Treatment of infectious diseases is an ever-increasing problem owing to the growing number of multi-drug resistant pathogens. Nosocomial infections are on the rise due to antibiotic-resistant microorganisms, which have led to increased morbidity and mortality in many hospitals. Cancer is a major disease worldwide[1]; colorectal cancer particularly, is the second leading cause of death by cancer in Western countries and has one of the highest mortality rates in both men and women. These cancer patients are especially susceptible to nosocomial infections; as a result of their immunosuppressed systems (due to the treatments associated with illness); which in turn leads to prolonged stays, disability and economic burden[2,3]. Therefore, the search for novel compounds that displayed a broad range of therapeutic activities that can address these major health problems is of outmost importance[4]. New therapeutic drugs that contain a heterocyclic core are on the forefront of pharmaceutical research[5,6]. Pyrazoles, (1,2-azoles) have proven to be a remarkable scaffold for the synthesis of biologically active compounds[7-9], thanks to their wide range of medical applications that center mainly on antimicrobial[10-12], antifungal[8,12,13], antiinflammatory[7], analgesic, and anxiolytic activities[13,14]. Some pyrazole derivatives have also shown antitumor activities towards several cancer cell lines, including leukemia (K562), HeLa cervix adenocarcinoma and Fem-x melanoma[14]. Consequently, in recent years pyrazole-base compounds have become a target of great interest.

A series of new Schiff base compounds derived from substituted 3-aminopyrazoles and dialdehydes were synthesized and characterized by ¹H, ¹³C nuclear magnetic resonance, Fourier-transform infrared, ultraviolet-visible, gas chromatography-mass spectrometry and high resolution mass spectrometry. The antimicrobial activity of the ligands was screened against the bacterial Gram-negative species Escherichia coli, Pseudomonas aeruginosa and the Gram-positive species Staphylococcus aureus, using gentamycin as a control. Minimal inhibitory concentration was determined by the microlodination method. The anticancer activity was evaluated against HCT116 colorectal cancer cells, with etoposide as a control, using MTS-PMS assay and expressed as IC₅₀ values. The pharmacological studies showed that in general ligands exhibited broad-spectrum antibacterial activity, which decreased in the following order Escherichia coli > Staphylococcus aureus > Pseudomonas aeruginosa. Bis(imino)pyridine Schiff bases (2a-e) have excellent activity towards Staphylococcus aureus; compounds 2a and 2d (3.125 µg/ml) are several times more potent than the control drug, whilst bis(imino)benzene compounds (3a-e) showed significant pathogenic activity toward Pseudomonas aeruginosa with MIC values of 6.25 µg/ml for 3c and 3e. Compound 2c showed higher cytotoxicity (0.40 µM) than etoposide. The results suggest that pyrazole ring as well as the substitution pattern on the heterocyclic moiety have effect on bioactivity.

Key words: Pyrazole, bioactivity, Schiff base, antibacterial activity, cytotoxic activity, bis(imino)pyridine, bis(imino)benzene

Iglesis et al.: Biological Activity of Pyrazole Schiff Base Compounds

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Schiff bases or imines, products of the condensation of carbonyl compound and primary amines, are important molecules that have been extensively studied owing to their broad range of industrial and biomedical applications\[15\]. The relative ease of their preparation, as well as the facile modification of the electronic and steric factor of the ligands; together with their chelating properties toward different metals, have made them attractive targets in the field of medicinal chemistry. Their growing importance stems from their diverse pharmacological properties: antibacterial, antifungal, antimalarial, antiinflammatory and antiviral\[16-18\]. Imines have also been found to possess cytotoxic and antiproliferative activity towards several cancer cell lines like leukemia, colorectal adenocarcinoma (Caco-2), and pancreatic cancer (Panc-1)\[19-21\], where the presence of the azomethine bond (CH=N) is believed to be critical to biological activity\[15,16,22\].

Inspired by the aforementioned and continuing our work on Schiff base compounds and their applications\[23-26\], the pyrazole nucleus was incorporated into the design and architecture of the imine ligand, with the goal of finding compounds that elicit and enhance bioactivity. The synthesis of two homologous families of NNN-bis(imo)pyridine and NN-bis(imo)benzene compounds derived from 3-aminopyrazole was reported, as well as their biological activity was evaluated. We expect that the presence of the different substituents on the pyrazole ring, together with the change in the aromatic system (pyridine, benzene) of the Schiff bases; may have an effect in the in vitro antibacterial and anticaner activity of these potential chemotherapeutics. Finally, despite the fact that transition metal complexes with bis(imo)pyridine ligands, have been extensively used for last two decades as highly active catalyst; especially towards the polymerization of numerous and diverse olefins\[27\], few reports have been devoted to explore their potential as chemotherapeutics. To the best of our knowledge, this is the first report of pyrazole containing bis(imo)-pyridine or benzene compounds, coupled with the evaluation of their pharmacological activity.

