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Evaluation of Antimicrobial and Antioxidant Activity of Newly Synthesized N-Mannich Bases from Methyl Salicylate

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Abstract: Isatin reacted with formaldehyde and a number of secondary amines in order to produce the N-Mannich bases of isatin derivatives. FT-IR, 1H-NMR, and mass spectrum data were used to demonstrate that their chemical structures were accurate. An agar diffusion method was used to examine the antibacterial activity of compounds against Gram (+) bacteria (*Staphylococcus aureus* and *Micrococcus luteus*) and Gram (-) bacteria (*Escherichia coli* and *Klebsiella pneumonia*), with streptomycin employed as control. The antifungal activity of fluconazole against *Candida albicans* and *Aspergillus niger* was measured against a benchmark of fluconazole's own performance. Compounds IVc, IVd, and IVe all exhibited potent antibacterial activity, but only compounds IVc and IVd were effective against fungal infections. Compounds IVc and IVe were found to have the highest potential as anti-oxidants among the chemicals that were investigated.

Keywords: Anti-oxidant, DPPH, Synthesis, Mannich bases, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Klebsiella pneumonia*

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Introduction

The indole-2,3-dione compound known as isatin, together with its Schiff and Mannich bases, have antimicrobial, antifungal, and anti-HIV properties. The isatin N-Mannich bases are the primary focus of the present investigation. The formation of these compounds involves the condensation of the acidic imino group of isatin with formaldehyde and secondary amines (Tahir *et al*., 2019). The elemental analysis of each component (Table 1) came back with positive results. IR and 1H-NMR spectra provided confirmation that the structures were correct (Bayati, 2012). The latest

developments of isatin dimers as pharmacologically relevant scaffolds and the structure-activity connection have established the direction for the creation and development of isatin dimers that have better efficiency and reduced toxicity (Zhang *et al*., 2020). Many approaches that may be used in the design and synthesis of several IST-based compounds with a variety of biological activities with SAR, which can favour future exploration and modification for the creation of new and more powerful entities (Chauhan *et al*., 2021). An agar diffusion cup-plate approach was used in order to examine each of the synthetic chemicals for their potential to inhibit the growth of bacteria and fungi.

By monitoring for shifts in the absorbance of a free radical solution, the antioxidant power of the newly synthesised compounds was evaluated against free radicals of three different types: DPPH, OH, and NO. A control was carried out using ascorbic acid.

Materials and Methods

In the course of this study, a number of N-Mannich bases were manufactured by subjecting them to reactions involving formaldehyde and secondary amines. The H-NMR and IR spectra both verified the structures that were given to them. The information on the physicochemical properties have been given in Table 1.

Preparation of 2-Hydroxybenzohydrazide (*II*):

In an RB flask with a capacity of 500 ml, 10 g of methyl salicylate and 50 ml of distilled alcohol were mixed together and swirled for a period of 5 min. After adding 20 ml of hydrazine hydrate to this, the contents of the flask were allowed to reflux for 3 h while TLC monitored whether or not the reaction was complete (Sridhar, 1999). After this, the product, which was crystalline and white in appearance, was filtered and washed multiple times with low volumes of cold alcohol. Recrystallization from methanol was done in order to accomplish drying and purifying of the product (yield 90 per cent, m.p. 251–254°C) (Scheme).

Synthesis of 2-Hydroxy-N'-(*2-oxoindolin-3-ylidene*) *benzohydrazide* (*III*):

Cooking a combination of suitable Indole-2,3dione (0.01 M) and 2-Hydroxybenzohydrazide (0.01 M) in methanol (50 ml) with glacial acetic acid for 6-7 h under reflux on a water bath resulted in the formation of the desired compound. After being cooled, the brightly coloured compounds were collected, filtered, and then washed with cold methanol before being dried. They were recrystallized from alcohol in order to achieve the desired level of cleanliness (Nagadeep *et al*., 2019)

Synthesis of N-Mannich bases derived from 2-Hydroxy-N'-(*2-oxoindolin-3-ylidene*) *benzohydrazide* (*IV*):

General technique for synthesis of compounds IVa through IVf:

In a flask containing 250 ml of RB buffer, place 0.01 mM solutions of secondary amine, 0.01 nM solutions of formaldehyde, and 0.01 nM solutions of 2-hydroxy-N'-(2-oxoindolin-3-ylidene) benzohydrazide (0.01 M). Added 15-30 ml of methanol or ethanol to this mixture. After a period of 12 to 16 h added two to three drops of glacial acetic acid. The contents were strained into ice cold water. Recrystallization from methanol was done in order to accomplish drying and purifying of the product (Pandeya, 2002).

