Synthesis, Characterization and Pharmacological Evaluation of Chalcones and its Derivatives for Antileishmanial activity.

Abstract:

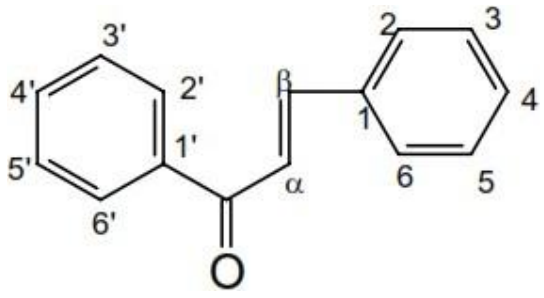
Medicinal chemistry is almost always geared toward drug discovery and development. The focus on development of new synthetic drug compounds has resulted in the incorporation of many other disciplines, such as biochemistry, combinatorial chemistry, chemical biology, phytochemistry, pharmacology, enzymology, pharmacognosy, statistics, physical chemistry and molecular biology into medicinal chemistry. In this view medicinal chemists are also trying to speed up drug discovery process for finding the lead molecule (Thomas *et al.* 1998). The compounds with chalcone as backbone have been reported to possess varied biological and pharmacological activities, including antimicrobial, anti-inflammatory, analgesic, cytotoxic, antitumor, antimalarial, antitubercular, antiviral, anti-HIV, antiulcerative, antileishmanial activities. Leishmaniasis is a vector-borne disease caused by protozoan parasites of the genus Leishmania. It is transmitted through the bite of female phlebotomine sandflies and can range from mild self-healing cutaneous lesions to lethal visceral leishmaniasis. Lic A **(1)**, efficiently inhibited the proliferation of *Leishmania donovani* and *Leishmania major* promastigotes and amastigotes *in vitro* by inhibiting fumarate reductase (Chen *et al.* 1993), a selective target present in the parasite mitochondria. The Lic C **(113)** inhibited the growth of the *L. major* parasite to the same extent as Lic A **(1)** (Nielson *et al.* 1995).

Key Words: Chalcone, Aldol condensation, Pharmacological -Biological activity.

Introduction

Medicinal chemistry is the application of chemical research techniques to the synthesis of pharmaceuticals. During the early stages of medicinal chemistry development, scientists were primarily concerned with the isolation of medicinal agents found in plants. Today, scientists in this field are also equally concerned with the creation of new synthetic drug. Chalcone is a member of the class of chalcones that is acetophenone in which one of the methyl hydrogens has been replaced by a benzylidene group. It has a role as a plant metabolite. It is a member of styrenes and a member of chalcones. Chalcones (1,3 diaryl-2 propen-1ones) are precursors for Flavonoids and isoflavonoids which are common simple chemical scaffolds found in many naturally occurring compounds. Many chalcones derivatives were also prepared due to their convenient synthesis.

General structure of chalcone:



Chalcones are one of the major classes of natural products which occur widely in nature particularly in colored flowers and wide spread distribution in fruits, vegetables, spices and tea. Various natural or synthetic chalcones have been found to posses diverse biological activites (Di Carlo *et al.* 1999).

All the chalcones give dark red coloration with concentrated sulphuric acid wilson test and violet red coloration with alcoholic ferric chloride solution. Chalcones on heating with traces of iodine in dimethylsulphoxide (DMSO) for two hours give the corresponding flavones.

Chalcones were converted into the corresponding flavonols by their oxidation using hydrogen peroxide in methanolic sodium hydroxide solution and these flavonols showed characteristic greenish yellow fluorescence in ethanolic solution as well as with concentrated sulphuric acid. In the past decade, synthetic or naturally occurring chalcones emerged as a new class of antileishmanial compounds. Leshmania is a genus of trypanosomes responsible for the disease leishmaniasis.leishmaniasis is spread through sand files of the genus Phlebotomus, primary hosts being the vertebrates.The Chalcone was evaluated against 29 promatigotes of Leshmania donovani exhibiting low toxicity against mammalian cells.

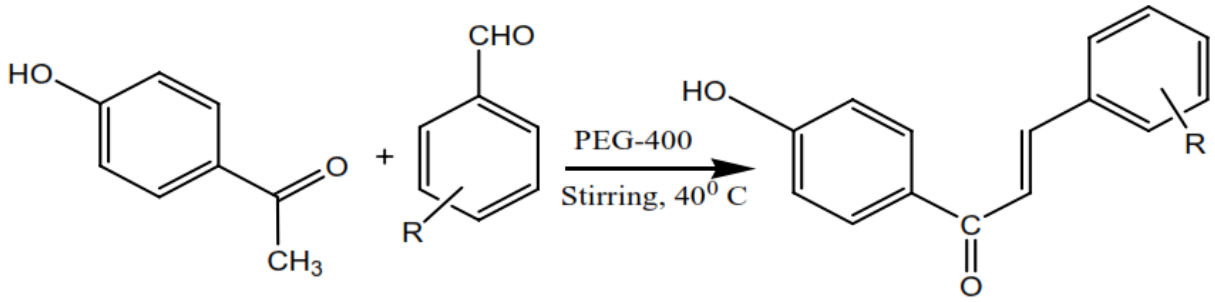
Material and Methods:

The following methods for synthesizing chalcones and Chalcones derivatives. All the synthetic compounds were acquired from Sigma-Aldrich, Spectrochem and High Media.. Melting point is determined by utilizing an open capillary and are uncorrected.TLC were performed on silica plates with observation under uv or iodine chamber . Infrared Infrared spectra were recorded on a FT-IR Shimadzu DZU 8400S spectrophotometer in KBr circles and Elemental examination were done on a Perkin-Elmer 2400C, H, N analyzer and values were viewed to be within satisfactory limits reaches of the determined qualities. The 1H-NMR spectra of the methodize mixtures in CDCl3/DMSO were recorded at 400 MHz by Bruker Advance II 400 NMR spectrometer. Chemical shift esteems are given in scale utilizing tetramethylsilane (TMS) as an inside norm. Huge 1H-NMR information are written all together: number of protons, assortment (b, wide; s, singlet; d, doublet; t, trio; m, multiplet), coupling constants in Hertz, task. The fab mass spectra (at room temperature) were recorded on tof MS-ES Mass spectrometer.

