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In Vitro Pharmacological Evaluation of (N^1E,N^2E)-$N^1.N^2$-BIS(4-FORMYLBENZYLIDENE) and its Complex Compounds

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ABSTRACT

The number of people suffering from life-threatening multidrug-resistant infections is sharply increasing in both developed and developing nations, leaving humanity without any choice but to search for new treatment options and strategies. Drug resistance is a well-known phenomenon that results when diseases become tolerant to pharmaceutical treatments. In order to address these serious medical problems, there is urgent need to discover new chemotherapeutic agents with novel mechanisms of action, higher activity and improved selectivity to combat the menace of pathogenic microorganisms, to reverse the adverse effect of oxidative stress and undesirable consequences of inflammation. Dihydrazones are compounds containing two hydrazone groups, RC=NNHCOCONHN=CR. Hydrazones and their complexes have wide applications as antitumor, antibacterial, antifungal, antioxidant antimalarial and antiviral. (N^1E,N^2E)-$N^1.N^2$-bis(4-formylbenzylidene)oxalohydrazide and its Mn(II), Fe(II) and Co(II) complexes were synthesized and characterized by spectral and physicochemical techniques. The compounds were screened against clinically isolated gram-positive and gram-negative bacteria (*Staphylococcus aureus, Escherichia coli and Salmonella typhi*) and three pathogenic fungal species (*Aspergillus flavus, Aspergillus fumigitus* and *Aspergillus niger*), ciprofloxacin and ketoconazole were used as positive control respectively. All the complexes show significant activities than the free (N^1E,N^2E)-$N^1.N^2$-bis(4-formylbenzylidene)oxalohydrazide. The antioxidant potency of the compounds was evaluated using DPPH radical scavenging activities which was significantly appreciable. Furthermore, all the compounds were subjected to *in-vitro* anti-inflammatory test by protein denaturation technique at different concentrations diclofenac sodium was employed as the standard drug

Keywords: Antibacterial, Antifungal, anti-inflammatory, Antioxidant and Oxalohydrazide

Introduction

Microbial infections are life-threatening diseases, they weaken the immune system and deteriorate most disease conditions. The burden of infections due to antimicrobial resistance, especially in Africa and Asia, is alarming. The use of conventional antibiotics as both preventive and curative measures against microbes has increased multidrug-resistant infections that have negatively impacted human being of all age categories (Racheal *et al.,* 2023). According to European Center for Disease Prevention and Control, about 33,000 people die annually from infections (Bamigboye *et al.,* 2021; Ejidike *et al.,* 2019). Antibiotic-resistant bacteria are one of the underlying conditions that have been increasing the mortality rate to date, millions of people have been affected according to the World Health Organization (2021) (Feldman and Anderson, 2021). Most approved antibiotics are inefficient in treating bacterial infections due to their ability to form biofilms, regularly change strains, and develop complex structures

with other pathogens (Racheal *et al.,* 2023).

Oxidative stress may deteriorate the health of patients already infected with bacteria or fungi, resulting in more severe conditions; these challenges call for potential novel drugs that will combat the challenges of infections and oxidative stress (Bamigboye *et al.,* 2021). Antioxidants are substances that can prevent or slow damage to cells caused by free radicals. Free radicals are unstable molecules/atoms with unpaired electrons; they can be from either endogenous or exogenous sources (Ejidike, 2018). The accumulation of free radicals has been strongly associated with several health conditions such as cancer, diabetes, cardiovascular diseases, and neurodegenerative diseases. Thus, there is need for an antioxidant to neutralize the effects of free radicals. Research has shown that antioxidants can reduce the risk of chronic diseases, including cancer and cardiovascular disease (Racheal *et al.,* 2023). Although the human biological system has antioxidant defence mechanisms that scavenge free radicals, but, they are not sufficient. Therefore, there is need for an external antioxidant.

