STUDY OF ANTIOXIDANT, ANTIMICROBIAL AND ANTHELMINTIC PROPERTIES OF 1-NICOTINOYL-4-ARYL-3-METHYL 3a,4-DIHYDROPYRAZOLE [3,4c] PYRAZOLE AND THEIR INCLUSION COMPLEXES WITH β-CYCLODEXTRIN

Sunakar Panda*, D. L. Singh

PG Department of Chemistry, Berhampur University, Bhanja Bihar-760007, Odisha, India.

ABSTRACT

Three different fused pyrazoles with nicotinyl moiety namely 1-Nicotinoyl-4-phenyl-3-methyl 3a, 4-dihydropyrazolo [3, 4-c] pyrazole, 1-Nicotinoyl-4-(2-chlorobenzylidene)-3-methyl-3a, 4-Dihydropyrazolo [3,4-c] pyrazole and 1-Nicotinoyl-4- (4-chlorobenzylidene) -3-methyl-3a,4-dihydropyrazolo[3,4-c] pyrazole have been synthesized and characterized spectroscopically. To enhance the solubility and minimise the side effects of the above synthesized compounds, their inclusion complexes have been prepared with β-cyclodextrin. The stability of inclusion complexes has been ascertained from the study of spectral and thermodynamic properties. Finally, the compounds and their inclusion complexes have been screened for antioxidant, antimicrobial and anthelmintic properties. It is found that there happens a significant increase in antioxidant, antimicrobial and anthelmintic activities after the formation of inclusion complexes.

Keywords: Fused pyrazoles, β-cyclodextrin, Inclusion complexes, Thermodynamic properties, Pharmacological properties.

INTRODUCTION

In recent years, the widespread use of antibacterial drugs has resulted in resistance to drug therapy against bacterial infections. Such resistance to antibacterial agents have prompted the researchers to design and modify the existing drugs or to synthesize the newer one. It is well-known that fused pyrazoles and their coupling with nicotinoyl moieties are important
pharmacophores that appear extensively in various types of pharmaceutical agents, widely implicated in biochemical processes and display diversity of pharmacological activities\cite{1-10}. The use of these compounds may result in local tissue irritation, interference with wound healing process, hypersensitivity reactions, systemic toxicity, narrow antimicrobial spectrum, development of resistance etc. All these negative effects can be minimised by forming inclusion complexes with β-cyclodextrin. Secondly, since the bio-accessibility of the compounds depends upon their solubility, one of the factors limiting the pharmacological activities of these compounds may be their poor solubility in polar medium\cite{11}. The solubility and bio-accessibility of these compounds may be enhanced by forming inclusion complex with β-cyclodextrin, a nontoxic cheaper oligosaccharide\cite{12-13}.

In the present work, an attempt has been made to synthesise some fused pyrazoles 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 the compounds and their inclusion complexes has been ascertained by the study of their physical and spectral characteristics. Thermodynamic properties of the inclusion complexes are also studied to know the stability of inclusion complex and type of interaction in between the host and guest. Finally antioxidant, antimicrobial and anthelmintic activities of the compounds and their inclusion complexes are studied to know whether the inclusion complex formation has any impact on the biological activities of the compounds.

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 an internal standard and chemical shifts were expressed in δ ppm. Purity of the synthesized compounds were checked by elemental analysis and homogeneity were checked by TLC using silica gel-G, as adsorbent. Melting points were recorded by open capillary method.

i) Synthesis of 1-Nicotinoyl-4-aryl-3-methyl 3a,4-dihydropyrazolo[3,4c]pyrazoles: The synthesis of the compounds were carried out in three steps as per the scheme given\cite{10}. 

\begin{verbatim}
Sunakar Panda et al. World Journal of Pharmacy and Pharmaceutical Sciences
\end{verbatim}
i) **Synthesis of 2-nicotinoyl-5-methyl-2,4-dihydro-3H-pyrazol-3-one**

A mixture of nicotinic hydrazide (pyridine-3-carboxyhydrazide) (1.4g, 0.01mole) and ethyl acetoacetate (1.3g, 0.01mole) was taken in dry ethanol (10mL) and refluxed for 40hr. Excess of solvent was distilled off and the resultant residue was poured on crushed ice to obtain the pale white coloured residue (Compound-1).

