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ORIGINAL ARTICLE

Evaluation of Antioxidant, Anti-Inflammatory, and Antimicrobial Activities of Synthesized Schiff and Mannich Base Derivatives: An *In Vitro* Study

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ABSTRACT
Inflammation and oxidative stress are critical contributors to numerous chronic and degenerative diseases. In this study, a series of synthesized Schiff base and Mannich base compounds were evaluated for their antioxidant, anti-inflammatory, and antimicrobial activities using various in vitro assays. Antioxidant potential was assessed via DPPH and ABTS radical scavenging assays, where all samples exhibited significant free radical scavenging activity, with compounds 5 and 6 showing the highest inhibition (90.27% and 90.02%, respectively). Anti-inflammatory activity was evaluated through inhibition of albumin denaturation and human red blood cell (HRBC) membrane stabilization assays. Compounds demonstrated dose-dependent inhibition, with maximum protein denaturation inhibition reaching 96.30% and HRBC protection up to 92.37%. Antimicrobial efficacy against Escherichia coli and Staphylococcus aureus was tested using the agar well diffusion method. Sample 5 showed a dose-dependent antimicrobial effect against E. coli with a maximum inhibition zone of 13 mm at 1000 µg/mL, though activity against **S.** aureus *was limited. The presence of functional groups like hydroxyl, imine, and aromatic rings, along with metal complexation, significantly influenced the biological activity of the compounds. These findings align with previous reports, indicating the promising therapeutic potential of Schiff and Mannich base derivatives as antioxidant and anti-inflammatory agents with moderate antimicrobial properties. Further investigation into their mechanisms of action and* **in vivo** *efficacy is warranted.*
***Keywords**: Antimicrobial, ABTS, DPPH, Inflammations, HRBC*

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INTRODUCTION

Inflammation is a vital biological process that occurs in response to injury or infection, marked by symptoms such as redness, heat, swelling, and pain. While inflammation is essential for the body's immune defense, prolonged inflammation can lead to chronic diseases such as arthritis, cardiovascular diseases, and autoimmune disorders [1]. Acute inflammation typically resolves quickly, but chronic inflammation can have detrimental effects on health. Key immune cells like neutrophils and lymphocytes are involved in this process, migrating to the site of injury through extravasation [2]. This migration is regulated by various mediators, including chemokines, cytokines, and lipid molecules that help manage the inflammation process.

Treatment of inflammation commonly involves corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs), which are effective in reducing inflammation but often come with side effects such as gastrointestinal issues and immune suppression [3]. As a result, natural compounds like white willow bark, curcumin, and omega-3 fatty acids are gaining interest due to their potential for managing inflammation with fewer side effects [4]. These natural compounds have demonstrated anti-inflammatory

effects by modulating key pathways involved in immune cell recruitment and inflammatory mediator production.

In addition to inflammation, oxidative stress plays a significant role in the development of degenerative diseases, as it causes damage to proteins, lipids, and DNA. Antioxidants help protect the body from such damage by neutralizing free radicals. These compounds, including phenolic acids, polyphenols, and flavonoids, act through mechanisms such as free radical scavenging, chain-breaking, and metal chelation [5]. The body also has its own antioxidant defense system, which includes enzymatic antioxidants like superoxide dismutase and non-enzymatic antioxidants like vitamins C and E, to counteract oxidative stress.

Schiff bases and Mannich bases, both of which are synthesized through condensation reactions involving amines, aldehydes, or ketones, have emerged as important compounds in medicinal chemistry. Schiff bases contain the azomethine group (-HC=N-) and are used in various applications, including the formation of metal complexes and as intermediates for amino acid synthesis [5]. Similarly, Mannich bases, produced through the Mannich reaction, are used in the synthesis of nitrogen-containing compounds and have shown potential for their anti-inflammatory, antioxidant, and antibacterial activities. The evaluation of these compounds' bioactivity offers insights into their therapeutic potential for treating a range of diseases, including inflammatory conditions, oxidative stress-related diseases, and bacterial infections.

