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Rational design of chalcone and pyrazole analogues inspired by curcumin: Synthesis and biological assessment

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Abstract

Chalcones, an important class of bioactive compounds, have gained significant attention due to their diverse pharmacological properties. In this study, a series of novel chalcone derivatives, inspired by the curcumin pharmacophore, were synthesized and further transformed into corresponding pyrazole derivatives. The structural modifications were aimed at enhancing their biological efficacy. The synthesized compounds were evaluated for their antibacterial and antifungal activity against various microbial strains, including drug-resistant bacteria. Notably, some derivatives exhibited promising antimicrobial potential, particularly against resistant pathogens. Additionally, selected molecules were screened for topoisomerase inhibition activity, revealing potential as enzyme inhibitors. The Structure-Activity Relationship (SAR) analysis highlighted key functional groups contributing to biological potency. The findings suggest that these novel chalcone and pyrazole derivatives could serve as promising scaffolds for antimicrobial and enzyme inhibitory drug development. Further investigations on their mechanistic aspects and pharmacokinetic properties may provide valuable insights into their therapeutic potential.

Keywords: Chalcone, antibacterial, antifungal, pyrazole, curcumin

Introduction

Chalcones are organic compounds belonging to the flavonoid family and are commonly recognized by their structure, characterized by two aromatic rings joined by a three-carbon α,β -unsaturated carbonyl system ^[1-3]. They have attracted significant attention due to their diverse biological and pharmacological activities. These molecules are well known to be found naturally in several plants and exhibited various beneficial properties, such as antioxidant, anti-inflammatory, antimicrobial, anticancer, and antiviral effects ^[4-9]. Because of their widespread presence in nature and multiple biological activities, chalcones have become important compounds for chemical synthesis and biological evaluations ^[10].

Curcumin is one of the most crucial naturally occurring chalcone analogue. It is the primary bioactive component present in turmeric and derived from the rhizomes of the *Curcuma longa* plant. Curcumin holds its special place to work for its medicinal properties, which include anti-inflammatory, antioxidant, antimicrobial, anticancer, and wound healing effects ^[11-15]. The pharmacological effects of Curcumin motivates researchers modify the curcumin structure in innovative manner to generate new chalcone derivatives. These modifications are based on the fact to alter activity of molecule, greater stability, and right bioavailability. The curcumin analogues are designed in a manner to inclusion of some specific moiety in the molecule like heterocyclic rings, aromatic rings or any bio-isosteric group ^[16]. Such chemical modifications can significantly influence the biological properties of the resultant compounds. The, α , β -unsaturated carbonyl group of chalcones is particularly reactive and serves as a vital intermediate for synthesizing various heterocyclic molecules. Among these, pyrazole derivatives are notably important because of their unique chemical characteristics and diverse pharmacological activities ^[17-21].

Pyrazoles represent a crucial class of five-membered nitrogen-containing heterocyclic compounds.

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Their synthesis involves the reaction of chalcones with hydrazine derivatives, particularly phenyl hydrazine and its analogues, resulting in cyclization to form pyrazole rings. Pyrazoles have garnered considerable attention in medicinal chemistry due to their extensive biological activities, including antimicrobial, anti-inflammatory, analgesic,

anticancer, antiviral, and antifungal properties. Pyrazole compounds have shown remarkable efficacy in treating microbial infections, making them promising candidates for drug development. Figure 1 represents the structures of various pyrazole-based drug molecules used in clinics for the treatment of different health ailments ^[22-24].

