# Chapter 5
# Experimental

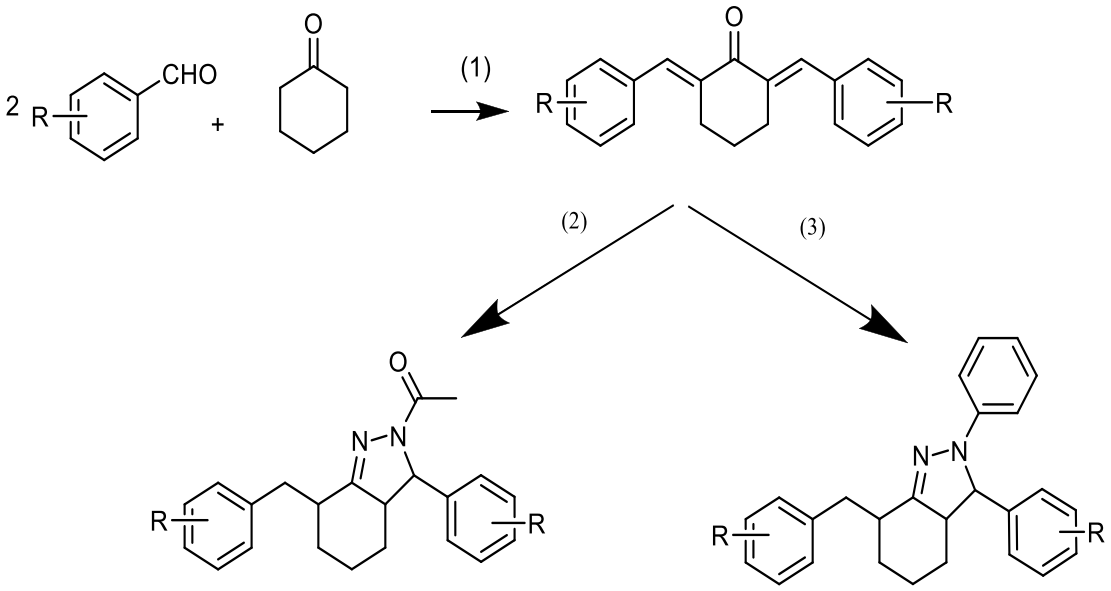
## 5. EXPERIMENTAL

All reagents used were obtained from commercial suppliers and were used without further purification. The progress of the reaction was monitored by thin layer chromatography (TLC) on pre-coated Merck alu-foil plate (silica gel 60F-254, 0.25 mm thickness). Melting points were determined on a Veego capillary melting point apparatus and are uncorrected. FT-IR spectra were recorded by using KBr pellets on Shimadzu FT-IR spectrophotometer. $^{1}H$ NMR and $^{13}C$ NMR spectra were recorded on a Bruker 300 MHz spectrophotometer. All NMR spectra were obtained in deuterated chloroform ($CDCl_3$); chemical shifts are reported in parts per million, and coupling constants in Hertz (Hz). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet). Mass spectra were recorded on a LC-MS-2010A spectrometer on ESI positive mode and molecular ion peaks are reported as m/z ratio. Elemental analysis of synthesized compounds was also recorded on EXETERTCE-440 elemental analyzer.

### 5.1. General procedure for the synthesis of hexahydroindazole analogues of curcumin (A1-B5)

These were prepared in two steps. In First step, respective dibenzylidiene cyclohexanones were synthesized. A mixture of ethanol (20ml) and sodium hydroxide solution (10%, 10 ml) was taken in a beaker, maintained at temperature between 15 to 25ºC. The solution was vigorously stirred and one half of the previously prepared mixture of appropriate aromatic aldehyde and cyclohexanone (in ratio of 2:1) was added to it. After 20 min, the remaining of the aromatic aldehyde-cyclohexanone mixture was added. The reaction mixture was further stirred for 45 minutes. The solid was filtered off, washed with distilled water, and subjected to further purification by recrystallization with ethyl alcohol

In second step, the substituted dibenzylidene cycloheaxanone (1.2 mmol) obtained from first step was dissolved in glacial acetic acid (5 mL), and hydrazine hydrate/ phenylhydrazine (1.5 mmol) was added to the solution. The solution was refluxed for 12 hours and monitored by TLC. The solvent was removed in vacuum and the residue was recrystallized with ethyl alcohol. The remaining analogues (Table: 5.1) were also prepared by the same procedure.




