

2- Materials and Methods

2.1- Materials

2.1.1-Test organisms

The synthesized compounds were screened for antibacterial and antifungal activities using the standard microorganisms shown in table (2.1).

Table 2.1: Test organisms

Ser. No	Micro organism	Type	Source
1	<i>Bacillus subtilus</i>	G+ve	NCTC 2836
2	<i>Staphylococcus aureus</i>	G+ve	ATCC 29213
3	<i>Pseudomonas aeroginosa</i>	G-ve	ATCC 27853
4	<i>Escherichia coli</i>	G-ve	ATCC 25922
5	<i>Aspergillus Niger</i>	Fungi	ATCC 9736
6	<i>Candida albicans</i>	Fungi	ATCC 7596

* NCTC. National collection of type culture, Colindale. England

** ATCC. American type culture collection, Maryland, USA

2.1.2-Chemicals and solvents

Analytical grade reagents (Table 2.2) were used . They were purchased from Sigma – Aldrich company (UK).

Table 2.2: Chemicals and solvents

Ser. No	Chemical
1	Dimedone
2	Dimethylamine
3	Absolute ethanol
4	Diethylamine
5	Methanol
6	Nutrient agar
7	Diethyl ether
8	Ethyl acetate
9	Diethylamine
10	Formalin
11	Byrrolidine
12	N-methylpiperazine
13	Morpholin
14	Chloroform
15	Diphenylamine

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16	Aniline
17	4-aminobenzoic acid
18	Benzamide
19	3-nitrophenol
20	Potassium bromide
22	Piperidine HCl
23	4-chloro-cesol
24	4-aminophenol
25	Ethylenediamine
26	3-nitroaniline
27	Acetylacetone
28	Sulphuric acid

2.2- Methods

2.2.1-Synthesis of Mannich base: 2-dimethylaminomethyl-5,5-dimethylcyclohexane-1,3-dione(1)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of dimedone(4.5g,0.0321mol) and dimethylamine(1.6ml, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 24 hours .Removal of the solvent under reduced pressure gave the product .

2.2.2- Synthesis of the Mannich base: 2-diethylaminomethyl-5,5-dimethylcyclohexane-1,3-dione(2)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of dimedone(4.5g,0.0321mol) and diethylamine(3.3ml, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 24 hours .Removal of the solvent under reduced pressure gave the product .

2.2.3- Synthesis of the Mannich base: 2-pyrrolidinomethyl-5,5-dimethylcyclohexane-1,3-dione(3)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of dimedone(4.5g,0.0321mol) and pyrrolidine(2.6ml, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 36 hours .Removal of the solvent under reduced pressure gave the product .

2.2.4- Synthesis of: 2-morpholinomethyl-5,5-dimethylcyclohexane-1,3-dione(4)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of dimedone(4.5g,0.0321mol) and morpholine(2.8ml, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 24 hours .Removal of the solvent under reduced pressure gave the product .

2.2.5-Synthesis of the Mannich base: 2-piperidinomethyl-5,5-dimethylcyclohexane-1,3-dione(5)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of dimedone(4.5g,0.0321mol) and piperidine HCl(2.7g, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 72 hours .Removal of the solvent under reduced pressure gave the product .

2.2.6-Synthesis of the Mannich base:

2-diphenylaminomethyl-5,5-dimethylcyclohexane-1,3-dione(6)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of dimedone(4.5g,0.0321mol) and diphenylamine(5.42g, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 36 hours .Removal of the solvent under reduced pressure gave the product .

2.2.7-Synthesis of the Mannich base: 2-piperidinomethyl-5-nitrophenol(7)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of 3-nitrophenol(4.465g,0.0321mol) and piperidine HCl(2.7g, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 72 hours .Removal of the solvent under reduced pressure gave the product .

2.2.8- Synthesis of the Mannich base:

2-diphenylaminomethyl-5-nitrophenol(8)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of 3-nitrophenol(4.465g,0.0321mol) and diphenylamine(5.42g, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 36 hours .Removal of the solvent under reduced pressure gave the product .

2.2.9-Synthesis of the Mannich base: 2-piperidinomethyl-4-chloro-5-methylphenol(9)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of 4-chloro-cresol(4.58g,0.0321mol) and piperidine HCl(2.7g, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 24 hours .Removal of the solvent under reduced pressure gave the product .