Spectral data of synthesized compounds (*IVa – IVf*):

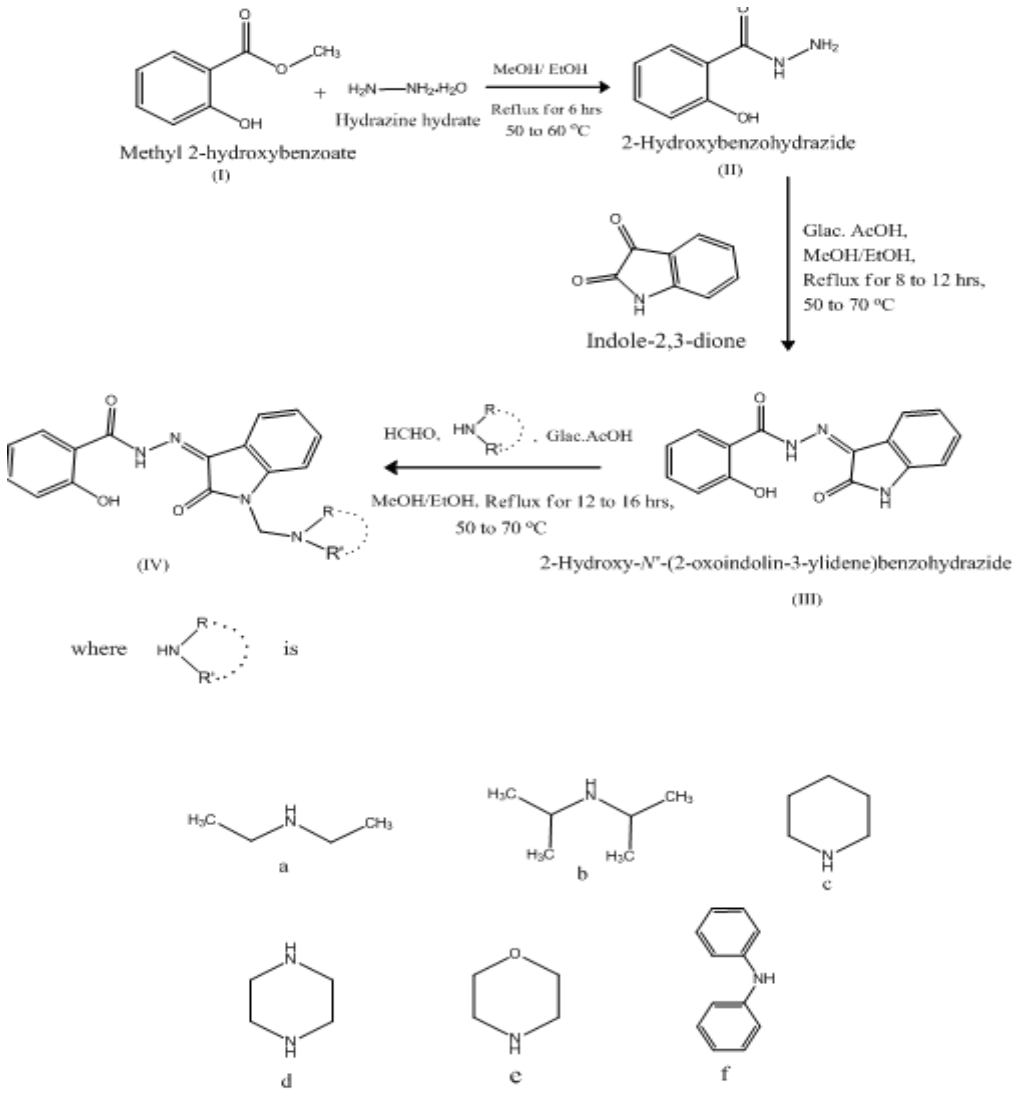
N'-(*1-((Diethylamino*) *methyl*)*-2-oxoindolin-3-ylidene*)*-2-hydroxybenzohydrazide* (*IVa*) :

IR (KBr $c^{m^{-1}}$): 3426.76 (-OH), 3090.21(CH-Aromatic), 2993.19 (CH-Aliphatic), 1659.79 (C=O)), 1017.14 (C-O).