Synthesis of hexahydroindazole analogues of curcumin (A1-A9) and (B1-B5). Reagents and conditions: (1) 10 % NaOH, EtOH, $H_2O$; (2) Hydrazine hydrate, Glacial acetic acid, 12 h reflux; (3) Phenyl hydrazine, Glacial acetic acid, 12 h reflux.

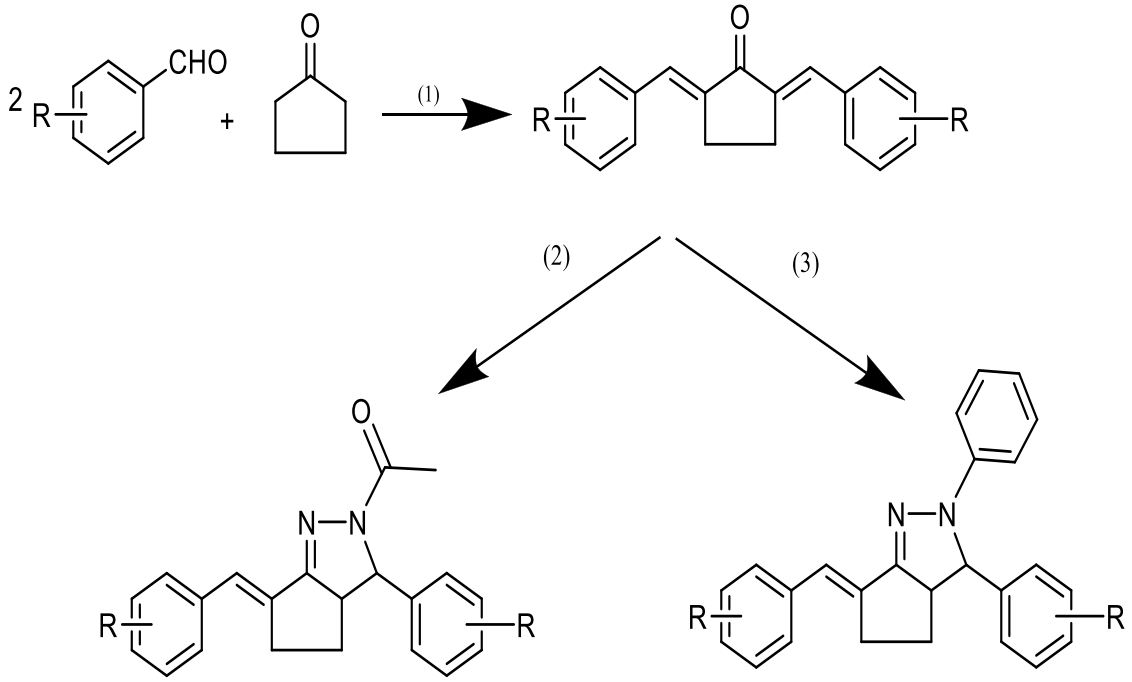
**Table: 5.1.** Different Substitutions on aryl ring of synthesized compounds(A1-B5)

| Comp. Code | Substitution (R) |
|---|---|
| **A1** | H |
| **A2** | 2-Cl |
| **A3** | 3-OH |
| **A4** | 4-Cl |
| **A5** | 2,4-di Cl |
| **A6** | 3-$NO_2$ |
| **A7** | 3-$OCH_3$ |

| Comp. Code | Substitution (R) |
|---|---|
| **A8** | 2,3,4-tri $OCH_3$ |
| **A9** | 4-$N(CH_3)_2$ |
| **B1** | 3-Cl |
| **B2** | 4-Cl |
| **B3** | 2,4-di Cl |
| **B4** | 4-$OCH_3$ |
| **B5** | 4-$N(CH_3)_2$ |