MATERIAL AND METHODS

Synthesis of the compounds: (Plate 1)

Compound 1 (Mannich Base): N -[(4-Hydroxy-3-methoxyphenyl)-(4-methoxyphenyl amino)-methyl] -nicotinamide

Preparation: To prepare Compound 1, dissolve nicotinamide (1.22 g) and p-anisidine (1.23 g) in ethanol to form a solution. Stir the solution for 10 minutes using a magnetic stirrer. Next, add vanillin (1.52 g), dissolved in ethanol, to the mixture. Continue stirring for 6 hours. After this, a dirty white solid precipitate will form. The precipitate is then washed, filtered, and recrystallized using toluene to obtain the final product.

Compound 2: $[Cd(NA)_2(4\text{-}AT)_2(H_2O)_2]$

Preparation: To prepare Compound 2, dissolve metal chloride (5 mM) [Cd(II) = 1 g] in ethanol. Gradually add this solution to a stirred ethanol solution of nicotinamide ligand (5 mM) (0.61 g). Next, add 4-aminotriazole (0.42 g) (5 mM), dissolved in methanol, to the reaction mixture in the metal-to-ligand molar ratio, with constant stirring. After the reaction, the solid complexes will precipitate. Filter the precipitate and wash it several times with methanol to remove any unreacted starting materials. Finally, dry the product and store it in an airtight container.

Compound 3: $[Cu(INH)_2(urea)_2]$

Preparation: Dissolve 2.37 g of copper sulfate in distilled water and mix it with 1.37 g of isoniazid in water. Heat and then cool the mixture. Next, add a solution of urea (0.76 g) in a 1:2:2 molar ratio to the reaction mixture and shake well. A precipitate will form, which is then filtered using Whatman filter paper. The precipitate is dried and stored in an airtight container.

Compound 4: $[Cd(INH)_2(NCS)_2]$ Complex

Preparation: Dissolve 2.37 g of cadmium chloride in distilled water and mix it with 1.37 g of isoniazid in water. Heat the mixture and then cool it. Afterward, add thiocyanate (0.76 g) in a 1:2:2 molar ratio and shake the mixture well. A precipitate will form, which is then filtered through Whatman filter paper. The precipitate is dried and stored in an airtight container.

Compound 5 (Schiff Base Compound): (1Z)-N-(3,5-dibromopyrazin-2-yl) ethanimine (ACBP1)

Preparation: Take 1.37 g of 3,5-dibromopyrazin-2-amine (0.01 M) and 1.56 g of acetaldehyde (0.01 M) in a 1:1 molar ratio. Dissolve both 3,5-dibromopyrazin-2-amine and acetaldehyde in ethanol and place the mixture in a round-bottom flask. Stir the solution constantly for three hours using a magnetic stirrer under ice-cold conditions. Afterward, a yellow-orange solid will separate out. The solid is washed several times with cold distilled water. The crude sample is then recrystallized from ethanol. The reaction scheme is provided below.

Compound 6: $[Co(INH)_2(NCS)_2]$

Preparation: Dissolve 2.37 g of cobalt chloride in distilled water and mix it with 1.37 g of isoniazid in water. Heat the mixture, then allow it to cool. After cooling, add a hydrated solution of ammonium thiocyanate (0.76 g) in a 1:2:2 molar ratio. Shake the mixture thoroughly. A precipitate will form, which should be filtered using Whatman filter paper. The precipitate is then dried and stored in an airtight container.

Preparation of the Working Standard for Assays: All the compounds were dissolved in DMSO (Dimethyl sulfoxide) at a concentration of 1 mg/mL. Four different concentrations were prepared for the assays, as follows:1000 µg/mL, 100 µg/mL, 50 µg/mL & 25 µg/ml.