^{1}H NMR($CDCl_3$, δ, ppm): 1.1 (t, 6H, 2X-CH_3), 2.6 (q, 4H, 2X-CH_2), 4.4 (2H, s, -CH_2), 6.9-7.8 (m, 8H, Ar-H), 5.2 (s, 1H, Ar-OH), 7.6 (s,1H, -CO-NH).

(EI-MS): MS: m/z 366.41 (M^+)

N'-(*1*((*Diisopropylamino*)*methyl*)*-2-oxoindolin-3-ylidene*)*-2-hydroxybenzohydrazide* (*IVb*):



Scheme

IR (KBr $c^{m^{-1}}$): 3826.76 (-OH), 3050.21(CH-Aromatic), 2995.19 (CH-Aliphatic), 1655.81 (C=O)), 1012.12 (C-O).

^{1}H NMR($CDCl_3$, δ, ppm): 1.2 (d, 12H, 4X-CH_3), 2.8 (m, 2H, 2X-CH), 4.3 (2H, s, -CH_2), 6.8-7.9 (m, 8H, Ar-H), 5.4 (s, 1H, Ar-OH), 7.8 (s,1H, -CO-NH).

(EI-MS): MS: m/z 394.47 (M^+)

2-Hydroxy-N'-(2-oxo-1-(piperidin-1-ylmethyl)indolin-3-ylidene)benzohydrazide (*IVc*):

IR (KBr $c^{m^{-1}}$): 3826.76 (-OH), 3050.21 (CH-Aromatic), 2995.19 (CH-Aliphatic), 1655.81 (C=O)), 1012.12 (C-O).

^{1}H NMR($CDCl_3$, δ, ppm): 1.4 (m, 2H. -CH_2), 1.7 (m, 4H, 2XCH_2), 2.4 (t, 4H,-CH_2-N-CH_2-), 4.4 (2H, s, -CH_2), 6.6-7.8 (m, 8H, Ar-H), 5.6 (s, 1H, Ar-OH), 7.6 (s,1H, -CO-NH).

(EI-MS): MS: m/z 378.42 (M^+)

2-Hydroxy-N'-(2-oxo-1-(piperazin-1-ylmethyl) indolin-3-ylidene)benzohydrazide (*IVd*):

IR (KBr $c^{m^{-1}}$): 3421.56 (-OH), 3031.21 (CH-Aromatic), 2996.19 (CH-Aliphatic), 1651.56 (C=O)), 1015.1 (C-O).

^{1}H NMR($CDCl_3$, δ, ppm): 2.5 (t, 4H, -CH_2-N-CH_2-), 2.7 (T,4H,-CH_2-NH-CH_2-), 3.1 (s, 1H, -NH-), 4.4 (2H,

s, -CH_2), 6.6-7.8 (m, 8H, Ar-H), 5.2 (s, 1H, Ar-OH), 7.7 (s,1H, -CO-NH).

(EI-MS): MS: m/z 379.41 (M^+)

2-Hydroxy-N'-(1-(morpholinomethyl)-2-oxoindolin-3-ylidene)benzohydrazide (*IVe*):

IR (KBr $c^{m^{-1}}$): 3476.52 (-OH), 3072.25 (CH-Aromatic), 2997.24 (CH-Aliphatic), 1660.19 (C=O)), 1011.12 (C-O).

^{1}H NMR($CDCl_3$, δ, ppm): 2.6 (t, 4H, -CH_2-N-CH_2-), 3.7 (t, 4H, -CH_2-O-CH_2-), 4.3 (2H, s, -CH_2), 6.7-7.9 (m, 8H, Ar-H), 5.6 (s, 1H, Ar-OH), 7.9 (s,1H, -CO-NH).

