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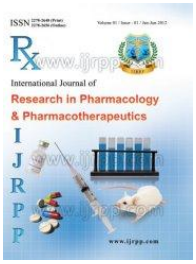

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In-vitro anti-tuberculosis, anti-inflammatory and anti-oxidant screening for certain synthesized N-Phenyl-3-Phenyl-5-Substituted Phenyl Pyrazoline and 4-Phenyl-6-Substituted phenyl-3, 4-Dihydro Pyrimidine-2-one analogues

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ABSTRACT

One series of N-phenyl pyrazoline analogues (K1-K5) were synthesized by reaction of substituted chalcones (C1-5) with phenyl hydrazine in acidic medium. The second series of 3, 4-dihydropyrimidine-2-one analogues (K6-K10) were synthesized by reaction of substituted chalcones (C1-5) with ethanolic urea in alkali medium. These substituted chalcones (C1-5) was prepared by reaction of acetophenone (a) and aromatic aldehydes (b1-5). The yield of the synthesized analogues ranged from 62-76%. The *in-vitro* anti-oxidant, anti-inflammatory and anti-tuberculosis screenings was performed. The result of all heterocyclic analogues (K1-K10) indicates that have significant *in-vitro* anti-oxidant, anti-inflammatory activity when compared to standard drugs. Among those analogues K1, K3, K6 & K8 were showed potent and analogues K2 & K7 were registered comparably good anti-oxidant and anti-inflammatory activities. The analogue K3 only exhibited potent activity against *Mycobacterium tuberculosis*. The analogues K1 and K6 exhibited moderate *in-vitro* anti-tuberculosis activity.

Keywords: Pyrazoline, Pyrimidine, Chalcones, Anti-oxidant, Anti-inflammatory and Anti-tuberculosis

INTRODUCTION

The nitrogen containing heterocyclic's drugs exhibited wide range of pharmacological properties owing to their high reactivity in various biochemical processes [1]. Among that pyrazole and pyrimidine derivatives are attracted due to its fascinating biological activities. The pyrimidine heterocyclic's derivatives comprise the ring system of numerous drugs barbiturates, zidovudine, 5- fluorouracil & idoxuridine [2] and it is found in a number of naturally occurring compounds such as water soluble vitamins riboflavin, thiamine and folic acid [3] are essential for the body. The presence of DNA and RNA pyrimidine base in thymine, cytosine and uracil, which are the essential building blocks of nucleic acids, is the major reason for their high

therapeutic activities. The pyrimidine nucleus derived synthetic products has different medical uses such as antihypertensive, antibacterial, antifungal, anticonvulsant, anti-inflammatory[4], cyclo oxygenase inhibitor[5], antitumor [6], antitubercular, alpha glucosidase inhibitors[7] and anti-oxidant properties. The chalcones derivatives are an important intermediate of synthetic pathways [8], has been shown to exhibit diverse biological and pharmacological activities [9,10, 11]. The pyrazole ring is present in a variety of leading drugs such as celecoxib, rimonabant, phenylbutazone, 2-lonazolac, 3-zubrin, ionazlac and difenamizole. The sildenafil (viagra) drug possess both pyrazole and pyrimidine fused ring system.The pharmacological importance of these pyrazole compounds lies in the fact that they can be effectively utilized as antimicrobial, analgesic, anti-Inflammatory [12], antiviral, antiparasitic, anthelmintic [13], antitubercular, anticancer [14], antiproliferative [15] and insecticidal agents. Some substituted pyrazolines and their derivatives have been reported to possess some interesting biological activities such as anticancer, insecticidal, antibacterial, antifungal, antidepressant, anticonvulsant, anti-inflammatory, antimalarial [16] and anti- tumor properties [17, 18, 19]. Hence the synthesis of new derivatives of pyrazolines and pyrimidine heterocyclic's is being continuously reported with specifying wide variety of pharmacological activities [20, 21, 22]. In this way our present work involves synthesis of N-phenyl pyrazoline with phenyl substitution at third position and different substituted phenyl attachments at fifth position. Also our present work involves the synthesis of 3, 4-dihydropyrimidine with phenyl substitution at fourth position and different substituted phenyl attachments at sixth position, in order to exhibit fascinating pharmacological activities [23]. Our research work has been extended to screen in-*vitro* anti-oxidant, anti-inflammatory and anti-tuberculosis screening methods for the above synthesized pyrazoline and pyrimidine heterocyclic's analogues.