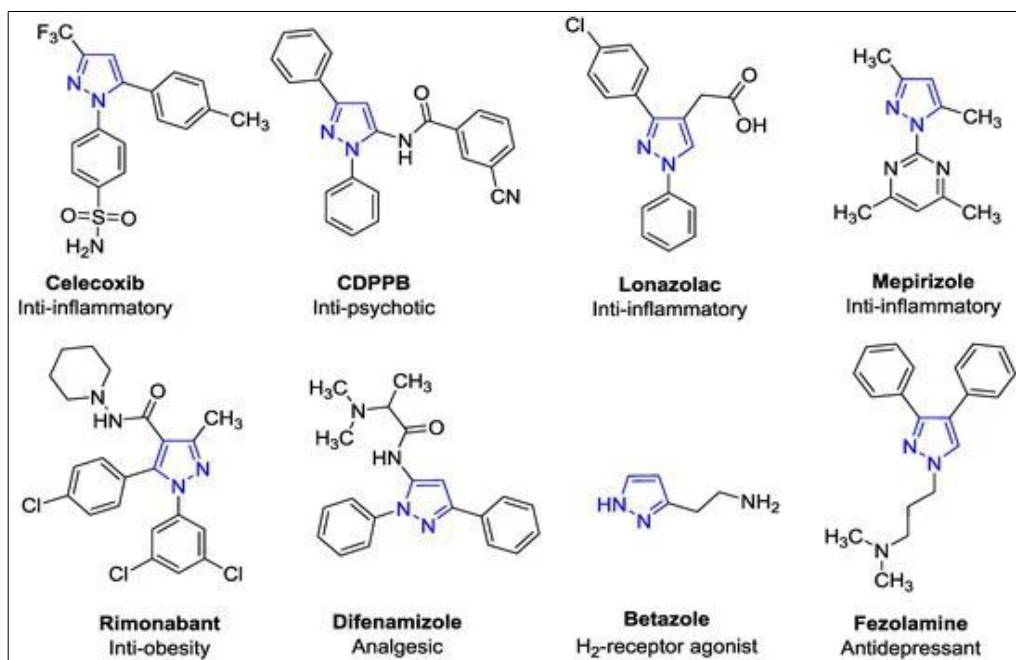


Fig 1: Structures of various pyrazole-based drug molecules

Considering the ongoing global health threats posed by antibiotic-resistant bacteria and fungi, there is an urgent need for new effective antimicrobial agents. Antibiotic resistance is becoming a severe problem worldwide, and conventional antimicrobial drugs are gradually losing their effectiveness. This issue has intensified the search for novel chemical entities with improved antimicrobial activity to overcome existing resistance mechanisms. Thus, the development of new chalcone and pyrazole derivatives with potent antimicrobial effects is of significant interest in modern medicinal chemistry.

In the current research, we have synthesized new chalcone derivatives inspired by the curcumin structure. Five unique chalcone compounds have been prepared, each containing different substituents on their aromatic rings to assess the impact of these variations on biological activity. Subsequently, these newly synthesized chalcones served as precursors for synthesizing dihydropyrazole derivatives. Specifically, four novel dihydropyrazole derivatives were synthesized by reacting the chalcone functionalities with phenyl hydrazine analogues. These reactions involved a straightforward cyclization method, leading to pyrazole rings attached to substituted aromatic frameworks.

The synthesized dihydropyrazole derivatives were then screened for antimicrobial properties, specifically targeting antibacterial and antifungal activities. Preliminary biological evaluations of these pyrazoles revealed promising results against various microbial strains. These findings underline the potential of chalcone-based pyrazole compounds as candidates for antimicrobial drug development. Moreover, the ease of synthesis makes these compounds attractive for further chemical modifications and extensive biological evaluations. The present study aims to contribute

significantly to the field of medicinal chemistry by providing new insights into the structure-activity relationships of chalcone-derived pyrazoles. Understanding these relationships will be beneficial in optimizing antimicrobial properties and developing more effective therapeutic agents. By exploring the biological activity of chalcone and pyrazole derivatives derived from curcumin structures, we hope to offer valuable contributions to combating microbial infections and addressing the global issue of antibiotic resistance.

Experimental

All the chemicals used in the synthesis of given compounds are purchased commercially from Sigma Aldrich and Spectrochem chemical company and did not purify before using. After isolation the final products were purified in silica gel column for by column chromatography. Solvent was concentrated on Buchi rotatory evaporator. Reactions were monitored by performing thin layer chromatography and plates were visualised using UV lamp. The products were analysed by recording ¹H NMR spectral detail on BRUCKER 300MHz instrument. Mass spectra was recorded on Micromass Quattro II instrument.

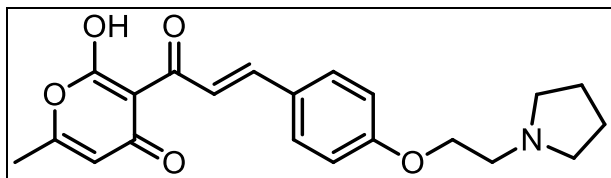
General procedure

General experimental procedure for synthesis of chalcone analogues: The dehydroacetic acid 1 mmol and the benzaldehyde aminoalkyl derivative 1 mmol were mixed in 100 ml round bottom flask in dry chloroform. Piperidine the organocatalyst 10 mol% was added and the reaction mixture was allowed to react at room temperature up to completion of reaction. After completion the reaction mixture was extracted using ethyl acetate and water. The

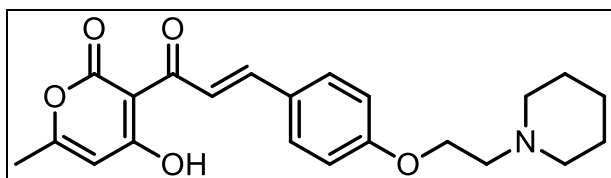
organic layer was separated and dried over anhydrous sodium sulphate. The dried organic layer was concentrated on rotavapor. The crude obtained was further purified by column chromatography on silica column. The purified chalcone derivatives were isolated as oil.