### 5.2. General procedure for synthesis of pyrazole analogues of curcumin (CP1-CP14)

These were prepared in two steps. In first step, respective dibenzylidiene cyclopentanones were synthesized. A mixture of ethanol (20 ml) and sodium hydroxide solution (10%, 10 ml) was taken in a beaker and was maintained at temperature between 15 to 25ºC. The solution was vigorously stirred and one half of previously prepared mixture of appropriate aromatic aldehyde and cyclopentanone (in molar ratio of 2: 1) was added to it. After 20 min, the remaining aromatic aldehyde-cyclopentanone mixture was added. The reaction mixture was further stirred for 45 minutes. The solid was filtered off, washed with distilled water, and subjected to further purification by crystallization with ethyl alcohol.




Synthesis of pyrazole analogues of curcumin (CP1-CP14); Reagents and conditions: (1) 10% NaOH, EtOH, $H_2O$; (2) Hydrazine hydrate, Glacial acetic acid, 14 h reflux; (3) Phenyl hydrazine, Glacial acetic acid, 14 h reflux.

In second step, synthesized substituted dibenzylidene cyclopentanone (1.2 mmol) from first step was dissolved in glacial acetic acid (5 ml), and hydrazine hydrate (1.5 mmol) was added to the solution. The solution was refluxed for 14 hours and monitored by TLC. The solvent was removed in vacuum and the residue was crystallized with ethyl alcohol. The remaining analogues were also prepared by the same procedure. Structures of all synthesized compounds are given in table (Table 5.2).

**Table 5.2.** Different Substitutions on aryl ring of synthesized compounds(CP1-CP14).

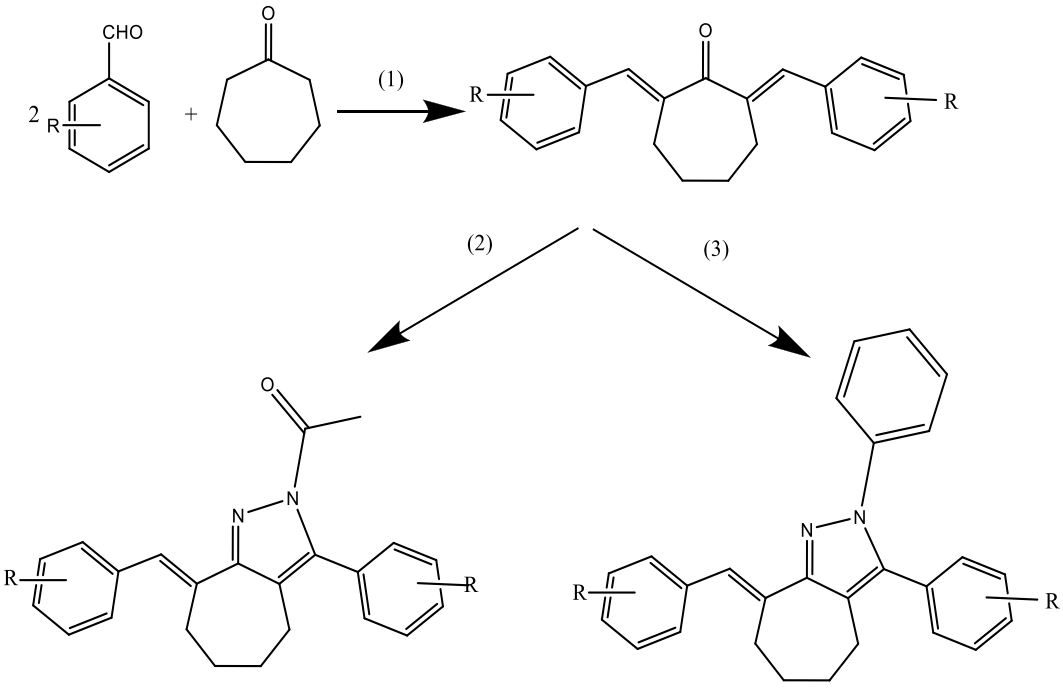
| Comp. Code | Substitution (R) |
|---|---|
| **CP1** | H |
| **CP 2** | 2-Cl |
| **CP 3** | 3-OH |
| **CP 4** | 4-Cl |
| **CP 5** | 2,4-di Cl |
| **CP 6** | 3-$NO_2$ |
| **CP 7** | 3-$OCH_3$ |