EXPERIMENTAL

Synthesis of 3-(Substituted phenyl)-1-phenylprop-2-en-1-one (Chalcones, C1-5)

The solution of 0.01mol acetophenone (a) and 0.01mol aromatic aldehydes (b1-5) named b1= 4-chloro benzaldehyde, b2 = 3,4,5-Trimethoxy benzaldehyde , b3 = 4-dimethyl benzaldehyde , b4=4-methoxy benzaldehyde, b5 = benzaldehyde, in ethanol (20 ml), sodium hydroxide (0.01M) was added at room temperature with constant stirring was maintained. The reaction mixtures were stirred further until a precipitate was formed. The reaction mixtures were diluted with ice water and neutralized by using (0.01M) diluted hydrochloric acid. The products 3-(4-chlorophenyl)-1-phenylprop-2-en-1-one (c1), 3-(3,4,5-methoxyphenyl)-1-phenyl prop-2-en-1- one (c2), 3-(4-(dimethyl amino) phenyl)-1-phenylprop-2-en-1- one (c3), 3-(4-methoxyphenyl)-1-phenylprop-2-en-1- one (c4) & 1,3-diphenylprop-2-en-1- one (c5) were filtered and recrystallized from ethanol.

Synthesis of 5-Substituted phenyl-1, 3-diphenyl-4, 5-dihydro-1H-pyrazoles (k 1-5).

The 0.01 mol of 3-(substituted phenyl)-1-phenyl prop-2-en-1-ones (C1-5) in 20 ml of 1, 4-dioxane and 0.024 mol of phenyl hydrazine was added. To these mixtures 2-3 drops of sulphuric acid was added and the contents were refluxed for 4 hrs, after the process 5ml of glacial acetic acid was added; again refluxed for next 2 hrs. On cooling to room temperature the contents were poured on crushed ice. As a result the solid products were 5-(4-chloro phenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole (k1), 5-(3,4,5-metoxyphenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole (k2), 5-(4-(dimethyl amino) phenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole (k3), 5-(4-methoxyphenyl)-1,3-diphenyl-4,5-dihydro-1H-pyrazole (k4) and 1,3,5-triphenyl-4,5-dihydro-1H-pyrazole (k5), were obtained which was recrystallized by using ethanol.

Synthesis of 6- substituted phenyl -4- phenyl -3,4-dihydropyrimidine-2-ones (k 6-10).

