CHAPTER 2

EXPERIMENTAL TECHNIQUES

2.1 PURIFICATION OF SOLVENTS AND REAGENTS

2.1.1 Solvents

Dimethylsulphoxide (DMSO), ethyl acetate, tetrahydrofuran (THF), toluene, chloroform, 1,4-dioxane, methanol, petroleum ether and ethanol were purified according to the standard methods (Vogel’s Text Book 1989) and distilled before use. Anhydrous sodium sulphate was used as the drying agent. All other solvents used were purified by distillation.

2.1.2 Reagents

Para formaldehyde (SRL), chloro acetic acid (Merck), o-phenylenediamine (Merck) were recrystallised from ethanol and dried over vaccum. Benzaldehyde (CDH), acetyl acetone (Merck), carbondisulphide (SRL) and morpholine (Merck) were distilled before use.

2.1.3 Ethyl methyl ketone (EMK)

To EMK (AR, SRL) potassium carbonate was added and was kept overnight. Then EMK solvent was distilled and the fraction boiling at 80 °C was collected and used.
2.1.4 Ethyl acetoacetate (EAA)

The EAA (AR, SRL) was allowed to stand for a day over freshly heated granular calcium chloride in a well-stoppered bottle, then filtered into a dry distilling flask and was then redistilled. Care was taken to maintain all parts of the apparatus, perfectly dry. The fraction boiling between 76-77 °C was collected.

2.2 PREPARATIONS

2.2.1 2,6-Diphenyl-piperidin-4-one [2,6-DPP]

![Chemical Structure of 2,6-DPP]

2,6-Diphenyl-piperidin-4-one (2,6-DPP) was prepared adopting the procedure of Balasubramaniam and Padma (1963). A mixture of acetone (5.8 g, 0.1 mol), benzaldehyde (21.2 g, 0.2 mol), anhydrous ammonium acetate (7.7 g, 0.1 mol) and absolute ethanol (20 mL) were taken in a three necked round bottom flask. A mechanical stirrer was attached to the center neck, a refluxing condenser was attached to the second neck and the third neck was stoppered. The RB flask was placed on a water bath and the contents was just heated to boil and allowed to stand overnight at room temperature. Concentrated hydrochloric acid (8 mL) was then added drop wise with constant stirring and the reaction mixer was stirred in cold condition for 2 h and then at room temperature for 1 h. The precipitated hydrochloride of 2,6-diphenyl-piperidin-4-one was removed by filtration. The hydrochloride obtained was suspended in acetone and treated with strong ammonia solution. The free base obtained after dilution with water was filtered, washed with distilled water and recrystallised from ethanol to get shining white flakes. Yield: 21.52 g (62%), mp. 92 °C. Reported mp. 91-93 °C
2.2.2 3-Methyl-2,6-diphenyl-piperidin-4-one [3-MDPP]

3-Methyl-2,6-diphenyl-piperidin-4-one (3-MDPP) was prepared adopting the procedure of Balasubramaniam and Padma (1963). A mixture of ethyl methyl ketone (7.2 g, 0.1 mol), benzaldehyde (21.2 g, 0.2 mol), anhydrous ammonium acetate (7.7 g, 0.1 mol) and absolute ethanol (20 mL) were taken in a two necked round bottom flask equipped with a mechanical stirrer and Leibeig condenser. The RB flask was placed on a water bath and the contents was just heated to boil and allowed to stand overnight at room temperature. Concentrated hydrochloric acid (8 mL) was then added drop wise with constant stirring and the reaction mixer was stirred in cold condition for 2 h and then at room temperature for 1 h. The precipitated hydrochloride of 3-methyl-2,6-diphenyl-piperidin-4-one was removed by filtration. The hydrochloride obtained was suspended in acetone and treated with strong ammonia solution. The free base was obtained after dilution with water. The product was filtered, washed with distilled water and recrystallised from ethanol to get shining white flakes. Yield: 23.46 g (65%), mp. 97°C. Reported mp. 95-97°C.

2.2.3 (4-Oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid [2,6-OEA]
2,6-Diphenyl-piperidin-4-one (10.04 g, 0.04 mol) and ether (150 mL) were taken in a two necked round bottom flask equipped with a mechanical stirrer and Leibeig condenser. The RB flask was placed in an ice bath to maintain the temperature at 15 °C. Chloro acetic acid (3.6 g, 0.04 mol) in 40 mL of absolute ethanol was added from the dropping funnel and the contents were stirred well. The reaction mixture was heated on a water bath under reflux for 4 h and it was allowed to stand overnight at room temperature. Dry ether (200 mL) was added drop wise to the reaction mixture with constant stirring. The reaction mixture was then placed in an ice bath for 2 h and the hydrochloride obtained was separated by filtration. It was then suspended in 100 mL of acetone and treated with strong solution of ammonia to get crude (4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid. The crude product was then recrystallised in petroleum ether to get yellow crystals. Yield: 8.84 g (71%), mp. 97 °C.

2.2.4 (3-Methyl-4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid [3-MDEA]

3-Methyl-2,6-diphenyl-piperidin-4-one (10.6 g, 0.04 mol) and ether (150 mL) were taken in a three necked round bottom flask. A mechanical stirrer was attached to the center neck, a Leibig condenser was attached to the second neck and a dropping funnel was attached to the third neck. The RB flask was placed in an ice bath to maintain the temperature at 15 °C. Chloro acetic acid (3.6 g, 0.04 mol) in 40 mL of absolute ethanol was
added from the dropping funnel and the contents were stirred well. The reaction mixture was heated on a water bath under reflux for 4 h and it was allowed to stand overnight at room temperature. Dry ether (200 mL) was added drop wise to the reaction mixture with constant stirring. The reaction mixture was then placed in an ice bath for 2 h and the hydrochloride obtained was separated by filtration. It was then suspended in 100 mL of acetone and treated with strong solution of ammonia to get crude (3-methyl-4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid. The crude product was then recrystallised in petroleum ether to get yellow crystals. Yield: 9.48 g (67%), mp. 97 °C.

2.2.5 (4-Hydrazone-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide [2,6-AEH]

(4-Hydrazone-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide (2,6-AEH) was prepared by the following procedure. A solution of (4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid (12.36 g, 0.04 mol) and hydrazine hydrochloric acid (4.00 g, 0.08 mol) in ethanol (100 mL) was taken in a three-necked flask equipped with a refluxing condenser, thermometer and a dropping funnel. The reaction mixture was refluxed over a hot water bath for 3 h with continuous stirring. The contents were cooled and poured into
crushed ice. The precipitate obtained was filtered, washed with water and recrystallised from absolute alcohol. Yield: 13.72 g (70 %), mp. 131-132 °C.

TLC: Rf = 0.62; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4).

Elemental analysis C_{19}H_{23}N_{5}O; M.W. 337 (%): C: 67.49 (Found): 67.63 (Calcd), H: 6.75 (Found): 6.87 (Calcd), N: 20.43 (Found): 20.76 (Calcd).