| Comp. Code | Substitution (R) |
|---|---|
| **CP 8** | 2,3,4-tri $OCH_3$ |
| **CP 9** | 4-$N(CH_3)_2$ |
| **CP 10** | 3-Cl |
| **CP 11** | 4-Cl |
| **CP 12** | 2,4-di Cl |
| **CP 13** | 4-$OCH_3$ |
| **CP 14** | 4-$N(CH_3)_2$ |

## 5.3. General procedure for Synthesis of cycloheptanones analogues of curcumin (C1-C14)

These were prepared in two steps. In first step, respective dibenzylidiene cycloheptanones were synthesized. A mixture of ethanol (20 ml) and sodium hydroxide solution (10%, 10 ml) was taken in a beaker and was maintained at temperature between 15 to 25ºC. The solution was vigorously stirred and one half of previously prepared mixture of appropriate aromatic aldehyde and cycloheptanone (in molar ratio of 2: 1) was added to it. After 20 min, the remaining aromatic aldehyde- cycloheptanones mixture was added. The reaction mixture was further stirred for 50 minutes. The solid was filtered off, washed with distilled water, and subjected to further purification by crystallization with ethyl alcohol.

In second step, synthesized substituted dibenzylidene cycloheptanones (1.2 mmol) from first step was dissolved in glacial acetic acid (5 ml), and hydrazine hydrate (1.5 mmol) was added to the solution. The solution was refluxed for 20 hours and monitored by TLC. The solvent was removed in vacuum and the residue was crystallized with ethyl alcohol. The remaining analogues were also prepared by the same procedure. Structures of all synthesized compounds are given in table (Table 5.3).




Synthesis of cycloheptanone analogues of curcumin (C1-C14); Reagents and conditions: (1) 10% NaOH, EtOH, $H_2O$; (2) Hydrazine hydrate, Glacial acetic acid, 20 h reflux; (3) Phenyl hydrazine, Glacial acetic acid, 20 h reflux.

**Table 5.3.** Different Substitutions on aryl ring of synthesized compounds(C1-C14).

| Comp. Code | Substitution (R) |
|---|---|
| **C1** | H |
| **C2** | 2-Cl |
| **C3** | 4-Br |
| **C4** | 2-OH |
| **C5** | 2,4-di Cl |
| **C6** | 3-$NO_2$ |
| **C7** | 3-$OCH_3$ |

| Comp. Code | Substitution (R) |
|---|---|
| **C8** | 3,4,5-tri $OCH_3$ |
| **C9** | 4-$N(CH_3)_2$ |
| **C10** | 2-Cl |
| **C11** | 4-Cl |
| **C12** | 2,4-di Cl |
| **C13** | 4-$OCH_3$ |
| **C14** | 3-$NO_2$ |

### 5.4. *In-vitro* Antimicrobial Study of curcumin analogues

Antimicrobial activities of synthesized compounds were first screened by disc diffusion method [Bharti *et al.*. 2010] against various Gram positive and Gram negative human pathogenic bacteria viz. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27893, *Salmonella typhi* MTCC 3216, *Escherichia faecalis* and *Staphylococcus aureus* ATCCC 25323 and different fungal strains of Candida according to the guidelines of national committee for clinical laboratory standards (NCCLS), 1997. Fresh grown bacteria were mixed in sterile saline (0.85%) and the turbidity was matched with McFarland No. 2 system to achieve concentration of $10^7$ CFU/ml. Sterile petri plates containing 20 mL of Mueller Hinton agar (MHA, Hi-Media) were used for all bacterial culture and Sabouraud's dextrose agar (SDA)/Potato dextrose agar (PDA) (Hi-Media) were used for all fungal culture. The bacterial inoculums suspension was spread on the surface of agar plates.