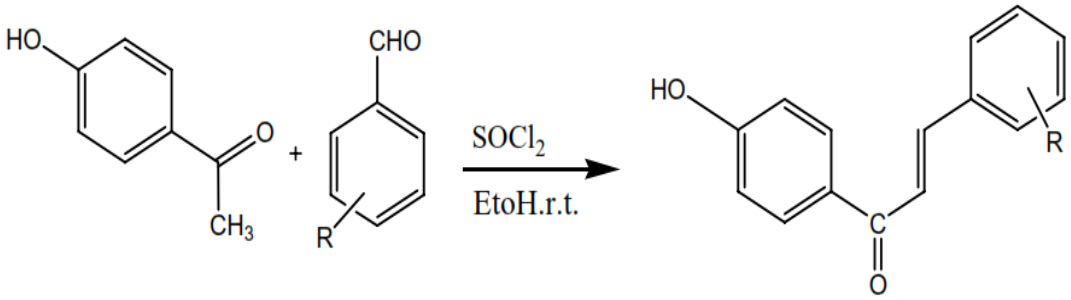
General methods of synthesis of chalcones

Chalcones are well known intermeadiates for synthesizing various heterocyclic compounds. They can be obtained by the acid or base catalyzed aldol condensation of acetophenones with benzaldehydes (Guida *et al.* 1997).

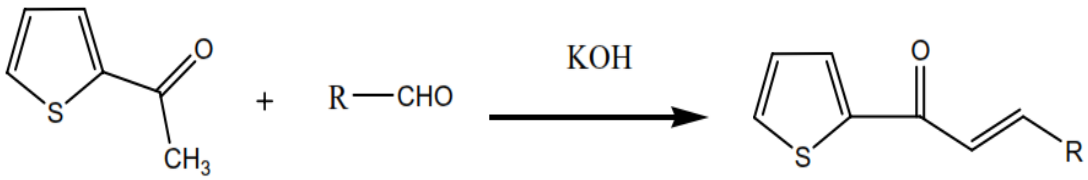
1) Claisen–Schmidt condensation between 4-hydroxy acetophenone and benzaldehyde was carried out in the presence of a base catalyst was stirred in PEG-400 as a recyclable solvent form 4’ -hydroxy chalcones (Sreedhar *et al.* 2010).



2) Stirred mixture of 4-hydroxy acetophenone and various benzaldehyde in the presence of thionylchloride in absolute ethanol form substituted 4’-hydroxy chalcones (Eddarir, 2003).



A mixture of 2-acetyl thiophene substituted aldehydes was stirred in ethnol then an aqueous solution of KOH was added to form chalcones (Romanelli *et al.* 2011).



1. General method of synthesis of chalcone derivatives (1a-1p)

Chalcones are synthesized by Claisen-Schmidt condensation (Furniss *et al.*, 1989; Kumar *et al.*, 2010) of aldehyde and ketone by base catalyzed or acid catalyzed followed by dehydration to yield chalcones (Figure 1).

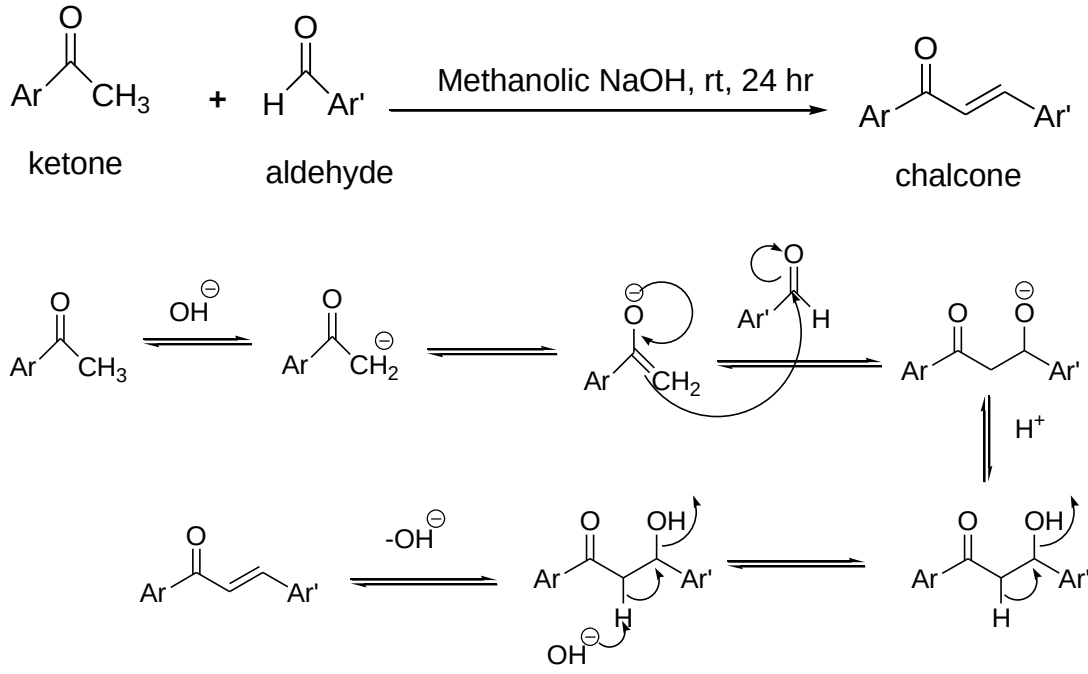


Figure 1. : Mechanism of reaction for synthesis of chalcone derivatives (1a-1p)

The synthesis of the designed compounds (**2a-2p, 3a-3p**) was performed in a manner as outlined in Figure 2-3 and Table 1.