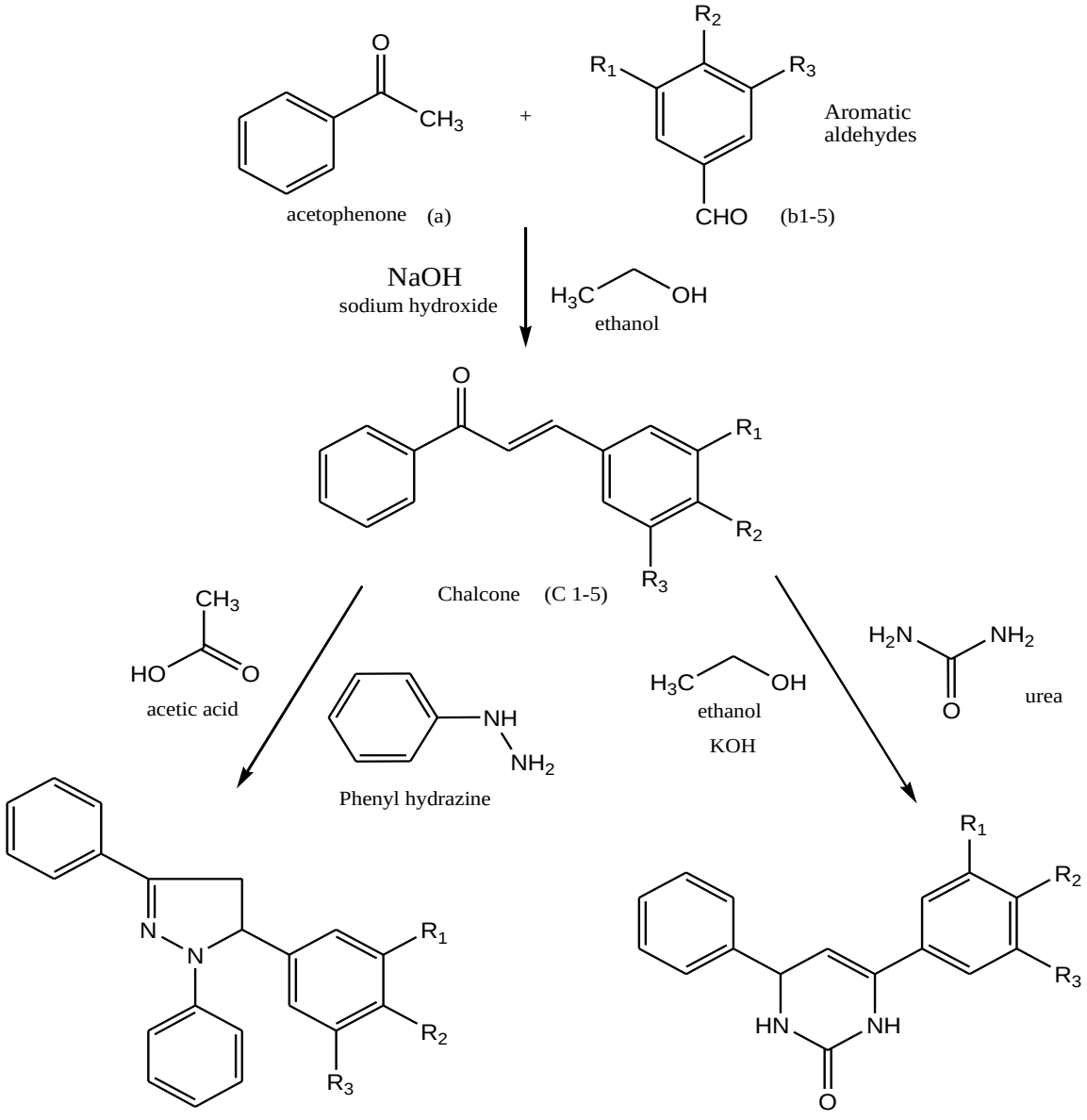
A mixture of 0.01Mol of 3-(substituted phenyl)-1-phenyl prop-2-en-1-ones (C1-5), 0.01Mol of urea in 25 ml absolute ethanol and 10% 5ml potassium hydroxide refluxed on a water bath for 8 hours. The reactions were monitored by TLC and the precipitation was recrystallized from absolute ethanol to give pure compounds named as 6-(4-chlorophenyl)-4-phenyl-3,4-dihydro pyrimidine-2(1H)-one (k6), 4-(phenyl-6-(3,4,5-trimethoxy phenyl)-3,4-dihydropyrimidin-2(1H)-one (k7), 6-[4-(dimethylamino)phenyl]-4-phenyl-3,4-dihydro pyrimidin-2(1H)-one(k8),6-(4-methoxyphenyl)-4-

phenyl-3,4-dihydropyrimidin-2(1H)-one(k9),4,6-diphenyl-3,4-dihydro pyrimidin-2(1H)-one (k10).

MATERIALS AND METHODS

The *in-vitro* anti-oxidant and anti-inflammatory screening methods were estimated by using ultraviolet-visible spectrophotometer. The Versa trek myco bottle, mycogrowth supplement, tubes with sterile saline, tuberculin syringes and sterile filter packs were used for *in-vitro* anti-tuberculosis screening. The melting points were taken by using an open capillary tube melting point apparatus. The analytical instruments such as FTIR spectra, 1HNMR, and MASS spectra were used for the characterization of the synthesized pyrazoline and pyrimidine heterocyclic's compounds (18). TLC analysis was carried out on commercially available silica gel plates of 0.5mm of thickness, as stationary phase. The mobile phase was used benzene: ethyl acetate in the ratio of 8:2 and the spots were visualized by UV chamber.