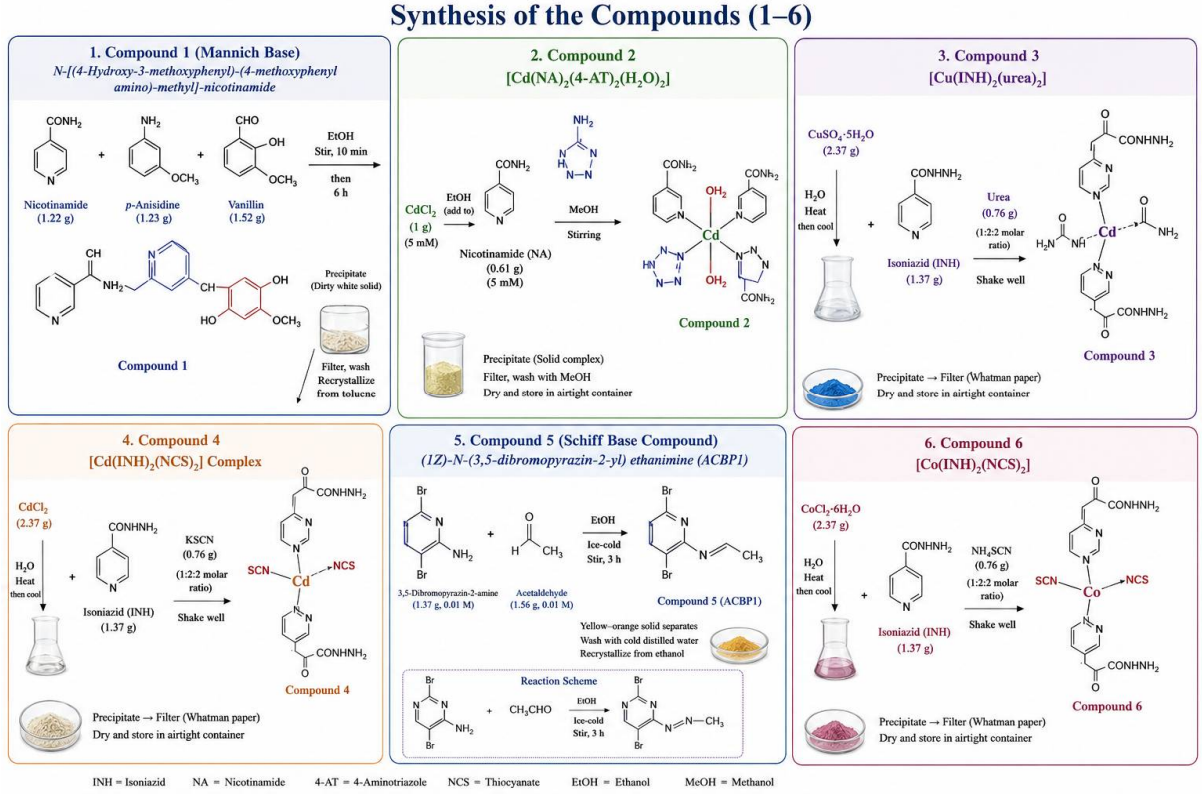


Plate 1: Synthesis of Compounds

In vitro Antioxidant and Free Radical Scavenging Assays

DPPH Radical Scavenging Assay

The scavenging activity was measured using the method described by Yen and Chen (1995). Briefly, 2.0 mL of test samples at varying concentrations (25 µg/mL, 50 µg/mL, 100 µg/mL, 1000 µg/mL) were mixed with 2.0 mL of 0.16 mM DPPH methanolic solution. The mixture was vortexed for 1 minute and then allowed to stand at room temperature for 30 minutes in the dark. The absorbance of each sample solution was measured at 517 nm. The concentrations used for the test samples were 25 µg/mL, 50 µg/mL, 100 µg/mL, and 1000 µg/mL, while varying concentrations (100–500 µg/mL) were used for the standard. The scavenging effect (%) was calculated using the following formula:

$$\text{Scavenging effect (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test solution}}{\text{Absorbance of control}} \times 100$$

ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] Radical Cation Scavenging Assay

Free radical scavenging activity was determined using the ABTS radical cationdecolorization assay, as described by Re et al. (1999). The ABTS radical cation was generated by mixing a 20 mM ABTS solution with a 70 mM potassium peroxodisulphate solution and allowing the mixture to stand in the dark at room temperature for 24 hours before use. To 0.6 mL of each extract at varying concentrations (25 µg/mL, 50 µg/mL, 100 µg/mL, and 1000 µg/mL), 0.45 mL of ABTS reagent was added. The absorbance of the solutions was then measured at 734 nm after 10 minutes.

$$\text{ABTS radical cation scavenging assay [\%]} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Inhibition of Albumin Denaturation