General experimental procedure for synthesis of corresponding dihydropyrazole analogues: The chalcone derivatives 1 mmol, phenyl hydrazine hydrochloride 1.2 mmol and anhydrous potassium carbonate 1.5 mmol were mixed in dry THF and reaction mixture was refluxed for 8 to 10 hrs. The progress of reaction was monitored by using Thin Layer Chromatography applying methanol: chloroform in 1:10 ratio. After completion the solvent in the reaction mixture was evaporated under reduced pressure. The crude residue obtained was extracted in ethyl acetate and water. The organic layer was dried over anhydrous sodium sulphate and residue was crystallized using ethanol and petroleum ether. The resulting dihydropyrazole analogues were isolated as dark brown coloured solid materials. The products were further characterized using spectroscopic and elemental analysis data.

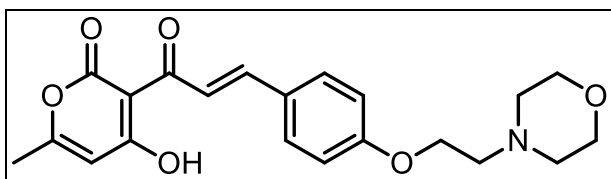
Characterization data for chalcone analogues



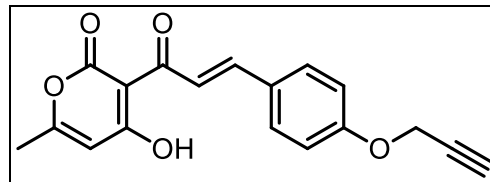
1. (E)-6-methyl-3-(3-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)acryloyl)-2H-pyran-2,4(3H)-dione: ^1H NMR: δ 10.14 (s, 1H); 8.28(d, $J=16\text{Hz}$, 1H), 7.95(d, $J=15.8\text{Hz}$, 1H) 7.72-7.65(m, 2H); 7.11 (t, $J=8\text{Hz}$, 2H), 5.97(s, 1H); 4.11(t, 2H); 2.86(t, 2H); 2.67(s, 3H); 2.51-2.48(m, 4H); 1.689-1.67(m, 4H).



2. (E)-6-methyl-3-(3-(4-(2-(piperidin-1-yl)ethoxy)phenyl)acryloyl)-2H-pyran-2,4(3H)-dione: ^1H NMR: δ 10.14 (s, 1H); 8.28(d, $J=16\text{Hz}$, 1H), 7.95(d, $J=15.8\text{Hz}$, 1H) 7.72-7.65(m, 2H); 7.11 (t, $J=8\text{Hz}$, 2H), 5.97(s, 1H); 4.11(t, 2H); 2.86(t, 2H); 2.67(s, 3H); 2.48-2.45(m, 4H); 1.59-1.53(m, 6H).

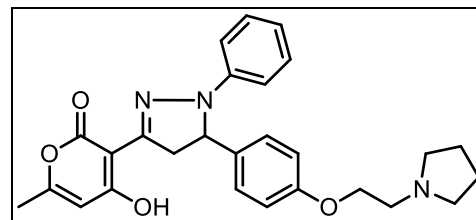


3. (E)-6-methyl-3-(3-(4-(2-(morpholinoethoxy)phenyl)acryloyl)-2H-pyran-2,4(3H)-dione: ^1H NMR: δ 10.14 (s, 1H); 8.28(d, $J=16\text{Hz}$, 1H), 7.95(d, $J=15.8\text{Hz}$, 1H) 7.72-7.65(m, 2H); 7.11 (t, $J=8\text{Hz}$, 2H), 5.97(s, 1H); 4.11(t, 2H); 3.65(t, 4H); 2.86(t, 2H); 2.67(s, 3H); 2.36(t, 4H).

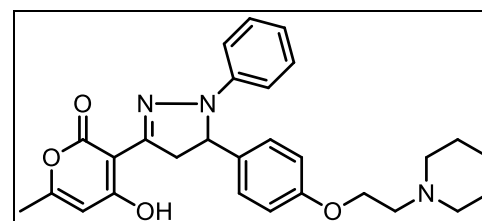


4. (E)-6-methyl-3-(3-(4-(prop-2-yn-1-yloxy)phenyl)acryloyl)-2H-pyran-2,4(3H)-dione: ^1H NMR: δ 10.14 (s, 1H); 8.28(d, $J=16\text{Hz}$, 1H), 7.95(d, $J=15.8\text{Hz}$, 1H) 7.72-7.65(m, 2H); 7.11 (t, $J=8\text{Hz}$, 2H), 5.97(s, 1H); 4.68 (s, 2H); 3.32(s, 1H).