IR(cm⁻¹) (KBr): 3101(CH str., ArH), 2948 (CHstr. CH₃), 1687, 1654 (C=Ostr.), 1600(C=Nstr.);

¹HNMR(ppm) (DMSO-d₆): 7.54-8.79(m, 4H, Ar-H), 4.89 (s, 2H, CH₂), 2.26 (s, 3H, CH₃)

---

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) and benzaldehyde (0.106g, 0.001mole) was added to it. The resultant reaction mixture was refluxed for 12hr, cooled, filtered and the filtrate was poured on crushed ice and kept for sometimes. Solid 4-(bezylidene)-2-nicotinoyl-5-methyl-2, 4-di-hydro-3H-pyrazol-3-one appeared gradually. It was filtered and dried (A).

IR(cm⁻¹) (KBr): 3101(C-Hstr., Ar-H), 2922(C-Hstr., CH), 1709(C=Ostr.), 1592(C=Nstr.);

¹HNMR (ppm) (DMSO-d₆): 7.09-8.01(m, 9H, Ar-H), 6.22(s, 1H, =CH-Ar), 2.10(s, 3H, CH₃).

Similarly, compound **B**: 4-(2-chlorobenzylidene)-2-nicotino-y1-5-methyl-2,4-dihydro-3H-pyrazol-3-one and compound **C**: 4-(4-chlorobenzylidene)-2-nicotino-y1-5-methyl-2,4-dihydro-3H-pyrazol-3-one were prepared. The characteristic spectral data of the above compounds were given below:

**Characteristics of (B)**

IR(cm⁻¹) (KBr): 3093(C-Hstr., Ar-H), 2913(C-Hstr., CH₃), 1711(C=Ostr.), 1600(C=Nstr.), 738(C-Clstr.);

¹HNMR(ppm) (DMSO-d₆): 6.94-7.94(m, 8H, Ar-H), 6.26(s, 1H, =CH-Ar), 2.12(s, 3H, CH₃).

**Characteristics of (C)**

IR(cm⁻¹) (KBr): 3093(C-Hstr., Ar-H), 2913(C-Hstr., CH₃), 1711(C=Ostr.), 1600(C=Nstr.), 738(C-Clstr.);
\(^1\)HNMR(ppm) (DMSO-\textit{d}6): 6.94-7.94(m, 8H, Ar-H), 6.26(s, 1H, =CH-Ar), 2.12(s, 3H, CH\text{\textsubscript{3}}).

iii) \textit{Synthesis of 1-nicotinoyl-4-phenyl-3-methyl-3a,4-dihydropyrazolo[3,4-c] pyrazole (K)}

Compound A (0.34g, 0.001mole) and hydrazine hydrate (0.002mole) were taken in dry ethanol (10mL) and a few drops of acetic acid (as catalyst) was added to it. Then the reaction mixture was refluxed for 9hr, concentrated, cooled and poured on crushed ice. The product obtained was washed several times with water and then dried (K). Similarly compound L, 1-Nicotinoyl-4- (2-Chlorobenzylidene) -3-methyl-3a,4-dihydropyrazolo[3,4-c] pyrazole and compound M, 1-Nicotinoyl-4-(4-chlorobenzylidene) -3-methyl-3a,4-dihydropyrazolo [3,4-c] pyrazole were prepared. Their characteristic spectral and analytical data were given in Table1 and 2.

\[\begin{align*}
\text{Pyridine} & + \text{Acetylacetone} \rightarrow \text{Product} \\
\Delta, 40 \text{ hrs.} & \\
\end{align*}\]
Aqueous Phase Solubility Measurements
The aqueous phase solubility of the compounds was studied as per 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 by using 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 \[15-19\]. 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 refrigerator. The precipitates obtained were filtered, washed with water and dried in open atmosphere for 24 hours.