Sterile disc (5mm) of Whatman paper no. 1 was then placed on the surface of the media and the test compounds (25µl/ml) was put and allowed to diffuse and plates were incubated for 24 h at 37ºC for bacterial cultures and 72 h at 25ºC for fungal culture. Ciprofloxacin (5µg/disc, Hi-Media) was used as positive control for bacteria and Fluconazole (10µg/disc, Hi-Media), was used as a positive control for fungi. Zone of inhibition was measured in millimeters after 24 h. All tests were performed in triplicate.

### 5.5. *In-vitro* Anti-malarial study of curcumin analogues

#### 5.5.1 Chemical and reagents

All reagents were procured from Sigma-Aldrich Chemical Company (St. Louis, Mo.) and maintained according to manufacturer's specifications until use. *Plasmodium falciparum* W2 strain was kindly provided by Dr. Luzia Helena Carvalho, Centro de Pesquisas René Rachou, FIOCRUZ-MG. Human plasma was donated by a blood bank.

### 5.5.2 Solubility of samples

Samples were diluted in Dimethyl Sulfoxide (DMSO) at a concentration of 50mg/ml. Samples A5, A6, A9, B3, B4, C5, C7 and CP1 showed precipitations in DMSO. Samples A8, B2 precipitated in RPMI medium. Samples A3, A4, B1, C4, C9 and B4 precipitated in DMSO and RPMI. The precipitated samples were submitted to centrifugation and the supernatants were used for testing.

### 5.5.3 *Plasmodium falciparum* cultivation

*Plasmodium falciparum* W2 strain, partially resistant to Chloroquine, was used in the anti-malarial assays that were performed as described by [Makler *et al.* 1993]

Prior to the experiments Chloroquine-resistant RKL9 **(***Plasmodium falciparum* W2 strain,) were cultivated by the method of Trager and Jensen [Trager & Jensen 1976] with minor modifications. Cultures were maintained in fresh $A^+$ human erythrocytes suspended at 5% hematocrit in RPMI 1640 containing sodium bicarbonate (21mM), D-glucose (11mM), HEPES (25mM), hypoxanthine (300µM) and Gentamicin (40µg/ml) and supplemented with 10% human plasma. They were incubated at 37°C under a gas mixture 5% $O_2$, 5% $CO_2$, and 90% $N_2$. Every day, infected erythrocytes were transferred into fresh complete medium to propagate the culture. The synchronization of the parasites was achieved by sorbitol [Lambros & Vanderberg 1979] treatment and parasitemias were determined microscopically in Giemsa-stained smears.

### 5.5.4 Determination of $IC_{50}$

The results were evaluated with the software Origin 8.0 for determination of the dose-response curves plotted with sigmoidal fit. The 50% inhibitory concentration growth of the parasites ($IC_{50}$) was determined by comparison with controls with standard drug (Chloroquine) and without drugs.

### 5.5.5 Cytotoxicity Tests With a Human Hepatoma cell line

The cytotoxicity assay was performed as described by Moss and collaborators with modifications [Mosmann 1983]. HepG2 liver cell line was used for the tests.

The hepatoma cells Hep G2A16 were maintained at 37ºC, 5% $CO_2$ in 75-$cm^2$ sterile culture flasks (Corning®) with RPMI culture medium supplemented with 5% FBS, Penicillin (10 U/ml), and Streptomycin (100 g/ml), with changes of medium twice a week. The cells were maintained in weekly passages (at 1:3 dilutions in sterile culture flasks) and

grown to confluence [Varotti *et al*. 2008]. They were used for experiments after being trypsinized (0.05% trypsin/0.5 mM EDTA) and plated on 96 wells plates (Corning®) [Madureira *et al.* 2002].

The results were evaluated with the software Origin 8.0 for determination of the dose-response curves plotted with sigmoidal fit. The 50% cytotoxic concentration of cells ($CC_{50}$) were determined by comparison with controls without drugs and standard drug (Chloroquine). The selectivity index (SI) was calculated based on ratio of $CC_{50}$ and $IC_{50}$ values.