Many drugs possess modified pharmacological and toxicological properties when administered in the form of metallic complexes (More *et al.,* 2019; Ibrahim *et al.,* 2021).Numerous studies demonstrated that bioactive compounds require trace amounts of metal ions incorporated into their structure for improvement of their potential to fight resistance aggressively as such new hydrazone/dihydrazone metal(II) complexes were found to be active chemotherapeutic agents against deleterious pathogenic microorganisms (Ashraf *et al.,* 2023). Number of metal complexes have a wide range of pharmaceutical and biological applications. For instance, complexes having "O" and "N" donor atoms are very essential due to their numerous biological applications; Antibacterial (Hashem *et al.,* 2022; Shi,2022), enzyme inhibitors (Noma, 2020), antifungal (Nfor *et al.,* 2023; Cukurovali, 2023; Bitu, 2019), antitumor (Liu *et al.,* 2023; Muhamed *et al.,* 2022), antioxidants (Musad *et al.,* 2023), anti-hypertensive (Ali *et al.,* 2020), anticonvulsant (Angelova, 2016), anti-inflammatory (Le and Thuy 2022) and antimalarial agents (Le and Thuy 2022; Shaikh *et al.,* 2022).

Fig. 1: Some important pharmacological applications of dihydrazone complexes

The majority of biological processes in our body involve the transition metal such as copper in the synthesis of redox enzymes and neurotransmitters, as well as aerobic and endocrine mechanisms (Venkateswarlu *et al.,* 2022). Zinc is considered a micronutrient that is required for vital biochemical processes and is recognized as a protein and DNA building block. Additionally, zinc-containing compounds have been investigated as potential cytotoxic agents against a variety of cell lines (Gurusamy 2022). Development of new transition metal-based medicines are an excellent approach to fight effectively in contradiction of the current problem of antibacterial, anticancer, antioxidant, antidiabetic resistance (Abu-Dief, *et al.,* 2020).

Materials and Methods

All the chemicals were of analytical grade and were used without further purification. The transition metal salts and other chemicals were obtained from either Merck or Sigma Aldrich. All glass wares were washed with detergent, rinsed with distilled water and dried in the oven at 110 ^{o}C. All weighing was carried out on electrical meter balance Toledo BI54. The *in vitro* antibacterial and antifungal screening were performed by well diffusion method. The antioxidant potency of the compounds was evaluated by DPPH radical scavenging activities. The *in-vitro* anti-inflammatory evaluation was conducted by denaturation technique.

Synthesis of (N^1E,N^2E)-$N^1.N^2$-Bis(4-formylbenzylidene)oxalohydrazide (Dihydrazone A)

Bis(terephthalaldehyde)oxalyldihydrazone was synthesized by refluxing ethanolic solution of oxalyldihydrazide (0.00l mol, 0.1181 g) and terephthalaldehyde (0.002 mol, 0.2683 g) for four (4) hours, after which the volume of the solution was reduced to one-third by slow evaporation on water bath, on cooling, the yellow coloured solid compound was filtered and washed several times with cold ethanol, followed by ether, then dried in desiccator over P_2O_5 and finally the yield was calculated (Ayman *et al.,* 2019).

Synthesis of (N^1E,N^2E)-$N^1.N^2$-Bis(4-formylbenzylidene)oxalohydrazide Complexes

The complexes were synthesized by conventional refluxing method. The dihydrazone A (0.002 mol, 0.700 g) was added to 50 cm^3 ethanol. The resulting solution was mixed with ethanolic solution of metal(II) chloride (0.001 mol) and heated under reflux for six (6) hours, after which, the volume was reduced to 20 cm^3 by evaporation on water bath. The resulting reaction mixture was cooled to room temperature, the coloured solid complexes were filtered off, washed several times with cold ethanol and acetone to remove any excess of unreacted component and finally dried in a desiccator over P_2O_5. The dried compound was weighed so as to calculate the yield. (Ahmed *et al.,* 2016).