**MATERIALS AND METHODS**

Reactions were carried out under an inert atmosphere or using standard Schlenk techniques. Solvents were dried according to standard techniques. 1,3-benzenedicarboxaldehyde, 2,6-pyridinedi methyl alcohol, 2,6-dimethylaniline and 3-aminopyrazoles were purchased from Aldrich and used as received without further purification. Melting point determinations were made on a Stuart SMP10 and are uncorrected. UV/Vis spectra were acquired at room temperature (Beckman Coulter model DU730). Fourier-transform infrared (FTIR) spectra were recorded on a Perkin-Elmer FTIR 1605 spectrophotometer (ATR mode).$^1$H, $^{13}$C nuclear magnetic resonance (NMR) spectra were acquired at 400 MHz with Bruker Avance III spectrometer at 30$^\circ$, chemical shifts were reported in ppm and referenced to residual solvent resonance. Gas chromatography-mass spectrometry (GC-MS) spectra were done by direct insertion on an Agilent Technologies 5975C. High resolution mass spectrometry (HRMS) was collected on a micrOTOF-Q III MS instrument with electrospray ionization using sodium formate as calibrant.

**Synthesis of 2,6-pyridinedicarboxaldehyde (1):**

To a solution of 2,6 pyridinedimethanol (5 g, 36 mmol) in 250 ml of CHCl$_3$, MnO$_2$ (48.98 g, 576 mmol) was added. The black slurry solution was refluxed for 5 h. After the reaction was allowed to cool down, the solution was filtered through a double layer celite bed. The yellow solution was evaporated under reduced pressure, washed (3×50 ml) with hexane and dried in vacuo to afford a white crystalline solid\[28\]. Yield: (2.68 g, 55 %), melting point (mp) 120-121$^\circ$, IR (ATR): 1715 cm$^{-1}$. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 10.18 (s, 2H), 8.18 (d, J=8 Hz, 2H), 8.09 (t, J=8 Hz, 1H).

**2,6-Bis(2,6-dimethyl-phenyl-iminomethyl)pyridine (2):**

Compounds 2-3 were synthesized with the following general procedure: 2,6 dimethylaniline (0.361 g, 2,984 mmol) and isophthaldehyde (0.100 g, 1,492 mmol), were dissolved in 25 ml of dry methanol, and reflux for 24 h; upon cooling a yellow precipitate was formed, filtered, dried in vacuo and used without further purification. Yellow bright solid, yield: (0.203 g, 80 %), mp 340-341$^\circ$. IR (cm$^{-1}$, ATR): 3307, 2180, 1637, 1471, 1030, 761, 581. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.33 (s, 2H, CH=N), 8.32 (d, J=7.6, 2H, Hb), 7.90 (t, J=7.6, 1H, Ha), 7.01 (d, 4H, Hf), 6.902 (t, 2H, Hg), 2.098 (s, 12H, He). $^{13}$C ($^1$H) NMR (101 MHz, CDCl$_3$): $\delta$ 163.17 (CH=N), 154.46, 150.27, 137.34, 128.16, 126.80, 124.18, 122.72, 18.32 (CH$_3$); UV/Vis (CH$_3$OH, 1×10$^{-4}$ M): $\lambda$ 244 nm, 2653 1 cm$^{-1}$mol$^{-1}$. MS (m/z, %), 341.2 (M$^+$, 100). HRMS (ESI-TOF) m/z: [M+Na]$^+$ calcd. for C$_{23}$H$_{23}$N$_3$Na 364.1783, found: 364.1784.
2,6-Bis(pyrazolyl-1H-iminomethyl)pyridine (2a):

Schiff bases were synthesized in a similar fashion using ligand 2a as an example: to a solution of 3-amine-
pyrazole (0.062 g, 0.740 mmol) in dry ethyl acetate,
2,6-pyridinedicarbalddehyde (0.050 g, 0.370 mmol) was
added and stirred at room temperature. The reaction was
followed by the disappearance of the aldehyde by thin-
layer chromatography (hexane:ethyl acetate 1:9), and
after 96 h the solution was evaporated under reduced
pressure, washed with acetone (3×5 ml) and dried
in vacuo to give a light yellow solid. Yield: (0.070 g,
35.0 %), mp 259°. IR (cm\(^{-1}\), ATR): 3217, 2946, 1649,
1574, 1409, 1109, 1017, 664. \(^1\)H NMR (400 MHz,
dimethyl sulfoxide (DMSO)-d\(_6\)): \(\delta\) 12.92 (s, 2H, N-H),
8.93 (s, 2H, HC-N), 8.24 (d, \(J=8\) Hz, 2H, Hb), 8.10
(t, \(J=7.6\) Hz, 1H, Ha), 7.44 (d, \(J=2\) Hz, 2H, Hf), 6.60
(d, \(J=2.4\) Hz, 2H, He). \(^13\)C\(^{\{1\}}\) NMR (101 MHz,
DMSO-d\(_6\)): \(\delta\) 159.4 (HC=N), 150.0, 138.5, 131.3, 130.9,
122.9, 96.8 (Ce). UV/Vis (DMSO, \(1\times10^{-4}\) M): \(\lambda\) 329 nm \(\varepsilon=640\)
1 cm\(^{-1}\) mol\(^{-1}\), \(\lambda\) 376 \(\varepsilon=523\) 1 cm\(^{-1}\) mol\(^{-1}\), MS
(m/z, %), 293.1 (M\(^+\), 100). HRMS (ESI-TOF) m/z: [M+Na]\(^+\)
called for C\(_{15}\)H\(_{15}\)N\(_7\)Na 316.1281, found: 316.1281.

