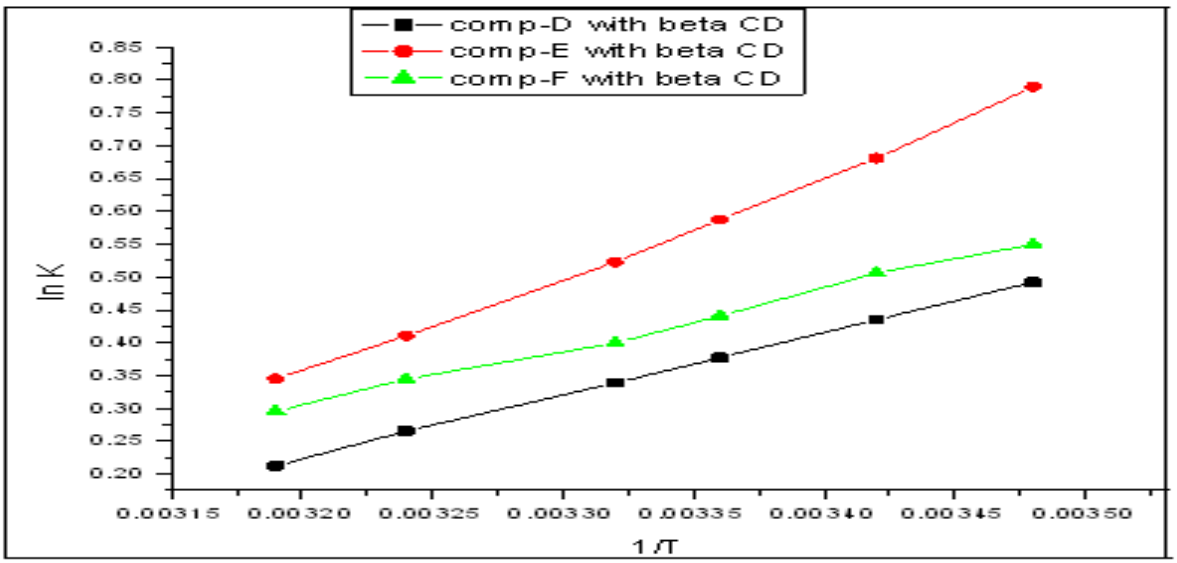


Fig. 3: Plot of ln K vs. 1/T

Further, it is found that in case of all three inclusion complexes, ΔH values were negative and ΔS values were positive (Table 2). The negative value of enthalpy change (ΔH) and positive value of entropy change (ΔS) indicated that all the three inclusion complex formations were energy allowed and entropy allowed processes. [24-27]

The antibacterial activities of the compounds and their inclusion complexes against *Escherichia coli* (MTCC 40),

Bacillus subtilis (MTCC 441), *Staphylococcus aureus* (MTCC 87) and *Proteus vulgaris* (MTCC 426) were shown in Table 4. The compounds and their inclusion complexes were susceptible to all the bacteria. However, the activity was found to undergo a slight increase after inclusion complex formation (Table 4). This may be attributed to enhanced solubility of the compounds after their inclusion complex formation which becomes more available to specific tissues leading to increased antibacterial activity. [28-30]

From the above results and discussion, it is clear that the formation of inclusion complexes of compounds (D, E and F) is thermodynamically allowed which can be a very good analytical tool for enhancing the bio accessibility of the drugs. The study further reveals that the formation of inclusion complex causes an increase in antibacterial activity (Table 4).

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