Antimicrobial Assay

The antibacterial and antifungal activity test of the synthesized dihydrazone and its respective complexes were carried out against three pathogenic bacterial isolates *Staphylococcus aureus, Escherichia coli and Salmonella typhi* and three deleterious fungal isolates, *Aspergillus flavus, Aspergillus fumigitus* and *Aspergillus niger* using well diffusion method by adopting the method reported by Mouayed and Abduljleel, (2024) and Laxman *et al.,* (2024) with slight modification. The result was shown in figure 2 and 3 respectively.

DPPH Radical Scavenging Assay

The stock solution of each standard control (Ascorbic acid), dihydrazone and the complexes were prepared (2 mg/mL each) and diluted to final concentration of 1000, 500, 250, 125, 62.5, 31.25, 15.63 and 7.81 µg/mL by serial dilution. 100 µL of 0.1mM 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) was used to evaluate the antioxidant potentials of the compounds. The absorbance was recorded at 517nm using a JASCO model V-550 UV-Vis spectrophotometer in triplicate (Bhagwat *et al.,* 2022). The percentage radical scavenging activities (% RSA) were calculated by using the relation shown in equation beneath;

$$\% \text{ RSA} = \frac{\text{Absorbance of Control} - \text{Absorbace of Sample}}{\text{Absorbance of Control}} \times 100$$

***In-Vitro* Anti-Inflammatory Evaluation**

The dihydrazone and its complexes were subjected to *in-vitro* anti-inflammatory test by protein denaturation technique at different concentrations; 62.5, 125, 250, 500, 1000 and 2000 µg/mL. The standard drug (diclofenac sodium) and the synthesized compounds were dissolved in a minimum amount of dimethylformamide (DMF) and diluted with phosphate buffer (0.2 M, pH 7.4). Final concentration of DMF in all the solution was less than 2%. 1 mL of the test solution containing different concentrations of the compounds were mixed with 1 mL of 1 mM egg albumin solution in phosphate buffer and incubated at $37^{o} \pm 2^{o}$ C for 15 min. Denaturation was induced by keeping the reaction mixture at $70^{o} \pm 2°$ C in water bath for 10 min. After cooling, the turbidity was measured at 660 nm. Each experiment was done in triplicate and average is taken. Diclofenac sodium was used as standard drug (positive control) to compare its inhibition with the inhibition of test samples. The absorbance of 1 mM egg albumin solution in phosphate buffer without the test samples was taken as the absorbance of control which was used in the computation of % inhibition (Karthik *et al.,* 2024). The percentage of inhibition of denaturation was calculated using the equation below;

$$\% \text{ Inhibition} = \frac{Ac - At}{Ac} \times 100$$

Where, A_c = Absorbance of control, A_t = Absorbance of test sample

Statistical Analysis

The IC50 values of the standard drugs (ascorbic acid and diclofenac

sodium), the dihydrazone and its complexes at different concentrations were statistically analysed by probit analysis using IBM SPSS software version 20 (Nighat *et al.,* 2020).

Results and Discussion

The results obtained from the *in-vitro* pharmacological evaluation of the dihydrazone and its complexes were presented in the below. Table 1 and figure 2, show the antibacterial Activity of Dihydrazone A and its metal(II) Complexes

Table 1: Antibacterial Activity of Dihydrazone A and its metal(II) Complexes

Test Organisms	***Staphylococcus aureus***				***Escherichia coli***				***Salmonella typhi***			
Conc. (µg/mL)	**125**	**250**	**500**	**1000**	**125**	**250**	**500**	**1000**	**125**	**250**	**500**	**1000**
DHZ A	11	13	16	17	10	12	14	17	13	15	18	20
$[MnA_2Cl_2]$	14	15	18	22	12	13	15	19	16	18	19	21
$[FeA_2Cl_2]$	13	16	20	28	14	15	19	20	14	18	20	23
$[CoA_2Cl_2]$	14	19	21	29	17	20	23	25	16	19	22	24
Ciprofloxacin (Positive control)				30				27				25