### 5.6. Molecular docking study of curcumin analogues against antimicrobial target Glucosamine-6-phosphate synthase

Automated docking was used to determine the orientation of inhibitors bound to the active site of GlcN-6-P synthase. A genetic algorithm method, implemented in the program AutoDock 4.0 in case of hexahydroindazole analogue and AutoDock 4.2 in case of pyrazole analogue of curcumin was employed [Morris *et al.* 2009]. The 3D structure file of all thirteen curcumin analogues and flucanozole molecule were loaded on to PRODRG server [Ghose *et al.* 1987] and PreADMET server for energy minimization and drug likeliness prediction respectively. The protein structure file 1JXA was downloaded from Protein Data Bank (www.rcsb.org/pdb) was edited by removing the heteroatoms, adding C-terminal oxygen [Binkowski *et al.* 2003] For docking calculations, Gasteigere-Marsili partial charges [Gasteiger *et al.* 1980] were assigned to the ligands and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The grid map was centered at the residues of the protein predicted from the CASTp server. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization using default parameters. The number of docking runs was 50, the population in the genetic algorithm was 250, the number of energy evaluations was 100,000, and the maximum number of iterations 10,000. The docking results for ligand molecules against glucosamine-6-phosphate synthase [PDB Id: 1JKA], showed minimum docking energy, binding energy, inhibition constant, intermolecular energy with 0.0 RMS as documented.

### 5.7. Quantitative Structure Activity Relationship (QSAR) Study for curcumin analogue as antimicrobial agent

Stepwise multiple linear regression analysis method was used to perform QSAR analysis employing in-house VALSTAT programme [Gupta *et al.* 2004]. The ± data within the

parentheses are the error of regression coefficients associated with corresponding regression coefficients in regression equation. The best model was selected on the basis of various statistical parameters such as correlation coefficient (r), standard error of estimation (std), sequential Fischer test (F). Quality of the each model was estimated from the cross-validated squared correlation coefficient ($q^2$), calculated root mean square error (SDEP), chance statistics evaluated as the ratio of the equivalent regression equations to the total number of randomized sets; a chance value of 0.001 corresponds to 0.1% chance of fortuitous correlation and boot-strapping square correlation coefficient ($r^2$bs), which confirm the robustness and applicability of QSAR equation.

### 5.7.1. Workstation used

Workstations are raster systems in which a computer with a full operating system and mass storage facility is integrated with graphical display. All the computational studies were performed on IBM PC running on Intel Pentium 4 processor.

### 5.7.2. QSAR tool -Valstat

For the present study VALSTAT, a PC based programme developed by using $C^{++}$ language was used. The program has provision of sequential and stepwise multiple regression analysis with linear and parabolic relationship to generate the QSAR model. Valstat compute statistical parameters such as correlation coefficient (r), standard deviation (std) and F-test for statistical significance (F). Additional special statistical parameters such as cross validation, squared correlation coefficient ($Q^2$), randomization test (Chance) and bootstrapping squared correlation coefficient ($r^2_{bs}$) were incorporated for selection and validation of best QSAR model. The program is validated for its accuracy and reproducibility.

### 5.7.3. General Methodology used for QSAR Analysis

The Compounds in the series were sketched using ChemDraw module of Chemoffice 2004.

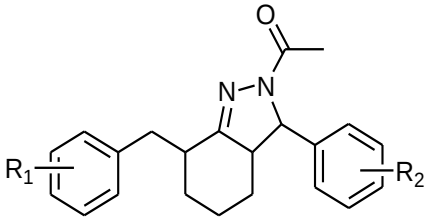
The structures were energy minimized to calculate electronic descriptors.

- MM2 server was used for energy minimization; minimum RMS gradient used was 0.100.
- In the subsequent step MOPAC server was used for energy minimization, minimum RMS gradient used was 0.0001.