The reaction mixture consisted of test samples at concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, and 1000 µg/mL, along with a 1% aqueous solution of bovine albumin. The pH of the reaction mixture was adjusted to pH 7 using a small amount of 1 N HCl. The samples were incubated at 37°C for 20 minutes,

followed by heating at 51°C for 20 minutes. After cooling, the turbidity was measured spectrophotometrically at 660 nm. Diclofenac sodium was used as a standard drug with varying concentrations of 100-500 µg/mL. The experiment was performed in triplicate. The percent inhibition of protein denaturation was calculated using the following formula:

$$\text{Scavenging effect (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test solution}}{\text{Absorbance of control}} \times 100$$

Human RBC Membrane Stabilization Method:

The human red blood cell (HRBC) membrane stabilization method was used to study in vitro anti-inflammatory activity. Blood was collected from a healthy human volunteer under aseptic conditions, who had not taken any Non-Steroidal Anti-inflammatory Drugs (NSAIDs) for two weeks prior to the experiment. The blood was mixed with an equal volume of AL sever solution (containing 2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% NaCl) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline (NaCl, pH 7.2), and a 10% suspension was prepared. To 0.5 mL of test samples at concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, and 1000 µg/mL, 1 mL of phosphate buffer (0.15 M, pH 7.4), 2 mL of hyposaline (0.36% NaCl), and 0.5 mL of HRBC suspension were added. The mixture was incubated at 37°C for 30 minutes and then centrifuged at 3,000 rpm for 20 minutes. The absorbance of the supernatant solution was measured spectrophotometrically at 560 nm. A control sample was taken without the test compound. Diclofenac sodium (100-500 µg/mL) was used as the reference standard.

$$\text{Percent of protection} = 100 - \frac{\text{OD of test}}{\text{OD of control}} \times 100.$$

Antimicrobial Activity:

Two bacterial cultures, ***Staphylococcus aureus*** and ***Escherichia coli***, were used in this study. The cultures were procured from the PG and Research Department of Biotechnology, Mohamed Sathak College of Arts and Science, Chennai, India. The bacterial strains were grown in nutrient broth at 37°C and stored on nutrient agar slants for future use. Antimicrobial activity of the samples was tested using a modified Agar well diffusion method [6].

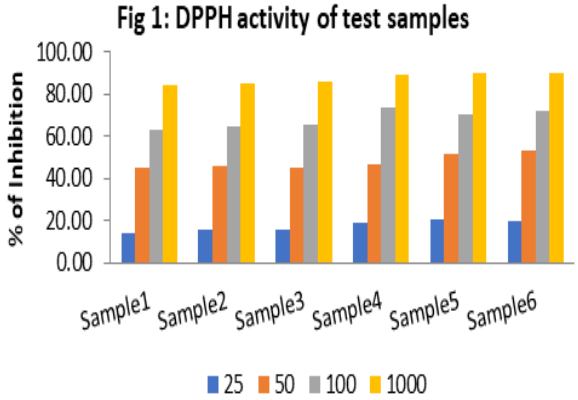
From the nutrient broth, the respective inoculum suspensions were swabbed uniformly over Mueller-Hinton Agar plates using sterile cotton swabs. Subsequently, using a sterile borer, four wells of 0.5 cm diameter and about 2 cm apart were made in each pathogen-inoculated agar plate. Then, 50 µL of the different concentrations (25 µg/mL, 50 µg/mL, 100 µg/mL, 1000 µg/mL) of each sample were aseptically filled into the wells, with one plate for each sample and one well for each concentration. A standard antibiotic, Ampicillin (10 µg), was placed as a disc at the center of all the agar plates.

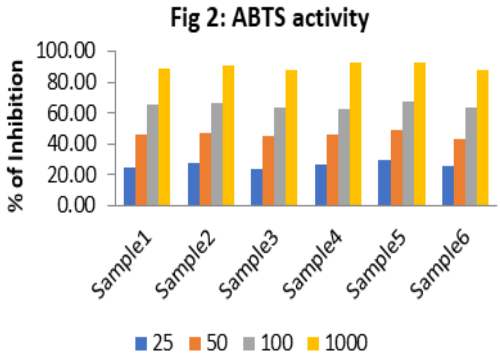
The plates were left at room temperature for one hour to allow the diffusion of the samples into the agar. The plates were then incubated for 24 hours at 37°C. After incubation, the results were recorded by measuring the diameter of the zone of inhibition (mm) at the end of 24 hours. Triplicates were maintained, and the experiment was repeated three times. For each replicate, the readings were taken in three different fixed directions, and the average values were recorded.

