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To

The Convener, ICCBCTFP-2016

PG & Research Department of Chemistry

Holy Cross College,

Tiruchirappalli-620 002. Tamil Nadu, India.

Respected Madam,

Sub: ICCBCTFP-2016-full paper of research work being submitted-reg.

Thank you vey much for accepting my paper. I am glad to submit the full paper of my research work for presentation in the International Conference to be held in your institution on July 27th and July 28th, 2016.

Thanking you,

Yours faithfully,

Sunitha.S

SYNTHESIS, CHARACTERIZATION AND CYTOTOXICITY STUDIES ON SCHIFF BASES OF PYRAZOLONES-A CASE OF LEAD COMPOUNDS IN PHARMACEUTICAL AND MEDICINAL INDUSTRIES

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INTRODUCTION:

Heterocycles form by far the largest of classical divisions of organic chemistry and are of immense importance biologically and industrially. The majority of pharmaceuticals and biologically active agrochemicals are heterocyclic while countless additives and modifiers used in industrial applications ranging from cosmetics, reprography, information storage and plastics are heterocyclic in nature. One striking structural features inherent to heterocycles, which continue to be exploited to great advantage by the drug industry, lies in their ability to manifest substituents around a core scaffold in defined three dimensional representations. For more than a century, heterocycles have constituted one of the largest areas of research in organic chemistry. They have contributed to the development of society from a biological and industrial point of view as well as to the understanding of life processes and to the efforts to improve the quality of life. Many natural drugs[1-4] such as papaverine, theobromine, quinine, emetine, theophylline, atropine, procaine, codeine, reserpine and morphine are heterocycles. Almost all the compounds we know as synthetic drugs such as diazepam, chlorpromazine, isoniazid, metronidazole, azidothymidine, barbiturates, antipyrine, captopril and methotrexate are also heterocycles. Some dyes (e.g. mauveine), luminophores, (e.g. acridine orange), pesticides (e.g. diazinon) and herbicides (e.g. paraquat) are also heterocyclic in nature. All these natural and synthetic heterocyclic compounds can and do participate in chemical reactions in the human body. Furthermore,

all biological processes are chemical in nature. Such fundamental manifestations of life as the provision of energy, transmission of nerve impulses, sight, metabolism and the transfer of hereditary information are all based on chemical reactions involving the participation of many heterocyclic compounds, such as vitamins, enzymes, coenzymes, nucleic acids, ATP and serotonin[5].

Synthetic heterocycles have widespread therapeutic uses such as antibacterial, antifungal, antimycobacterial, trypanocidal, anti-HIV activity, antileishmanial agents, genotoxic, antitubercular, antimalarial, herbicidal, analgesic, antiinflammatory, muscle relaxants anticonvulsant, anticancer and lipid peroxidation inhibitor, hypnotics, antidepressant, antitumoral, anthelmintic and insecticidal agents[6-12].

Among different five-membered heterocyclic systems pyrazolones and their derivatives have gained importance as they constitute the structural features of many bioactive compounds. Pyrazolone is a five membered lactam ring and is a derivative of pyrazole that has an additional keto group. The synthesis of new derivatives of pyrazolones has been attracting considerable attention because of various biological properties such as: antibacterial[13-14,] antifungal[13,16], anti-tubercular[13,17,18], antiviral[13,19], antioxidant[13,20], antitumoral[13,21], anti-inflammatory[13,22,23], anticonvulsant[13,24] etc. Heterocyclic systems bearing the pyrazolyl moiety show antibacterial, antifungal, anti-inflammatory and enzyme-inhibitory activity

Pyrazolones are important class of heterocyclic compounds that occur in many drugs and is a non-steroidal anti-inflammatory agent used in the treatment of arthritis and other musculoskeletal and joint disorders. They are biologically important group of compounds having different activities like antibacterial, antifungal, anti-inflammatory, antidiabetic, analgesic, antipyretic, immunosuppressive agents, hypoglycemic, antiviral, antineoplastic activity and other biological activities.[25,26]

Materials and Methods:

Chemicals used:

Ethylacetoacetate and phenylhyrazine were purchased from Merck, Worli Mumbai. Calcium hydroxide was purchased from Qualigens Fine Chemicals Mumbai. Diethyl ether was purchased from Lobo Chemie. Benzoyl chloride was purchased from s.d. fine chemicals limited, Mumbai. Hydrochloric acid was purchased from Ranbaxy fine chemicals limited. Ethanol was purchased from Changshu Yangyuan Chemical, China. Methanol, sulfanilic acid and sulfadiazine were purchased from Research Lab Fine Chem. Industries. Chloroform and DMSO were purchased from Merck Specialities Limited. These compounds were used as purchased without any further purification.