2,6-Bis(5-methyl-pyrazolyl-1H-iminomethyl)
pyridine (2b):

Light beige solid, yield: (0.065 g, 60.6 %), mp >300°.
IR (cm\(^{-1}\), ATR): 3213, 2920, 1636, 1578, 1554, 1517,
1452, 1318, 1080, 995, 759, 597. \(^1\)H NMR (400 MHz,
DMSO-d\(_6\)): \(\delta\) 12.58 (s, 2H, N-H), 8.84 (s, 2H, HC-N),
8.21 (d, \(J=7.6\) Hz, 2H, Hb), 8.04 (t, \(J=7.6\) Hz, 1H, Ha),
6.33 (s, 2H, He), 2.24 (s, 6H, -CH\(_3\)). \(^13\)C\(^{\{1\}}\) NMR
(101 MHz, DMSO-d\(_6\)): \(\delta\) 158.7 (HC=N), 121.9, 96.1
(Ce), 11.20 (CH\(_3\)). UV/Vis (DMSO, \(1\times10^{-4}\) M): \(\lambda\) 303
nm \(\varepsilon=3029\) 1 cm\(^{-1}\) mol\(^{-1}\), \(\lambda\) 350 nm \(\varepsilon=2291\) 1 cm\(^{-1}\) mol\(^{-1}\),
MS (m/z, %), 293.1 (M\(^+\), 100). HRMS (ESI-TOF) m/z: [M+Na]\(^+\)
called for C\(_{15}\)H\(_{15}\)N\(_7\)Na 316.1281, found: 316.1286.

2,6-Bis(5-tert-butyl-pyrazolyl-1H-iminomethyl)
pyridine (2c):

Orange solid, yield: (0.056 g, 85.0%), mp 278-280°
with decomp, IR (cm\(^{-1}\), ATR): 3205, 2962, 1619, 1567,
1484, 1260, 1089, 1015, 794, 692. \(^1\)H NMR (400 MHz,
DMSO-d\(_6\)): \(\delta\) 12.56 (s, 2H, N-H), 8.94 (s, 2H, HC-N),
8.27 (d, \(J=7.8\) Hz, 2H, Hb), 8.01 (t, \(J=7.8\) Hz, 1H, Ha),
6.35 (s, 2H, He), 1.38 (s, 18H, -C(CH\(_3\))\(_3\)). \(^13\)C\(^{\{1\}}\) NMR
(101 MHz, DMSO-d\(_6\)): \(\delta\) 158.8 (HC=N), 153.9,
140.0, 139.0, 125.9, 123.1, 94.0 (Ce), 31.9 -C(CH\(_3\))\(_3\),
30.4 -C(CH\(_3\))\(_3\). UV/Vis (DMSO, \(1\times10^{-4}\) M): \(\lambda\) 266 nm
\(\varepsilon=4657\) 1 cm\(^{-1}\) mol\(^{-1}\), \(\lambda\) 329 nm \(\varepsilon=3423\) 1 cm\(^{-1}\) mol\(^{-1}\), MS
(m/z, %), 377.2 (M\(^+\), 10), 139.1 (50), 124.1 (100).
1,3-Bis(5-methylpyrazol-1H-iminomethyl) benzene (3b):

Beige solid, yield: (0.144 g, 63.8 %), mp with decomp. >300°. IR (cm⁻¹, ATR): 3239, 3199, 2960, 1618, 1589, 1578, 1507, 1488, 1453, 1152, 1025, 1000. ¹H NMR (400 MHz, DMSO-d₆): δ 12.50 (s, 2H, N-H), 8.86 (s, 2H, CH=N), 8.25 (s, 2H, CH=N), 8.05 (dd, J=7.7, 1.7 Hz, 2H, Hₗ), 7.68 (t, J=7.7 Hz, 1H, Ha), 6.27 (s, 2H, He), 2.29 (s, 6H, -CH₃). ¹³C({¹H}NMR (101 MHz, DMSO-d₆): δ 159.1 (CH=N), 158.8, 140.1, 137.3, 131.4, 129.8, 95.7 (Ce) 11.27 (s, 2H, N-H), 8.86 (s, 2H, CH=N), 8.25 (s, 2H, CH=N), 8.05 (dd, J=7.7, 1.7 Hz, 2H, Hₗ), 7.68 (t, J=7.7 Hz, 1H, Ha), 6.27 (s, 2H, He), 2.29 (s, 6H, -CH₃). UV/Vis (DMSO, 1×10⁻⁴ M): λ 304 nm ε=2768 l cm⁻¹ mol⁻¹, 351 nm ε=2768 l cm⁻¹ mol⁻¹, 403 nm ε=3430 l cm⁻¹ mol⁻¹. HRMS (ESI-TOF) m/z: [M+Na]⁺ calefd for C₁₄H₁₂N₆Na 287.1015, found: 287.1007.