2.2.6 (4-Hydrazone-3-methyl-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide [3-MAEH]

(4-Hydrazone-3-methyl-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide (3-MAAH) was prepared by the following procedure. In a three-necked flask equipped with a refluxing condenser, thermometer and a dropping funnel, a solution of (3-methyl-4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid (12.92 g, 0.04 mol) and hydrazine hydrochloric acid (4.00 g, 0.08 mol) in ethanol (100 mL) was taken. The reaction mixture was refluxed over a water bath for 3 h with continuous stirring. The contents were cooled and poured into crushed ice. The crude product obtained was filtered, washed with water and recrystallised from absolute alcohol. Yield: 11.48 g (68%), mp. 137 -139 °C.
TLC: Rf = 0.56; Solvent = Benzene; Eluent = Ethyl acetate : pet-ether (1:4).

Elemental analysis C_{20}H_{25}N_{5}O; M.W. 351 (%): C: 68.46 (Found): 68.35 (Calcd), H: 7.36 (Found): 7.17 (Calcd), N: 19.81 (Found): 19.93 (Calcd).

### 2.2.7 (4-Oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide [2,6-OEH]

(4-Oxo-2,6-diphenyl-piperidin-1-yl)-methanoic acid hydrazide (2,6-OEH) was prepared in two steps. In the first step hydrazine - ethanoic acid was prepared. In a three-necked flask equipped with a refluxing condenser, thermometer and a dropping funnel, mixed solution of chloro acidic acid (9.45 g, 1 mol) and hydrazine hydrochloric acid (5 g, 1 mol) in ethanol (200 mL) was taken. The reaction mixture was refluxed on a hot water bath for 3 h with continuous stirring. In the second step (4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide (2,6-OEH) was prepared. To a solution of hydrazino-ethanoic acid (4.4 g, 0.04 mol) taken in a three-necked flask, a solution of 2,6-diphenyl-piperidin-4-one (10.04 g, 0.04 mol) in 200 mL of ether and 40 mL of absolute ethanol taken in a dropping funnel was added dropwise with constant stirring. The temperature was maintained
below 15 °C by keeping the reaction mixture in an ice bath. Then the reaction mixture was heated under reflux condition for 4 h on a hot water bath, and allowed to stand overnight at room temperature. Then dry ether (200 mL) was added and the reaction mixer was stirred in cold condition for 2 h. The residue obtained was filtered, washed with ethanol and recrystallised from petroleum ether. Yield 10.12 g (70%), mp. 85 - 89 °C.

TLC: Rf = 0.91; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4). Elemental analysis C_{19}H_{21}N_{3}O_{2}; M.W. 323 (%): C: 71.79 (Found): 70.57 (Calcd), H: 5.76 (Found): 5.55 (Calcd), N: 10.67 (Found): 12.99 (Calcd).

2.2.8 (3-Methyl-4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide [3-MOEH]

(3-Methyl-4-Oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide (3-MOEH) was prepared in two steps. In the first step hydrazino-ethanoic acid was prepared. In a three-necked flask equipped with a refluxing condenser, thermometer and a dropping funnel, a solution of chloro acidic acid (9.45 g, 1 mol) and hydrazine hydrochloric acid (5 g, 1 mol) in ethanol (200 mL) was taken. The reaction mixture was refluxed over a water bath for
3 hours with continuous stirring. In the second step (3-methyl-4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide (3-MOEH) was prepared. To a solution of hydrazino-ethanoic acid (4.4 g, 0.04 mol) taken in a three-necked flask, a solution of 3-methyl-2,6-diphenyl-piperidin-4-one (10.6 g, 0.04 mol) in 200 mL of ether and 40 mL of absolute ethanol taken in a dropping funnel was added dropwise with constant stirring. The temperature was maintained below 15 °C by keeping the reaction mixture in an ice bath. Then the reaction mixture was heated under reflux condition for 4 h on a hot water bath, and allowed to stand overnight at room temperature. Then dry ether (200 mL) was added and the reaction mixer was stirred in cold condition for 2 h. The residue obtained was filtered, washed with ethanol and recrystallised from petroleum ether. Yield: 10.2 g (68%), mp. 83 -87 °C.

TLC: Rf = 0.78; Solvent = Benzene; Eluent = Ethyl acetate: pet ether (1:4).

Elemental analysis C_{20}H_{23}N_{3}O_{2}; M.W. 337 (%): C: 71.43 (Found): 71.19 (Calcd), H: 6.52 (Found): 6.87 (Calcd), N: 12.54 (Found): 12.45 (Calcd).

**2.2.9 5-(2,6-Diphenyl-4-hydrazono-piperidin-1-ylmethyl)-3H-[1,3,4]oxadiazole-2-thione [2,6-MOT]**

A solution of (4-hydrazono-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide (10.11 g, 0.03 mol) in ethanol (200 mL) was taken along with
a solution of KOH (2.52 g, 0.045 mol) in ethanol (40 mL) and CS$_2$ (40 mL) in
a three necked round bottom flask. A mechanical stirrer was attached to the
center neck, a Leibig condenser was attached to the second neck and the third
neck was stoppered. The reaction mixture was heated under reflux condition
for 8 h on a steam water bath. Then RB flask was placed in an ice bath and
the contents were stirred well. Dilute hydrochloric was added dropwise
through the dropping funnel with constant stirring. The residue obtained was
washed successively with distilled water and finally with little cold alcohol to
remove the unreacted materials. The pure solid product was obtained by
recrystallization in petroleum ether. Yield: 10.26 g (81.3%), mp. 169 -171 °C.

TLC: Rf = 0.84; Solvent = Benzene; Eluent = Ethyl acetate : pet-
ether (1:4).

Elemental analysis C$_{20}$H$_{21}$N$_5$OS; M.W. 378 (%): C: 63.61
(Found): 63.32 (Calcd), H: 5.66 (Found): 5.54 (Calcd), N: 18.45 (Found):
18.46 (Calcd).

2.2.10 5-(2,6-Diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-
3H-[1,3,4]oxadiazole-2-thione [3-MMOT]

A solution of (3-methyl-4-oxo-2,6-diphenyl-piperidin-1-yl)-
ethanoic acid hydrazide (10.53 g, 0.03 mol) in ethanol (200 mL) and a
solution of KOH (2.52 g, 0.045 mol) in ethanol (40 mL) were mixed with CS$_2$
(40 mL) in a two necked round bottom flask. A mechanical stirrer was attached to the center neck and a refluxing condenser was attached to the second neck. The reaction mixture was heated under reflux condition for 8 h on a water bath. Then the RB flask was placed in an ice bath and the contents were stirred well. Then the reaction mixture was treated with dilute hydrochloric acid with constant stirring. The residue obtained was washed successively with distilled water and finally with little cold alcohol to remove the unreacted materials. The product was recrystallized in petroleum ether to get the pure product. Yield: 10.26 g (81.3%), mp. 169 -171 °C.

TLC: Rf = 0.74; Solvent = Benzene; Eluent = Ethyl acetate : pet-ether (1:4).

Elemental analysis C$_{21}$H$_{23}$N$_{5}$OS; M.W. 378 (%): C: 64.24 (Found): 64.12 (Calcd), H: 5.94 (Found): 5.85 (Calcd), N: 17.93 (Found): 17.81 (Calcd).