Scheme



K1 & K6 – R1& R3= H, R2 = Cl, K2 &K7– R1& R2& R3= OCH3, K3 & K8 – R1& R3= H, R2 =-N-(CH3)2, K4 & K9 – R1& R3= H, R2 = OCH3, K5 & K10 –R1& R2& R3= H.

Table -1 Physical data of the synthesized compounds

Analogues	Molecular Formula	Nature of Crystals	Soluble in	Molecular Weight	% Yield
K1	$C_{21}H_{17}ClN_2$	Brown solid	DMSO	332.82	74
K2	$C_{24}H_{24}N_2O_3$	Brown solid	DMSO	388.45	76
K3	$C_{23}H_{23}N_3$	Brown solid	DMSO	341.44	72
K4	$C_{22}H_{20}N_2O$	Brown solid	DMSO	328.40	69
K5	$C_{21}H_{18}N_2$	Brown solid	DMSO	298.38	65
K6	$C_{16}H_{13}C_lN_2O$	Yellow solid	DMSO	284.74	73
K7	$C_{19}H_{20}N_2O_4$	Yellow solid	DMSO	340.37	74
K8	$C_{18}H_{19}N_3O$	Orange solid	DMSO	293.36	68
K9	$C_{17}H_{16}N_2O_2$	Yellow solid	DMSO	280.32	65
K10	$C_{16}H_{14}N_2O$	Yellow solid	DMSO	250.29	62

PHARMACOLOGICAL SCREENING

IN-VITRO ANTI-OXIDANT ACTIVITY

Evaluation of antioxidant capacity by phosphomolybdenum method

The *in-vitro* antioxidant activity of the compounds was evaluated by the phosphor molybdenum method (19, 20). The assay is based on the reduction of Mo (VI) – Mo (V) by the analogues and subsequent formation of a green phosphate /Mo (V) complex at acid pH. A 0.3ml of analogues (100µg/ml, 200µg/ml and 300µg/ml) was combined with 3ml of reagent solution (0.6M sulfuric acid, 28M sodium phosphate and 4M ammonium molybdate). In case of blank 0.3ml of ethanol was used in place of compounds. The tubes containing the reaction solution were capped and incubated in a boiling thermostatically controlled water bath at 950C for 90 min. After cooling to room temperature, the absorbance of the solution was measured at 695nm using a UV-Visible spectrophotometer. The antioxidant capacity of each sample was compared with the absorbance of ascorbic acid as standard. The results displayed in Table 2 and Figure 1.

Table-2: *In-vitro* Anti-oxidant activity: Absorbance of synthesized analogues compared with standard Ascorbic acid

Analogues	Absorbance of different concentration		
	100µg/ml	200µg/ml	300µg/ml
K1	1.62±0.02	1.72±0.03	1.89±0.02
K2	1.34±0.06	1.63±0.06	1.82±0.09
K3	1.63±0.07	1.77±0.04	2.02±0.07
K4	1.34±0.03	1.45±0.04	1.62±0.04
K5	1.37±0.04	1.43±0.04	1.53±0.03
K6	1.53±0.03	1.62±0.03	1.88±0.02
K7	1.48±0.04	1.69±0.02	1.81±0.01
K8	1.5±0.03	1.8±0.03	2.01±0.05
K9	1.49±0.06	1.53±0.06	1.59±0.09
K10	1.16±0.05	1.35±0.06	1.55±0.06
Ascorbic acid	1.68±0.04	1.87±0.06	2.15±0.05