1,3-Bis(5-phenylpyrazol-1H-iminomethyl) benzene (3e):

Beige solid, yield: (0.217 g, 66.4 %), mp >300°. IR (cm⁻¹, ATR): 3206, 1629, 1582, 1472, 1157, 1070, 797, 756, 693. ¹H NMR (400 MHz, DMSO-d₆): δ 13.30 (s, 2H, N-H), 9.05 (s, 2H, CH=N), 8.59 (s, 1H, Hc), 8.08 (dd, J=7.7, 1.6 Hz, 2H, b), 7.82 (d, J=7.5 Hz, 5H, Ar), 7.48 (t, J=7.5 Hz, 1H, Ha), 6.97 (s, 2H, He). ¹³C({¹H}NMR (101 MHz, DMSO-d₆): δ 160.8 (CH=N), 159.8, 143.7, 137.3, 129.4, 128.8, 127.7, 125.5, 125.2, 95.0 (Ce). UV/Vis (DMSO, 1×10⁻⁴ M): λ 383 nm ε=1,844 l cm⁻¹ mol⁻¹, 375.2 (M⁺, %), 415.1(M⁻-1, 12), 159 (100). HRMS (ESI-TOF) m/z: [M+Na]⁺ calefd for C₂₆H₂₀N₆Na 439.1642, found: 439.1652.

**Antimicrobial assay:**

The antimicrobial activity test was performed using the serial dilution method[26] and carried out by inoculating the pathogenic strains *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923), previously incubated for 24 h at 35° in Mueller-Hinton medium (MHB at 23 g/l) in 96 microwell plates, where the inoculated medium was added, selecting the sample at a concentration of 10 mg/ml in DMSO.

Controls (gentamicin as a positive control and inoculated MHB culture medium and DMSO as negative controls) were handled in parallel to verify the inhibition and normal growth of the pathogen. After the inoculation was completed, the absorbance of each well was read at 492 nm using a microplate photometer (Thermo Scientific Multiskan). Subsequently, the microplates were incubated at 35° for 24 h, in order to observe the inhibitory effect on the strains of the samples.

A volume of 96 µl of MHB medium was inoculated with the pathogen strain to be tested and deposited in row A of the microplate, to which 4 µl of the sample at a concentration of 10 mg/ml in DMSO) was added and homogenized. From row B to H, a volume of 50 µl of inoculated MHB medium was deposited. A volume of 50 µl of the homogenate from row A was taken and homogenized. From row B to H, a volume of 50 µl of the homogenate from row A was transferred to row B by homogenizing and completing a total volume of 100 µl, of which a volume of 50 µl was taken, repeating this procedure consecutively until reaching the wells of row F, from which the remaining 50 µl were discarded. Eight dilutions of the sample of interest were obtained from the dilution process, and incubated at 35°C for 24, 48 and 72 h, after which the
absorbance of the microwell plate was measured at 492 nm, to calculate the inhibitory effect for each of the established concentrations.

An inhibition curve was constructed at 24, 48 and 72 h of exposure, separately calculating dose-response at these times through a Probit analysis, which would establish the minimum inhibitory concentration (MIC) for each sample. For this Probit analysis, Minitab 17 Statistical Software® package was used. All experiments were done in triplicate.

Anticancer assay:
HCT-116 (ATCC® CCL-247™) colorectal cancer cells were used to evaluate the anticancer activity of the samples[26,29,30]. For this, a cell suspension was cultured at a concentration of 2.5 to 3.0×10^4 cells/ml using Roswell Park Memorial institute medium (RPMI 1640), 10 % bovine fetal serum and 1 % solution of penicillin-streptomycin-amphotericin B solution at 37° and 5 % CO₂. After adding the samples at a concentration of 1000 μg/ml, the cultured cells were incubated for 24 h and the indicator, which help to evaluate the efficiency in cell metabolism, was added using a prepared tetrazolium compound 3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) 20:1 solution (2 mg/ml) and 1-methoxy-phenazine-methosulfate (0.92 mg/ml) in Dulbecco's phosphate-buffered saline. Trials were performed in triplicate and etoposide (10 mg/ml) was used as the positive control.

Subsequently, samples were read at 490 nm in a microplate reader. The mean inhibitory concentration (IC₅₀) representing the concentration of a compound required for 50 % cell inhibition in vitro was calculated with Softmax® PRO software (Molecular Devices Corporation, EU).