Study of thermodynamic properties
The stability constant of the complexes K_T has been calculated with increasing temperature. From the slope and intercept of the linear plot of ln K_T vs. 1/T, ΔH and ΔS were calculated by using Vant Hoff’s equation \[18-19\].

\[
\ln K = -\Delta H/RT + \Delta S/R
\]

Evaluation of antioxidant activity
The antioxidant activity of the synthesized compounds was studied as per DPPH (2, 2-Diphenyl-1-picrylhydrazyl) scavenging assay method described by Tagashira and Ohtake \[20\]. Test sample solution was prepared in 500, 100 and 50µg/ml concentration in ethanolic DPPH. After vortexing, the mixture was incubated for 10 minutes at room temperature. The absorbances of the samples were measured at 517 nm. The activity of the sample was calculated by finding the difference of absorbance between a test sample and a control. Ascorbic acid was used as reference substance. The percentage of inhibition was calculated as per the equation given below

\[
\% \text{ Inhibition} = \left( A_o - A_1 \right) / A_o \times 100
\]

where A_o was the absorbance of the control and A_1 was the absorbance of the sample.
Evaluation of antibacterial activity
The antibacterial activity of compounds was studied as per cup-plate method. The solutions of the test compounds were prepared in dimethyl sulphoxide (DMSO) at a concentration of 500µg/ml. The bacterial strains 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 as per Bollela et al (1999) [21]. Well of uniform diameter (6mm) was made on agar plates, after inoculating them separately with the test organisms aseptically. The drug, control and the test compounds 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 zones of inhibition were measured by using venire scale. The results were reported by comparing the zone of inhibition shown by the test compounds with standard drug Tetracycline.

Evaluation of anthelmintic activity
The synthesized compounds were screened for anthelmintic activity by using Indian earth worms. Six earth worms of nearly equal size and weight were placed in standard drug solution and test compound solutions at room temperature. Normal saline was used as control. The standard drug and test compounds were dissolved in minimum quantity of DMSO and the volume was adjusted up to 15 ml with normal saline solution to get the concentration of 0.5% w/v. Albendazole (20mg/ml) was used as a standard drug. 10 ml of each suspension was added in separate petridishes keeping six earth worms in each Petridish. Observations were made for the time taken to cause paralysis and death of worms. Paralysis was said to occur when the worms did not receive any sense even in normal saline. Death was concluded when the worms lost their motility followed by fading away of their body colour, when dipped in warm water (50°C). The compounds were evaluated for the time taken for complete paralysis and death of earthworms. The mean lethal time for each test compound was recorded and compared with standard drug.

RESULTS AND DISCUSSION
The synthesis of compounds has been confirmed from physical (Table 1) and spectral data (Table 2). The elemental composition matches with theoretical data (Table-1). The Infra-Red and NMR data indicate the presence of expected bonds and groups in the newly synthesized compounds. The inclusion complex formation has been ascertained from significant changes.
in colour, melting point (Table-1), a shift in UV-Visible absorption maximum and Infra-Red signals of characteristic absorption peaks (Table-2). The higher melting point of the 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 compounds 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.[18,19].

The aqueous phase solubility plots of the compounds in β-cyclodextrin solution (Fig. 1) show a linear increase in solubility of these compounds with increasing concentration of β-cyclodextrin up to 0.005M. Since the slopes of all the plots are less than unity, the stoichiometry of these complexes may be 1:1 as proposed earlier.[18, 19]. The thermodynamic stability constants (K_T) of inclusion complexes have been determined by using Benesi-Hilderband relation[22]. Good linear correlations are obtained for a plot of 1/ΔA verses [β-CD] for all the three compounds (Fig. 2). The values of K_T for all the complexes earth worms have been calculated by using the following relation

\[ K_T = \text{Intercept}/\text{Slope} \]