2.2.10-Synthesis of the Mannich base:

2-diphenylaminomethyl-4-chloro-5-methylphenol(10)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of 4-chloro-cresol(4.58g,0.0321mol) and diphenylamine(5.42g, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 24 hours .Removal of the solvent under reduced pressure gave the product .

2.2.11-Synthesis of the Mannich base:

2-N-methylpiperazinomethyl-5,5-dimethylcyclohexan-1,3-dione(11)

Formaldehyde(0.44ml,0.0161mol)was added dropwise with stirring to a mixture of dimedone(2.25g,0.0161mol) and N-methylpiperazine(1.8ml, 0.0161mol) in absolute ethanol(50ml) at room temperature.The mixture was left for 24 hours. Removal of the solvent under reduced pressure gave the product .

2.2.12-Synthesis of the Mannich base:

3-piperidinomethylpentane-2,4-dione(12)

Formaldehyde(0.89ml,0.0321mol) was added dropwise with stirring to a mixture of acetylacetone(3.29ml,0.0321mol) and piperidine (2.7ml, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 36 hours .Removal of the solvent under reduced pressure to gave the product .

2.2.13-Synthesis of the Mannich base:

2-diphenylaminomethyl-4-aminophenol(13)

Formaldehyde(0.88ml,0.0321mol) was added dropwise with stirring to a mixture of 4-aminophenol(3.5g,0.0321mol) and diphenylamine(5.42g, 0.0321mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 24 hours .Removal of the solvent under reduced pressure gave the product .

2.2.14- Synthesis of the Mannich base:

2-N-methylpiperzinomethyl-5-nitrophenol(14)

Formaldehyde(0.44ml,0.0161mol) was added dropwise with stirring to a mixture of 3-nitrophenol(2.23g,0.0161mol) and N-methylpiperizine (1.8ml, 0.0161mol) in absolute ethanol(50ml) at room temperature.The mixture was left for 72 hours .Removal of the solvent under reduced pressure gave the product .

2.2.15- Synthesis of the Mannich base: 2-morpholinomethyl-5-nitrophenol(15)

Formaldehyde(0.88Cm³,0.032mol) was added dropwise with stirring to a mixture of 3-nitrophenol(4.465g,0.032mol) and morpholine (2.8 Cm³,0.032mol) in absolute ethanol (50ml) at room temperature .The mixture was left for 24hours .Removal of the solvent under reduced pressure gave the product .

2.2.16- Synthesis of the Schiff base:

(E)-2[(dimethylaminomethyl)-5,5-dimethyl-3-(phenylimino)]cyclohexanone(16)

Mannich base I (1.5g,0.0076mol) and aniline (0.7Cm³, 0.0076mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 24hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.17-Synthesis of the Schiff base:

(E)-2-[(diethylaminomethyl)-5,5-dimethyl-3-(phenylimino)]cyclohexanone(17)

Mannich base 2 (2.0g,0.0088mol) and aniline (0.8Cm³, 0.0088mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with

stirring . The mixture was left for 24hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.18- Synthesis of the Schiff base: (E)-5,5-dimethyl-3-(phenylimino)-2-(pyrrolidin-1-yl)cyclohexanone(18)

Mannich base 3 (2.0g,0.00896mol) and aniline (0.83Cm³, 0.00896mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 36 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.19-Synthesis of the Schiff base: (E)-N-[2-(dimethylaminomethyl)-5,5-dimethyl-3-oxocyclohexylidene]benzamide(19)

Mannich base 1 (1.8g,0.009mol) and benzamide (1.1g, 0.009mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 24 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.20- Synthesis of the Schiff base: (E)-N-[2-(diethylaminomethyl)-5,5-dimethyl-3-oxocyclohexylidene]benzamide(20)

Mannich base 2 (2.0g,0.0088mol) and benzamide (1.066g, 0.0088mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 36 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.21- Synthesis of the Schiff base: (E)-N-[5-dimethyl-3-oxo(pyrrolidin-1-methyl)cyclohexylidene]benzamide (21)

Mannich base 3 (2.0g,0.00896mol) and benzamide (1.085g, 0.00896mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 72 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.22-Synthesis of the Schiff base: (E)-4-[2-(dimethylaminomethyl)-5,5-dimethyl-3-oxocyclohexylideneamino]benzoicacid(22)

Mannich base 1 (1.8g,0.009mol) and 4-aminobenzoic acid (1.234g, 0.009mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 36 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.23-Synthesis of the Schiff base: (E)-4-[2-(diethylaminomethyl)-5,5-dimethyl-3-oxocyclohexylideneamino]benzoicacid (23)