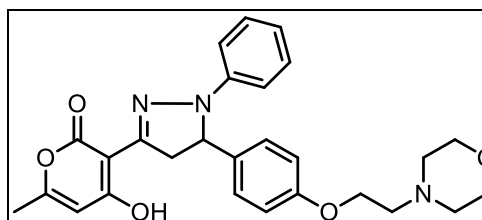
Characterization data for Pyrazole analogues



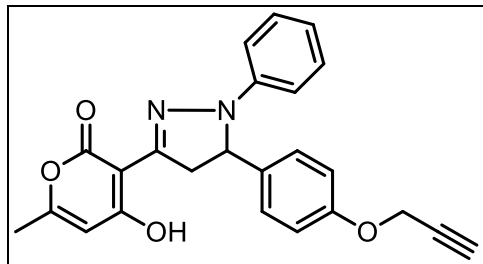
1. 4-hydroxy-6-methyl-3-(1-phenyl-5-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-pyran-2-one: ^1H NMR (CDCl_3 , 300 MHz): 7.29-7.13 (m, 4H); 6.92-6.78 (m, 3H); 6.71-6.67 (m, 2H); 6.17(s, 1H); 5.07 (dd, 1H, $J=8\text{Hz}$, $J=12\text{Hz}$); 4.12 (dd, 1H, $J=12\text{Hz}$, $J=18\text{Hz}$); 3.50 (dd, 1H, $J=8\text{Hz}$, $J=22\text{Hz}$); 2.26 (s, 3H); 2.86(t, 2H); 2.67(s, 3H); 2.51-2.48(m, 4H); 1.689-1.67(m, 4H).



2. 4-hydroxy-6-methyl-3-(1-phenyl-5-(4-(2-(piperidin-1-yl)ethoxy)phenyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-pyran-2-one: ^1H NMR (CDCl_3 , 300 MHz): 7.29-7.13 (m, 4H); 6.92-6.78 (m, 3H); 6.71-6.67 (m, 2H); 6.17(s, 1H); 5.04 (dd, 1H, $J=8\text{Hz}$, $J=12\text{Hz}$); 4.09 (dd, 1H, $J=10\text{Hz}$, $J=18\text{Hz}$); 3.52 (dd, 1H, $J=8\text{Hz}$, $J=19\text{Hz}$); 2.26 (s, 3H); 2.86(t, 2H); 2.67(s, 3H); 2.48-2.45(m, 4H); 1.59-1.53(m, 6H).



3. 4-hydroxy-6-methyl-3-(5-(4-(2-(morpholinoethoxy)phenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)-2H-pyran-2-one: ^1H NMR (CDCl_3 , 300 MHz): 7.29-7.13 (m, 4H); 6.92-6.78 (m, 3H); 6.71-6.67 (m, 2H); 6.17(s, 1H); 5.10 (dd, 1H, $J=8\text{Hz}$, $J=10\text{Hz}$); 4.16 (dd, 1H, $J=12\text{Hz}$, $J=16\text{Hz}$); 3.56 (dd, 1H, $J=8\text{Hz}$, $J=20\text{Hz}$); 4.11(t, 2H); 3.65(t, 4H); 2.86(t, 2H); 2.67(s, 3H); 2.36(t, 4H).



4. 4-hydroxy-6-methyl-3-(1-phenyl-5-(4-(prop-2-yn-1-yloxy)phenyl)-4,5-dihydro-1H-pyrazol-3-yl)-2H-pyran-2-one: ^1H NMR (CDCl_3 , 300 MHz): 7.29-7.13 (m, 4H); 6.92-6.78 (m, 3H); 6.71-6.67 (m, 2H); 6.17(s, 1H); 5.03 (dd, 1H, $J=8\text{Hz}$, $J=11\text{Hz}$); 4.11 (dd, 1H, $J=12\text{Hz}$, $J=16\text{Hz}$); 3.48 (dd, 1H, $J=8\text{Hz}$, $J=18\text{Hz}$); 4.68 (s, 2H); 3.32(s, 1H).

Antimicrobial Activity

Minimum inhibitory concentration (MIC) and IC_{50} values are determined using standard broth microdilution technique as per NCCLS guidelines. The bacterial strains were grown on nutrient agar at 37°C . After 24 h of incubation, bacterial cells were suspended in normal saline containing Tween 20 at 0.05% at a concentration of approximately $1.0\text{-}2.0 \times 10^7$ cells/mL by matching with 0.5 McFarland standards. The activity of compounds was determined as per NCCLS protocol using Mueller Hinton broth (Becton Dickinson, USA) in 96-well tissue culture plates. Proper growth control, drug control and the negative control were adjusted onto the plate. The fungal strains are grown in Sabouraud Dextrose Agar slants at 35°C . For yeast like fungi (eg *Candida* sp.) the cells are suspended in normal saline after

24 h growth in case of sporulating mycelial fungi 3-7 days old slant cultures are used and approximately $1.0\text{-}2.0 \times 10^6$ cells/mL by matching with 0.5 McFarland standards. This suspension serves as stock fungal cell suspension. The activity of compounds is determined by two-fold micro broth dilution method using RPMI 1640 (1.04 g/100ml) buffered with MOPS (3.47 gm per 100 ml RPMI) for fungus and Mueller Hinton broth (Becton Dickinson, USA) for bacteria in 96-well tissue culture plates.

