3. EXPERIMENTAL

3.1. PREPARATION OF COMPOUNDS

All chemicals and solvents used were of AnalaR grade. The melting points were taken in open capillaries in an electrical apparatus and are uncorrected.

3.1.1. Synthesis of 3-alkyl/3,5-dialkyl-2r,6c-diarylpiperidin-4-ones and their oximes

All the substituted piperidin-4-ones and their oximes were synthesized according to literature procedures [1] and [157].

3.1.2. Synthesis of 3t-Pentyl-2r,6c-diarylpiperidin-4-one thiosemicarbazones (100-106)

General procedure

To a boiling solution of the respective 3t-pentyl-2r,6c-diarylpiperidin-4-one (0.01 mol) in 45 ml methanol, a few drops of conc. HCl were added. There-after, thiosemicarbazide (previously dissolved in 20 ml methanol) solution (0.01 mol) was added dropwise with stirring. The reaction mixture was refluxed for 3 h on a heating mantle. After cooling, the solid product obtained was filtered off and recrystallised from methanol. The dried compound was purified by column chromatography using neutral alumina; benzene:ethyl acetate mixture (4.8:0.2) as eluting solvent. The above procedure was adopted to prepare the following compounds.
3\textit{t}-pentyl-2\textit{r},6\textit{c}-diphenylpiperidin-4-one thiosemicarbazone (100)

3\textit{t}-pentyl-2\textit{r},6\textit{c}-di(4-fluorophenyl)piperidin-4-one thiosemicarbazone (101)

3\textit{t}-pentyl-2\textit{r},6\textit{c}-di(4-chlorophenyl)piperidin-4-one thiosemicarbazone (102)

3\textit{t}-pentyl-2\textit{r},6\textit{c}-di(4-nitrophenyl)piperidin-4-one thiosemicarbazone (103)

3\textit{t}-pentyl-2\textit{r},6\textit{c}-di(4-methoxyphenyl)piperidin-4-one thiosemicarbazone (104)

3\textit{t}-pentyl-2\textit{r},6\textit{c}-di(3,4-dimethoxyphenyl)piperidin-4-one thiosemicarbazone (105)

3\textit{t}-pentyl-2\textit{r},6\textit{c}-di(4-methylphenyl)piperidin-4-one thiosemicarbazone (106)

3.1.3. (\textit{E})-2-(3-Pentyl-2\textit{r},6\textit{c}-diaryl)piperidin-4-ylidene)-\textit{N}-phenylhydrazine carbothioamides (107-112)

**General procedure:**

A mixture of 3-pentyl-2\textit{r},6\textit{c}-diaryl)piperidin-4-one (0.01 mol) and 4\textsuperscript{'}-phenylthiosemicarbazide (0.01 mol) in the presence of a few drops of conc. hydrochloric acid in methanol was refluxed for about 2 to 3 h. After the completion of reaction, the reaction mixture was cooled and poured into crushed ice and kept at room temperature. The solid product formed was separated by filtration and washed with water and recrystallized by methanol. Thus the following compounds were prepared.
(\(E\))-2-(3-Pentyl-2\(r\),6\(c\)-diphenylpiperidin-4-ylidene)-\(N\)-phenylhydrazine carbothioamides \(107\)

(\(E\))-2-(3-Pentyl-2\(r\),6\(c\)-di(4-fluorophenyl)piperidin-4-ylidene)-\(N\)-phenylhydrazine carbothioamides \(108\)

(\(E\))-2-(3-Pentyl-2\(r\),6\(c\)-di(4-chlorophenyl)piperidin-4-ylidene)-\(N\)-phenylhydrazine carbothioamides \(109\)

(\(E\))-2-(3-Pentyl-2\(r\),6\(c\)-di(4-bromophenyl)piperidin-4-ylidene)-\(N\)-phenylhydrazine carbothioamides \(110\)

(\(E\))-2-(3-Pentyl-2\(r\),6\(c\)-di(4-methoxyphenyl)piperidin-4-ylidene)-\(N\)-phenylhydrazine carbothioamides \(111\)