Mannich base 2 (2.0g,0.0088mol) and 4-aminobenzoic acid (1.21g, 0.0088mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 24 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.24- Synthesis of the Schiff base:

(E)-4-[5,5-dimethyl-3-oxo-2-(pyrrolidin-1-ylmethyl)cyclohexylideneamino]benzoic acid (24)

Mannich base 3 (2.0g,0.00896mol) and 4-aminobenzoic acid (1.23g, 0.00896mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 24 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.25- Synthesis of the Schiff base: (Z)-5,5-dimethyl-2-(morpholinomethyl)-3-(phenylimino)cyclohexanone (25)

Mannich base 4 (1.5g,0.006mol) and aniline (0.5Cm³, 0.006mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 24 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.26-- Synthesis of the Schiff base: (E)-4-[5,5-dimethyl-2-(morpholinomethyl)]-3-(oxocyclohexylideneamino)benzene sulphonic acid (26)

Mannich base 4 (1.5g,0.006mol) and sulfanilic acid (1.04g, 0.006mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 72 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.27-- Synthesis of the Schiff base: (E)-5,5-dimethyl-3-(phenylimino)-2-(piperidin-1-ylmethyl)cyclohexanone (27)

Mannich base 5 (1.0g,0.004mol) and aniline (0.4Cm³, 0.004mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 24 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

**2.2.28- Synthesis of Schiff base:
(E)-2-(diphenylaminomethyl)-5,5-dimethyl-3-(phenylimino)cyclohexanone(28)**

Mannich base 6 (1.5g,0.005mol) and aniline (0.5Cm³, 0.005mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 48 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product .

2.2.29- Synthesis of Schiff base: (Z)-3-[(2-aminomethylimino)-2-(diphenylaminomethyl)]cyclohexanone(29)

Mannich base 6 (1.5g,0.005mol) and ethylenediamine (0.3Cm³, 0.005mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring . The mixture was left for 24 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product.

2.2.30- Synthesis of Schiff base: (Z)-5,5-dimethyl-2-(morpholinomethyl)-3-(3-nitrophenylimino)cyclohexanone (30)

Mannich base 4 (1.5g,0.006mol) and 3-nitroaniline (0.83g, 0.006mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring. The mixture was left for 36 hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product.

2.2.31-Synthesis of the Schiff base: (Z)-2-(diphenylaminomethyl)-5,5-dimethyl-3-(3-nitrophenylimino)cyclohexanone(31)

Mannich base 6 (1.5g,0.005mol) and 3-nitroaniline (0.83g, 0.005mol) were dissolved in absolute ethanol (50ml). Then 3 drops of concentrated sulphuric acid were added dropwise with stirring. The mixture was left for 24hours with stirring at room temperature. Removal of the solvent under reduced pressure gave the product.

2.2.32-Screening the synthesized compounds for biological activity

2.2.32.1-Preparation of the test organisms

2.2.32.1.1-Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto hutment agar slopes and incubated at 37°C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about 10⁸-10⁴ colony

forming units per ml .The suspension was stored in the refrigerator at 4°C until used. The average number of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volumes (0.02 ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drop to dry, and then incubated at 37°C for 24 hours.

2.2.32.1.2-Preparation of fungal suspension

Fungal cultures were maintained on sabouraud dextrose agar incubated at 25°C for four days.The fungal growth was harvested and washed with sterile normal saline, and the suspension was stored in the refrigerator until used.

2.2.33-Testing for antibacterial activity

The cup-plate agar diffusion method was adopted with some minor modifications, to assess the antibacterial activity of the targeted molecules. 2ml of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45°C in a water bath.

(20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes, the agar was left to settle in each of these plates which were divided into two halves. Two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed for one compound . Separate Petri dishes were designed for standard antibacterial chemotherapeutic (ampicillin and gentamycin).

The agar discs were removed. Cups were filled with 0.1 ml sample of each compound using adjustable volume microtiter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for hours.

The above procedure was repeated for different concentrations of synthesized compounds and the standard antibacterial chemotherapeutics. After incubation, the diameters of the resultant growth inhibition zones were measured.

2.2.34-Testing for antifungal activity

The same method used for bacteria was adopted, but instead of nutrient agar, Sabouraud dextrose agar was used. Samples were used here by the same concentrations used above.