Compounds were dissolved in DMSO at a concentration of 1mg/mL (synthetic compound) or 10mg/ml (natural extract) and $20\mu\text{L}$ of this was added to each well of 96-well tissue culture plate having $180\mu\text{L}$ Mueller Hinton broth. From here the solution was serially diluted resulting in twofold dilution of the test compounds in subsequent wells. $100\mu\text{L}$ of Mc Farland matched bacterial or fungal suspension was diluted in 10 ml of media and then $100\mu\text{L}$ of it was added in each well and kept for incubation. The maximum concentration of compounds tested was $500\mu\text{g/mL}$. The micro-titer plates were incubated at 35°C in a moist, dark chamber and MICs were recorded visually.

Results and Discussion

We started our work by synthesis of the chalcone based structures inspired by curcumin pharmacophores. Designing of chalcone derivatives was based on the fact that the structural feature of the curcumin molecule is responsible for the potent bioactivity of the molecule. The 1, 3 diketo functionality (Figure 2) of curcumin moiety was selected and was develop in the form of chalcone molecule by using dehydroacetic acid and tailored Benz aldehyde derivatives.

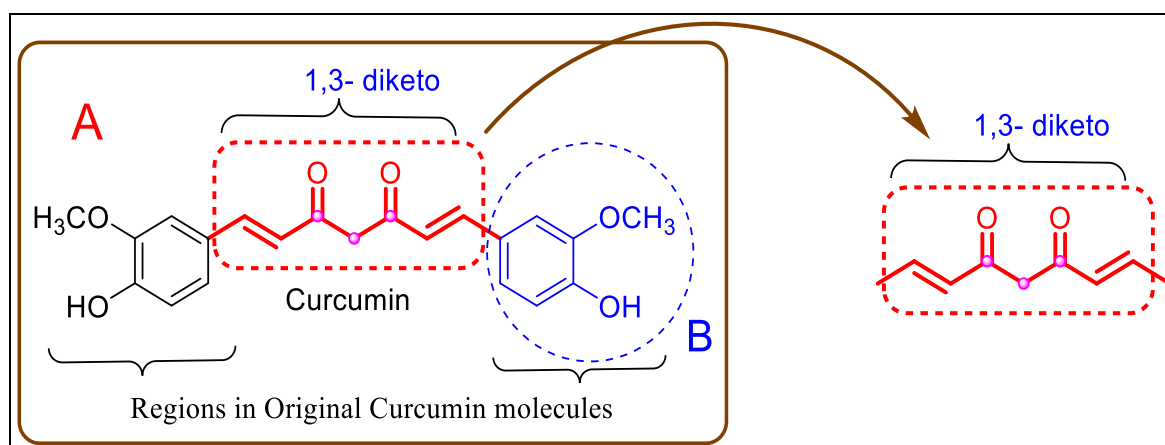


Fig 2: The bioactive 1, 3-dikeo functionality in Curcumin structure

Further, to enhance the receptor binding ability and then the biological activity of the molecule different pharmacophoric groups were attached with the Benz aldehyde molecules a shown in the figure.

The Figure 3 clearly demonstrates that the 1, 3 diketo finctionality was selected from the curcumin molecule. Further both the ketonic groups are designed to possess the chalcone type characteristic in the structure (Figure 3).

The rationale behind the synthesis of chalcone functionality is due to the fact that the chalcone class of compounds have proved their vital applications in crucial sectors like imaging agents, medicines, agrochemicals, dying agents as well as

enhanced inhibition potential against range of pathogens. Pharmacophoric aminoalkyl chains consisting of piperidine, Pyrrolidine and Morpholine heterocycles were attached with the benzaldehyde molecule before applying them for the synthesis of chalcone derivatives.

The chalcone derivatives were synthesized by adopting the conventional Claisen-Schmidt condensation reaction. The reaction Substituted benzaldehydes reacted with dehydroacetic acid in the presence of piperidine base as an organocatalyst. Initial experiments tested various solvents such as ethanol, methanol, and water.