RESULT

In the present study, all samples exhibited varying degrees of ability to scavenge DPPH radicals. For Compound A, the highest percentage of inhibition was 84.23% at 1000 µg/mL. For Compound B, the highest percentage of inhibition was 84.96% at 1000 µg/mL. For Compound C, the highest percentage of inhibition was 85.80% at 1000 µg/mL. For Compound D, the highest percentage of inhibition was 89.14% at 1000 µg/mL. For Compound E, the highest percentage of inhibition was 90.02% at 1000 µg/mL. For Compound F, the highest percentage of inhibition was 90.27% at 1000 µg/mL (Fig 1).

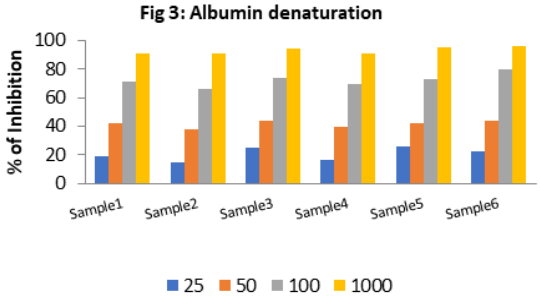
In the present study, all samples exhibited the ability to scavenge the ABTS Radical Cation to varying extents. For Sample 1, the highest scavenging percentage was 88.38%, corresponding to a concentration of 1000 µg/ml. For Sample 2, the highest scavenging percentage was 90.59%, also at 1000 µg/ml. For Sample 3, the highest scavenging percentage was 87.52%, corresponding to 1000 µg/ml. For Sample 4, the highest scavenging percentage was 93.10%, at 1000 µg/ml. For Sample 5, the highest scavenging percentage was 93.10%, corresponding to 1000 µg/ml. For Sample 6, the highest scavenging percentage was 87.85%, at 1000 µg/ml (Fig 2).

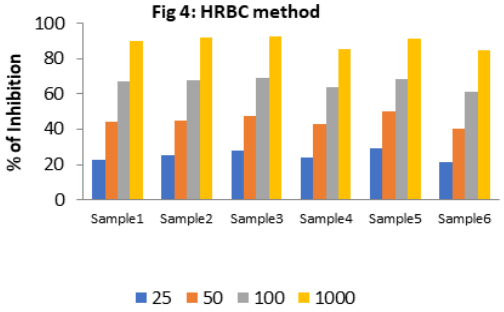




In the present study, all samples demonstrated the ability to inhibit albumin denaturation to varying extents. For Sample 1, the highest inhibition percentage was 91.15%, corresponding to a concentration of 1000 µg/ml. For Sample 2, the highest inhibition percentage was 90.51%, also at 1000 µg/ml. For Sample 3, the highest inhibition percentage was 94.49%, corresponding to 1000 µg/ml. For Sample 4, the highest inhibition percentage was 90.88%, at 1000 µg/ml. For Sample 5, the highest inhibition percentage was 94.94%, corresponding to 1000 µg/ml. For Sample 6, the highest inhibition percentage was 96.30%, at 1000 µg/ml (Fig 3).

The present study evaluated the human red blood cell (HRBC) membrane stabilization activity of various samples. All samples exhibited HRBC membrane stabilization properties to varying extents. The highest protective effect was observed at a concentration of 1000 µg/ml for each sample, with Sample 1, 2, 3, 4, 5, and 6 showing maximum protection of 90.27%, 91.60%, 92.37%, 85.29%, 91.52%, and 84.90%, respectively (Table 7 & 8 & Fig 4)





Anti-bacterial activity

The antimicrobial activity of Sample 5 and Sample 6 against *E. coli* and *S. aureus* was evaluated using the zone of inhibition assay. The results showed that Sample 5 exhibited a dose-dependent increase in antimicrobial activity against *E. coli*, with zone of inhibition diameters ranging from 9 mm at 25 µg/ml to 13 mm at 1000 µg/ml. In contrast, Sample 6 showed a decrease in antimicrobial activity against E. coli at higher concentrations. Against *S. aureus*, both samples showed limited antimicrobial activity, with zone of inhibition diameters ranging from 3-6 mm. The standard antibiotic ampicillin (10 mcg) exhibited zone of inhibition diameters of 14 mm and 8 mm against *E. coli* and *S. aureus*, respectively.(Table 1 & Fig 5).