(\(E\))-2-(3-Pentyl-2\(r\),6\(c\)-di(3,4-dimethoxyphenyl)piperidin-4-ylidene)-\(N\)-phenylhydrazine carbothioamides \(112\)

3.1.4. *Synthesis of 3-alkyl/3,5-dialkyl-2\(r\),6\(c\)-diaryl piperidin-4-one and their oxime picrates (113-146)*

**General procedure:**

All the picrates were prepared by mixing equimolar solutions of the corresponding 3-alkyl/3,5-dialkyl-2\(r\),6\(c\)-diaryl piperidin-4-one and their oximes (0.01 mol) in ethanol with picric acid (0.01 mol) in ethanol and stirring the solution for 30 mins. The yellowish crystals formed were filtered. The yield of the product was found to be 70-95%. The harvested crystals were recrystallized repeatedly to get excellent quality crystals. The compounds thus prepared were:

3\(t\)-pentyl-2\(r\),6\(c\)-diphenylpiperidin-4-one picrate \(113\)

3\(t\)-pentyl-2\(r\),6\(c\)-di(4-fluorophenyl)piperidin-4-one picrate \(114\)

3\(t\)-pentyl-2\(r\),6\(c\)-di(4-chlorophenyl)piperidin-4-one picrate \(115\)
3t-pentyl-2r,6c-di(4-bromophenyl)piperidin-4-one picrate (116)
3t-pentyl-2r,6c-di(4-methoxyphenyl)piperidin-4-one picrate (117)
3t-pentyl-2r,6c-di(3,4-dimethoxyphenyl)piperidin-4-one picrate (118)
3t-pentyl-2r,6c-di(4-methylphenyl)piperidin-4-one picrate (119)
3t-pentyl-2r,6c-diphenylpiperidin-4-one oxime picrate (120)
3t-pentyl-2r,6c-di(4-fluorophenyl)piperidin-4-one oxime picrate (121)
3t-pentyl-2r,6c-di(4-chlorophenyl)piperidin-4-one oxime picrate (122)
3t-pentyl-2r,6c-di(4-bromophenyl)piperidin-4-one oxime picrate (123)
3t-pentyl-2r,6c-di(4-methoxyphenyl)piperidin-4-one oxime picrate (124)
3t-pentyl-2r,6c-di(3,4-dimethoxyphenyl)piperidin-4-one oxime picrate (125)
3t-pentyl-2r,6c-di(2,3,4-trimethoxyphenyl)piperidin-4-one oxime picrate (126)
3,5-diethyl-2r,6c-diphenylpiperidin-4-one picrate (127)
3,5-diethyl-2r,6c-di(4-fluorophenyl)piperidin-4-one picrate (128)
3,5-diethyl-2r,6c-di(4-chlorophenyl)piperidin-4-one picrate (129)
3,5-diethyl-2r,6c-di(2-bromophenyl)piperidin-4-one picrate (130)
3,5-diethyl-2r,6c-di(4-hydroxyphenyl)piperidin-4-one picrate (131)
3,5-diethyl-2r,6c-di(4-methoxyphenyl)piperidin-4-one picrate (132)
3,5-diethyl-2r,6c-di(3,4-dimethoxyphenyl)piperidin-4-one picrate (133)
3,5-diethyl-2r,6c-di(4-methylphenyl)piperidin-4-one picrate (134)
3,5-diethyl-2r,6c-diphenylpiperidin-4-one oxime picrate (135)
3,5-diethyl-2r,6c-di(4-fluorophenyl)piperidin-4-one oxime picrate (136)
3,5-diethyl-2r,6c-di(4-chlorophenyl)piperidin-4-one oxime picrate (137)
3,5-diethyl-2r,6c-di(4-bromophenyl)piperidin-4-one oxime picrate (138)
3,5-diethyl-2r,6c-di(2-bromophenyl)piperidin-4-one oxime picrate (139)
3,5-diethyl-2r,6c-di(4-methoxyphenyl)piperidin-4-one oxime picrate (140)
3t-methyl-2r,6c-di(naphthalene-1-yl)piperidin-4-one picrates (141)
3,5-dimethyl-2r,6c-di(naphthalene-1-yl)piperidin-4-one picrates (142)
3t-isopropyl-2r,6c-di(naphthalene-1-yl)piperidin-4-one picrates (143)
3t-butyl-2r,6c-di(naphthalene-1-yl)piperidin-4-one picrates (144)
3t-pentyl-2r,6c-di(naphthalene-2-yl)piperidin-4-one picrates (145)
3t-methyl-2r,6c-di(naphthalene-2-yl)piperidin-4-one picrates (146)