Statistical analysis:
Each data point was obtained by making at least 3 independent measurements. Data were analyzed by analysis of variance (p<0.05) and the means separated by one-way ANOVA. To reject null hypothesis F value> F critical and p<α (0.05)

RESULTS AND DISCUSSION
Schiff bases were synthesized from substituted 3-aminopyrazoles and the corresponding dialdehyde, according to fig. 1, to afford bis(imino)pyridine (2a-e) and bis(imino)benzene (3a-e) compounds, in moderate to good yields. In general, the compounds have high mp and very low solubility in the majority of organic solvents. The latter can be attributed to intra or inter molecular hydrogen bonding with N(1)-H bond[31]. Alkylation at the N(1)-H position (-R₁) as in compound 2d and 3d (fig. 1) decreased the mp and increased the solubility of the ligands. This methylation appeared to disrupt N-N···H interactions, thus preventing the formation of hydrogen bonds, which might lead to the formation of supramolecular structures[32]. Increasing the steric factor at position -R₂ in the heterocyclic ring with aromatic or branch alkyl groups, increased the solubility only slightly (fig. 1).

The diagnostic peak for the CH=N vibration appears in the range of 1612-1649 cm⁻¹, in agreement with other related imine heterocyclic compounds[17,19,33,34]. The ν(C=N) corresponding to the breathing or ring stretching modes of pyrazole ring were observed (Table 1) at 1509-1602 cm⁻¹[13,35-39]. Although some

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Fig. 1: Synthesis of bis(imino)pyridine and bis(imino)benzene compounds
a: Reflux/5 h; b: ethyl acetate/room temperature

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\(\nu(\text{HC} = \text{N})\) of the new compounds coincided with the vibration of the \(\text{C} = \text{N}\) band in amino-pyrazole parent compound; further evidence of the formation of the imine bond\(^{40,41}\), came from the disappearance of the \(\nu(\text{NH}_2)\) bands due to the asymmetric and symmetric stretch of the 3-aminopyrazole starting material and by the presence of \(N-H\) peak absorption as a broad band between 3205-3239 cm\(^{-1}\)\(^\text{[18]}\). In compounds 2d and 3d, this latter band was absent by cause of the methyl group substitution at \(N(1)\) position. Absorption peaks \(\nu(\text{C-H})\) were seen in the range of 2902-2980 cm\(^{-1}\) for alkyl groups. No significant pattern between the two families was observed.

The UV spectra data for all the pyrazole-derived Schiff bases in DMSO is summarized in Table 2. The compounds in general have two main peaks between 290-343 and 299-383 nm, attributed to \(\pi-\pi^*\) aromatic and \(n-\pi^*\) imine transitions, respectively\(^{13,38,42}\). In contrast, the \(\lambda_{\text{max}}\) of the free 3-aminopyrazoles showed one absorption band between 290-304 nm corresponding to \(\pi-\pi^*\) transition of the pyrazole ring\(^{35}\). In general, this peak suffered a bathochromic shift upon the formation of the azomethine bond. There was no apparent correlation between substitution in the heterocyclic ring and the \(\lambda_{\text{max}}\) of the new compounds, in agreement with the other relative few reports of the electronic absorption of pyrazole-Schiff base ligands and 3-aminopyrazoles\(^{32,38,43}\).

In general, the signal for the iminic proton \(\text{CH}=\text{N}\) in the \(^1H\) NMR appeared as a singlet between 8.84 and 9.05 ppm (Table 3) in accordance with other reported bis(imino)pyridine and related ligands\(^{44-46}\). The aromatic ring of the bis(imino)benzene ligands (3a-e), was observed as an \(\text{AB}_2\) system. Proton \(H_b\) was split in to a doublet of doublets (\(^3J_{\text{Hb-Ha}}\), \(^4J_{\text{Hb-Hc}}\)) in the range of 7.93-8.07 ppm, while proton \(H_a\) was observed as a triplet (\(^1J_{\text{Hb-Hc}}\)) at 8.39-8.59 ppm. Proton \(H_a\) in compounds 3a-e appears as a triplet in the range of 7.48-7.68 ppm due to the expected \(\text{ortho}\) coupling with \(H_b\) protons (\(^1J_{\text{Hb-Ha}}\) = 7.7 Hz). In contrast, for bis(imino)pyridine family (2a-e), an \(\text{AB}_2\) spin system was expected, (fig. 2) proton \(H_b\) appeared as a doublet at 8.21-8.33 ppm, and \(H_a\) is a triplet at 7.60-8.03 ppm. The multiplicity for proton \(H_e\) was determined by the type of substituent at -R2 position of the pyrazole ring, regardless of the aromatic system (fig. 1, Table 3). For example, proton \(H_e\) in 2b and 3b appeared as a singlet at 6.27-6.33 ppm (R2=CH3), whereas for 2d and 3d the signal split in to a doublet at 6.32-6.42 ppm (R2=H).

\(\text{H}_2\) in compounds 3a-e appears as a triplet in the range of 7.48-7.68 ppm due to the expected \(\text{ortho}\) coupling with \(H_b\) protons (\(^1J_{\text{Hb-Ha}}\) = 7.7 Hz). In contrast, for bis(imino)pyridine family (2a-e), an \(\text{AB}_2\) spin system was expected, (fig. 2) proton \(H_b\) appeared as a doublet at 8.21-8.33 ppm, and \(H_a\) is a triplet at 7.60-8.03 ppm. The multiplicity for proton \(H_e\) was determined by the type of substituent at -R2 position of the pyrazole ring, regardless of the aromatic system (fig. 1, Table 3). For example, proton \(H_e\) in 2b and 3b appeared as a singlet at 6.27-6.33 ppm (R2=CH3), whereas for 2d and 3d the signal split in to a doublet at 6.32-6.42 ppm (R2=H).