(EI-MS): MS: m/z 380.40 (M^+)

N'-(1-((diphenylamino)methyl)-2-oxoindolin-3-ylidene)-2-hydroxybenzohydrazide (*IVf*):

^{1}H NMR($CDCl_3$, δ, ppm): 4.3 (2H, s, -CH_2), 6.5-7.6 (m, 18H, Ar-H), 5.4 (s, 1H, Ar-OH), 7.8 (s,1H, -CO-NH).

IR (KBr $c^{m^{-1}}$): 3452.26 (-OH), 3040.71 (CH-Aromatic), 2996.89 (CH-Aliphatic), 1655.39 (C=O)), 1011.18 (C-O).

(EI-MS): MS: m/z 462.50 (M^+)

In vitro antibacterial activity:

The antibacterial activity of N-Mannich bases of isatin derivatives was examined using the Cup-plate agar diffusion technique, with the zone of inhibition being measured in centimetres. These derivatives were tested against four different bacteria strains (Jaishetty, 2016).

Organisms chosen:

Gram –positive bacteria: *Staphylococcus aureus*, *Bacillus cereus*.

Gram-negative bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*.

Procedure:

It was decided to introduce the bacterial strains. The organisms being tested were subcultured in a medium consisting of nutrient broth. After being cleaned, the bacterial strains were placed in tubes that contained sterile medium. After being incubated for 24 h at 371 ℃, they were then put in the refrigerator. The original culture was preserved. A loopful of culture was added to the nutrient broth that was contained inside a conical flask in order to produce bacterial inoculum. Before beginning the experiment, the flask was given a 48 h incubation at 37 ℃.

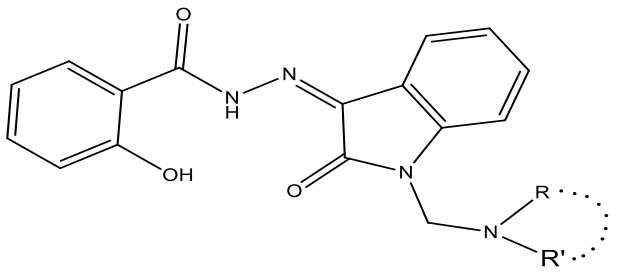
Methanol was used to dissolve the test chemicals so that the desired concentrations could be achieved (50 g/ml, 100 g/ml, and 500 g/ml). When preparing the test compounds, the same amounts of amoxicillin were used throughout. The nutritious agar medium was autoclaved at a temperature of 121℃ for 15 min. Petri plates, tubes, and flasks that had cotton plugs placed in them were heated in an oven at 80 ℃ for 1 h to achieve sterilisation. Approximately 25 ml of molten nutritional agar medium that had been infected with the necessary bacteria strains was poured onto each sterile petri dish (Singh *et al*., 2007). The plates were allowed to cool down to room temperature so that the solidification process could take place. A sterile borer was used to create cups with a diameter of 10 mm from each plate. After that, an aseptic addition of 100 g/ml of the test solution was made, and the cups were given their appropriate labels. In order to give the solution enough time to adequately diffuse into the nutrient agar medium, the plates were placed in the refrigerator and left there undisturbed for at least 2 h. After incubation the plates at 37 ℃ for 24 h, the width of the zone of inhibition that surrounded each cup was measured using a scale and recorded (Tables 2, 3, 4 5).

In Vitro Antifungal Activity:

The Agar Well Diffusion Method was used in order to evaluate the antifungal activity of the sample, and the zone of inhibition was evaluated in mm.

Candida albicans and *Aspergillus niger* were the species that were tested. In order to prepare cultures of fungus for testing, the organisms being tested were first sub-cultured in SDA broth medium. The fungal strains were placed in tubes

Table 1: Physical constants of the synthesized compounds



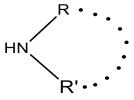
S. No.	Compound		Molecular formula	Molecular weight	Melting Point (°C)	% Yield
1	IVa	$C_4H_{11}N$	$C_{20}H_{22}N_4O_3$	366.41	311	71
2	IVb	$C_6H_{15}N$	$C_{22}H_{26}N_4O_3$	394.47	291	65
3	IVc	$C_6H_{15}N$	$C_{21}H_{22}N_4O_3$	378.42	175	68
4	IVd	$C_4H_{10}N_2$	$C_{20}H_{21}N_5O_3$	379.41	321	73
5	IVe	C_4H_9NO	$C_{20}H_{20}N_4O_4$	380.40	223	61
6	IVf	$C_{12}H_{11}N$	$C_{28}H_{22}N_4O_3$	462.50	337	65