3.2. SPECTRAL MEASUREMENTS

3.2.1. IR SPECTROSCOPY

IR spectra were recorded on AVATAR-330 FT-IR spectrometer
(Thermo Nicolet range 4000-400 cm\(^{-1}\)) as KBr pellets and iS5 FT-IR
spectrometer (Thermo Nicolet range 4000-650 cm\(^{-1}\)) as KBr pellets
and ATR (Attenuated Total Reflectance) method and only noteworthy
absorption levels (reciprocal centimeters) are listed.

3.2.2. MASS SPECTROMETRY

Electron Impact Mass Spectra (EIMS) were recorded on mass
engine JEOL GC MATE II mass spectrometer and VARIAN-
SASTURAN 2200 GC-MS Mass spectrometer.

3.2.3. ELEMENTAL ANALYSIS

Elemental analyses were carried out on VARIOMICRO V2.2.0
CHN analyser.

3.2.4. NMR SPECTROSCOPY

\(^1\)H NMR spectra were recorded on a BRUKER AVANCE III 400
MHz NMR spectrometer operating at 400.13 MHz. \(^{13}\)C NMR, DEPT
spectra were recorded on a BRUKER AVANCE III 400 MHz NMR spectrometer operating at 100.61 MHz. HOMOCOSY, NOESY, HSQC, HMBC and DEPT spectra were recorded on a BRUKER AVANCE III 400 MHz NMR spectrometer using standard parameters. All the NMR measurements were made using 5 mm NMR tubes. For recording $^1$H NMR spectrum of compounds, solutions were prepared by dissolving about 10 mg of the compound in 0.5 mL of CDC$_3$/DMSO-$d_6$ while for $^{13}$C NMR spectra, about 50 mg of the compound was dissolved in the same volume of the respective solvents. TMS was used as an internal standard.

3.2.5. UV–vis spectroscopy

The ultraviolet–visible spectra were recorded on UV–vis spectrophotometer (Perkin Elmer, Lambda 35) and corrected for background due to solvent absorption.

3.2.6. Raman spectroscopy

Fourier transform-Raman spectra were recorded with an integral microscope Raman system BRUKER RFS27 spectrometer (50-5000 cm$^{-1}$) equipped with 1024 X 256 pixels liquefied nitrogen-cooled germanium detector. The 1064 nm line of the Nd:YAG laser (red laser) was used to excite. To avoid intensive heating of the sample, the laser power at the sample was not higher than 15 mW.
3.3. SINGLE CRYSTAL X-RAY DIFFRACTION

Data collection: The crystal was grown by the slow evaporation technique in ethanol solvent. Diffraction data were collected on a Bruker SMART APEX CCD diffractometer using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) at 298 (2) K with crystal size of 0.35 x 0.30 x 0.25 mm. The structure was solved by direct methods and successive Fourier difference synthesis (SHELXS-97) [158] and refined by full matrix least square procedure on F² with anisotropic thermal parameters. All non-hydrogen atoms were refined (SHELXL-97) [159] and placed at chemically acceptable positions. A total of 361 parameters were refined with 5028 unique reflections which covered the residuals to R₁=0.0722. Crystallographic data have been deposited with the Cambridge Crystallographic data centre as supplementary publication number CCDC 1002105 for 113. Copies of the data can be obtained free of charge via http://www.ccdc.cam.ac.uk or from the Cambridge Crystallographic data centre, 12 union road, Cambridge CB2 1EZ, UK; fax: +44 1223 336 033: or e-mail: deposit@ccdc.cam.ac.uk.