Instrumentation

The melting point of the compounds were determined on a digital melting point apparatus. The infrared spectra were recorded on Lab India spectrophotometer as KBr pellet at National College Instrumentation Facility, National College(Autonomous), Tiruchirapalli. Lab India UV-Visible spectrophotometer was used for recording electronic spectrum of 10^{-4} M solution using various solvents DMSO, acetone, 1,4-dioxane and chloroform also at National College Instrumentation Facility, National College(Autonomous), Tiruchirapalli. The mass spectra were recorded on a JEOL GCMATE II GC-MS spectrometer which is a high resolution, double focusing instrument at Sophisticated Analytical Instrumentation Facility, Indian Institute of Technology, Madras, Chennai. The source of ionization used was electron ionization. The proton magnetic resonance spectra was recorded on a Bruker AV 400 MHZ spectrometer at Annamalai University, Chidambaram. The biological studies were carried out at Periyar Maniymmai Pharmaceutical College, Tiruchirapalli using Kirby Bauer disc diffusion method.

Synthetic procedure:

3-methyl-1-phenyl-5-pyrazolone was synthesized by literature method[27]. It was benzoylated using Jensen's procedure[28]. It was condensed with sulphanilic acid and sulphadiazine separately in 1:1 molar ratio and refluxed for three hours. The solid formed on cooling was filtered at the pump, washed with the solvent first and then with petroleum benzene and air dried.

Results and discussion:

Infrared spectral details of S1:

The bands at 3066 and 2870 cm^{-1} are due to asymmetric and symmetric stretching frequencies of the CH_3 group. Two frequencies[1] arise from the bending of the hydrogen atoms of the CH_3 group about the carbon atom, the asymmetrical mode giving rise to a sharp and strong band at 1495 cm^{-1} absorption, whilst the symmetrical mode is responsible for the band at 1395cm^{-1} . The sharp and strong bands at 1601 cm^{-1} and 1644 cm^{-1} are due to >C=N and >C=O stretching vibrations respectively. Intra molecular association with the nitrogen atom of >C=N- double bonds occurs readily in Schiff's bases and related compounds. Aromatic-type compounds give rise to a number of very sharp, characteristic bands, so that their identification by infrared methods is usually straight forward; furthermore, the changes in certain regions which result from substitution are largely independent of the nature of the substituents, so that it is usually possible to determine, also, the degree and type of the substitutions present. The presence of an aromatic - type structure is best recognized by the presence of the =C-H stretching vibrations near 3030 cm^{-1} and the ring breathing vibrations in the1644-1601cm^{-1} region. The three sharp bands between 1500 and 1600 cm^{-1} can be attributed to ring breathing vibrations. They are found to be merged with the >C=O and >C=N- stretching frequencies. The strong bands at 1353 and 1187 cm^{-1} are due to asymmetric and symmetric stretching of SO_2 group. For most aromatic materials

one of the ring breathing vibrations occur within the 1625-1575 cm^{-1} region. With para substituiton there is a small shift towards higher frequencies (1650-1585 cm^{-1}). The band at 1601 cm^{-1} may be due to ring breathing vibration. The sharp bands at 608, 681, 750 , 801 and 829 cm^{-1} are due to out -of-plane deformation vibrations of the hydrogen atoms remaining on the phenyl ring. The strong bands in the range 937-608 cm^{-1} are attributed to Ar-CH out-of-plane bending vibration in 6-membered aromatic rings. In the same range are found =C-H out –of-plane , C-O-C γ and C-N-C γ in saturated heterocyclics, and N-H δ. The absence of band in the 3555-3364 cm^{-1} region indicate the absence of intra molecular association further proving that it does not exist as the OH tautomer. The absorption range for the OH valence-stretching vibration of an unbonded hydroxyl group is usually quoted as being 3700-3500 cm^{-1}. However, this overall range is considerably greater than is found experimentally using unassociated alcohols or phenols in nonpolar solvents. The frequencies range for each class is very narrow and the overall spread is no greater than 3650-3590 cm^{-1}. The absence of any band in the region 3644 and 3605 cm^{-1}. indicates that the Schiff base does not exist as the O-H tautomer. In compounds involving a chelated hydroxyl group and a heterocyclic nitrogen atom, an OH frequency of 2600cm^{-1} has been found. The absence of any peak near 2600 cm^{-1} indicate the absence of chelated hydroxyl group and a heterocyclic nitrogen atom further giving proof for the fact that the Schiff base does not exist as the -OH tautomer. The absence of a broad band between 3450 and 3550 cm^{-1} indicates the absence of a hydrogen bonded OH group.