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 are within 100 to 1000 M^{-1}(ideal values) indicating appreciable stabilities for the inclusion complexes through host-guest interactions[22-23]. The thermodynamic parameters associated with the interaction of the compound with β-cyclodextrin for 1:1 stoichiometry have also been calculated by determining stability constant (K_T - values) at different temperatures. The K_T - values are 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 are calculated using van’t Hoff’s equation (Table 3). In case of all the inclusion complexes, ΔG values are negative. These data clearly demonstrate that formation of inclusion complexes of compounds K, L and M with β-cyclodextrin is spontaneous. Further, it is also found that in case of all three inclusion complexes, ΔH values are negative and ΔS values are positive (Table-2). The negative value of enthalpy change (ΔH), free energy change (ΔG) and positive value of entropy change (ΔS)
indicate that all the three inclusion complex formations are energy allowed and entropy allowed processes [24-30].

The synthesized compounds (K, Land M) and their inclusion complexes have been tested for their antioxidant potential from lower to higher concentration (50-500 µg/ml). All the compounds and their inclusion complexes exhibit DPPH radical scavenging activity in a dose dependent manner. The RSA of the compounds increases significantly after the formation of inclusion complex (Fig.4-6). This can be correlated to the higher bio-accessibility of the compounds after inclusion complex formation. Higher the bio-accessibility of the compounds, higher becomes the ability of compound to trap the reactive oxygen species (free radicals) there by increasing the antioxidant activity of the compounds.

The antibacterial activities of the compounds and their inclusion complexes against S. aureus, E.coli, B. Subtilis and P. vulgaris are shown in (Fig.7-10). The compounds and their inclusion complexes are susceptible to all the bacteria. However, the inclusion complexes increase the antibacterial activity significantly as compared to their corresponding compounds. 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 [28-29].

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

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Compound/complex</th>
<th>Colour</th>
<th>Melting Point (°C)</th>
<th>Elemental Analysis (%) Theoretical (Experimental)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Compound- K</td>
<td>Bright white</td>
<td>221</td>
<td>C 67.1 (67.2) H 4.6 (4.7) N 23.1 (23.0) O 5.2 (5.1)</td>
</tr>
<tr>
<td>2</td>
<td>Compound- K With β-CD</td>
<td>Dull White</td>
<td>273</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Compound- L</td>
<td>Bright yellow</td>
<td>215</td>
<td>60.1 (60.1) 4.1 (4.0) 20.6 (20.7) 4.7 (4.8)</td>
</tr>
<tr>
<td>4</td>
<td>Compound- L With β-CD</td>
<td>White</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Compound- M</td>
<td>Light brown</td>
<td>218</td>
<td>60.1 (60.1) 4.1 (4.0) 20.6 (20.7) 4.7 (4.8)</td>
</tr>
<tr>
<td>6</td>
<td>Compound- M With β-CD</td>
<td>Pale White</td>
<td>279</td>
<td></td>
</tr>
</tbody>
</table>
**Compound-K:** 1-Nicotinoyl-4-phenyl-3-methyl-3a,4-dihydropyrazolo[3,4-c]pyrazole  
**Compound-L:** 1-Nicotinoyl-4-(2-Chlorobenzylidene)-3-methyl-3a,4-dihydropyrazolo[3,4-c]pyrazole  
**Compound-M:** 1-Nicotinoyl-4-(4-chlorobenzylidene)-3-methyl-3a,4-dihydropyrazolo[3,4-c]pyrazole  