2.2.11 4-Amino-5-(2,6-diphenyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol [2,6-ATT]

For the preparation of 4-amino-5-(2,6-diphenyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol (2,6-ATT) a mixture of 5-(2,6-diphenyl-4-hydrazono-piperidin-1-ylmethyl)-3H-[1,3,4]oxadiazole-2-thione (11.37 g, 0.03 mol) and 99% hydrazine hydrate (6 mL, 0.09 mol) in absolute ethanol (40 mL) were taken in a two necked round bottom flask. A mechanical stirrer was attached to the center neck and a refluxing condensor was attached to the second neck. The reaction mixture was heated under reflux condition for 6 h on a hot water bath. Then the RB flask was placed in an ice bath and the contents were stirred well. The solvent and the excess hydrazine hydrate were removed using rotary evaporator. The residue obtained was washed with cold ethanol to remove the unreacted materials.
The pure product was obtained by recrystallization in petroleum ether. Yield: 7.44 g (65.5%), mp. 177 -179 °C.

TLC: Rf = 0.62; Solvent = Benzene; Eluent = Ethyl acetate : pet-ether (1:4).

Elemental analysis C_{20}H_{23}N_{7}S; M.W. 393 (%): C: 61.23 (Found): 61.07 (Calcd), H: 5.89 (Found): 5.85 (Calcd), N: 24.73 (Found): 24.94 (Calcd).

2.2.12 4-Amino-5-(2,6-diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol, [3-MATT]

For the preparation of 4-amino-5-(2,6-diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol (3-MATT) a mixture of 5-(2,6-diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-3H-[1,3,4] oxadiazole-2-thione (11.79 g, 0.03 mol) and 99% hydrazine hydrate (6 mL, 0.09 mol) in absolute ethanol (40 mL) were taken in a two necked round bottom flask. A mechanical stirrer was attached to the center neck and a refluxing condensor was attached to the second neck. The reaction mixture was heated under reflux condition for 6 h on a steam water bath. Then the RB flask was placed in an ice bath and the contents were stirred well. The solvent
and the excess hydrazine hydrate were removed using rotary evaporator. The residue obtained was washed with cold ethanol to remove the unreacted materials. The pure product was obtained by recrystallization in petroleum ether. Yield: 7.14 g (60.6%), mp. 142 -144 °C.

TLC: Rf = 0.58; Solvent = Benzene; Eluent = Ethyl acetate : pet-ether (1:4).

Elemental analysis C_{21}H_{25}N_{7}S; M.W. 407 (%): C: 61.79 (Found): 61.91 (Calcd), H: 6.58 (Found): 6.42 (Calcd), N: 24.16 (Found): 24.08 (Calcd).

2.2.13 5-(2,6-Diphenyl-4-hydrazono-piperidin-1-ylmethyl)-3-morpholin-4-ylmethyl-3H-[1,3,4]oxadiazole-2-thione [2,6-MMT]

A solution of paraformaldehyde (4.5 g, 0.005 mol) and morpholine (6.5 g, 0.075 mol) in absolute ethanol (100 mL) was taken in a two necked round bottom flask equipped with a mechanical stirrer and a refluxing condenser. The reaction mixture was heated on a hot water bath under reflux condition until complete solubilization of the paraformaldehyde was observed. Then the RB flask was placed in an ice bath and the contents were stirred well for 1h. A solution of 5-(2,6-diphenyl-4-hydrazono-piperidin-1-ylmethyl)-3H-[1,3,4]oxadiazole-2-thione (7.55 g, 0.02 mol) in absolute
ethanol (100 mL) was heated and then added to the reaction mixture. The contents were then refluxed for 2 h. The residue obtained after filtration was extracted with chloroform and the solution was washed successively with distilled water to remove the unreacted materials. Finally, the chloroform layer was dried over anhydrous sodium sulphate and then it was evaporated using rotary evaporator. The solid product was recrystallised from absolute alcohol to get white crystals of pure 5-(2,6-diphenyl-4-hydrazone-piperidin-1-ylmethyl)-3-morpholin-4-ylmethyl-3H-[1,3,4] oxadiazole-2-thione (2,6-MMT). Yield: 14.05 g (76%), mp. 165 -167 °C.

TLC: Rf = 0.64; Solvent = Benzene; Eluent = Ethyl acetate : pet-ether (1:4).

Elemental analysis C_{25}H_{30}N_{6}O_{2}S; M.W. 478 (%): C: 63.29 (Found): 63.39 (Calcd), H: 6.33 (Found): 6.55 (Calcd), N: 16.91 (Found): 17.06 (Calcd).

2.2.14 5-(2,6-Diphenyl-3-methyl-4-hydrazone-piperidin-1-ylmethyl)-3-morpholin-4-ylmethyl-3H-[1,3,4]oxadiazole-2-thione [3-MMMT]

A solution of paraformaldehyde (4.5 g, 0.005 mol) and morpholine (6.5 g, 0.075 mol) in absolute ethanol (100 mL) was taken in a two necked
round bottom flask fitted with a mechanical stirrer and a refluxing condenser.

The reaction mixture was heated on a hot water bath under reflux until complete solubilization of the paraformaldehyde was observed. Then the RB flask was placed in an ice bath and the contents were stirred well for 1h. A solution of 5-(2,6-diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-3H-[1,3,4] oxadiazole-2-thione (7.55 g, 0.02 mol) in absolute ethanol (100 mL) was heated and then added to the reaction mixture. The contents were then refluxed for 2 h. The residue obtained after filtration was extracted with chloroform and the solution was washed successively with distilled water to remove the unreacted materials. Finally, the chloroform layer was dried over anhydrous sodium sulphate and decanted. The solution was then evaporated using rotary evaporator. The solid product obtained was recrystallised from absolute alcohol to get white crystals of pure 5-(2,6-diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-3-morpholin-4-ylmethyl-3H-[1,3,4]oxadiazole-2-thione (3-MMMT). Yield: 2.78 g (75%), mp. 120 -122 °C.

TLC: Rf = 0.67; Solvent = Benzene; Eluent = Ethyl acetate : pet-ether (1:4).
Elemental analysis $C_{26}H_{32}N_{6}O_{2}S$; M.W. 492 (%): C: 63.22 (Found): 63.39 (Calcd), H: 6.48 (Found): 6.51 (Calcd), N: 16.95 (Found): 17.06 (Calcd).

2.2.15 4-(Benzylidene-amino)-5-(2,6-diphenyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol [2,6-BTT]

A solution of 4-amino-5-(2,6-diphenyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol (11.79, 0.03 mol) in absolute ethanol (50 mL) and benzaldehyde (3.81 g, 0.036 mol) were taken in a two necked round bottom flask. A mechanical stirrer was attached to the center neck and a refluxing condenser was attached to the second neck. The reaction mixture was heated under reflux condition for 4 h on a water bath. Then the RB flask was placed in an ice bath and the contents were stirred well for 30 minutes. The residue obtained after filtration was extracted with chloroform and the solution was washed successively with distilled water to remove the unreacted materials. Finally, the chloroform layer was dried over anhydrous sodium sulphate and decanted. Then it was evaporated using rotary evaporator. The solid product was recrystallised in absolute ethanol to get 4-(benzyldene-amino)-5-(2,6-diphenyl-4-hydrazono-piperidin-1-ylmethy)-4H-[1,2,4]triazole-3-thiol (2,6-BTT). Yield: 11.76 g (75.3%), mp. 163 - 165 °C.
TLC: Rf = 0.48; Solvent = Benzene; Eluent = Ethyl acetate : petrol ether (1:4).