3.4. ANTIMICROBIAL STUDIES [160-162]

The term microbiological assay designates a type of biological assay, specifically, a biological assay performed with microorganisms like bacteria, yeasts, molds etc. This involves the measurement of the relative potency and activity of compounds by determining the amount required to produce a stipulated effect on a suitable
organism under standard condition. The procedure employed in microbial assay may be divided into two broad classifications.

1. Disc diffusion method

2. Two-fold serial dilution method

3.4.1a Disc diffusion method

The disc diffusion assay of drug potency is based on the measurement of the diameters of zones of microbial growth inhibition surrounding the disc impregnated with test compounds (which are placed on the surface of solid nutrient previously inoculated with a culture of a suitable microorganism). Inhibition produced by the test compound is classified into three categories depending upon the zone of inhibition (>15 mm - active, 10-15 mm - moderately active and <10 mm - less active or inactive) and also compared with that produced by known concentration of a standard.

3.4.1b Two-fold serial dilution method

The Minimum Inhibitory Concentration (MIC in $\mu$g/mL) value for a particular compound is determined by serial dilution method. MIC is the lowest concentration of the test compound, which results in no visible growth on the respective medium in the petri dish. To ensure that the solvent has no impact on the microbial growth, a control was carried out with test medium supplemented with DMSO at the same dilution as used in the experiment.
3.4.2. ANTIBACTERIAL STUDIES [163, 164]

The following Gram positive and Gram negative strains have been used for the study.

1. *Staphylococcus aureus* (Gram positive)
2. *Bacillus subtilis* (Gram positive)
3. *Enterococcus faecalis*
4. *Vibrio cholere* (Gram negative)
5. *Escherichia coli* (Gram negative)
6. *Pseudomonas aeruginosa* (Gram negative)
7. *Salmonella typhi* (Gram negative)

PREPARATION OF TEST INOCULUM

(a) Sub-culture (preparation of seeded broth)

The strains of *Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis, Vibrio cholere, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi* were inoculated in conical flasks containing 100 mL of sterile nutrient broth. These conical flasks were incubated at 37 ± 1 °C for 24 h. This was referred as seeded broth.

(b) Standardization of seeded broth (viable count)

(i) Dilutions

One mL of 24 h seeded broth of each strain was diluted with 99 mL of sterilized normal saline containing 0.05% tween 80. From that, 1 mL is further diluted to 10 mL with sterile normal saline. This was continued to $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ until $10^{-10}$ mL of the dilution of seeded broth was obtained.
(ii) Incubation of nutrient agar petridishes

The dilutions were studied by inoculating 0.2 mL of each dilution on to the solidified nutrient agar medium by spread plate method after incubation at 37 ± 1 °C for 24 h. The numbers of well-formed colonies on the plates were counted. The seeded broth was then suitably diluted to have between $10^5$-$10^7$ microorganisms per milliliter or cfu/mL. This was designated as the working stock and used for the antibacterial studies.

**PREPARATION OF SOLUTION OF TEST COMPOUNDS**

The solution of test compounds was prepared by dissolving the same in dimethyl sulfoxide in specific gravity bottle and stored in refrigerator. The solution was removed from the refrigerator 1 h prior to its use and allowed to warm upto room temperature. The test compounds were prepared at a concentration of 200 µg/mL. Similarly, the standard drug solutions of Ciprofloxacin and Amphotericin B/Cetramazole were used respectively at a concentration 200 µg/mL for finding the minimum inhibitory concentration. Solvent control of DMSO was also maintained throughout the experiment simultaneously.

**PREPARATION OF CULTURE MEDIA**

The media used for the growth of bacteria were

a. Nutrient agar medium

b. Nutrient broth medium
The media were sterilized by autoclaving at a pressure of 15 lb/sq at 121°C for 20 min.

(a) Nutrient agar medium (Hi-media)

The nutrient agar medium was prepared by dissolving 28 g of nutrient agar (procured from Hi-media, Mumbai) in 1000 mL of distilled water.