Electronic spectral details of S1:

The bands at 243 nm and at 286 nm are attributed to K band of substituted benzene resulting from $\pi \rightarrow \pi^*$ transition..

Proton magnetic resonance spectrum of S1:

The signal due to methyl group of pyrazolone ring is observed at δ 2.105 *ppm* as a singlet in the spectrum corresponding to three hydrogen atoms. The -CH proton in

position 4 of the pyrazolone ring resonates at δ 7.443 *ppm* as a singlet corresponding to one hydrogen atom. A weak signal at δ 7.985 *ppm* is due to the $–SO_2NH_2$ protons. In the aromatic region, the aryl protons of three benzene rings resonate in the range of δ 7.50-7.65 *ppm* as multiplets. The protons on C(7) and C(11) give rise to a doublet at δ 7.592 *ppm*. The protons on C(14) and C(18) give rise to another doublet at δ 7.456 *ppm*. The protons on C(21) and C(23) give rise to another doublet at δ 7.456 *ppm*. The four protons on C(8), C(10), C(15) and C(17) give rise to a doublet of doublet δ 7.891 *ppm.* The proton on C(16) gives rise to a triplet at δ 7.291 *ppm*. The protons on C(20) and C(24) give rise to a doublet at δ 6.455 *ppm*. The proton on C(9) gives rise to a triplet at δ 7.317 *ppm* . The integral intensities of each signal were found to agree with the number of different types of protons present.

^{13}C magnetic resonance spectral details of S1:

. The carbon atoms of the methyl group appear at δ 15.79 *ppm*. The carbon atoms of the three benzene rings exhibit eleven signals in the range δ 120.72–191.95 *ppm.* In the low field region, three signals were observed around δ 137.55, 147.91 and 161.46 *ppm*, which are associated with the carbon atoms of the heterocyclic ring. The most deshielded signal (δ 191.95 *ppm*) is due to the C=O of the pyrazolone. The singlet appearing for all ligands at δ 103.55 *ppm* is assigned to the carbon atom of the >C-N- moiety.

Mass spectral details of S1:

. The mass spectrum of SB1 is in good agreement with the proposed structure. It shows a molecular ion peak at m/z 432.0252 which is also the base peak.. Fragments at m/z values 198, 103, 89 and 75 are observed which confirm the proposed structure.

Infrared spectral details of S2:

Two frequencies[1] arise from the bending of the hydrogen atoms of the CH_3 group about the carbon atom, the asymmetrical mode giving rise to a sharp and strong band at 2924 cm^{-1} absorption, whilst the symmetrical mode is responsible for the band at