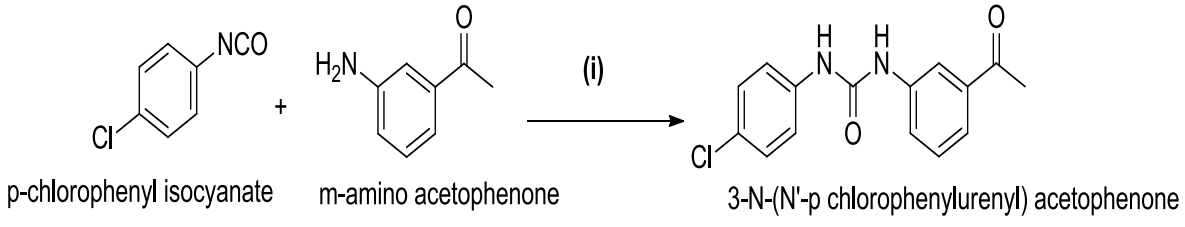


Figure. 2: The synthesis of the designed compounds 1a-1h, 2a-2h, 3a-3h (i) Me_2CO, rt, 6 hr (ii) substituted benzaldehyde, methanolic NaOH, stirred at room temperature, 24 hr (iii) *n*-butanol, reflux (iv) thiosemicarbazide, EtOH, ACOH, reflux.

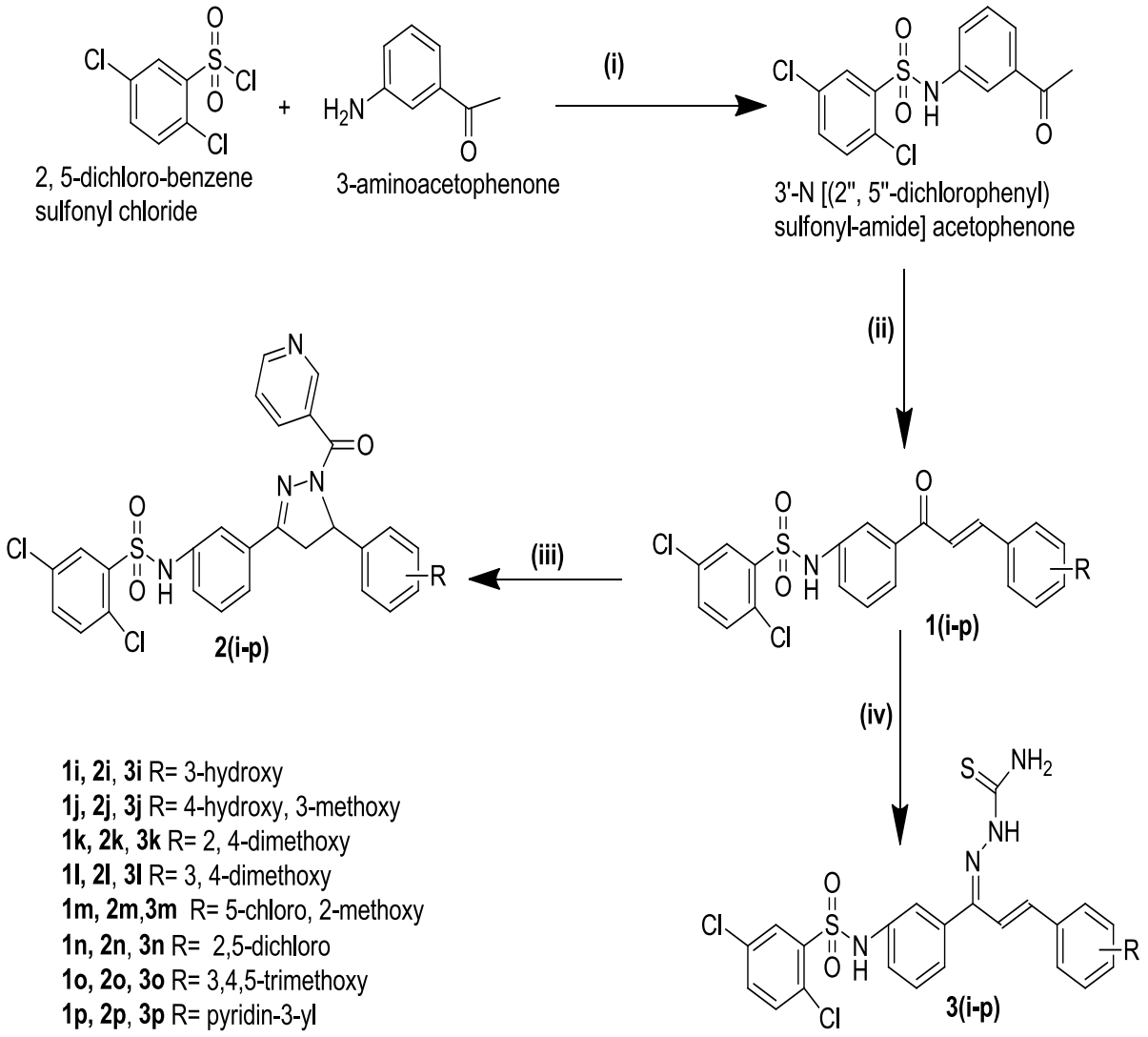
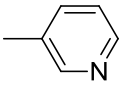
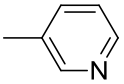


Figure. 3: The synthesis of the designed compounds 1i-1p, 2i-2p, 3i-3p (i) $CHCl_3$, rt, 3-6 hrs (ii) substituted benzaldehyde, methanolic NaOH, stirred at room temperature, 24 hr (iii) *n*-butanol, reflux (iv) thiosemicarbazide, EtOH, ACOH, reflux.