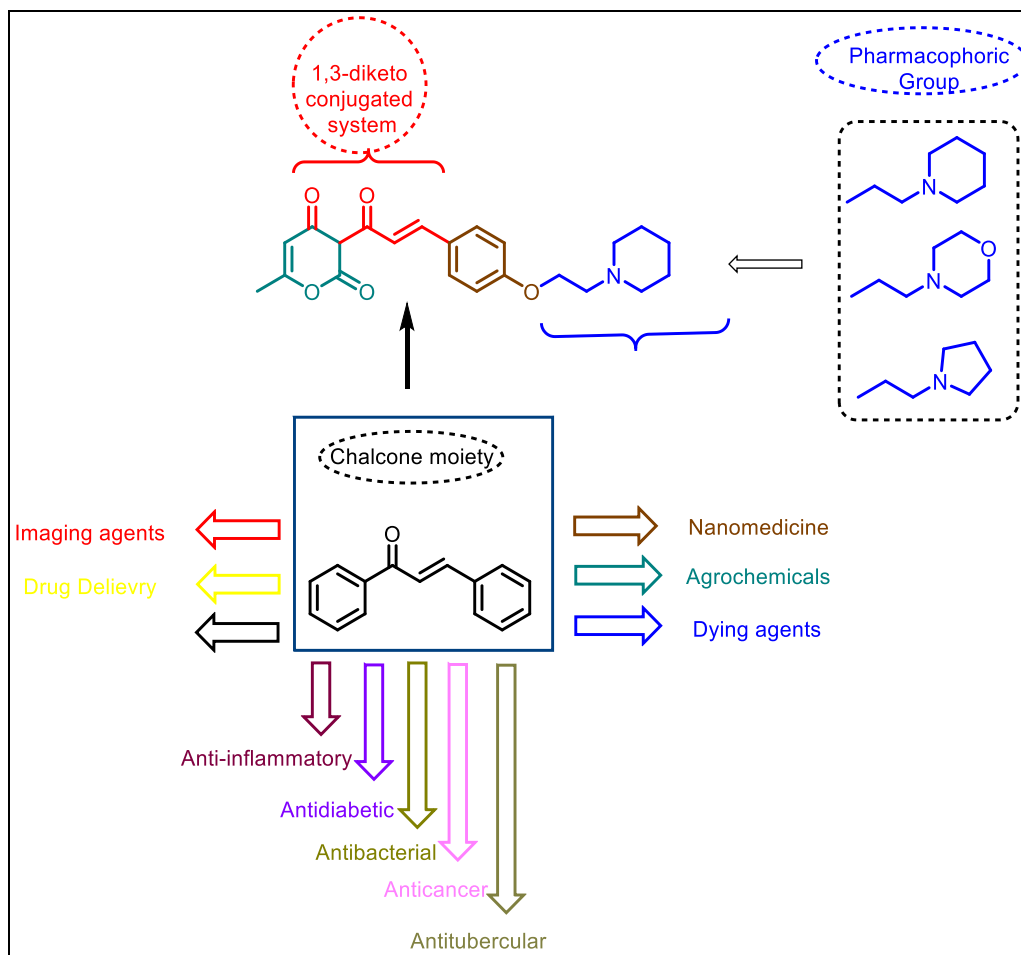
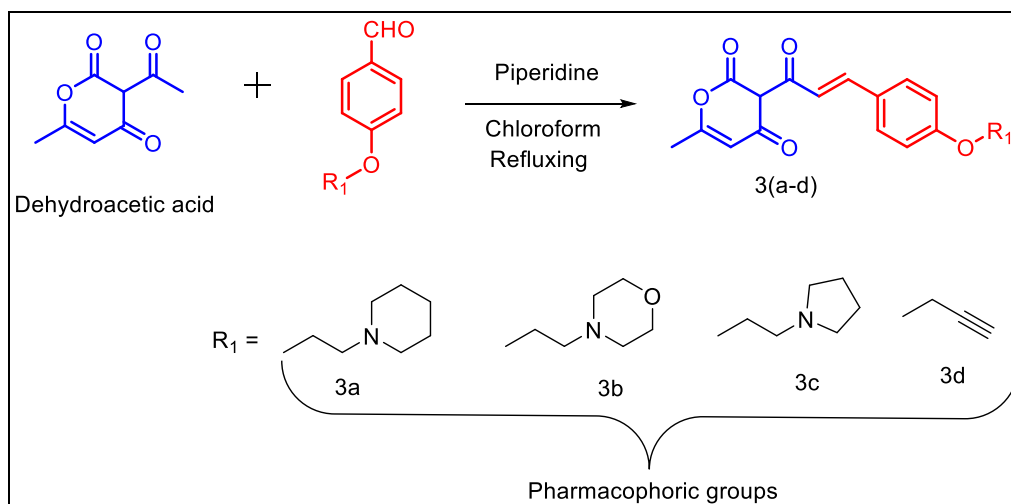


Fig 3: Schematic representation of designing of chalcone molecules

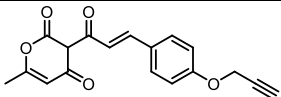
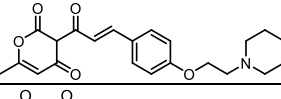
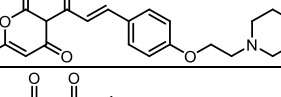
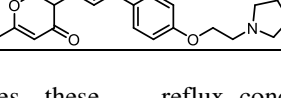


Scheme 1: Synthesis of chalcone analogues

Initially we picked up the reaction of dehydroacetic acid and benzaldehyde as a model reaction to finalize the reaction conditions. We screened all common table solvents like THF, Ethanol, Methanol, Dichloromethane, Chloroform, Acetonitrile and water. We screened few organocatalysts ephedrine, L-proline, Piperidine and Pyrrolidine. We observe the best results on using ethanol as solvent for Claisen-Schmidt condensation reaction and piperidine as an organocatalyst provided superior yields, ranging between