Evaluation of antioxidant capacity by phosphomolybdenum method

Absorbance of synthesized analogues compared with standard Ascorbic acid

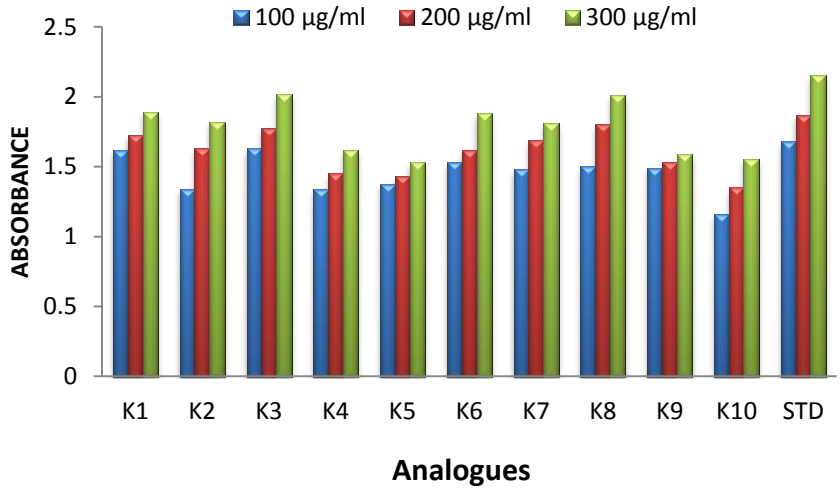


Figure 1. Evaluation of antioxidant capacity by phosphomolybdenum method Absorbance of synthesized analogues compared with standard Ascorbic acid

Evaluation of *in-vitro* anti-inflammatory activity by Membrane Stabilization Assay [21]

Preparation of HRBC suspension in isosaline

The human erythrocytes suspension was used for the *in-vitro* membrane stabilization assay. Blood was collected from healthy volunteers who had not consumed any NSAIDs for two weeks prior to the experiment. The blood was mixed with equal volume of Alsever solution (2% dextrose, 8.0% sodium citrate, 0.5% citric acid and 0.42% sodium chloride) and centrifuged at 3000 rpm. The packed cells were washed with isosaline and a 10% v/v erythrocyte suspension in isosaline was prepared.

Method

The assay mixture consist of 2 ml of 0.36% w/v hyposaline and 1ml of 0.2M sodium phosphate buffer (pH 7.4) and varying volumes of the extract (0.1 to 0.5 ml) and 0.5ml of 10% v/v HRBC suspension in isosaline, then the final volume were made up with isosaline up to 4.5 mL. The analogues 200µg/ml, 400µg/ml and 600 µg/ml concentrations were used. The control was prepared as mentioned above except the drug was omitted, while drug control was also prepared similarly but without HRBC suspension. The reaction mixture was incubated at 56ºC for 30 min in a water bath, then the tube was cooled under running water. Then the absorbance of the released hemoglobin was measured at 560 nm. Diclofenac 50µg/mL was used as a reference standard. The percentage of membrane stabilisation activity of the analogues were determined by the formula

% membrane stabilization = [A control- (A test - A product control)] / A control x 100

A control -Absorbance in control

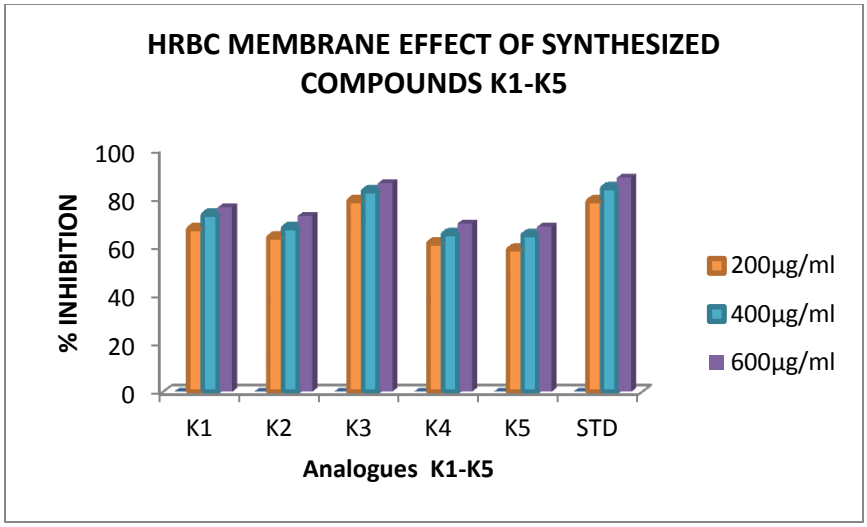
A test -Absorbance in test

A product control -Absorbance in product control

The results obtained for *in-vitro* membrane stabilization effect is presented in Table 3 & Figure 2