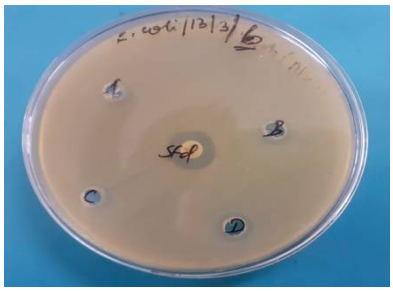
Table 1. Anti-bacterial activity of Compound 5 and 6

Name of the bacteria									
	Sample 5 in (µg/ml)				Sample 6 in (µg/ml)				Ampicillin
	25	50	100	1000	25	50	100	1000	10 mcg
E. coli	9	10	11	13	11	9	8	7	14
S. aureus	6	5	4	3	5	3	4	3	8

Zone of inhibition was seen for *E.coli* and *S.aureus* using compound 5 and compound 6

Fig 5.Zone of inhition of the compound

COMPOUND 5
A - 1000 µg / ml
B - 100 µg / ml
C - 50 µg / ml
D – 25 µg / ml
Standard – Ampicillin (10 mcg)



COMPOUND 6
A - 1000 µg / ml
B - 100 µg / ml
C - 50 µg / ml
D – 25 µg / ml
Standard – Ampicillin (10 mcg)
Bacteria used – *E.coli*

DISCUSSION

The antioxidant activity of an inhibitor is primarily based on its ability to neutralize radical sites generated in biological systems, either by donating an electron or a hydrogen bond. The structure and properties of the inhibitor are critical to the manifestation of this activity. These findings are consistent with previous studies on metal complexes [7], which showed that the ligand exhibits antioxidant activity and that the metal portion enhanced this activity.

The evaluation of antioxidant activity through the stable DPPH radical scavenging model is quite common, since it allows obtaining results in a shorter time than other methods. DPPH, a stable free radical, is capable of accepting either an electron or a hydrogen radical, allowing it to become a stable diamagnetic molecule. Because it presents an unpaired electron, DPPH has a notable absorption band at 517 nm. Absorption decreases stoichiometrically when this electron is paired, depending on the number of electrons that have been incorporated. This variation in absorbance during the reaction has been widely used to evaluate the effectiveness of different molecules as free radical scavengers. All compounds were evaluated in their interaction with the DPPH free radical [8]. In the present study, all samples evaluated showed the ability to capture DPPH to different degrees, with compounds 6, 5, and 4 being the most effective (90.27%, 90.02%, and 89.14%, respectively). Compound 6 exhibited the highest activity at 1000 µg/ml. Beena et al. (9) suggested that Schiff bases scavenge DPPH free radicals as antioxidants through electron and proton transport mechanisms. In the electron transfer mechanism, Schiff bases lose a proton and form ions, which then transfer electrons to the DPPH radical, converting it into a charged species. This species, upon neutralization by the lost proton, transforms the Schiff base into a radical. Similarly, Oladipo et al.(10) investigated the antioxidant potential of Schiff base compounds H1–H6 using 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO), and ferric reduction capacity (FRAP) assays. DPPH and NO radical scavenging assays demonstrated that all compounds exhibited excellent antioxidant results, with some of them outperforming catechin.

A significant attribute of antioxidants is their ability to scavenge proton radicals. A well-known protonated radical, such as 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), exhibits characteristic absorbance maxima at 734 nm, which decrease upon capture of proton radicals. Mannich base and Schiff base metal complexes were shown to be moderately effective in scavenging the ABTS radical, and this activity was comparable to that of BHT, which is used as a standard drug. Lower concentrations of the test samples were found to be more effective in neutralizing ABTS+ radicals in the system. The capture of ABTS+ radical by the Schiff base and its metal complexes was observed to possess moderate to high activities compared to the standards (BHT). The research of Turan et al. (11) indicated that the ABTS and DPPH radical scavenging activities of base-Pd(II) Schiff complexes showed results

approaching those of standard oxidants. Similar findings have also been reported in the literature for metal complexes and ligands. Antioxidant properties vary depending on the presence of hydroxyl, imine, and aromatic rings. Furthermore, the central metal ion plays a crucial role in antioxidant capacity [12–14]. Overall, the evaluation of the results of this study is consistent with the results of previous research