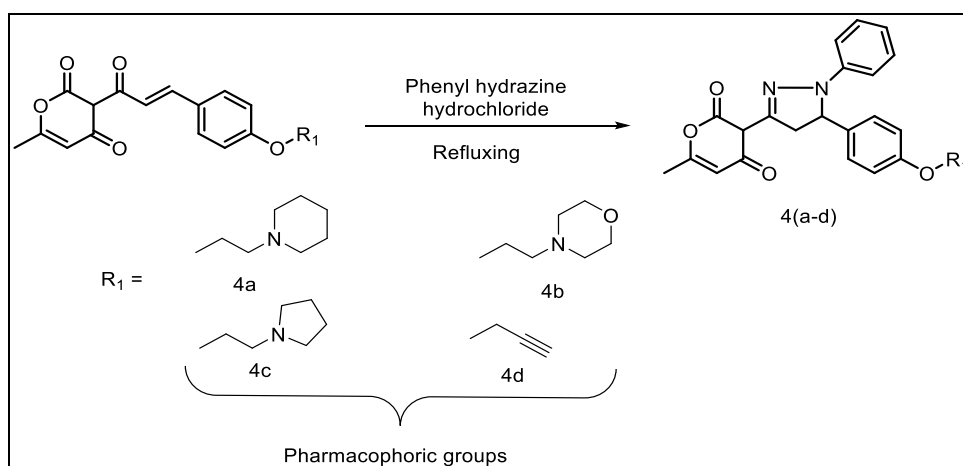
85% and 95%. The progress of reaction was monitored by using Thin Layer Chromatography. Reaction completion occurred optimally within 8-12 hours at room temperature. Structural characterisation was conducted using infrared (IR) spectroscopy, nuclear magnetic resonance (NMR), and mass spectrometry. IR spectra confirmed characteristic absorption peaks near 1650 cm^{-1} for $\text{C}=\text{O}$ stretching vibrations, while NMR provided clear proton signals corresponding to aromatic and alkene protons.

Table 1: Synthesis of chalcones derivatives

S. No.	Comp. No.	Structure	Yield (%)	Mp °C
1	3a		94	167
2	3b		90	158
3	3c		92	170
4	3d		90	195

Following the successful synthesis of chalcones, these compounds were converted to dihydropyrazole analogues by reaction of prepared chalcone derivatives and phenyl hydrazine hydrochloride using triethylamine as base in the reaction. We found that the reaction result was excellent in using ethanol with triethyl amine as reaction medium. Reaction conditions were optimized using ethanol under

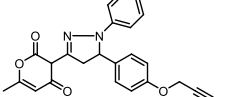
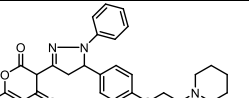
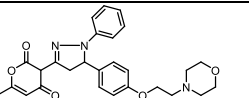
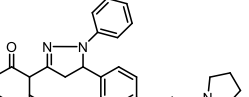
reflux conditions, providing yields of approximately 78-88% within 6-10 hours. The IR, NMR, and mass spectrometry analyses confirmed the successful cyclization into pyrazole rings, with IR spectra exhibiting characteristic peaks around 1560 cm^{-1} corresponding to C=N stretching vibrations.

**Scheme 2:** Synthesis of pyrazole analogues

Optimization of Reaction Conditions Detailed investigation into reaction optimization was conducted to maximize yields and purities. Ethanol consistently delivered higher yields compared to other solvents. Sodium hydroxide proved superior to potassium hydroxide in catalyzing

chalcone formation, suggesting stronger base efficacy for condensation reactions. Optimal reaction times were critical, with shorter or longer durations resulting in lower yields or impure products.

Table 2: Synthesis of Dihydropyrazoles

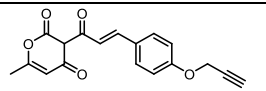
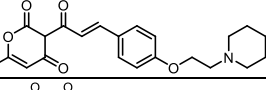
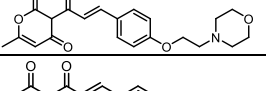
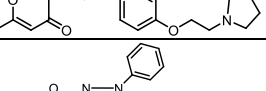
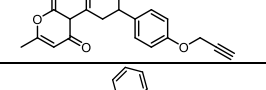
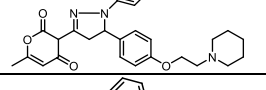
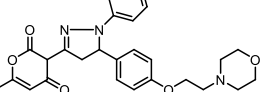
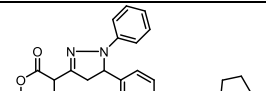
S. No.	Comp. No.	Structure	Yield (%)	m.p. (°C)
1	4a		88	176
2	4b		85	158
3	4c		86	165
4	4d		88	191

Antibacterial and Antifungal Activity

Antibacterial antifungal evaluation of the synthesized chalcones as well as dihydropyrazoles analogues were investigated against range of gram positive and gram negative bacterial strains. The *Staphylococcus aureus*, *Staphylococcus pyogenes* strains were used as gram positive bacterial cell lines and *E. coli*, *P. aeruginosa* cell lines were tested from gram negatives bacterial cell lines. To test the

antifungal activity *Candida albicans* and *Aspergillus niger* fungal species were tested. All the four chalcone derivatives as well as all the four pyrazole analogues were tested against all the bacterial as well as fungal cell lines. The bacterial strains were grown on nutrient agar at 37°C and results are calculated in form of minimum inhibitory concentration by using dilution method.