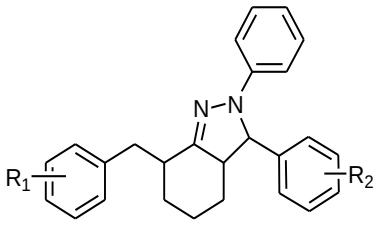
Biological activity was taken as dependent variable and descriptors were taken as independent variables. A correlation analysis was performed between dependent and independent variables. The statistical measures used for the evaluating the generated models are correlation coefficient (r), squared correlation coefficient ($r^2$), Fischer ratio value and standard deviation. The selected models were validated by Leave One Out (LOO) method employing validation software VALSTAT and the validation parameters ($q^2$ $r^2$pred, SPRESS and SDEP) were calculated for generated models.

Table 5.4-5.5 Shows the structural features of both hexahydroindazole and pyrazole analogues of curcumin along with their biological activities (MIC µg/ml) taken out from experimental data and descriptors included in final QSAR model:

**Table: 5.4** Structural features of both hexahydroindazole analogues of curcumin for QSAR model 1




| Comp. code | R1 | R2 | pMIC |
|---|---|---|---|
| A1 | H | H | NA |
| A2 | 2-Cl | 2-Cl | 7.34416 |
| A3 | 3-OH | 3-OH | 7.46125 |
| A4 | 4-Cl | 4-Cl | 7.45142 |
| A5 | 2,4-di Cl | 2,4-di Cl | 7.46129 |
| A6 | 3-$OCH_3$ | 3-$OCH_3$ | 7.57221 |
| A7 | 2,3,4-tri-$OCH_3$ | 2,3,4-tri-$OCH_3$ | 7.51124 |




| Comp. code | R1 | R2 | pMIC |
|---|---|---|---|
| B1 | 3-Cl | 3-Cl | 7.78206 |
| B2 | 4-Cl | 4-Cl | 7.51707 |
| B3 | 2,4-di Cl | 2,4-di Cl | 7.51707 |
| B4 | 4-$N(CH_3)_2$ | 4-$N(CH_3)_2$ | 7.61876 |

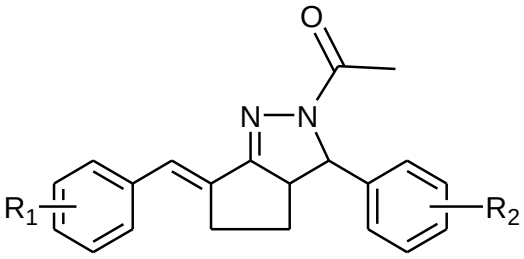
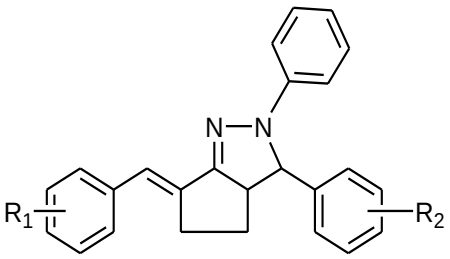



**Table: 5.5** Structural features of both pyrazole analogues of curcumin for QSAR model 2

| Comp. code | R1 | R2 | pMIC |
|---|---|---|---|
| CP1 | 2-Cl | 2-Cl | 7.5804 |
| CP 2 | 3-OH | 3-OH | 7.49285 |
| CP 3 | 4-Cl | 4-Cl | 7.48299 |
| CP 4 | 2,4-di Cl | 2,4-di Cl | 7.49287 |
| CP 5 | 3-$NO_2$ | 3-$NO_2$ | 7.59916 |
| CP 6 | 3-$OCH_3$ | 3-$OCH_3$ | NA |
| CP 7 | 4-$N(CH_3)_2$ | 4-$N(CH_3)_2$ | 7.63628 |




| Compound code | R1 | R2 | pMIC |
|---|---|---|---|
| CP10 | 3-Cl | 3-Cl | 7.53819 |
| CP11 | 4-Cl | 4-Cl | 7.56392 |
| CP12 | 2,4-di Cl | 2,4-di Cl | 7.54405 |
| CP13 | 3-$OCH_3$ | 3-$OCH_3$ | NA |
| CP14 | 4-$N(CH_3)_2$ | 4-$N(CH_3)_2$ | 7.56468 |