The heat-induced albumin denaturation inhibitory activity of the compound showed IC50 values ranging from 25 µg/ml to 1000 µg/ml. In this assay, the Schiff base compounds (1Z)-N-(3,5-dibromopyrazin-2-yl)ethanamine (ACBP1) and $Co(INH)_2(NCS)_2$ demonstrated the highest activity, comparable to that of diclofenac sodium. A previous study indicated that although a complete correlation could not be established, several acetamido[(phenyl-4'-yl)-oxymethylene]-2-(p-phenylamino)-1,2,4-triazoles and -1,3,4-thiadiazoles compounds that exhibited good denaturation inhibition also showed remarkable anti-inflammatory activity in vivo in carrageenan-induced paw edema of rats [15]. Anti-inflammatory agents act by inhibiting cyclooxygenase enzymes, which are responsible for the conversion of arachidonic acid into prostaglandins. Since human red blood cell (HRBC) membranes are similar to the components of lysosomal membranes, prevention of hypotonicity-induced HRBC membrane lysis was used as an indicator of anti-inflammatory activity. The anti-inflammatory activity performed by human red blood cells (HRBC) shows that DFS stabilizes the membrane, thereby reducing hemolysis. Therefore, increasing the concentration of this component prevents leakage of cellular contents, resulting in a decrease in DO as the DFS concentration increases, thereby reducing the tonicity effect induced by hypotonic saline solutions. HRBC membrane stabilization method was used to evaluate the anti-inflammatory activity. The results of in vitro membrane stabilization activity of the synthesized Mannich and Schiff base compounds are presented in Fig. 3. According to these results, all the compounds showed dose-dependent inhibition of hemolysis. The Schiff base compounds of (1Z)-N-(3,5-Dibromopyrazin-2-yl)ethanimine (ACBP1) and $Co(INH)_2(NCS)_2$ exhibited very prominent activity as compared to the standard Diclofenac sodium. Similarly, Swathi et al. (16), reported that the in vitro anti-inflammatory activity of synthesized thiazolidinone derivatives showed good results at concentrations of 50 and 100 µg/ml using Diclofenac sodium as a standard by HRBC membrane stabilization method.

The antimicrobial activities at concentrations of 25 µg/ml and 1000 µg/ml are presented in Table 5. The zones of inhibition against the test organisms are illustrated in Figure 5. Sample 5 showed a dose-dependent increase in antimicrobial activity against *E. coli*, with inhibition zone diameters ranging from 9 mm at 25 µg/ml to 13 mm at 1000 µg/ml. Similarly, Yusuf et al. (2020) [17] reported the synthesis and antimicrobial evaluation of three Schiff bases, (E)-1-(2-nitrophenyl)-N-(o-tolyl) methanimine (11), (E)-2-isopropyl-N-(2-nitrobenzylidene) aniline (12) and (E)-2-((2-nitrobenzylidene) amino)phenol (13). Compounds 11–13 were tested against *E. coli, Salmonella typhimurium*and *P. aeruginosa.* Then, compounds 11–13 were docked into the active site pocket as observed experimentally of crystalized structures from *E. coli, S. typhimurium, P. aeruginosa and S. aureus.*

CONCLUSION

The present study highlights the significant biological potential of synthesized Schiff base and Mannich base derivatives, particularly in the areas of antioxidant, anti-inflammatory, and antimicrobial activities. The compounds demonstrated strong free radical scavenging abilities in both DPPH and ABTS assays, suggesting effective antioxidant properties. Their capacity to inhibit protein denaturation and stabilize red blood cell membranes indicates promising anti-inflammatory activity, comparable to standard drugs like diclofenac sodium. Additionally, selected compounds exhibited moderate antimicrobial activity, especially against *Escherichia coli***,** with a dose-dependent response observed. The structural features of the compounds, including the presence of aromatic rings, hydroxyl, and imine groups, played a key role in enhancing their bioactivity. These findings suggest that Schiff and Mannich base derivatives could serve as potential lead compounds for the development of novel therapeutic agents targeting oxidative stress, inflammation, and bacterial infections. Further in vivo studies and mechanistic evaluations are recommended to validate their therapeutic potential.

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