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

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Compound/Complex</th>
<th>UV $\lambda_{Max}$ (nm)</th>
<th>IR(KBr) cm$^{-1}$</th>
<th>$^1$H NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Compound- K</td>
<td>262</td>
<td>3392 (N-H str), 3019 (C-H str, Ar), 1650 (C=O str), 1541 (C=N str)</td>
<td>$\delta$ 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)</td>
</tr>
<tr>
<td>2</td>
<td>Compound- K With $\beta$-CD</td>
<td>264</td>
<td>3401 (N-H str), 1651 (C=O str), 1403 (C=N str), 771 (C-Chlstr)</td>
<td>$\delta$ 7.264 (s, 1H, NH), 2.3 (d), 2.329 (s), 1.576 (s), 0.859 (t)</td>
</tr>
<tr>
<td>3</td>
<td>Compound- L</td>
<td>261</td>
<td>3435 (N-H str), 3019 (C-H str, Ar), 1643 (C=O str), 1565 (C=N str), 771 (C-Chlstr)</td>
<td>$\delta$ 9.1 (s, 1H, NH), 6.90-8.20 (m, 8H, Ar-H), 4.84-4.85 (dd, 2H, CH-CH), 2.16 (s, 3H, CH$_3$)</td>
</tr>
<tr>
<td>4</td>
<td>Compound- L With $\beta$-CD</td>
<td>262</td>
<td>3401 (N-H str), 3019 (C-H str, Ar), 1650 (C=O str), 1525 (C=N str), 758 (C-Chlstr)</td>
<td>$\delta$ 9.1 (s, 1H, NH), 6.90-8.20 (m, 8H, Ar-H), 4.84-4.85 (dd, 2H, CH-CH), 2.16 (s, 3H, CH$_3$)</td>
</tr>
<tr>
<td>5</td>
<td>Compound- M</td>
<td>261</td>
<td>3408 (N-H str), 3020 (C-H str, Ar), 1656 (C=O str), 1565 (C=N str), 758 (C-Chlstr)</td>
<td>$\delta$ 8.71 (s, 1H, NH), 6.94-7.92 (m, 8H, Ar-H), 4.84-4.85 (dd, 2H, CH-CH), 2.16 (s, 3H, CH$_3$)</td>
</tr>
<tr>
<td>6</td>
<td>Compound- M With $\beta$-CD</td>
<td>262</td>
<td>3400 (N-H str), 3019 (C-H str, Ar), 1650 (C=O str), 1525 (C=N str), 757 (C-Chlstr)</td>
<td>$\delta$ 7.26 (s, 1H, NH), 6.94-7.92 (m, 8H, Ar-H), 4.84-4.85 (dd, 2H, CH-CH), 1.57 (s, 3H, CH$_3$)</td>
</tr>
</tbody>
</table>
Fig. 1: Aqueous Phase Solubility of the compounds

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

Fig. 3: Plot of ln K_T vs. 1/T
Fig. 4: % of Inhibition at Conc. (500µg/ml) of sample with DPPH

Fig. 5: % of Inhibition at Conc.(100µg/ml) of sample with DPPH

Fig. 6: % of Inhibition at Conc.(50µg/ml) of sample with DPPH
Fig. 7: Antibacterial activity of the test substances against *E. Coli*

Fig. 8: Antibacterial activity of the test substances against *B. Subtilis*

Fig. 9: Antibacterial activity of the test substances against *S. aureus*
Fig. 10: Antibacterial activity of the test substances against *P. Vulgaris*

Fig. 11: Paralysis study of earthworms

Fig. 12: Death study of earthworms
The synthesized compounds and their inclusion complexes have been evaluated for their anthelmintic activity by using *Pheretima posthuma* (Indian earthworm) at the concentration of 0.5% w/v (Fig. 11-12). It is seen that both the compounds and their inclusion complexes are capable of causing the paralysis and death of earth worms. However, the inclusion complexes are more efficient in causing the paralysis and death of earth worms as compared to their corresponding compounds. 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 anthelmintic activity.

**CONCLUSION**

From the above results and discussion, it is clear that the formation of inclusion complexes of compounds (K, L and M) is thermodynamically allowed which can be a very good analytical tool for enhancing the bioaccessibility of the drugs. The study further reveals that the formation of inclusion complex causes a significant increase in antibacterial (Fig. 7-10), antioxidant (Fig. 4-6) and anthelmintic activities (Fig. 11, 12).

**ACKNOWLEDGEMENT**

The authors are thankful to Dr. J R Panda, Department of Pharmaceutical Science, Roland Institute of Pharmaceutical Sciences, Berhampur, Odisha, India for studying the antioxidant, antibacterial and anthelmintic activities. Thanks to the Director CDRI, Lucknow, India for analysis of sample for IR and NMR. Financial assistance from UGC is also thankfully acknowledged.

**REFERENCES**