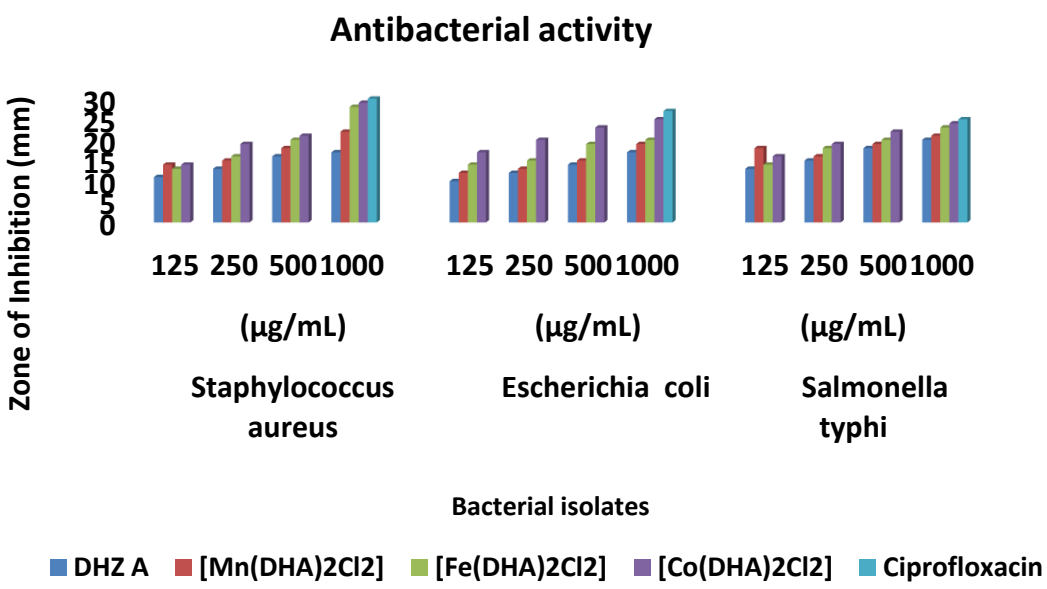


Fig. 2: Bar chart showing the antibacterial activity of dihydrazone A, its Mn(II), Fe(II) and Co(II) complexes

The *in-vitro* antibacterial evaluation of the dihydrazones A and its Mn(II), Fe(II) and Co(II) complexes were conducted by well diffusion method on nutrient agar. All the compounds were tested against three clinically isolated pathogenic bacteria (*Staphylococcus aureus, Escherichia coli* and *Salmonella typhi*) at four different concentrations, using ciprofloxacin as positive control and dimethylsulphoxide (DMSO) as negative control, the results were presented in tables figure 2. Zones of

inhibition around each well were measured in millimetre (mm) and recorded. The activity of the free dihydrazone ligands and their individual complex compounds were generally increasing with increase in concentration of the tested compounds. From the results, dihydrazone A, has a zone of inhibition of 11, 13, 14 and 17 mm at 125, 250, 500 and 1000 μg/mL respectively, so also $[FeA_2Cl_2]$ has a zone of inhibition of 13, 16, 20 and 28 mm at the same concentration as above against *Staphylococcus aureus.*

These were in good accord with the results reported by Jadhav *et al.,* (2022). Furthermore, the results indicated that the metal(II) complexes exhibited higher antibacterial activities than their respective free (N^1E,N^2E)-$N^1.N^2$-bis(4-formylbenzylidene)oxalohydrazide.