Elemental analysis C\(_{28}\)H\(_{29}\)N\(_{7}\)S; M.W. 495 (%): C: 67.96 (Found): 67.88 (Calcd), H: 5.92 (Found): 6.60 (Calcd), N: 16.89 (Found): 19.80 (Calcd).

2.2.16  4-(Benzylidene-amino)-5-(2,6-diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethy)-4H-[1,2,4]triazole-3-thiol [3-MBTT]

A solution of 4-amino-5-(2,6-diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol (8.14 g, 0.02 mol) in absolute ethanol (50 mL) and benzaldehyde (2.54 g, 0.026 mol) were taken in a two necked round bottom flask fitted with a mechanical stirrer and a refluxing condenser. The reaction mixture was heated under reflux condition for 4 h on a hot water bath. Then the RB flask was placed in an ice bath and the contents were stirred well for 30 minutes. The residue obtained after filtration was extracted with chloroform and the solution was washed successively with distilled water to remove the unreacted materials. Finally, the chloroform layer was dried over anhydrous sodium sulphate and decanted. Then it was evaporated using rotary evaporator. The solid product was recrystallised in absolute ethanol to get 4-(benzylidene-amino)-5-(2,6-diphenyl-3-methyl-4-
hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol (3-MBTT). Yield: 7.02 g (65.7 %), mp. 184 -186 °C.

TLC: Rf = 0.41; Solvent = Benzene; Eluent = Ethyl acetate : pet-ether (1:4).

Elemental analysis C_{29}H_{31}N_{7}S; M.W. 509 (%): C: 68.49 (Found): 68.34 (Calcd), H: 6.17 (Found): 6.13 (Calcd), N: 19.32 (Found): 19.24 (Calcd).

2.2.17 1-[2-(3-Methyl-5-oxo-4,5-dihydro-pyrazol-1-yl)-2-oxo-ethyl]-2,6-diphenyl-piperidin-4-one [2,6-MPO]

![Diagram](image-url)

1-[2-(3-Methyl-5-oxo-4,5-dihydro-pyrazol-1-yl)-2-oxo-ethyl]-2,6-diphenyl-piperidin-4-one (2,6-MPO) was synthesised by heating a mixture of (4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanolic acid hydrazide (9.0 g, 0.028 mol) and ethyl acetoacetate (3.6 g, 0.028 mol) in absolute ethanol (100 mL) in a two necked round bottom flask. A mechanical stirrer was attached to the center neck and a refluxing condenser was attached to the second neck. The reaction mixture was heated under reflux condition for 3 h on a water bath. Then the RB flask was placed in an ice bath and the contents were stirred well. The crude product obtained was filtered off and washed successfully
with water and finally with little cold ethanol. The solid product was recrystallised in absolute ethanol to get white crystals. Yield: 8.8 g (70%), mp. 65 -68 °C.

TLC: Rf = 0.82; Solvent = Benzene; Eluent = Ethyl acetate : pet-ether (1:4).

Elemental analysis C_{23}H_{23}N_{3}O_{3}; M.W. 389 (%): C: 71.04 (Found): 70.93 (Calcd), H: 5.86 (Found): 5.95 (Calcd), N: 10.86 (Found): 10.79 (Calcd).

2.2.18 1-[2-(3-Methyl-5-oxo-4,5-dihydro-pyrazol-1-yl)-2-oxo-ethyl]-2,6-diphenyl-3-methyl-piperidin-4- one [3-MMPO]

1-[2-(3-Methyl-5-oxo-4,5-dihydro-pyrazol-1-yl)-2-oxo-ethyl] -2,6-diphenyl-3-methyl-piperidin-4-one (3-MMPO) was synthesised by heating a mixture of (3-methyl-4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanolic acid hydrazide (9.4 g, 0.028 mol) and ethyl acetoacetate (3.6 g, 0.028 mol) in absolute ethanol (100 mL) in a two necked round bottom flask. A mechanical stirrer was attached to the center neck and a refluxing condenser was attached to the second neck. The reaction mixture was heated under reflux condition for 3 h on a water bath. Then the RB flask was placed in an ice bath and the
contents were stirred well. The crude product obtained was filtered off and washed successfully with water and finally with little cold ethanol. The crude product was recrystallised in absolute ethanol to get white crystals. Yield: 9.6 g (74%), mp. 77-79 °C.

TLC: Rf = 0.84; Solvent = Benzene; Eluent = Ethyl acetate: petrol ether (1:4).


2.2.19 1-[2-(3,5-Dimethyl-pyrazol-1-yl)-2-oxo-ethyl]-2,6-diphenylpiperidin-4-one [2,6-EDP]

A solution of (4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanolic acid hydrazide (9.0 g, 0.028 mol) and acetylacetone (2.8 g, 0.028 mol) in absolute ethanol (100 mL) was taken in a round bottom flask fitted with Liebig condenser. The flask was kept in an oil bath which was placed over a magnetic stirrer. The solution was stirred under reflux condition for 3 h. Then the oil bath was removed and the RB flask was placed in an ice bath. The contents were stirred well for 30 minutes. The product obtained was filtered off and washed with water and finally with cold ethanol. The solid product was recrystallised in
petroleum ether to get pure 1-[2-(3,5-dimethyl-pyrazol-1-yl)-2-oxo-ethyl]-2,6-diphenyl-piperidin-4-one (2,6-EDP). Yield: 8.3 g (71%), mp. 72-75°C.

TLC: Rf = 0.66; Solvent = Benzene; Eluent = Ethyl acetate : petroleum ether (1:4).

Elemental analysis C_{24}H_{25}N_{3}O_{2}; M.W. 389 (%): C: 74.25 (Found): 74.39 (Calcd), H: 6.65 (Found): 6.50 (Calcd), N: 10.86 (Found): 10.84 (Calcd).

2.2.20 1-[2-(3,5-Dimethyl-pyrazol-1-yl)-2-oxo-ethyl]-2,6-diphenyl-3-methyl-piperidin-4-one [3-MEDP]

A solution of (3-methyl-4-oxo-2,6-diphenyl-piperidin-1-yl)-ethanoic acid hydrazide (9.4 g, 0.028 mol) and acetyl acetone (2.8 g, 0.028 mol) in absolute ethanol (100 mL) was taken in a round bottom flask fitted with a Liebig condenser. The flask was kept in an oil bath which was placed over a magnetic stirrer. The solution was stirred under reflux condition for 3 h. Then the oil bath was removed and the RB flask was placed in an ice bath. The contents were stirred well for 30 minutes. The product obtained was filtered off and washed with water and then with cold ethanol. The solid product was recrystallised in petroleum ether to get pure 1-[2-(3,5-dimethyl-pyrazol-1-yl)-2-oxo-ethyl]-2,6-diphenyl-3-methyl-piperidin-4-one (3-MEDP). Yield: 9.2 g (75%), mp. 82-87°C.
TLC: Rf = 0.91; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4).