Table.1: Different substitutions on new synthesized substituted Chalcones and pyrazolines compounds (1a- 1p, 2a-2p, 3a-3p)

S.No	Comp. No.		R_2	R_3	R_4	R_5
1	**2a**	**3a**	-	OCH_3	-	-
2	**2b**	**3b**	-	OCH_3	OH	-
3	**2c**	**3c**	OCH_3	-	OCH_3	-
4	**2d**	**3d**	-	OCH_3	OCH_3	-
5	**2e**	**3e**	OCH_3	-	-	Cl
6	**2f**	**3f**	Cl	-	-	Cl
7	**2g**	**3g**	-	OCH_3	OCH_3	OCH_3
8	**2h**	**3h**				
9	**2i**	**3i**	-	OCH_3	-	-
10	**2j**	**3j**	-	OCH_3	OH	-
11	**2k**	**3k**	OCH_3	-	OCH_3	-
12	**2l**	**3l**	-	OCH_3	OCH_3	-
13	**2m**	**3m**	OCH_3	-	-	Cl
14	**2n**	**3n**	Cl	-	-	Cl
15	**2o**	**3o**	-	OCH_3	OCH_3	OCH_3
17	**2p**	**3p**				

2. Synthesis of intermediates

Synthesis 3-N-(N'-p-chlorophenylurenyl)acetophenone

Synthesis of methyl ketone derivative was carried out by making *m*-amino acetophenone react with the *p*-chlorophenyl isocyanate. A mixture of the *m*-aminoacetophenone (2.7 g, 20 mmol) and p-chlorophenyl isocyanate (3 g, 20 mmol) was dissolved in dry acetone (100 mL). The mixture was stirred for 6-7 hr at room temperature, filtered, and the crude compound urenylacetophenone was recrystallized using ethanol (Sonmez *et al.*, 2011).

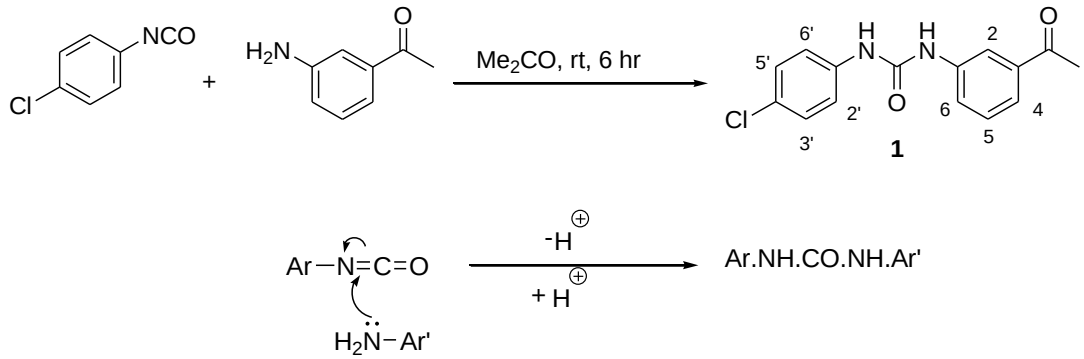


Figure 4: Scheme for synthesis of 3-N-(N'-p-chlorophenylurenyl)acetophenone

Yield 3.3 g, 58%, White solid; mp 272-274 ^{o}C; IR(KBr) ν_{max} /cm^{-1} 3372 (N-H), 3056 (ArC-H), 2962 2872 (C-H), 1711 ($COCH_3$), 1645 (C=O), 1614, 1534, 1461 (Ar C=C), 1515, 1290, 1185 (ArC-N), 1147 (Ar-Cl) 756, 687 (Ar); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ_H 9.12 (br s, 1H, NH), 8.91 (br s, 1H, NH); 8.18 (1H, s, H-2), 7.78 (1H, d, *J* 5.9, H-6), 7.53 (3H, m, H-4, 2', 6'), 7.30 (1H, t, *J* 6.30, H-5), 7.21 (2H, d, *J* 6.65, H-3', 5'), 2.53 (s, 3H, 3-$COCH_3$).

Synthesis of 3'-N [(2'', 5''-dichlorophenyl) sulfonyl-amide] acetophenone

The intermediate compound 3'-N[(2'',5''-dichlorophenyl) sulfonyl-amide] acetophenone was synthesized adopting the procedure described by Leon *et al*. (2007) with some modifications (Figure 4).

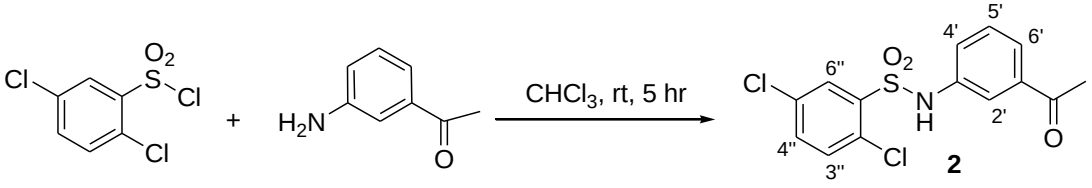


Figure 5 : Scheme for synthesis of 3'-N[(2'',5''-dichlorophenyl) sulfonyl-amide] acetophenone

A mixture of 3-aminoacetophenone (2.7 g, 20 mmol) and 2, 5-dichloro-benzene sulfonyl chloride (4.9 g, 20 mmol) in 5 mL of chloroform was stirred at room temperature (rt) for 3–6 hr. The resulting precipitate was washed with acetone, filtered, and the crude

material obtained was recrystallized in acetonitrile to give pure compound 3'-N[(2'',5''-dichlorophenyl) sulfonyl-amide] acetophenone.

Yield 3.6 g, 52%, Brown crystals; mp 230–232 °C; IR 3216 (N-H); 1667 (C=O); 1715 ($COCH_3$), 1337, 1270 (SO_2), 1142 (Ar-Cl), 3060 (Ar-H), 2967 (C-H), 1584, 1461, 1357, 1297, 1273, 1166, 993, 852, 819, 795, 720 (Ar); ^{1}H-NMR: δ_H 11.38 (s, 1H, NH), 7.94 (1H, s, H-6´´), 7.70 (1H, d, *J* 8.44, H-3´´), 7.25-7.44 (3H, m, H-2´, 5´, 6'), 7.71 (d, 1H, *J* 6.42, H-4´´), 6.94 (1H, d, *J* 8.91, H4'), 2.51 (s, 3H, CH_3CO).