Table 3: ***In-vitro*** **anti-inflammatory activity by membrane stabilization assay**

Analogues	Percentage of activity		
	200µg/ml	**400µg/ml**	**600µg/ml**
K1	67.41±0.14	73.29±0.12	76.16±0.05
K2	63.69±0.08	67.71±0.09	72.66±0.08
K3	78.97±0.08	82.97±0.08	86.10±0.09
K4	61.44±0.05	65.13±0.07	69.54±0.06
K5	58.77±0.06	64.72±0.04	68.17±0.08
K6	70.09±0.08	74.41±0.09	78.20±0.09
K7	64.10±0.09	68.22±0.06	73.19±0.07
K8	76.25±0.02	80.19±0.06	84.22±0.08
K9	60.34±0.09	65.23±0.05	69.11±0.1
K10	56.96±0.08	62.27±0.09	66.37±0.08
Diclofenac	78.74±0.06	84.21±0.05	88.43±0.07



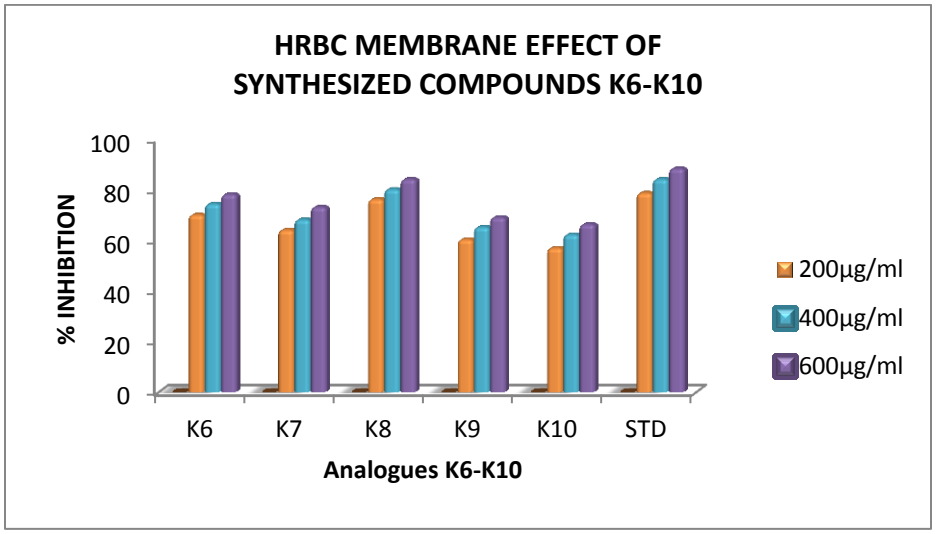


Figure 2 HRBC membrane effect of Synthesized compounds

In-vitro anti-tuberculosis activity

Evaluation of anti-tuberculosis activity by Mycobacterium tuberculosis susceptibility testing (22,23)

Preparation of drug solution (INH and Synthesized compounds)

25ml of sterile distilled water was added to each of three drug containing bottles. Swirl to dissolve the contents. Dilute 1:1 with sterile distilled water. Remove 5ml of the rehydrated drug solution and add to a sterile tube containing 15ml of sterile distilled water. Label as above (0.1µg/ml).

Preparation of Inoculum

Prepare a suspension of the test organism in tubes containing sterile saline and glass beads. Vortex well and allow the larger particles to settle for at least 30 minutes. Remove the upper half of suspension to a sterile tube and adjust with sterile saline, to a turbidity matching that of a 1.0 McFarland Standard. Dilute 1:10 with sterile saline. This suspension serves as the inoculum.