Table 2: Antibacterial activity against *Staphylococcus aureus*- (Zone of inhibition in mm)

Compound	50µg/ml (in mm)	100µg/ml (in mm)	500µg/ml (in mm)
IVa	4	7	12
IVb	3	8	14
IVc	5	8	15
IVd	5	10	18
IVe	3	8	14
IVf	4	7	13
Amoxicillin	8	14	22

Table 3: Antibacterial activity against *Micrococcus luteus* (Zone of inhibition in mm)

Compound	50 µg/ml (in mm)	100 µg/ml (in mm)	500 µg/ml (in mm)
IVa	3	7	12
IVb	4	7	15
IVc	4	8	16
IVd	2	6	14
IVe	3	7	16
IVf	2	5	13
Amoxicillin	7	12	18

Table 4: Antibacterial activity against *Escherichia coli* (Zone of inhibition in mm)

Compound	50 µg/ml (in mm)	100 µg/ml (in mm)	500 µg/ml (in mm)
IVa	2	6	15
IVb	2	5	12
IVc	3	7	14
IVd	2	5	11
IVe	4	9	17
IVf	3	7	14
Amoxicillin	7	15	21

Table 5: Antibacterial activity against *Klebsiella pneumoniae*- (Zone of inhibition in mm)

Compound	50µg/ml (in mm)	100µg/ml (in mm)	500µg/ml (in mm)
IVa	4	7	16
IVb	3	6	15
IVc	5	9	19
IVd	2	7	14
IVe	5	10	19
IVf	2	6	17
Amoxicillin	7	12	23

that contained sterile media before they were sterilised. They were chilled in the refrigerator after a 48 h incubation at 37 ℃. It was necessary to maintain stock cultures. Conical flasks were used to produce inoculums by adding culture from a loop to the nutrient broth. This process formed the inoculum. Before beginning the experiment, the flasks were given a 48 h incubation at 37 °C.

Test sample preparation:

In order to get the test compounds ready for use, we dissolved them in methanol at the requisite concentrations, which were 50 g/ml, 100 g/ml, and 500 g/ml. The fluconazole was used as a reference standard for both of the fungi, and the methanol was used as a control. The fluconazole was administered at the same dosages as the test compounds. When looking at the potential effectiveness of test compounds as antifungal agents, the agar well diffusion technique was used. Approximately 25 ml of SDA medium was sterilised by being autoclaved at a temperature of 120 ℃ for 20 min, and then it was poured onto sterile petri dishes to solidify. A sterile borer was used to spread the fungal suspension throughout the surface of the agar plates which resulted in the formation of fungal lawns (6 mm). The control and the test sample concentrations were both added to the SDA medium before the experiment was carried out. After incubating the plates at 37 ℃ for 48 h, the diameter of the zone of inhibition in each well was manually measured, and the results were recorded using a scale that measures in millimetres. There were three separate attempts made to complete the experiment. To determine how the fungal growth was impacted by the solvent, methanol was employed as a control. A scale was used to measure and record the diameter of the zone of inhibition that surrounded each cup (Tables 6, 7).

Antioxidant Activity: Antioxidant features of Isatin derivatives generated N-Mannich bases were explored for their involvement in this study. The idea described by Varma (2000) serves as the foundation for this strategy. Scavenging of 1,1-diphenyl-2-picrylhydrazyl (1,1-diphenyl-2-picrylhydrazyl) radicals is used as a model to evaluate the activity.

Procedure:

Preparation of an ascorbic acid standard solution In order to produce an ascorbic acid stock solution with a concentration of 1 mM, the needed quantity of ascorbic acid was first accurately weighed before being diluted in distilled water. From a stock solution of 1 mM ascorbic acid, solutions of varying concentrations (1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 1 M, 3 M, 10 nM, 30 nM, 100 nM, 300 M) were produced. The concentrations ranged from 1 nM to 1M.