General procedure for the synthesis of chalcone derivatives (1a-1p)

To a solution of substituted acetophenone (16 mmol) in 10 mL of methanol on an ice bath, freshly prepared 2 N methanolic NaOH solution (60 mL) was added and stirred for 10 min. To this, appropriate aldehyde (16 mmol) was added and stirred at room temperature for 12-24 hr. The reaction mixture was cooled on an ice bath, neutralized with diluted HCl and the precipitate was washed three times with 50 mL distilled water to give the crude product. The product was recrystallized from methanol or ethanol/ water.

The purity of the product was checked by TLC using ethyl acetate and hexane (4:6) as mobile phase and iodine vapors as detecting agent.

(E)-1-(4''-chlorophenyl)-3-(3-(3'-(3-hydroxyphenyl)acryloyl)phenyl)urea (1a)

(E)-1-(4''-chlorophenyl)-3-(3-(3'-(4-hydroxy-3-methoxyphenyl)acryloyl)phenyl)urea (1b)

Synthesis of (E)-1-(4''-chlorophenyl)-3-(3-(3'-(2,4-dimethoxyphenyl)acryloyl) phenyl)urea (1c)

Synthesis of (E)-1-(4''-chlorophenyl)-3-(3-(3'-(3,4-dimethoxyphenyl)acryloyl) phenyl)urea (1d)

Synthesis of (E)-1-(3-(3-(5-chloro-2-methoxyphenyl)acryloyl)phenyl)-3-(4-chloro-phenyl)urea (1e)

Synthesis of (E)-1-(4''-chlorophenyl)-3-(3'-(3-(2,5-dichlorophenyl)acryloyl) phenyl)urea (1f)

Synthesis of (E)-1-(4''-chlorophenyl)-3-(3'-(3-(3,4,5-trimethoxyphenyl) acryloyl) phenyl)urea (1g)

Synthesis of (E)-1-(4''-chlorophenyl)-3-(3'-(3-(pyridin-3-yl)acryloyl) phenyl) urea (1h)

Synthesis of (E)-2'',5''-dichloro-N-(3'-(3-(3-methoxyphenyl)acryloyl)phenyl)benzene sulfonamide (1i)

Synthesis of (E)-2'',5''-dichloro-N-(3'-(3-(3-hydroxy,4-methoxyphenyl)acryloyl)phenyl) benzenesulfonamide (1j)

Synthesis of (E)-2",5"-dichloro-N-(3'-(3-(2,4-dimethoxyphenyl)acryloyl)phenyl) benzene sulfonamide (1k)
Synthesis of (E)-2",5"-dichloro-N-(3'-(3-(3,4-dimethoxyphenyl)acryloyl) phenyl) benzene sulfonamide (1l)
Synthesis of (E)-2",5"-dichloro-N-(3'-(3-(5-chloro-2-methoxyphenyl)acryloyl)phenyl) benzenesulfonamide (1m)
Synthesis of (E)-2",5"-dichloro-N-(3'-(3-(2,5-dichlorophenyl)acryloyl)phenyl) benzenesulfonamide(1n)
Synthesis of (E)-2",5"-dichloro-N-(3'-(3-(3,4,5-trimethoxyphenyl)acryloyl)phenyl)benzene sulfonamide (1o)

Synthesis of (E)-2",5"-dichloro-N-(3'-(3-(pyridin-3-yl)acryloyl)phenyl) benzene sulfonamide (1p)**]**

General method for synthesis of 1, 3, 5-trisubstituted pyrazolines (2a-2p)

1,3,5-trisubstituted pyrazolines (**2a-2p**) were synthesized according to the scheme depicted in Figure 5 (Ozdemir *et al.*, 2008). In this method, chalcone and nicotinic acid hydrazide were refluxed in *n*-butanol in order to synthesize the desired product (Kini and Gandhi, 2008). Factors such as the structure and position of the substituents have profoundly influenced the rate of the reaction. The generally accepted interpretation of this reaction, involves the initial formation of an aryl hydrazone with subsequent nucleophilic attack of nitrogen upon the carbon-carbon double bond at position. Hence the electropositive nature of carbon may control the overall rate of the reaction. The electropositive nature of carbon is controlled by the aromatic ring directly connected to it. Halogens being electron withdrawing in nature significantly increase the positive character of carbon lead to faster reaction while electron donating alkyl and alkoxy groups contributed for slower reaction.

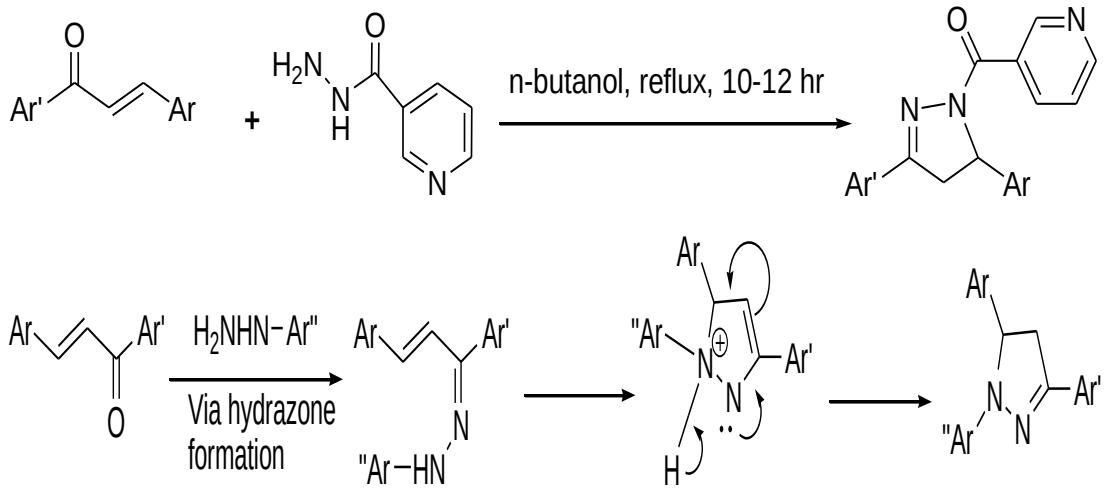


Figure 6. Scheme and mechanism of reaction for synthesis of compounds (2a-2p)

To the solution of the appropriate chalcone **1a-1p** (4 mmole) in 10 mL of *n*-butanol, (0.55 g, 4 mmole) of nicotinic acid hydrazide was added and the reaction mixture was refluxed for 8–10 hr. The excess of solvent was removed under reduced pressure and the reaction mixture was cooled on an ice bath. The products precipitated out at low temperature were washed five times with 50 mL distilled water, reconstituted in minimum amount of methanol and dried under reduced pressure. This product was further purified by crystallization from the ethanol-DMF mixture (1:1). Purity of the products was checked by TLC using mixture of acetone and petroleum ether (40:60 V/V) as mobile phase.