These were comparable with the results reported by (Gazwan *et al.,* 2021). From the results, at 1000 μg/mL, ciprofloxacin inhibited the growth of *Staphylococcus aureus* by 30 mm, whereas, the dihydrazones A to F inhibited the growth of the same bacteria by 17 mm, while for the complexes there were enhancement, which was observed between 24 – 29 mm. These bear a resemblance to the results reported by Fayed *et al.,* (2020) and (Gazwan *et al.,* 2021). Among all the complex compounds, cobalt complex $[CoA_2Cl_2]$, was found to have the highest zone of inhibition (29 mm) against gram-positive *Staphylococcus aureus* which is comparable to that of standard drug, ciprofloxacin (30 mm). Similar to the results described by Abu *et al.,* (2019). The presence of metal ions increases the activity of the compounds, since, incorporation of metal ions into the ligands increases its liposolubility (lipid soluble) (Laxman *et al.,* 2024). The complex compounds get adsorbed on the surface of the cell wall of microorganisms and disturb the respiration process of the cell and thus block the synthesis of the proteins and restricts further growth of the organisms. Such increased activity of the complexes can be explained on the basis of Overtone's concept and Tweedy's Chelation theory. This increased in lipophilicity enhances the penetration of the complexes into lipid membrane and restricts further multiplication of the microorganisms (Anna *et al.,* 2015).

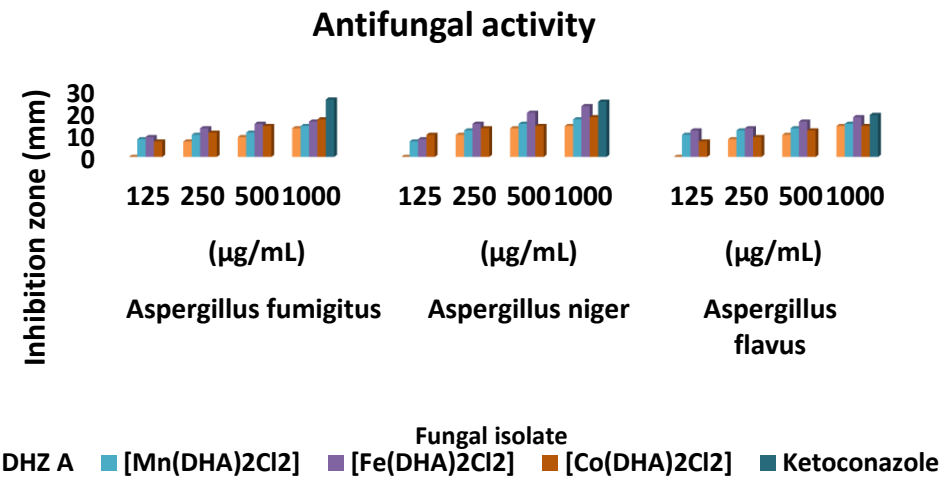
The variation in the effectiveness of different compounds against different organisms depends either on the permeability of the microbial cells or on differences in ribosome of microbial cells. That is the reason why some compounds are more potent against microbes than others (Karthika *et al.,* 2024). All the compounds were found to be more potent against gram-positive *staphylococcus aureus* than gram-negative *Escherichia coli* and *Salmonella typhi,* this is because, lipid and lipoprotein content is low in the cell wall of gram positive bacteria which facilitate the penetration of the compound through the cell of the microbes which may eventually resulted in the death of the cells. Hence, the cell wall of the gram positive bacteria is more prone to disruption by the tested compounds. These very much resembles the results reported by Gurusamy *et al.,* (2023) and Sridevi, (2015).