2862 cm^{-1} . The sharp and strong bands at 1642 cm^{-1} and 1584 cm^{-1} are due to >C=N and >C=O stretching vibrations respectively. The strong bands at 1308 and 1188 cm^{-1} are attributed to NO_2 asymmetric stretching and NO_2 symmetric stretching frequencies respectively. Intra molecular association with the nitrogen atom of >C=N- double bonds occurs readily in Schiff's bases and related compounds. Aromatic-type compounds give rise to a number of very sharp, characteristic bands, so that their identification by infrared methods is usually straight forward; furthermore, the changes in certain regions which result from substitution are largely independent of the nature of the substituents, so that it is usually possible to determine, also, the degree and type of the substitutions present. The presence of an aromatic - type structure is best recognized by the presence of the =C-H stretching vibrations near 2924 and 2862 cm^{-1} and the ring breathing vibrations in the1600-1390 cm^{-1} region. The three sharp bands between 1535 and 1584 cm^{-1} can be attributed to ring breathing vibrations. They are found to be merged with the >C=O and >C=N- stretching frequencies. For most aromatic materials one of the ring breathing vibrations occur within the 1625-1575 cm^{-1} region. With para substituiton there is a small shift towards higher frequencies (1650-1585 cm^{-1}). The band at 1584 cm^{-1} may be due to ring breathing vibration. The sharp bands at 607, 696, 753 , 801, 830 and 938 cm^{-1} are due to out -of-plane deformation vibrations of the hydrogen atoms remaining on the phenyl ring. The strong bands in the range 940-600 cm^{-1} are attributed to Ar-CH out-of-plane bending vibration in 6-membered aromatic rings. In the same range are found =C-H out –of-plane , C-O-C γ and C-N-C γ in saturated heterocyclics, and N-H δ. The absence of band in the 3555-3364 cm^{-1} region indicate the absence of intra molecular association further proving that it does not exist as the OH tautomer. The absorption range for the OH valence-stretching vibration of an unbonded hydroxyl group is usually quoted as being 3700-3500 cm^{-1}. However, this overall range is considerably greater than is found experimentally using unassociated alcohols or phenols in nonpolar solvents. The frequencies range for each class is very narrow and the overall spread is no greater than 3650-3590 cm^{-1} The absence of any band in the region 3644 and 3605 cm^{-1} indicates that the Schiff base does not exist as the O-H tautomer. In compounds

involving a chelated hydroxyl group and a heterocyclic nitrogen atom, an OH frequency of 2600cm^{-1} has been found. The absence of any peak near 2600 cm^{-1} indicate the absence of chelated hydroxyl group and a heterocyclic nitrogen atom further giving proof for the fact that the Schiff base does not exist as the -OH tautomer. The absence of a broad band between 3450 and 3550 cm^{-1} indicates the absence of a hydrogen bonded OH group.

Electronic spectral details of S2:

The bands at 243 nm and at 286 nm are attributed to K band of substituted benzene resulting from $\pi \rightarrow \pi^*$ transition

Proton magnetic resonance spectrum of S2:

The signal due to methyl group of pyrazolone ring is observed at δ 1.590 *ppm* as a singlet in the spectrum corresponding to three hydrogen atoms. The -CH proton in position 4 of the pyrazolone ring resonates at δ 7.263 *ppm* as a singlet corresponding to one hydrogen atom. A weak signal at δ 12.680 *ppm* is due to the $-SO_2NH_2$ protons. In the aromatic region, the aryl protons of three benzene rings resonate in the range of δ 7.1-7.9 *ppm* as multiplets. The protons on C(7) and C(11) give rise to a doublet at δ 6.827 *ppm*. The protons on C(14) and C(18) give rise to another doublet at δ 7.354 *ppm*. The protons on C(21) and C(23) give rise to another doublet at δ 7.380 *ppm*. The four protons on C(8), C(10), C(15) and C(17) give rise to a doublet of doublet δ 7.862 *ppm*. The proton on C(16) gives rise to a triplet at δ 7.263 *ppm*. The protons on C(20) and C(24) give rise to a doublet at δ 7.999 *ppm*. The proton on C(9) gives rise to a triplet at δ 7.192 *ppm* . The proton on C(26) gives rise to a triplet at δ 7.573ppm. The protons on C(25) and C(27) gives rise to a singlet at 7.386 ppm. The integral intensities of each signal were found to agree with the number of different types of protons present.

^{13}C magnetic resonance spectral details of S2:

The carbon atoms of the methyl group appear at δ 15.80 *ppm*. The carbon atoms of the three benzene rings exhibit eight signals in the range δ 115.81–148.08 *ppm.* The most deshielded signal (δ 191.99 *ppm*) is due to the >C=O of the pyrazolone .The singlet appearing for all ligands at δ 97.26 *ppm* is assigned to the carbon atom of the >C-N- moiety.

Mass spectral details of S2:

The mass spectrum of SB1 is in good agreement with the proposed structure. It shows a molecular ion peak at m/z 510.01 which is also the base peak.. Fragments at m/z values 446, 351.4, 261.5, 200.6, 105.8, 91.8, 77.1 and 68.0 are observed which confirm the proposed structure. The cleavage of the bond between C(22) and the $–SO_2NH_2$ group results in the fragment ($C_{23}H_{17}N_3O$) with the m/z value 351.1658. When the pyrazolone moiety is lost from the molecular ion by the cleavage of the bond between C(4) and C(12), the fragment ($C_{13}H_{13}N_2O_2S$)which results has the m/z value 261.1497. Two bonds in this fragment can undergo cleavage to form a fragment($C_6H_5N^+$) at m/z value 91.0990. When this fragment loses a nitrogen atom, the resulting phenyl fragment($C_6H_5^+$) fragment gives rise to a peak at 77.1090.