The appearance of signals at 2.24, 2.29 and 3.90, 3.93 ppm confirmed the presence of methyl groups for 2b, 3b and 2d, 3d, respectively (fig. 2). The latter low field resonance was a result of the EW nature of the pyrrole-like \(N(1)\) in the pyrazole ring. For both families the \(N-H\) proton resonance appeared as a broad singlet signal in the range 12.50-13.30 ppm. This fluxional

### Table 1: Selected Infrared Frequencies (cm\(^{-1}\)) for Schiff Bases

<table>
<thead>
<tr>
<th>Compound</th>
<th>(\text{N-H})</th>
<th>(\text{Alk})</th>
<th>(\text{HC} = \text{N})</th>
<th>(\text{C} = \text{C})</th>
<th>(\text{C} = \text{N})</th>
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<tbody>
<tr>
<td>2a</td>
<td>3217</td>
<td>--</td>
<td>1649</td>
<td>1574</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>3213</td>
<td>2920</td>
<td>1636</td>
<td>1572</td>
<td></td>
</tr>
<tr>
<td>2c</td>
<td>3205</td>
<td>2962</td>
<td>1619</td>
<td>1567</td>
<td></td>
</tr>
<tr>
<td>2d</td>
<td>--</td>
<td>2980</td>
<td>1612</td>
<td>1581</td>
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<tr>
<td>2e</td>
<td>3224</td>
<td>--</td>
<td>1653</td>
<td>1570</td>
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</tr>
<tr>
<td>3a</td>
<td>3209</td>
<td>--</td>
<td>1621</td>
<td>1602</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>3239</td>
<td>2960</td>
<td>1618</td>
<td>1589</td>
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</tr>
<tr>
<td>3c</td>
<td>3205</td>
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<td>1618</td>
<td>1567</td>
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<tr>
<td>3d</td>
<td>--</td>
<td>2902</td>
<td>1621</td>
<td>1590</td>
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</tr>
<tr>
<td>3e</td>
<td>3206</td>
<td>--</td>
<td>1629</td>
<td>1582</td>
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### Table 2: UV/VIS Spectra of Compounds in DMSO \(1\times10^{-4}\) M

<table>
<thead>
<tr>
<th>Ligand</th>
<th>(\lambda) nm, (\varepsilon = L) cm(^{-1}) mol(^{-1})</th>
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<tbody>
<tr>
<td>2a</td>
<td>290, 1650, 299, 1360</td>
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<tr>
<td>2b</td>
<td>303, 3029, 350, 2291</td>
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<tr>
<td>2c</td>
<td>266, 4657, 329, 3423</td>
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<tr>
<td>2d</td>
<td>322, 640, 376, 523</td>
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<tr>
<td>2e</td>
<td>372, 7880</td>
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<tr>
<td>3a</td>
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<tr>
<td>3b</td>
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<tr>
<td>3c</td>
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</tr>
<tr>
<td>3d</td>
<td>343, 1690, 364, 2320</td>
</tr>
<tr>
<td>3e</td>
<td>383, 8443</td>
</tr>
</tbody>
</table>

### Table 3: \(^1H\) and \(^13C\) NMR (PPM) Data for Compounds in DMSO

<table>
<thead>
<tr>
<th>Compound</th>
<th>(\text{CH}=\text{N}) ((s, 2H, H_d))</th>
<th>(\text{N-H}) ((s, 2H))</th>
<th>(\text{-H}) ((s, 2H))</th>
<th>(\text{CH}=\text{N})</th>
<th>(\text{C} = \text{C}) ((C_e))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>8.96</td>
<td>12.92</td>
<td>6.60 (d)</td>
<td>159.4</td>
<td>96.8</td>
</tr>
<tr>
<td>2b</td>
<td>8.84</td>
<td>12.58</td>
<td>6.334</td>
<td>158.9</td>
<td>96.1</td>
</tr>
<tr>
<td>2c</td>
<td>8.94</td>
<td>12.56</td>
<td>6.352</td>
<td>159.4</td>
<td>96.8</td>
</tr>
<tr>
<td>2d*</td>
<td>8.92</td>
<td>---</td>
<td>6.42 (d)</td>
<td>158.7</td>
<td>97.5</td>
</tr>
<tr>
<td>2e</td>
<td>8.98</td>
<td>13.00</td>
<td>6.39</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3a</td>
<td>8.98</td>
<td>12.77</td>
<td>6.48 (d)</td>
<td>158.9</td>
<td>96.3</td>
</tr>
<tr>
<td>3b</td>
<td>8.95</td>
<td>12.50</td>
<td>6.27</td>
<td>159.1</td>
<td>97.8</td>
</tr>
<tr>
<td>3c</td>
<td>8.86</td>
<td>12.53</td>
<td>6.22</td>
<td>157.8</td>
<td>91.6</td>
</tr>
<tr>
<td>3d*</td>
<td>8.86</td>
<td>---</td>
<td>6.32 (d)</td>
<td>158.9</td>
<td>97.7</td>
</tr>
<tr>
<td>3e</td>
<td>9.05</td>
<td>13.30</td>
<td>6.97</td>
<td>160.8</td>
<td>95.0</td>
</tr>
</tbody>
</table>