Table 2 and figure 3, show the antifungal Activity of Dihydrazone A and its metal(II) Complexes

Table 2: Antifungal Activity of Dihydrazone A and its metal(II) Complexes

Test Organisms	***Aspergillus fumigitus***				***Aspergillus niger***				***Aspergillus flavus***			
Conc. (µg/mL)	**125**	**250**	**500**	**1000**	**125**	**250**	**500**	**1000**	**125**	**250**	**500**	**1000**
DHZ A	6	7	9	13	6	10	13	14	6	8	10	14
$[MnA_2Cl_2]$	8	10	11	14	7	12	15	17	10	12	13	15
$[FeA_2Cl_2]$	9	13	15	16	8	15	20	23	12	13	16	18
$[CoA_2Cl_2]$	7	11	14	17	10	13	14	18	7	9	12	14
Ketoconazole (Positive control)				26				25				19

Fig. 3: Bar chart showing the antifungal activity of dihydrazone A, its Mn(II), Fe(II) and Co(II) complexes



The *in-vitro* antifungal activity of the compounds was studied on three deleterious fungal strains (*Aspergillus fumigitus, Aspergillus niger* and *Aspergillus flavus*) by well diffusion method using solidified Potato Dextrose Agar (PDA) at different concentrations (125, 250, 500 and 1000 µg/mL). Ketoconazole was used as positive control and dimethylsulphoxide (DMSO) as negative control, the results were displayed in figure 3. Zones of inhibition around each well were measured in millimetre (mm) and recorded. Similarly, the results for antifungal tests indicated that the metal(II) complexes exhibit higher activities than the free (N^1E,N^2E)-$N^1.N^2$-bis(4-formylbenzylidene)oxalohydrazide. The activity is concentration dependent, it increases generally, with increase in concentrations. Iron complex $[FeA_2Cl_2]$ demonstrated the maximum activities against *Aspergillus niger* displayed in figure 3. These were comparable with the results reported by Singh *et al.,* (2021) and Suleiman *et al.,* (2023).

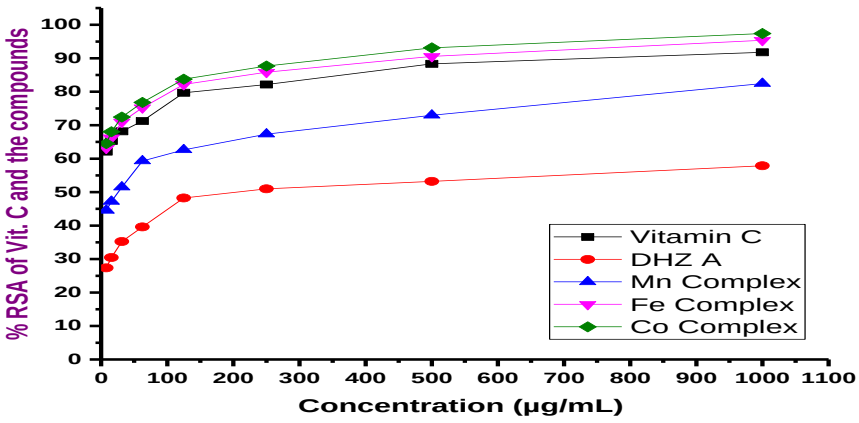


Fig. 4: Percentage RSA of Ascorbic Acid, Dihydrazone A and its Complexes

The compounds were screened for their antioxidant potential using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. The percentage radical scavenging capabilities of the dihydrazones (ligands) A, the complexes and standard drug (vitamin C i.e. ascorbic acid), were computed from their respective absorbances at different concentrations. The percentage radical scavenging activity of the ascorbic acid was found between 62.11 – 91.79 %, whereas, that of the (N^1E,N^2E)-$N^1.N^2$-bis(4-formylbenzylidene)oxalohydrazide was obtained in the range of 27.36 – 57.36 % indicating its lower percentage radical scavenging power than ascorbic acid especially at lower concentrations presented in figure 4. Analogous to the results reported by Yakan (2020) and Bhagwat *et al.,* (2022). However, upon complexation of the dihydrazones with metal ions, remarkable enhancement in activity were observed, some of the complex compounds have been found to have comparable radical scavenging activities with standard drug at higher concentrations (Adole *et al.,* 2020).