Inoculation of bottles

Add 0.5 ml of inoculum to each of the drug containing and control bottles. Inoculate a 7 H11 agar plate with a few drops of the inoculum to serve as a purity check. Invert the bottle several times to mix the contents. Each bottle place onto a connector.

Bottle Accessioning and Reading

Accession each Versa trek Myco bottle into the ESP Myco Culture system .Record the time to the nearest 9 days. Within 9 days drug and control bottle signals positive, remove from the system and confirm the presence of AFB by performing a kinyoun stain. The results displayed in Table 4 and Figure 3.

In-vitro Anti-tuberculosis Activity

Table-4: Anti-tuberculosis activity of analogues K1, K3, K6, K8

Analogues	Concentration	Day to positive	Result
K1	10µg/ml	5 days	Resistant
K3	10µg/ml	10 days	Sensitive
K6	10µg/ml	5 days	Resistant
K8	10µg/ml	4days	Resistant
Isoniazid	10µg/ml	11 days	Sensitive
Control	10µg/ml	4 days	NIL

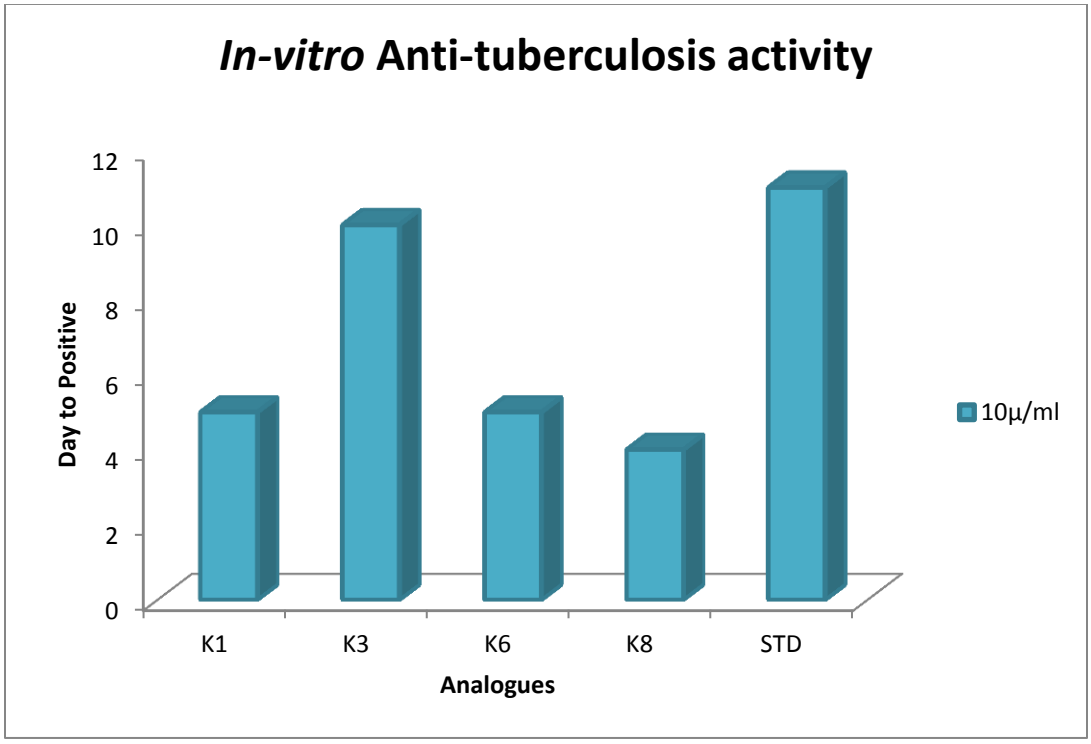


Figure: 3 In-vitro Anti-tuberculosis activity

RESULTS AND DISCUSSION

The two serious of N-phenyl pyrazoline derivatives (K1-K5) and 3,4-dihydropyrimidine derivatives (K6-K10) were synthesized from substituted chalcones (C1-5). The structures of the synthesized heterocyclic analogues were verified by IR, 1H-NMR, mass spectral data and physical analysis.