[a-(4”-chlorophenyl)-c-(3-(5''-(3'-hydroxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)urea (2a)

a-(4”-chlorophenyl)-c-(3-(5''-(4’-hydroxy,3'-methoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)urea (2b)

a-(4”-chlorophenyl)-c-(3-(5-(2',4'-dimethoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)urea (2c)

a-(4”-chlorophenyl)-c-(3-(5-(2',4'-dimethoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)urea (2d)

a-(4”-chlorophenyl)-c-(3-(5-(3',4'-dimethoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)urea (2e)

a-(4”-chlorophenyl)-c-(3-(5-(2',5'-dichloroyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)urea (2f)

a-(4”-chlorophenyl)-c-(3-(5-(3',4’,5’-trimethoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)urea (2g)

a-(4”-chlorophenyl)-c-(3-(5-(pyridine-3’-yl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)urea (2h)

2”,5”-dichloro-N-(3-(5-(3'-hydroxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzenesulphonamide (2i)

2”,5”-dichloro-N-(3-(5-(4’-hydroxy,3'-methoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzenesulphonamide (2j)

2”,5”-dichloro-N-(3-(5-(2',4'-dimethoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzenesulphonamide (2k)

2”,5”-dichloro-N-(3-(5-(3',4'-dimethoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzenesulphonamide (2l)

2”,5”-dichloro-N-(3-(5-(5’-chloro,2'methoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzenesulphonamide (2m)

2”,5”-dichloro-N-(3-(5-(2’,5’-dichlorophenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzenesulphonamide (2n)
2”,5”-dichloro-N-(3-(5-(3',4’,5’-trimethoxyphenyl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzenesulphonamide (2o)
2”,5”-dichloro-N-(3-(5-(pyridine-3’-yl)-1-nicotinoyl-4,5-dihydro-1H-pyrazol-3-yl)phenyl)benzenesulphonamide (2p)**]**

General procedure for synthesis of thiosemicarbazide derivatives (3a-p)

A mixture of chalcones (**3a**–**3p**) (0.5 mmol) and thiosemicarbazide (0.5 mmol) in hot ethanol (50 mL) had a few drops of concentrated hydrochloric acid added. The reaction mixture was stirred at reflux temperature for 2–6 h, and monitored by TLC using hexane:ethyl acetate (8:2) as the eluent. Afterwards, the precipitate was filtered off and the crude product purified by recrystallization from ethanol, resulting in the target compounds (**3a**–**p**).

(Z)-2-((E)-1-(3-(3-(4-chlorophenyl)ureido)phenyl)-3-(3hydroxyphenyl)allylidene) hydrazine carbothioamide (3a)
(Z)-2-((E)-1-(3-(3-(4-chlorophenyl)ureido)phenyl)-3-(4-hydroxy-3-methoxyphenyl) allylidene)hydrazine carbothioamide (3b)
(Z)-2-((E)-1-(3-(3-(4-chlorophenyl)ureido)phenyl)-3-(2,4-dimethoxyphenyl)allylidene) hydrazinecarbothioamide (3c)
(Z)-2-((E)-1-(3-(3-(4-chlorophenyl)ureido)phenyl)-3-(3,4-dimethoxyphenyl)allylidene) hydrazinecarbothioamide (3d)
(Z)-2-((E)-3-(5-chloro-2-methoxyphenyl)-1-(3-(3-(4-chlorophenyl)ureido)phenyl) allylidene)hydrazinecarbothioamide (3e)
(Z)-2-((E)-1-(3-(3-(4-chlorophenyl)ureido)phenyl)-3-(2,5-dichlorophenyl)allylidene) hydrazinecarbothioamide (3f)
(Z)-2-((E)-1-(3-(3-(4-chlorophenyl)ureido)phenyl)-3-(3,4,5-trimethoxyphenyl) allylidene)hydrazinecarbothioamide (3g)
(Z)-2-((E)-1-(3-(3-(4-chlorophenyl)ureido)phenyl)-3-(pyridin-3-yl)allylidene)hydrazine carbothioamide (3h)
(Z)-2-((E)-1-(3-(2,5-dichlorophenylsulfonamido)phenyl)-3-(3-hydroxyphenyl) allylidene)hydrazinecarbothioamide (3i)

(Z)-2-((E)-1-(3-(2,5-dichlorophenylsulfonamido)phenyl)-3-(4-hydroxy-3methoxy phenyl) allylidene)hydrazinecarbothioamide (3j)

(Z)-2-((E)-1-(3-(2,5-dichlorophenylsulfonamido)phenyl)-3-(2,4-dimethoxyphenyl) allylidene)hydrazinecarbothioamide (3k)

(Z)-2-((E)-1-(3-(2,5-dichlorophenylsulfonamido)phenyl)-3-(2,4-dimethoxyphenyl) allylidene)hydrazinecarbothioamide (3l)

(Z)-2-((E)-3-(5-chloro-2-methoxyphenyl)-1-(3-(2,5-dichlorophenylsulfonamido) phenyl)allylidene)hydrazinecarbothioamide (3m)

(Z)-2-((E)-3-(2,5-dichlorophenyl)-1-(3-(2,5-dichlorophenylsulfonamido)phenyl) allylidene) Hydrazine carbothioamide (3n)