Cytotoxicity in Umbilical cord Mesenchymal Stem Cells

MTT assay was performed to assess the inhibitory concentration (IC-50). Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number

of assay steps has been minimized as much as possible to expedite sample processing. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

The drug S1 and S2 stock were initially prepared in DMSO, and then they were diluted to the required level by DMEM media. Prior to drug addition the stem cell culture were maintained in the 96 well plate at incubator with the condition of 37ºC and 5%CO_2 in the humidified atmosphere. After the treatment of drugs for the period of 24hours the cell viability were estimated by MTT assay. The MTT dye is dissolved in cell culture medium and added to the 96 well plate with the working concentration of 1mg/ml which is further incubated at 37ºC for 4 hours in order to facilitate the formazan crystals. After the incubation period the formazan crystals were dissolved with acidic isopropanol and then the optical density were measured at 570nm with ELISA well plate reader (Robonik india pvt ltd).

Antibacterial Activity

The antibacterial activity of the compounds were assessed. The compounds were initially dissolved in 0.1% DMSO and the required dilutions were made with nutrient broth medium which is added to the 96-well plate. Each row in the 96 well plate were loaded with serially diluted concentration of the drug MM4, which allows to screen the antibacterial activity from 5mM to 5µM concentration. All the bacterial cultures which were grown in nutrient broth medium were carefully added to each well in row wise in the 96-well plate. In order to avoid the cross contamination of the rows with different bacterial species, all rows of the 96 well plate, except the current row were closed with the separate Lids, which allows to screen the drug in 7different bacterial species at the same time. After the completion of the drug addition with bacterial culture the 96-well plates were sealed with parafilm in order to avoid the external contamination which is

incubated at 37ºC in the CO_2 incubator and before that the Zero Hour Optical Density were recorded in the ELISA well plate reader (Robonik India -read well) at 630nm aseptically. After 24 hours of incubation the 96-well plate were read at the same wavelength and the Optical Density were recorded.

Results and Discussion:

Cytotoxicity in Human Umbilical cord Mesenchymal stem cell:

The S1 and S2 drugs at nano Molar range of concentrations did not show significant inhibitory effect compared to control. However the drugs at milli molar concentrations showed significant inhibitory effect dose dependently. The linearity between dose and cell viability confirmed by regression plot and the inhibitory concentration of 50% cell viability (IC50) identified as 0.5 to 1mM.

Antibacterial activity

Antibacterial effects of S1 and S2 have been identified based on the difference between OD measurement at Zero Hour and 24th hour reading.

In the case of S1, the Gram positive pathogenic bacteria culture (Staphylococcs Aureus and Enterococcus Faecalis) showed growth inhibition between the ranges between 2.5mM to 5mM which is closer to the IC_{50} value of this drug.

However the Gram negative bacteria (E. coli, Klepsiella Pneumoniae, Proteus Mirabilis, enterobacter Species and Pseudomonas Aeroginosa) showed slightly increased concentration such as 5mM, except the enterobacterial species which showed the Minimal Inhibitory Concentration same as that of the Gram positive bacteria. This results showed that S1 can be an effective drug both to Gram positive bacteria and Gram negative bacteria between the ranges of 1.25mM to 2.5mM.

In the case of S2, the Gram positive pathogenic bacteria culture (Staphylococcus aureus and Enterococcus Faecalis) showed growth inhibition between the ranges between 625µM to 1.25mM which is closer to the IC_{50} value of this drug.

However the Gram negative bacteria (E. coli, Klepsiella Pneumoniae, Proteus Mirabilis, Enterobacter Species and Pseudomonas Aeroginosa) showed slightly increased concentration such as 2.5mM, except the enterobacterial species which showed the Minimal Inhibitory Concentration same as that of the Gram positive bacteria. These results showed that S1 can be an effective drug both to Gram positive bacteria and Gram negative bacteria between the ranges of 1.25mM to 2.5mM.

The results indicate that the compounds synthesized and characterized, S1 and S2 can be used as lead compounds in pharmaceutical and medicinal industries.

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