Elemental analysis C_{25}H_{27}N_{3}O_{2}; M.W. 401 (%): C: 74.64 (Found): 74.79 (Calcd), H: 6.96 (Found): 6.78 (Calcd), N: 10.36 (Found): 10.47 (Calcd).

### 2.2.21 2,6-Diphenyl–piperidin-4–semicarbazone [2,6-DPS]

A solution of semicarbazide hydrochloride (5.5 g, 0.05 mol) and 2,6-diphenyl-piperidin-4-one (12.0 g, 0.05 mol) in ethanol (100 mL) was placed in a RB flask fitted with a Leibig condenser. The solution was stirred well using a magnetic stirrer. The RB flask was then placed over an oil bath and was heated under reflux condition for 3 h. Then the RB flask was placed in an ice bath and the contents were stirred well. The precipitate obtained was filtered off and washed with water and then with cold ethanol. The resulting solid was washed with water and recrystallised from absolute ethanol to get pure 2,6-diphenyl-piperidin-4–semicarbazone (2,6-DPS). Yield: 10.5 g (60%), mp. 89 °C.

TLC: Rf = 0.66; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4).
Elemental analysis (%): C: 70.54 (Found): 70.58 (Calcd.), H: 5.81 (Found): 5.88 (Calcd), N: 18.11 (Found): 18.30 (Calcd).

2.2.22 3-Methyl-2,6-diphenyl–piperidin-4-semicarbazone [3-MDPS]

A solution of semicarbazide hydrochloride (5.5 g, 0.05 mol) and 3-methyl-2,6-diphenyl-piperidin-4-one (13.15 g, 0.05 mol) in ethanol (100 mL) was placed in a RB flask fitted with a Leibig condenser. The solution was stirred well using a magnetic stirrer. The RB flask was then placed over an oil bath and was heated under reflux condition for 3 h. Then the RB flask was placed in an ice bath and the contents were stirred well. The precipitate obtained was filtered off and washed with cold water and then with cold ethanol. The resulting solid was recrystallised from absolute ethanol to get pure 3-methyl-2,6-diphenyl–piperidin-4-semicarbazone (3-MDPS). Yield: 13.61 g (73%), mp. 91 °C.

TLC: Rf = 0.68; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4).

Elemental analysis (%): C: 70.10 (Found): 70.25 (Calcd.), H: 6.21 (Found): 6.25 (Calcd), N: 17.11 (Found): 17.50 (Calcd).
2.2.23  4,6-Diphenyl-4,5,6,7-tetrahydro-3-selena-1,2,5-triazo-indene [STI]

A solution of 2,6-diphenyl-piperidin-4-semicarbazone (10 g) in dioxane (10 mL) and an aqueous solution of selenium dioxide (2.5 g in 4.0 mL water) were taken in a three necked round bottom flask equipped with a mechanical stirrer, thermometer and a dropping funnel. The reaction mixture was kept under continuous stirring at room temperature for 3 h. The precipitate obtained was filtered off and washed with cold water and then with cold ethanol. Then the resulting solid was recrystallised from ethanol to get pure 4,6-diphenyl-4,5,6,7-tetrahydro-3-selena-1,2,5-triazo-indene (STI). Yield: 7.5 g (60%), mp. 105 °C.

TLC: Rf = 0. 82; Solvent =Benzene; Eluent = Ethyl acetate: pet-ether (1:4).

Elemental analysis (%): C: 59.87 (Found): 60.00 (Calcd.), H: 4.39 (Found): 4.41 (Calcd), N: 12.30 (Found): 12.35 (Calcd).

2.2.24  4,6-Diphenyl-7-methyl-4,5,6,7-tetrahydro-3-selena-1,2,5-triazo-indene [MSTI]

A solution of 3-methyl-2,6-diphenyl-piperidin-4-semicarbazone (10 g) in dioxane (10 mL) and an aqueous solution of selenium dioxide (2.5 g
in 4.0 mL water) were taken in a three necked round bottom flask equipped with a mechanical stirrer, thermometer and a dropping funnel. The reaction mixture was kept under continuous stirring at room temperature for 3 h. The precipitate obtained was filtered off and washed with cold water and then finally with cold ethanol. Then the resulting solid was recrystallised from ethanol to get pure 4,6-diphenyl-7-methyl-4,5,6,7-tetrahydro-3-selena-1,2,5-triazolo-indene (MSTI). Yield: 8.05 g (65 %), mp. 92°C.

TLC: Rf = 0.78; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4).

Elemental analysis (%): C: 60.91 (Found): 61.02 (Calcd.), H: 4.71 (Found): 4.80 Calcd), N: 11.81 (Found): 11.86 (Calcd).

2.2.25 2-Chloromethyl benzimidazole [2-CB]

2-Chloromethyl benzimidazole (2-CB) was prepared by adopting the method of Bloom and Day (1939). Chloroacetic acid (14.2 g, 0.15 mol), o-phenylenediamine (10.8 g, 0.1 mol) and 4N acetic acid (100 mL) were taken in a two necked round bottom flask fitted with a mechanical stirrer, refluxing condensor. The reaction mixture was heated under reflux condition for 45 minutes on a water bath with constant stirring. Then the RB flask was
placed in an ice bath and the contents were stirred well. The reaction mixture was allowed to stand overnight and diluted with 200 mL of cold water. Then ammonium hydroxide solution (6N) was added carefully dropwise with constant stirring for neutralization. The residue obtained was filtrated, extracted with chloroform and the solution was washed successively with cold water to remove the unreacted materials. Finally, the chloroform layer was dried over anhydrous sodium sulphate and then, it was evaporated using a rotary evaporator. The solid product was recrystallized in dioxane. Yield: 16.8 g (67%), mp. 165-166 °C. Reported mp. 165 °C.

2.2.26 1-(1H-Benzimidazol-2-ylmethyl)-2,6-diphenyl-piperidin-4-one [BMD]

A solution of 2,6-diphenyl-piperidin-4-one (9.96 g, 0.04 mol) in 100 mL of ether and 2-chloromethyl benzimidazole (6.68 g, 0.04 mol) in 40 mL of absolute ethanol were taken in a two necked round bottom flask. A mechanical stirrer was attached to the center neck and a Leibig condensor was attached to the second neck. The RB flask was placed in an ice bath and the contents were stirred well to maintain the temperature below 15 °C. The reaction mixture was then heated under reflux condition for 4 h on a water bath and allowed to stand overnight at room temperature. Dry ether (100 mL) was added to the reaction mixture and it was placed in an ice bath for 2 h. Then the reaction mixture was neutralized with strong ammonia and filtered. The residue obtained was thoroughly washed with water and then with little
cold ethanol. The solid product was recrystallized in methanol-ethyl acetate mixture (1:1) to get pure 1-(1H-benzoimidazol-2-ylmethyl)-2,6-diphenylpiperidin-4-one (BMD). Yield: 9.0 g (67%), mp. 135 -136 ºC.