(Z)-2-((E)-1-(3-(2,5-dichlorophenylsulfonamido)phenyl)-3-(3,4,5-trimethoxyphenyl) allylidene)hydrazinecarbothioamide (3o)

(Z)-2-((E)-1-(3-(2,5-dichlorophenylsulfonamido)phenyl)-3-(pyridin-3-yl)allylidene) hydrazine carbothioamide (3p)

3. Pharmacological Evalution

Antileishmanial activity

3.1 In vitro screening Anti-promastigote activity

The effect of compounds on the viability of Leishmania promastigotes was assessed by monitoring the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] metabolism (Sigma Chemical Co.) after a 96 h culture period in the presence of the respective compounds. Parasites in stationary culture stage were seeded at 1 × 106 /100 μL medium 199 per well in 96-well flat bottom microtitre plates (Cellstar). Further 100 μL of medium 199 per well with different concentrations of test compounds or drug standard, dissolved in DMSO (Gupta et al., 2005) were added in triplicate to achieve desired concentrations (12.5 – 200 μg mL–1). Parallel dilutions of DMSO alone did not affect the parasite growth. The plates were incubated at 25 °C for 92 h prior to MTT (20 μL per well of a 5 mg mL–1 PBS stock) addition and then for further 4–5 hours. MTT processing was

stopped and formazan crystals solubilized by adding 50 µL per well acidified 20% SDS (Qualigens, India) and incubating overnight at 37 °C. The relative amount of formazan per well produced by viable cells was measured photometrically at 570 nm. Two independent experiments were performed for the determination of sensitivity of each compound. As a control, the activity of each compound was determined, and no substantial interaction was found.

3.2 *Cytotoxicity assay*

The cell viability was determined using the MTT assay. J774.A-1 cell line were maintained in RPMI medium (Sigma), supplemented with 10% Foetal Calf Serum and 40mg/mL gentamycin. Exponentially growing cells (1 $\times 10^4$ cells /100µL/well) were incubated with different drug concentrations for 72 hours and were incubated at 37ºC in a humidified mixture of CO_2 and 95 % air in an incubator. Stock solutions of compounds were initially dissolved in DMSO and further diluted with fresh complete medium. After incubation, 25 µL of MTT reagent (5mg/mL) in PBS medium, followed by syringe filtration were added to each well and incubated at 37°C for 2 hours. At the end of the incubation period, the supernatant were removed by tilting plate completely without disturbing cell layer and 150 µL of pure DMSO are added to each well. After 15 min of shaking the readings were recorded as absorbance at 544 nm on a micro plate reader. The cytotoxic effect were expressed as 50% lethal dose, i.e., as the concentration of a compound which provoked a 50% reduction in cell viability compared to cell in culture medium alone.

3.3 *Anti amastigote (semi - in vivo) activity*

For assessing the activity of compounds against the amastigote stage of the parasite, mouse macrophage cell line (J-774A.1) infected with promastigotes in stationary culture

stage were used. Cells were seeded in a 16 well chamber slides (Nunc) ($5x10^4$ cell/100µL/well) in RPMI1640 containing 10% foetal calf serum and the slides were incubated at 37°C in a CO_2 incubator. After 24h, the medium was replaced with fresh medium containing stationary– phase promastigotes ($2.5x10^5$ /100µL/well). Promastigotes invade the macrophage and are transformed into amastigotes. At 24hr of internalization of promastigotes, test material in appropriate concentrations (2.5-50µg/mL) in complete medium was added after replacing the previous medium and the plates were incubated at 37^oC in a CO_2 incubator for 72 hrs. After incubation, the drug containing medium was decanted and cells are fixed with methanol and stained with 5% geimsa stain for 45 min and at least 100 infected macrophages per sample were counted under optical microscope. Efficacy was expressed as percent inhibition of amastigote multiplication using formula:

$$\text{Percentage Inhibition (PI)} = \frac{\text{PT} \times 100}{\text{PC}}$$

PI : Percent inhibition of amastigote multiplication.

PT : Average number of amastigotes/100 macrophage cells in treated groups

PC : Average number of amastigotes/100 macrophage cells in control groups

3.4 *In vivo evaluation:*

For *in vivo* evaluation of compounds, the method of Beveridge (1963) as modified by Bhatnagar *et al.* (1989) and Gupta *et al.* (2002) was employed. Male hamsters weighing 35–40 g each were infected with 1×10^7 amastigotes and the intensity of infection after 20 days was assessed by spleen biopsy. Animals with 2^+ infections (5–15 amastigotes per 100 cell nuclei) were selected for screening the compounds. The infected animals were randomized into several groups on the basis of their parasitic burdens. Usually four to six animals were used for each compound and the same numbers were kept as untreated controls. The drug

treatment was given by intraperitoneal route / oral route for five consecutive days at 50 mg kg^{-1} dose level. To assess the effect of test compounds, spleen biopsies were performed on each animal after 7 days of last drug administration and amastigote counts were assessed by Giemsa staining. The percentage inhibition in amastigote multiplication was calculated using the following formula:

P.I. = 100 − ANAT × 100/ INAT × TIUC

P.I. = Percentage inhibition of amastigote multiplication.

ANAT = Actual no. of amastigotes in treated animal.

INAT = Initial no. of amastigotes in treated animals.

TIUC = Times increase of parasites in untreated control animals.

Selective Index: The selective Index (S.I.) were calculated using the following equation:

S.I. = IC_{50} (J774.A-1Cells) / IC_{50} *(Leishmania amastigotes*)

3.5 *Data analysis*

IC_{50} was calculated by Probit analysis (Finney, 1971). Compounds with more than 15 mg/ml IC_{50} were considered as inactive while compounds with IC_{50} between 15 and 5 mg/ml were considered as moderately active and less than 5 mg/ml are highly active compounds.

Antileishmanial Activity

The antileishmanial activity of the pyrazoline derivatives **(2a-2p, 3a-3p)** against a clinically derived strain of *L.donovani* is shown in Table 1.