Table 3: Antibacterial and Antifungal activity of Chalcones and dihydropyrazoles

Compound No.	Structure	Antibacterial activity minimum Inhibitory Concentration $\mu\text{g/ml}$				Antifungal activity minimum inhibitory concentration $\mu\text{g/ml}$	
		Gram +Ve Bacteria		Gram -Ve Bacteria			
		<i>S. aureus</i>	<i>S. pyogenes</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	<i>A. niger</i>
3a		6.25	6.25	12.5	25	25	12.5
3b		6.25	3.12	6.25	12.5	25	12.5
3c		25	12.5	50	50	50	50
3d		50	50	>50	>50	>50	>50
4a		50	50	50	>50	>50	>50
4b		6.25	6.25	12.5	25	25	12.5
4c		6.25	3.12	6.25	12.5	25	12.5
4d		25	12.5	50	50	50	50

Structure-Activity Relationship (SAR)

All the prepared chalcone analogues and pyrazole derivatives were screened for their antibacterial and antifungal activity. Both gram positive (*S. aureus*, *S. pyogenes*) and gram negative strains (*E. coli*, *P. aeruginosa*) of bacteria were used to investigate the antifungal and antibacterial activity. *A. Niger* and *C. albicans* antifungal cell lines were used to screen the antifungal activity of molecules. The results obtained from antibacterial antifungal property evaluation SAR analyses revealed distinct patterns linking chemical structures with biological activities. The results of the biological evaluations have revealed that in chalcone derivatives compound 3b with the piperidine ring as substitution was found to be most potent molecule in the chalcone series. The chalcone 3b exhibited the MIC value of the 6.25 $\mu\text{g/ml}$ against *S. aureus* and MIC value of 3.25 $\mu\text{g/ml}$ against *S. pyogenes* (both gram +ve strain of bacteria). Interestingly this chalcone derivative showed potent activity for the gram-negative bacterial strain *E. coli* and *P. aeruginosa* with the MIC value of 6.25 and 12.5 $\mu\text{g/ml}$ respectively. On the other hand, the chalcone derivative having morpholine substitution exhibited the inhibitory potential with the MIC values of 25 $\mu\text{g/ml}$ for the gram-positive strain of (*S. aureus*), and more than 25 $\mu\text{g/ml}$ for *S. pyogenes*. This result concludes that the piperidine

substitution is more crucial for the biological activity. This result was also established in the biological activity result of fungal strains *C. albicans* and *A. Niger* where the MIC was found to be 50 $\mu\text{g/ml}$. The biological potential of pyrazole analogues has exhibited similar pattern of antibacterial and antifungal activity with MIC value of 6.25 $\mu\text{g/ml}$ for both gram positive bacterial strains. While 12.5 $\mu\text{g/ml}$ for the fungal cell line. This result showed that the piperidine ring in the dihydropyrazole derivatives also enhances the biological potential of the molecule. Additionally, the dihydropyrazole derivative with the morpholine substitution in phenyl ring have shown good antibacterial activity with the MIC value of as low as 6.25 $\mu\text{g/ml}$ for the molecule 4c, whereas the same substitution was showing higher values of MIC against antibacterial and antifungal cell lines. The pyrazole-based molecule 4d having pyrrolidine ring in the phenyl group was also not showing any prominent activity and resulted in higher values of the inhibitory concentration. The molecule 4a with the propargyl substitution was proved to be less active compound on pyrazole series of molecules with MIC value of as high as 50 $\mu\text{g/ml}$. The SAR study showed that the prepared compounds exhibited the varied class of antibacterial and antifungal activity. The results of the biological study could be of great significance for

investigating potential of other derivatives of the synthesized molecules.

Conclusion

In present work we are demonstrating the designing of new chalcone derivatives inspired by curcumin pharmacophore with an objective to enhance the biological activity of molecule. We prepared aminoalkyl derivatives of chalcone molecules and further the dihydropyrazole analogues were synthesized by reaction of prepared chalcone derivatives and phenylhydrazine molecule. The prepared chalcone derivatives and dihydropyrazole analogues were investigated of their antibacterial and antifungal activity against both gram positive and gram negative cell lines. The activity results concluded that the synthesized molecule with piperidine substitution both in chalcone and dihydropyrazole analogue with MIC value of as low as 6.25 µg/ml.

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