TLC: Rf = 0.56; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4).

Elemental analysis C_{25}H_{23}N_{3}O; M.W. 381 (%): C: 78.62 (Found): 78.74 (Calcd), H: 6.46 (Found): 6.51 (Calcd), N: 11.35 (Found): 11.02 (Calcd).

2.2.27 1-(1H-Benzimidazol-2-ylmethyl)-2,6-diphenyl-3-methylpiperidin-4-one [MBMD]

A solution of 3–methyl-2,6–diphenyl-piperidin-4-one (10.6 g, 0.04 mol) in 100 mL of ether and 2-chloromethyl benzimidazole (6.68 g, 0.04 mol) in 40 mL of absolute ethanol were taken in a two necked round bottom flask equipped with a mechanical stirrer and a Leibig condensor. The RB flask was placed in an ice bath and the contents were stirred well to maintain the temperature below 15 ºC. The reaction mixture was then heated under reflux condition for 4 h on a water bath and allowed to stand overnight at room temperature. Dry ether (100 mL) was added to the reaction mixture and it was placed in an ice bath for 2 h. Then the reaction mixture was neutralized with strong ammonia and filtered. The residue obtained was
thoroughly washed with water and then with little cold ethanol. The solid product was recrystallized in methanol-ethyl acetate mixture (1:1) to get pure 1-(1H-benzoimidazol-2-ylmethyl)-2,6-diphenyl-3-methyl-piperidin-4-one (MBMD) Yield: 9.8 g (70%), mp. 131 -132 °C.

TLC: Rf = 0.62; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4).

Elemental analysis C_{26}H_{25}N_{3}O; M.W. 395 (%): C: 78.84 (Found): 78.98 (Calcd), H: 6.20 (Found): 6.32 (Calcd), N: 10.30 (Found): 10.63 (Calcd).

2.2.28 [1-(1H-Benzimidazol-2-ylmethyl)-2,6-diphenyl-piperidin-4-ylidine]-hydrazine [BDH]

A solution of 1-(1H-benzoimidazol-2-ylmethyl)-2,6-diphenyl-piperidin-4-one (7.6 g, 0.02 mol) and hydrazine hydrate (1.0 g, 0.02 mol) in ethanol (50 mL) was taken in a two necked round bottom flask. A mechanical stirrer was attached to the center neck and a Leibig condenser was attached to the second neck. The reactant mixture was then heated under reflux for 3 h on a water bath with continuous stirring. The crude product obtained was filtered, washed with cold water and recrystallised from absolute ethanol to
get pure \[1-(1\text{-H}-\text{benzoimidazol-2-ylmethyl})-2,6\text{-diphenyl-piperidin-4-ylidine}\]-hydrazine (BDH). Yield: 5.4 g (62%), mp 128 -131°C.

TLC: Rf = 0.62; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4).

Elemental analysis (%): C: 75.9. (Found): 75.94 (Calcd.), H: 6.31 (Found): 6.32 (Calcd), N: 17.69 (Found): 17.72 (Calcd).

2.2.29 \[1-(1\text{-H}-\text{benzoimidazol-2-ylmethyl})-2,6\text{-diphenyl-3-methyl-piperidin-4-ylidine}\]-hydrazine [MBDH]

A solution of \(1\text{-}(1\text{-H}(\text{benzimidazol-2-ylmethyl})\text{-3-methyl-2,6-diphenyl piperidin-4-one (7.95 g, 0.02 mol) and hydrazine hydrate (1.0 g, 0.02 mol)}}\) in ethanol (50 mL) was taken in a two necked round bottom flask fitted with a mechanical stirrer and a Leibeg condenser. The reaction mixture was then heated on a water bath with continuous stirring under reflux condition for 3 h. The product obtained was filtered, washed with cold water and recrystallised from absolute ethanol to get pure \[1-(1\text{-H-benzoimidazol-2-ylmethyl})-2,6\text{-diphenyl-3-methyl-piperidin-4-ylidine}\]-hydrazine (MBDH). Yield: 5.6 g (64%), mp. 100°C.
TLC: Rf = 0.66; Solvent = Benzene; Eluent = Ethyl acetate: pet-ether (1:4).

Elemental analysis (%): C: 76.20 (Found): 76.28 (Calcd.), H: 6.53 (Found): 6.60 (Calcd), N: 17.07 (Found): 17.11 (Calcd).

2.3 ANALYTICAL TECHNIQUES

2.3.1 Elemental Analysis

Micro elemental analysis of the synthesised compounds was performed with Perkin-Elmer 240C - CHN Elemental Analyzer.

2.3.2 Melting Point Analysis

All the melting points were taken in open capillaries on a Gallenkamp apparatus.

2.3.3 Infrared Spectra

The FT-IR spectra of the synthesised compounds were recorded in Nicolet Avator 60 FI-IR spectrophotometer using KBr pellets.

2.3.4 $^1$H-NMR Spectra

$^1$H-NMR spectra of the synthesised compounds were run on Hitachi 400 MHZ NMR spectrometer. The spectra were recorded at room temperature as 15-20 % (w/v) solution in CDCl$_3$. Tetramethylsilane (TMS) was used as the internal reference.
2.3.5 \textbf{\textsuperscript{13}C-NMR Spectra}

\textsuperscript{13}C-NMR spectra were recorded on Bruker DRX 125.77 MHz FT-NMR spectrometer. Samples were examined using 10-15 \% (w/v) solutions of the compounds in DMSO using tetramethylsilane (TMS) as the internal standard.

2.3.6 \textbf{Mass Bauer Spectra}

Mass Bauer spectra were recorded on MALDI Quadrupole ion trap time of flight mass analyser system. The spectrometer was calibrated using a N.B.S. Standard absorber: Na\textsubscript{2}Fe(CN)\textsubscript{5}NO\textsubscript{.}2H\textsubscript{2}O (\(\Delta = 1.172\) mm, sec\textsuperscript{-1}, \(\delta\) (\textsuperscript{57}Co/Pd) = -0.442 mm sec\textsuperscript{-1}). Na\textsubscript{2}Fe(CN)\textsubscript{5}NO\textsubscript{.}2H\textsubscript{2}O absorbers were maintained at 80 \(\pm\) 1 \textdegree K and \textsuperscript{57}Co/Pd source at room temperature.

2.3.7 \textbf{Thin Layer Chromatography}

Thin layer chromatography was performed using glass plates coated with silica gel (ACME sample) of 0.25 mm thickness. Spots were visualized using iodine chamber and ultra violet light chamber.

2.4 \textbf{PHARMACOLOGICAL SCREENING}

Pharmacological screening implies the evaluation of multiple samples adopting standardised techniques in order to have reproducibility of results. This screening is also associated with a real risk of missing unexpected unique activities because of experimental bias. Since a battery of tests is proportionately more expensive and time consuming, emphasis in recent years has been placed upon the use of a single standardised, multipurpose and discriminating procedure for initial screening. Several such
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reports presently exist. If unknown drugs are to be evaluated, the ideal requirements for preliminary screening in the order of importance are:

1. Extrapolation of results must be possible to human speculate either directly or by analogy with clinically effective drugs which should have been screened by the same procedure.