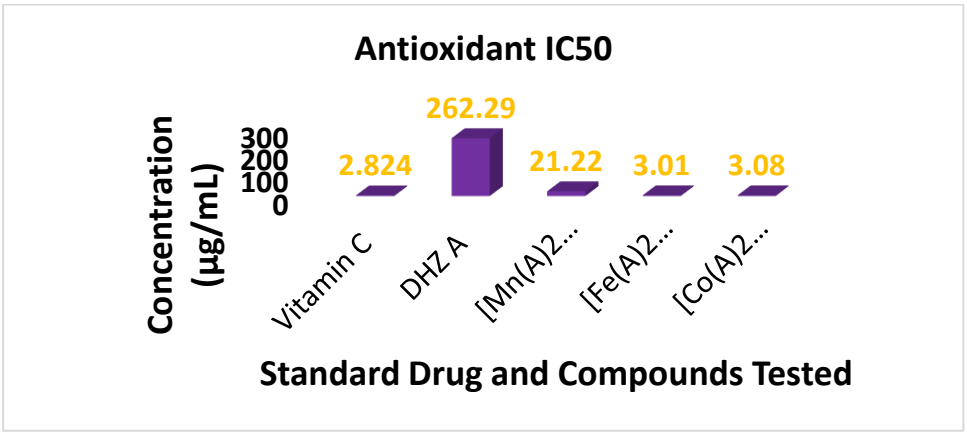


Fig. 5: Antioxidant IC50 Bar chat of ascorbic acid, dihydrazone A and its complexes

The IC50 (Half-maximal inhibitory concentration) value is a measure of the concentration of a drug or a compound required to inhibit a particular biological or biochemical process by 50% (David and Jeanet, 2023). It is a crucial metric in pharmacological research and drug development (Madhuranga & Samarakoon, 2023). Smaller value of IC50 indicates that, little amount of drug/test compound is required to inhibit the oxidative stress (in case of antioxidant evaluation). Whereas, the higher value of the IC50 require large amount of the drug/test compound to inhibit the biochemical process. As such, the smaller the IC50 value the better is the antioxidant activity. Larger IC50 value of a test compound indicates its poor antioxidant capacity (Nighat *et al.,* 2020). The ascorbic acid has an IC50 value of 2.824 µg/mL. The (N^1E,N^2E)-N^1.N^2-bis(4-formylbenzylidene)oxalohydrazide was found to have IC50 values of 262.29 µg/mL shown in figure 5. These were similar to the result reported by (Racheal *et al.,* 2023).

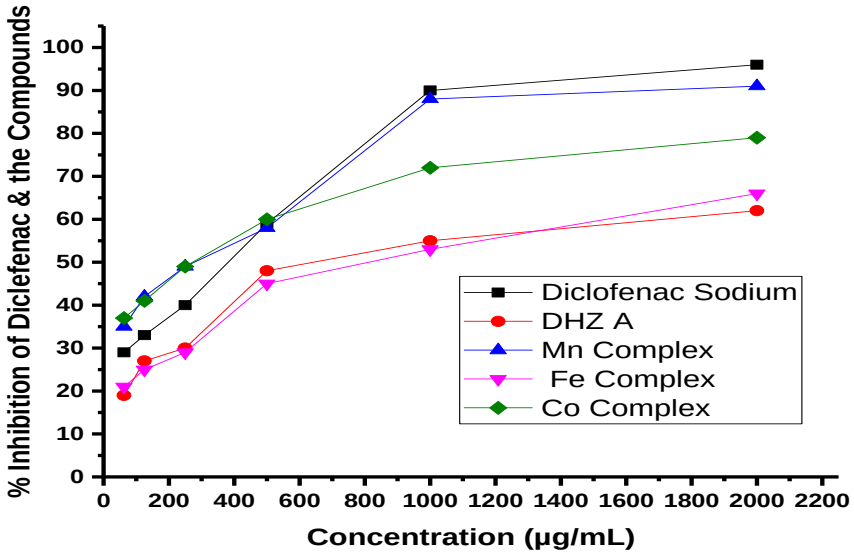


Fig. 6: Anti-inflammatory percentage inhibition of diclofenac sodium, dihydrazone A and its complexes