The *in-vitro* anti-oxidant and anti-inflammatory property for all the analogues showed positive significant results compared with standards. Among those screening methods, para dimethyl amino (K3) & para chloro (K1) substituted pyrazoline and para dimethyl amino (K8) & para chloro (K6) substituted pyrimidine analogues showed excellent potent anti-oxidant and anti-inflammatory activities when compared to the standard drugs. The trimethoxy

substituted pyrazoline (K2) and trimethoxy substituted pyrimidine (K7) analogues were also registered comparably good anti-oxidant and anti-inflammatory activities to the *in-vitro* screening methods. The data also indicates that para methoxy substituted pyrazoline (K4) & para methoxy substituted pyrimidine (K9) analogues also exhibited moderately considerable *in-vitro* anti-oxidant and anti-inflammatory activity than the substituted pyrazoline (K5) & substituted pyrimidine (K10) analogues when compared to the standard drugs ascorbic acid diclofenac respectively.

The analogues K1, K3, K6 & K8 showed more potent anti-oxidant activity so based on that *In-vitro* antioxidant activity the analogues K1, K3, K6 & K8 were selected and evaluated for *In-vitro* anti-tuberculosis activity. The para dimethyl amino substituted pyrazoline (K3) analogue only endowed with potent activity against *Mycobacterium tuberculosis*. The para chloro substituted pyrazoline (K1) and para chloro substituted pyrimidine (K6) analogues exhibited moderate *in-vitro* anti-tuberculosis activity than the para dimethyl amino substituted pyrimidine analogue (K8) when compared to the standard drug Isoniazid.

The structure activity relationship studies revealed that electron donating para dimethyl amino group substituted pyrazoline (K3) & pyrimidine (K8) analogues showed more potent anti-oxidant and anti-inflammatory activities than electron withdrawing para chloro group substituted pyrazoline (K1) & pyrimidine (K6) substituted analogues. The electron donating trimethoxy substituted pyrazoline (K2) and pyrimidine (K7) analogues were gave high anti-oxidant and anti-inflammatory activities than para-methoxy substituted pyrazoline (K4) and pyrimidine (K9) analogues. Any way electron donating and electron withdrawing substituent's (K1-K4 & K6-K9) were elevating *in-vitro* anti-oxidant and anti-inflammatory activities than unsubstituted analogues (K5 & K10).

The pyrazoline analogue (K3) exhibited peak activity against *Mycobacterium tuberculosis* than pyrimidine analogue (K8). Among this para dimethyl amino substituted pyrazoline analogue (K3) gives more *in-vitro* anti-tuberculosis activity than the para chloro substituted pyrazoline analogue (K1).

CONCLUSION

The molecules were designed by the software tools and the lead molecules of chalcone were synthesized by "*Claisen-Schmidt reaction*" followed by phenyl hydrazine and urea treatment forms N-phenyl pyrazoline and 3,4-dihydropyrimidine respectively. The formation of molecules was confirmed by TLC. Spectral structural elucidation details were conforming the molecular formula of all the synthesized heterocyclic's analogues.

In-vitro anti-oxidant and anti-inflammatory activities of all the compounds was evaluated and compared with standards. The analogues such as K1, K3, K6 & K8 showed more potent activity. Hence N-phenyl pyrazoline and 3,4-dihydropyrimidine with P-Chloro phenyl, para dimethyl amino phenyl substitution at fifth and sixth position respectively showed better activity. In Future, the analogues K1, K3, K6 & K8 can be studied for *in-vivo* anti-oxidant and anti-inflammatory activity as it exhibited significant *in-vivo* anti-oxidant and anti-inflammatory activity. Out of the above mentioned analogues only K3, showed high *in-vitro* anti-tuberculosis activity. Hence N-phenyl pyrazoline with para dimethyl amino phenyl substitution at fifth position exhibited better activity.

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