2. Potentially useful pharmacological activity must be detectable even when the activity is either unrevealing or unique.

3. The probable nature of the activity should be revealed so that subsequent exploration can be organised in a suitable manner to establish the action beyond doubt.

4. The procedure must be unbiased and allow for the coding of all samples including both reference materials and unknown test compounds.

5. Results obtained should be reproducible if the experiments are repeated at any period of time.

6. The screening method should detect both rapid onset and delayed onset of activity.

7. The procedure should be a multidose response experiment.

8. The procedure for setting up the experiment and collecting data should be standardised so as to allow comparison with known pure drugs or compounds.

9. The drug’s efficacy should be shown by the least quantity required/kg of body weight.

10. Potential toxicity should be expressed by the procedure so that subsequent research does not ignore this aspect.
11. In case of insoluble medicaments the solvent or vehicle should be selected in such a way that it does not affect the screening results both qualitatively and quantitatively.

12. The screening should be done on unanaesthetised test animals so that all the body systems are exposed to the compound tested similar to clinical situations.

13. The test animals should be readily obtainable, easily handled, bred and should be resistant to infections.

2.4.1 The use of multiple techniques – multi results

In this approach a variety of procedures are used to detect specific activity as well as multiple activity by Throp (1951) and Hooper and Leonard (1965). A compound is tested for its pharmacological effects on isolated organ preparations such as guinea pig ileum, rat uterus, rat hind limb, frog rectus and abdominal muscles. These tests clearly indicate the exact and specific actions of the test compounds. By employing the above mentioned pharmacological screening procedures one can obtain reasonably a good amount of data to understand the type of activity of the compound. It is customary to test the new compounds primarily in smaller animals such as mice or rats and depending upon the results observed on them, multidimensional secondary screening is done using dogs or cats. If the results in the secondary screening are satisfactory and encouraging, then complete screening is undertaken for understanding the entire spectrum of pharmacological activities of the new compound.
2.5 COMPOUNDS FOR SCREENING

The following compounds were synthesised and analysed chemically and subjected to preliminary pharmacological screening. The following abbreviations are used in the screening of the compounds.

1. 5-(2,6-Diphenyl-4-hydrazono-piperidin-1-ylmethyl)-3H-[1,3,4]oxadiazole-2-thione [2,6-MOT]
2. 5-(2,6-Diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-3H-[1,3,4]oxadiazole-2-thione [3-MMOT]
3. 4-Amino-5-(2,6-diphenyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol [2,6-ATT]
4. 4-Amino-5-(2,6-diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol, [3-MATT]
5. 5-(2,6-Diphenyl-4-hydrazono-piperidin-1-ylmethyl)-3-morpholin-4-ylmethyl-3H-[1,3,4]oxadiazole-2-thione [2,6-MMT]
6. 5-(2,6-Diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-3-morpholin-4-ylmethyl-3H-[1,3,4]oxadiazole-2-thione [3-MMMT]
7. 4-(Benzylidene-amino)-5-(2,6-diphenyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol [2,6-BTT]
8. 4-(Benzylidene-amino)-5-(2,6-diphenyl-3-methyl-4-hydrazono-piperidin-1-ylmethyl)-4H-[1,2,4]triazole-3-thiol [3-MBTT]
9. 1-[2-(3-Methyl-5-oxo-4,5-dihydro-pyrazol-1-yl)-2-oxo-ethyl]-2,6-diphenyl-piperidin-4-one [2,6-MPO]
DMSO was used as a vehicle for the administration of solubility and it is the third best solvent next to alcohol and chloroform. Alcohol and chloroform are not used as vehicles due to their own adverse effects. Moreover, DMSO is not highly toxic and is well tolerated by animals at reasonable concentrations.

2.6 EXPERIMENTAL ANIMALS

The test compounds and the standard drugs were administered in the form of a suspension (1% carboxymethyl cellulose as vehicle). Inbred Wistar Albino mice (20 – 30 g) was used for the tests. They were kept in colony cages at 25 ± 2 °C, relative humidity 45 - 55% under 12 h light and dark cycles. The animals were fed with standard animal feed and water ad libitum. All the animals were acclimatized for a week before use.
2.7 **PRELIMINARY SCREENING**

The following studies were undertaken to ascertain the possible pharmacological activities of these compounds.

1. Anti bacterial studies using Well diffusion method
2. Anti fungal studies using Poison plate method
3. Local anaesthetic studies using Nerve block method
4. Analgesic studies using Tail clip method
5. Anti-inflammatory studies using Carrageenin induced hind paw Oedema in mice method
6. Anticancer activity using Fibrosarcoma -20

### 2.7.1 Antimicrobial drugs and test compounds

Two standard antibacterial agents Amoxicillin (Am) and Streptomycin (S) were taken in a disc for comparative studies. The disc concentration levels were under National Committee for Clinical Laboratory Standards (NCCLS) column. The antibacterial disc were obtained from Hi-Media Laboratories Pvt. Limited, Mumbai ‘86, India.

#### 2.7.1.1 Stock solution preparation of test compounds

Stock solutions were prepared by dissolving 10 mg of each test compound in 10 mL of 100% dimethyl sulfoxide (DMSO). From the stock solution different measurements such as 50 µL, 100 µL, 150 µL and 200 µL were taken and diluted so that the sample contains 41.6 µg, 83.3 µg, 124.9 µg and 166.5 µg, respectively. These diluted drugs were immediately dispensed into each agar wells of culture inoculated plates using sterilized micropipette.
2.7.1.2 Clinical test microorganisms

The most clinically important multi drug resistant organisms were isolated, identified by standard methods and stored in the Department of Clinical Medical Microbiology, Apollo Main Hospital (AMH), Greams Lane, Chennai – 06, Tamil Nadu, India. From the Department of Culture collection, clinically important ten bacterial pathogens were obtained and they were classified as follows: Two gram positive Bacillus subtilis and Staphylcoccus aureus, and eight gram negative Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi ‘A’, Salmonella paratyphi ‘H’, Klebsiella pneumoniae, Proteus mirabilis, Proteus vulgaris, Escherichia coli strains were used. Bacterial isolates were stored frozen in skim milk containing 50% glycerol at 70 °C. Stored clinical isolate cultures were revived in the Brain heart infusion broth incubated at 35 °C for 24 h and then subcultured onto BHIA medium. All strains were recent clinical isolates collected between 2006 and 2007.

2.7.1.3 Inoculum preparation

The inoculum was prepared using gram positive and gram negative pathogens from 24 h old cultures on BHIA. With a sterile loop, the top portions of four to five colonies were transferred to a tube containing 5 mL of Mueller Hinton broth or Brain Heart infusion broth. The tube was incubated at 35 °C for 24 h. The turbidity of the culture suspension was adjusted with broth or a sterile saline solution (0.85 – 0.9%). The density of these cultures were adjusted with spectrophotometer at 660 nm to turbidity equivalent to a 0.5 McFarland standard and the final bacterial inoculum size of approximately of $5 \times 10^5$ CFU/mL.
2.7.1.4 Testing culture media

Mueller Hinton Agar (MHA) Casein acid hydrolysate: 17.5 g; Beaf Heart infusion: 2 g; Starch soluble 1.5 g; (pH 7.3 ± 0.2) Agar 17 g; H₂O 1000 mL). The medium was mixed well, heated in a water bath to dissolve the medium completely and autoclaved at 15 lbs pressure (121 °C) for 15 minutes. After removal from the autoclave the sterilized medium was immediately cooled in a 50-55 °C-water bath. The cooled medium was poured into sterile petri plates to a uniform depth of 4 mm; this is equivalent to approximately 20 mL in a 90 mm plate.