The *in vitro* efficacy of the synthesized compounds on promastigotes and amastigotes of *leishmania donovani* were assessed by a previously described method in chapter 4. The antileishmanial activity of the pyrazoline derivatives **(2a-2p, 3a-3p)** against a clinically

derived strain of *L.donovani* is shown in Table 2 while compounds having promastigote inhibition more than 80% were screened against amastigotes and their IC_{50} was calculated in as shown in Table 2

Table 2 Antileishmanial *in vitro* activity against luciferase–promastigote system

S.No.	Compound	% Inhibition at 10 µg/ml promastigote
1	2a	90
2	2b	87.99
3	2c	94.93
4	2d	88.79
5	2e	90.92
6	2f	NI
7	2g	NI
8	2h	100
9	2i	100
10	2j	97.83
11	2k	NI
12	2l	NI
13	2m	NI
14	2o	100
15	2p	100
16	3a	83.94
17	3b	NI
18	3c	97.63
19	3d	98.37
20	3e	87.65
21	3f	NI
22	3g	NI
23	3h	85.98
24	3i	100
25	3j	98.87
26	3k	NI
27	3l	NI
28	3m	NI
29	3o	100
30	3p	100

NI: no inhibition.

Pentamidine shows 85–90% inhibition against promastigotes at 0.5 mg/ml.
SSG (sodium stibogluconate) shows 40–50% inhibition against promastigotes at 940 mg/ml.

The data in Table 3 suggests that pyrazoline derivatives represent interesting leads as antileishmanial agents. All the pyrazoline derivatives showed 80-100% inhibition against promastigotes.

Table 3: *in vitro* (against MQ amastigotes) and *in vivo* antileishmanial activity

S. no.	Compound no.	In vitro antiamastigote activity IC_{50} (µg/ml)	Cytotoxicity against J774A.1 cell lines CC_{50} (µg/ml)	S.I. (selectivity index) CC_{50}/IC_{50}
1	2a	11.83	20.81	
2	2b	6.93	17.92	
3	2c	10.83	NA	
4	2d	5.93	NA	
5	2e	13.84	27.92	
8	2h	8.37	NA	
9	2i	NA	10.52	
10	2j	NA	27.37	
14	2o	16.18	NA	
15	2p	9.73	NA	
16	3a	9.28	13.86	
18	3c	15.92	18.56	
19	3d	17.29	NA	
20	3e	NA	82.65	
23	3h	3.81	NA	
24	**3i**	13.37	NA	
25	3j	15.47	NA	
29	3o	16.85	50.72	
30	**3p**	16.97	15.34	
	Pentamidine	13.37	35.92	
	SSG	55.72	307.82	

4. Conculsion

In conclusion, novel pyrazoline derivatives **(2a-2p, 3a-3p)** were synthesized and their antileishmanial activity against *Leishmania donovani* was evaluated. Compound **2p** and **3p**

showed better activity in comparison to Pentamidine and Sodium Stibogluconate. As a consequence of the above results and considerations, these molecules can serve as promising prototypes for the development of potent antileishmanial agents.

Reference:

- Di Carlo, G.; Mascolo, N.; Izzo, A.A.; Capasso, F. Flavonoids: Old and new aspects of a class of natural therapeutic drugs. *Life Sci.*, **1999**, *65*, 337-353.
- Eddarir, S.; Cotelle, N.; Bakkour,Y.; Rolando, C. An efficient synthesis of chalcones based on the Suzuki reaction. Tetrahedron Lett., 2003, 44, 5359-5363.
- Furniss, B.S.; Hannaford, A.J.; Rogers, V.; Smith, P.W.G.; Tatchell, A.R. (1989). Vogel's Textbook of Practical Organic Chemistry, 5th ed.; Longman Group Limited: New York, p. 1034.
- Guida, A.; Lhouty, M. H.; Tichit, D.; Figueras, F.; Geneste, P. Hydrotalcites as base catalysts. Kinetics of Claisen-Schmidt condensation, intramolecular condensation of acetonylacetone and synthesis of chalcone. *Appl. Catal. A*, **1997**, *164*, 251-264
- Kini, S.; Gandhi, A.M. (2008). Novel 2-pyrazoline derivatives as potential antibacterial and antifungal agents. Indian J. Pharm. Sci. 70, 105-108.
- Leon, C.; Rodrigues, J.; Dominguez, N.; Rosenthal, P.J.; Dominguez, J.N. (2007). Synthesis and evaluation of sulfonylurea derivatives as novel antimalarials. Eur. J. Med. Chem. 42, 735-742.
- Nielsen, S.F.; Boesen, T.; Larsen, M.; Schønning K.; Kromann, H. Antibacterial chalcones—bioisosteric replacement of the 4'-hydroxy group. *Bioorganic & Medicinal Chemistry*, **2004**, 12, 3047–3054
- Ozdemir, A.; Turan-Zitouni, G.; Kaplancikli, Z.M. (2008). Novel analogues of 2-pyrazoline: Synthesis and antimycobacterial evaluation. Turk. J. Chem. 32, 529-538.

- Romagnoli, R.; Baraldi, P.G.; Carrion, M.D.; Cruz-Lopez, O.; Cara, C.L.; Balzarini, J.; Hamel, E.; Canella, A.; Fabbri, E.; Gambari, R.; Basso, G.; Viola, G. Hybrid α-bromoacryloylamido chalcones. Design, synthesis and biological evaluation. Bioorganic & Medicinal Chemistry Letters, 2009,19, 2022–2028.

- Sonmez, F.; Sevmezler, S.; Atahan, A.; Ceylan, M.; Demir, D.; Gencer, N.; Arslan, O.; Kucukislamoglu, M. (2011). Evaluation of new chalcone derivatives as polyphenol oxidase inhibitors. Bioorg. Med. Chem. Lett. 21, 7479-7482.

- Sreedhar NY, MR Jayapal,K Seenivasa Prasad and P Reddy Prasad. Res. J. Pharm Bio. Chemical Sci. 2010; 1(4): 480-484.

- Thomas L, David A Willams, Victoria F Roche, Willams S Zito. Foyes Principle of Medicinal chemistry. 6th ed. Newyork: Lippincott publicators; 1998.