The DPPH solution was prepared by dissolving 19.71 mg of DPPH in 100 ml of methanol which resulted in 0.5 mM of DPPH being produced. In order to prevent DPPH from being oxidised, the solution was stored away from direct sunshine.

Creating a 1mM stock solution required dissolving the necessary quantity of test chemicals in methanol. This resulted in the production of test compounds. A range of concentrations from 1 nM to 1 mM was used to produce the test compounds.

Estimation of the antioxidant activity of ascorbic acid:

The antioxidant activity of ascorbic acid was estimated by adding 0.2 ml of DPPH solution to 2.8 ml of ascorbic acid solution in a test tube wrapped in aluminium foil. The mixture was then allowed to incubate for 30 min at room temperature before the absorbance was measured at 517 nm using a UV-Visible double beam spectrophotometer. This procedure was repeated three times. Following the procedure of plotting the data on a graph, the IC_{50} values were calculated.

Estimation of the Antioxidant Capacity of Test Compounds In order to determine the IC_{50} values of the test compounds, we used a procedure that was exactly the same as the one described under ascorbic acid. The findings are shown in Table 8.

Results and Discussion

Using the agar diffusion technique, each of the

Table 6: Antifungal activity against *Candida albicans*- (Zone of inhibition in mm)

Compound	50 µg/ml (in mm)	100 µg/ml (in mm)	500 µg/ml (in mm)
IVa	2	4	11
IVb	-	3	9
IVc	3	6	14
IVd	2	4	13
IVe	1	4	12
IVf	2	5	11
Fluconazole	5	9	18

Table 7: Antifungal activity against *Aspergillus niger*- (Zone of inhibition in mm)

Compound	50 µg/ml (in mm)	100 µg/ml (in mm)	500 µg/ml (in mm)
IVa	2	5	11
IVb	-	3	7
IVc	1	4	8
IVd	2	6	13
IVe	2	4	9
IVf	1	5	12
Fluconazole	4	10	18

Table 8: Antioxidant activity

S. No.	Compound	IC_{50} (□M)
1	IVa	64.8
2	IVb	91.3
3	IVc	28.1
4	IVd	31.4
5	IVe	26.3
6	IVf	37.2
7	Standard (Ascorbic acid)	6.5

Table 9: Antibacterial activity

Organism	Compound	Zone of inhibition (in mm)
S. aureus	IVd	18
M. luteus	IVc, IVe	16, 16
E. coli	IVe	17
K. pneumonia	IVc, IVd	19, 19

compounds that were created was examined *in vitro* to see whether or not they have antibacterial activity (Table 9). Tables 2 and 3 provide the minimum inhibitory concentrations (MICs) of the chemicals in relation to four different pathogenic bacteria. The activity of the reference chemical has also been included. All of the chemicals that were put to the test showed just a slight to moderate ability to kill the bacteria (Renukadevi and Biradar, 1999). Dissolving each of the test compounds in methanol in order to obtain concentrations of 50 g/ml, 100 g/ml, and 500 g/ml for gram-positive and gram-negative bacteria, respectively, was the method that was used to get all of the test compounds ready for testing (Kanchana *et al*., 2014). Amoxicillin is considered to be the best antibiotic available. The antifungal activity of fluconazole against *Candida albicans* and *Aspergillus niger* was measured against a benchmark of fluconazole's own performance (Sridhar *et al*., 1999). All synthesized compounds has shown high activity for Antibacterial activity when comparing with standard Amoxicillin (21 mm) at 500 µg/ml.

Conclusion

All of the compounds were investigated at concentrations ranging from 1 nM to 1 mM, and the results were compared to those obtained when the standard treatment (ascorbic acid) was administered at the same levels. The half-maximal inhibitory concentrations or IC_{50} values, of all of the test compounds that were synthesised varied from 26.3 to 91.3 microM. Compounds IVc and IVe, both of which are a part of this group, exhibited strong antioxidant activity at concentrations of 26.3 and 28.1 M, respectively, while IVb showed only a very modest level of antioxidant activity at 91.3 M.

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