2.8 ANTIBACTERIAL SUSCEPTIBILITY TESTS

2.8.1 Well diffusion method

The in vitro activities of the test compounds were determined by the well diffusion, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS). The well diffusion test method has been used in earlier days by Anand Manoharan et al (2003). Later few modifications were carried out by Jenkins (2006) and they compared the well diffusion method with NCCLS broth micro-dilution method using several antifungal drugs. The well diffusion test was performed using Mueller Hinton Agar and Casitone agar. 20 mL of the nutrient agar medium was poured into the sterile Petri dishes. To the solidified plates, wells of 10 mm diameter were made using a sterile cork borer. The 24 h subcultured bacteria were inoculated in the nutrient broth medium. After inoculating, the different concentrations of the compounds were dissolved separately with the DMSO solvent and poured in to the wells with varying concentrations ranging from 62.5 µg/100 µL, 125 µg/100 µL, 250 µg/100 µL, and 500 µg/100 µL using a micropipette. The plates were left over for 25 h at 37 °C. The antibiotic Streptomycin was used as a standard for comparative study.
The percentage of inhibition was calculated by the formula,

\[
\% \text{ Inhibition} = \frac{I (\text{Diameter of the inhibition zone}) \times 100}{90 (\text{Diameter of the Petri-dish in mm})}
\]

2.9 ANTIMICROBIAL ACTIVITY TESTING

2.9.1 Poison plate method

All the compounds were dissolved separately in DMSO solvent for screening of their antifungal activity. The compounds were poured into the sterile Petri-dishes at varying concentrations ranging from 62.5 µg/100 µL, 125 µg/100 µL, 250 µg/100 µL, and 500 µg/100 µL using a pipette. 20 mL of the sterilized Sabourud's agar medium, was poured into each Petri-dish. After solidifying the medium, the fungal mycelia, 8 mm in diameter, was plunged from the fresh culture plate and inoculated in the center of the plate. The solvent DMSO was used as a standard for comparative study. The plate with the solvent and the mycelia was kept as the control. The plates were incubated at room temperature and after 21 days, the growth of the mycelia was measured. The percentage of inhibition was calculated by the following formula.

\[
\text{Diameter of the inhibition zone} - \frac{\text{Total diameter of the plate} \times (I - 90) \times 100}{\text{Total diameter of the plate in mm}}
\]

2.10 LOCAL ANAESTHETIC ACTIVITY

2.10.1 Nerve block anaesthesia method

The local anaesthetic activity was determined by nerve block anaesthesia in frogs (n = 6). The animal was decerebrated and the upper part of the spinal cord was destroyed with a pithing needle. The abdomen was cut
open and all the abdominal organs were removed and a pouch (sac) was made 
to expose the spinal nerves. The animal was fixed on a frog board with two of 
its hind legs hanging free from the board. The right leg was immersed in a 
beaker containing 0.1 N hydrochloric acid and the time taken for absence of 
withdrawal was noted. The same procedure was repeated for the left leg also. 
The sac was filled with 2 mL of the test compounds at 0.5, 1, 2 \% \text{ w/v} 
(suspended in 1 \% carboxymethyl cellulose) and the absence of withdrawal 
reflex was observed at an interval of 30 seconds. The leg was washed with 
normal saline between exposures to acid. Lignocaine (0.5, 1, 2\% \text{ w/v}) was 
taken as the standard drug for comparison.

2.11 ANALGESIC STUDIES

2.11.1 Tail clip method

Analgesic studies were done by the tail clip method (Usha et al 1972). Albino rats weighing 80 g -100 g were used for the studies. Food and 
water were withdrawn for 24 h, prior to drug administration. All the rats were 
screened by applying a tail clip (a bull-dog clamp, arms of which were 
enclosed in a rubber tube) to the base of the tail. Those animals that did not 
commence continuous efforts to remove the clip within 15 seconds were 
rejected. The rats showing positive response were selected and divided into 
six groups of six rats each. The tail clip was applied to the base of the tail of 
the animal and observations were made till 120 minutes after the 
administration of the test compound. Time taken by the animals to remove the 
clip from its tail was noted as reaction time. Prolongation of the reaction time 
with respect to control gives the analgesic effect.
2.12 ANTI-INFLAMMATORY STUDIES

2.12.1 Carrageenin induced hind paw oedema in mice method

The following methods were adopted for the evaluation of the anti-inflammatory studies.


The anti-inflammatory activities of the compounds were studied by the method of Carrageenin induced hind paw oedema in mice. The animals were divided into different groups of six animals each. Mice were treated with different doses of the compounds and 30 minutes later 0.03 mL of Carrageenin in normal saline was injected into the right hind paw. The animals were sacrificed 4 h after Carrageenin administration. The hind paw was cut at the ankle joint level and weighed. The percentage of inhibition of hind paw oedema was calculated using control and compared with the standard phenylbutazone administered animals. The phenylbutazone was used as a standard for anti- Ekta Bansal et al 2001). The animals were tested with 50 mg/Kg and 100 mg/Kg of the test compounds. These compounds were dissolved in 2 mL DMSO. At this dose level, DMSO was found to have no anti-inflammatory activity of its own.
2.13 ANTICANCER ACTIVITY

2.13.1 Fibrosarcoma- 20 method

Fibrosarcoma is the common sarcoma of connective tissue. Fibrosarcoma may arise in the extremities, trunk, head and neck. They arise from subcutaneous fibrous tissues, from deep connective tissues around tenden sheaths or from other areas where connective tissues are found.

In the present study methylcholanthrene induced fibrosarcoma cells were used. The experimental rats were induced with fibrosarcoma according to the method of Asokan et al (1993). Methylcholanthrene induced fibrosarcoma was maintained in albino rats by several implantations. Minced tumour cell in 0.2 mL suspension in physiological saline was injected through a puncture in inquinal region. The transplanted tumour took about a week to become palpable. After 20 days of induction of fibrosarcoma, the rats were divided into different groups of six animals each and the efficacy of the compounds was tested.

The first group served as control, which contains 6 animals of fibrosarcoma-induced rats. Second group served as a negative control, which received 0.2 mL of DMSO for 20 days. The synthesised compounds were dissolved in 0.2 mL of DMSO and given at a dose level of 10 mg/Kg body weight orally to the third and fourth groups of rats respectively for 20 days. Fifth, sixth, seventh and eighth groups of animals received 20 mg/Kg body weight of the synthesised compounds for 20 days. The animals were bleeding through the jugular vein to death at the end of 20th day and the reduction in the size of the fibrosarcoma tumour was measured.