**Formula**

- Peptone : 1%
- Sodium chloride : 0.5%
- Beef extract : 1%
- Agar : 2%
- pH : 7.4 ± 0.2

(b) Nutrient broth medium (Hi-media)

The nutrient broth medium was prepared by dissolving 13 g of nutrient broth (procured from Hi-media, Mumbai) in 1000 mL of distilled water.

**Formula**

- Peptone : 1 %
- Sodium chloride : 0.5%
- Beef extract : 1%
- pH : 7.4 ± 0.2
Determination of antibacterial activity by Disc-Diffusion method

Nutrient agar plates were prepared under sterilized conditions and incubated overnight to detect contamination. About 0.2 mL of working stock culture was transferred into separate nutrient agar plates and spreaded thoroughly using a glass spreader. Whatman No. 1 discs (6 mm in diameter) were impregnated with the test compounds dissolved in DMSO (200 μg/mL) for about half an hour. Commercially available drug disc (Ciprofloxacin 10 μg/disc) was used as positive reference standard. Negative controls were also prepared by impregnating the disc of same size with DMSO solvent. The discs were placed on the inoculated agar plates and incubated at 37 ± 1 °C for about 18-24 h. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organism.

Determination of Minimum Inhibitory Concentration (MIC) of test compounds using two-fold serial dilution method

Testing was done in the seeded broth (10^{-6} to 10^{-7} cfu/mL). The test compounds were taken at different concentrations like 200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.25 μg/mL and 3.13 μg/mL for finding minimum inhibitory concentration by using seeded broth as diluent. Similarly, the
standard solution of Ciprofloxacin drug was prepared at the concentrations of 200 μg/mL, 100μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.25 μg/mL and 3.13 μg/mL with sterile distilled water and DMSO were maintained throughout the experiment simultaneously as control.

The study involves a series of 7 assay tubes for the test compounds against each strain. In the first assay tube, 1.6 mL of seeded broth was transferred and 0.4 mL of the test solution was added followed by mixing thoroughly to obtain a concentration 200 μg/mL. To the remaining 6 assay tubes, 1 mL of seeded broth was transferred. From the first assay tube 1 mL of the content was pipetted out and added into the second assay tube followed by thorough mixing. This type of dilution was repeated up to 7th assay tube serially. The same procedure was followed for standard drugs too. Duplicates were also maintained. These were done under aseptic conditions.

The racks of assay tubes were placed inside the incubator at 37 ± 1 °C for 24 h. At the end of 24th h, assay tube concentrations were again streaked into nutrient agar plate due to the turbidity of drug-microorganism mixture. The lowest concentration of the test compounds, which caused apparently a complete inhibition of growth of organisms, was taken as minimum inhibitory concentration.
The solvent control tube was also observed to find whether there was any inhibitory action. The sterile distilled water and DMSO did not show any inhibition.

3.4.3. Antifungal Studies [163, 164]

The following fungal strains were used for the study.

1. *Candida albicans*
2. *Aspergillus niger*
3. *Aspergillus flavus*
4. *Trichophyton mentagrophytes*
5. *Trichophyton rubrum*

Sabouraud’s dextrose agar (SDA) medium was used for the growth of fungi and testing was done in Sabouraud’s dextrose broth (SDB) medium.

The sub-culture and the viable count were carried out by the same procedure as done in antibacterial studies except for the temperature which should be maintained at 28 ± 1 °C for about 72 h. Similarly, for disc diffusion method, the petridishes were incubated at 28 ± 1 °C for about 72 h. The same concentration of the test compound, solvent (DMSO) and Amphotericin B/ Cetramazole (standard) prepared previously were used for the antifungal studies.
PREPARATION OF CULTURE MEDIA

(a) SDA medium

**Formula**

- Dextrose : 40 g
- Peptone : 10 g
- Agar : 15 g
- Distilled water : 1000mL
- pH : 5.4

(a) SDB medium

**Formula**

- Dextrose : 40 g
- Peptone : 10 g
- Distilled water : 1000mL
- pH : 5.4