\(^1H\) and \(^13C\) NMR in CDCl3

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\(^1H\) and \(^13C\) NMR in CDCl3
behavior was probably due to a tautomer equilibrium between the \( N(2) \) pyridine-like and \( N(1) \)-H nitrogen of the 1,2 azole ring \[31-32,47\]. No tautomerism existed when \( N(1) \) was substituted as in compound 2d and 3d (fig. 2A).

The \(^{13}\text{C} \) NMR spectra of the compounds (2a-d, 3a-e) displayed a signal corresponding to the azomethine carbon \( HC=\text{N} \) in a narrow range from 157.8-160.8 ppm (fig. 2B). The aromatic carbons in the benzene and pyridine rings were observed between 125-138 ppm, as expected for other heterocyclic Schiff base ligands \[45,46,48\]. For the pyrazole ring, carbon \( C_2 \) appears between 91.6-97.8 ppm \[35,49\] in both families. The methyl group labeled as -R 2 in 2b and 3b was observed as a singlet at 11.2 and 11.8 ppm, respectively. In contrast, when the methyl group was attached to \( N(1) \) as in 2d and 3d the singlet is shifted downfield to 38.4 and 39.2 ppm.

In order to better assess the role of the pyrazole ring on bioactivity, Schiff bases without an aromatic heterocyclic moiety were synthesized 2, 3 (experimental section). The 3-aminopyrazoles were substituted with an aniline derivative, therefore maintaining the aromatic character while eliminating the heterocyclic ring (fig. 3). Although these compounds were reported earlier as catalyst for polymerization reactions \[50\], their cytotoxic properties nonetheless were not evaluated.

The newly synthesized \( NNN \) and \( NN \) Schiff base ligands were screened for antimicrobial activity. MIC was determined using the micro dilution assay \[26\] (Table 4). All compounds showed broad spectrum activity against the pathogenic Gram-negative bacteria \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa} and Gram-positive bacteria \textit{Staphylococcus aureus}, compared to the standard drug gentamicin, an aminoglycoside mainly effective against Gram-negative bacteria \[51\].

Both bis(amine) pyridine and bis(amine) benzene derived pyrazole ligands presented activity against \textit{E. coli}, with MIC values in the range of 400-3.125 µg/ml. \( NNN \) tridentate ligands, (2c) displayed comparable antibacterial activity with the standard against \textit{E. coli} at 3.125 µg/ml. When comparing to the control imine 2 (400 µg/ml) all of the compounds except 2b and 2d exhibited higher bioactivity (fig. 3). However for 2d effectiveness was maintained for 72 h, in contrast to compound 2 and gentamicin that lost activity after 24 and 48 h, respectively; despite this, a higher concentration than the control drug was needed (400 µg/ml) to maintain bioactivity.

In the case of benzene pyrazole derivatives; 3a had an equivalent activity to gentamicin with a MIC of 3.125 µg/ml. Control ligand 3 showed no activity against \textit{E. coli}. (Table 4). Therefore, all compounds in this series had better bioactivity than the benzene aniline derivative 3 (fig. 3). These results suggest that for this bacterial strain, the incorporation of the heterocyclic fragment enhances the bioactivity of pyrazole bis(amine) compounds, when compared to the ligands derived from 2,6 dimethyl aniline (fig. 2). Substitutions on the pyrazole ring also have an effect on bioactivity: compounds 2d, 3d (R 1=methyl, R 2=H), exhibited higher activity than isomers 2b, 3b (R 1=H, R 2=methyl). Interestingly, 2b and 3b were the least bioactive compounds in the two families. These results suggest that the inclusion of a methyl group at

![Fig. 2: (A) \(^1\text{H} \) NMR, (B) \(^{13}\text{C} \ \{\text{H}\} \) NMR of compound 3d](image)

![Fig. 3: Structures of bis(amine)pyridine and bis(amine)benzene Bis(amine)pyridine (2) and bis(amine)benzene (3), Schiff bases derived from 2,6-dimethylaniline](image)
-R₂ in the heterocyclic moiety diminished bioactivity compared with the other pyrazole substitutions (fig. 1). With respect to the pyridine or benzene ring in the compounds, there’s no apparent difference between these aromatic systems i.e. 2e and 3e have same MIC values.

Among the screened samples, only 50 % displayed inhibitory activity against *P. aeruginosa*. In general, NNN-bidentate ligands 3b, 3c and 3e were more effective (6.25-400 µg/ml), than the NNN tridentate ligands 2d, 2e (12.5-400 µg/ml). For instance, the activity of 3e (6.25 µg/ml) was greater than 2e (12.5 µg/ml); although there both several orders of magnitude more potent than the commercial drug. A similar behavior was observed in the control ligands; 2 had no activity against *P. aeruginosa*, while 3 only exhibited activity at the highest concentration (400 µg/ml) for just 24 h. Thus, the difference in cytotoxicity can be attributed to the incorporation of the pyrazole fragment or benzene ring into the Schiff base compounds.