A dug or a substance that reduces/inhibit inflammation (redness, swelling and pain) in the body is known as anti-inflammatory agent. Anti-inflammatory agent blocks certain substance/process in the body that cause inflammation. They are used to treat many different conditions. Some anti-inflammatory agents are being used in the prevention and treatment of cancer. The percentage anti-inflammatory inhibition abilities of the diclofenac sodium (standard drug), the (N^1E,N^2E)-N^1.N^2-bis(4-formylbenzylidene)oxalohydrazide and its complex compounds at six different concentrations, that is; 62.5, 125, 250, 500, 1000 and 2000 µg/mL were computed from their respective absorbance. Their percentage (%) anti-inflammatory inhibition potentiality was evaluated and compared with that of the standard drug (diclofenac sodium).

The results were displayed in figure 6. The diclofenac sodium has a percentage anti-inflammatory inhibition between 29 – 96 % at the above concentrations, whereas, the percentage anti-inflammatory inhibition of the free (N^1E,N^2E)-N^1.N^2-bis(4-formylbenzylidene)oxalohydrazide was obtained between 19 – 62 %. At lower concentrations up to 1000 µg/mL, Mn(II) complex was found to have higher percentage inhibition than the standard drug, indicating its strength as potent anti-inflammatory agents, at 2000 µg/mL, it shows anti-inflammatory activity, that was comparable or equipotent with the standard drug, (diclofenac sodium). (Anna, *et al.,* 2015).

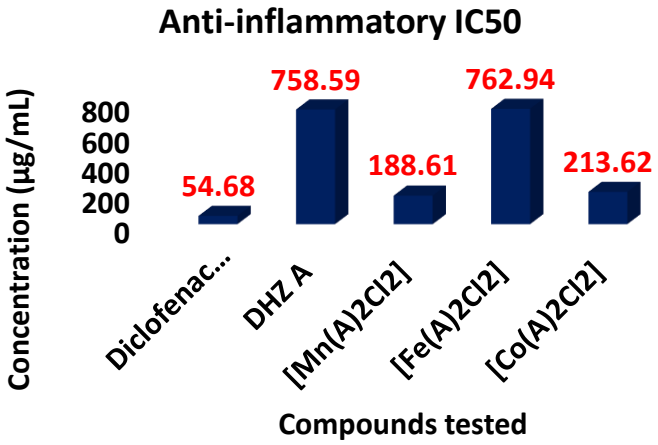


Fig. 7: Anti-inflammatory IC50 Bar-chat of diclofenac sodium, dihydrazone A and its respective complexes

The standard anti-inflammatory drug (diclofenac sodium) has an IC50 value of 54.68 µg/mL. The free (N^1E,N^2E)-N^1.N^2-bis(4-formylbenzylidene)oxalohydrazide 758.49 µg/mL. The IC50 values of all the complexes were found to be lower than that of their respective dihydrazones (ligands), implying enhancement in anti-inflammatory activity upon incorporation of metal ions into the dihydrazones. Among all the compounds, manganese complex displayed exceptionally significant anti-inflammatory activity due to its smallest IC50 value 188.61 µg/mL. The results were also displayed in figure 7. (Anna, *et al.,* 2015).

Conclusion

dihydrazone and all the complexes display appreciable activities against pathogenic bacterial and fungal species, as such the compounds are potent antimicrobial agents. Furthermore, they exhibit remarkable radical scavenging and significant anti-inflammatory activities, indicating their potency as good antioxidant and effective anti-inflammatory agents.

Competing Interest

The authors declared that, they have no competing interests with respect to publishing this article.

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