In general, NNN bis (imino)-derived pyrazole compounds exhibited excellent activity against *S. aureus* (3.125-400 µg/ml), with activities comparable or several times more potent that the displayed by gentamicin (400 µg/ml). The compounds with strongest cytotoxicity were 2a and 2d with MIC values of 3.125 µg/ml; but for the latter, activity was maintained for 72 h in contrast to 2a and the standard drug. Moreover, control 2 exhibited a similar antibacterial activity to gentamicin (400 µg/ml) and appears to be most active toward this Gram-positive bacterium (Table 4). Thus the increase in activity for 2a and 2d could be attributed to the pyrazole ring.

NN-Bidentate-bis(imino) compounds on the other hand, possess higher MIC values in the range of 100-400 µg/ml; the most active compound 3b (100 µg/ml) was four times more active than the standard drug gentamicin (400 µg/ml) at 24 h. Control ligand 3, was inactive towards *Staphylococcus aureus*, thus the cytotoxicity exhibited by 3a, 3b, and 3d could be attributed to the pyrazole moiety. From the controls 2 and 3, it could be perceived that the pyridine ring played a role in eliciting activity. Since control compound 2 exhibited the same cytotoxic activity than 2c, 2e, 3a, and 3d, therefore, the azomethine bond appeared to be responsible for the observed activity. In general, for this Gram-positive bacterium, bulky substituents (-Bu, Ph) at -R₂ of the pyrazole ring (2c, 2e, 3c, 3e) lacked or had no effect on activity (fig. 1). Finally, the absence of specificity between Gram-positive and Gram-negative bacteria might suggest a similar mode of action in the cell.

In this study, *in vitro* cytotoxicity against HCT-116 colorectal cancer cells was tested for all compounds.
and reported as IC$_{50}$, representing the concentration of a compound required for 50% of cell inhibition in µM$^{[26,29,30]}$. Etoposide was used as the standard drug. Results from Table 5, showed that anticancer activity for compound 2c (0.40 µM) is higher than the standard drug (0.50 µM), and also greater than the controls 2 and 3 at a concentration of 1.24-1.32 µM, respectively; followed by the Schiff base 3b (0.97 µM) with almost double of the concentrations of etoposide. Compounds 2a, 3a and 3d were also effective at higher concentrations of 3.40 µM. Finally, ligands 2b, 2d, 3c, 3e had no effect against this cell line.

To understand if these results were statistically significantly, a one-way ANOVA was performed, on all the compounds including the controls; the results indicated that there’s a significant difference between the activity exerted by all the Schiff bases in both bis(imino) pyridine (2a, 2c, 2e) and bis(imino) benzene (3a, 3b, 3d) compounds. Further analysis on bis(imino) pyridine compounds revealed that there was a significant difference between all the Schiff bases, including the control 2. This suggested that the difference in cytotoxicity within the group could be ascribed to substituents (-R$_2$, -R$_1$) on the pyrazole ring of the compounds (fig. 1).

Results from ANOVA indicated that for bis(imino) benzene compounds only 3b showed a significant difference in activity in this group; and although statistically different with control 3, this analysis suggested that in general, the pyrazole ring was not crucial to elicit bioactivity. On assessing the role of the pyridine/benzene aromatic systems on cytotoxicity, results from Table 5 (2a,3a), suggested that there was no difference in cytotoxicity of these compounds against HCT-116 cells. Given that a similar behavior was exhibited by the controls 2 and 3 (ANOVA), this suggested that pyridine/benzene aromatic systems have no clear effect on cytotoxic activity, so biological activity can be attributed mainly to the HC=N bond or the pyrazole ring. Finally, these result suggested that a t-butyl group in -R$_2$ position of the pyrazole ring (2c) is required to enhanced the anticancer activity compared to the control ligands 2, this behavior is similar to the results for E. coli.

Two new families of pyrazole derived NNN and NN Schiff bases were synthesized and characterized by spectral studies. The biological studies suggest that the compounds exhibit broad-spectrum antimicrobial activity. Bioactivity for these compounds appeared to be a combination of mainly two factors, inclusion and substitutions on the pyrazole ring, and the presence of the azomethine bond. The antimicrobial results showed a dependence on the type of bacteria, which suggested that compounds 2c, 2d, 3c, 3e, warranted further investigation as promising new chemotherapeutics. Furthermore, our preliminary studies showed that anticancer activity within a group depended on the pyrazole fragment, but the control group demonstrated that the bioactivity is also dependent on the azomethine bond, nonetheless the enhancement in biological activity on compounds 2c and 3b demonstrated that substitutions in the pyrazole moiety were also responsible for bioactivity. Work is currently underway to evaluate these compounds against other pathogenic bacteria and yeast.

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**Conflict of interest:**

The authors declared that they have no conflict of interest.

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