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Synthesis and biological evaluation of novel pyrazole scaffold

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Abstract: synthesis of a pyrazole containing compound was achieved by reacting phenyl hydrazine with (*E*)-2-((4-bromophenyl) diazinyl)-1-phenylbutane-1,3-dione to produce 4-((4-bromophenyl) diazinyl)-5-methyl-1,3-diphenyl-pyrazole and characterization using mass spectrometer, ^{1}H NMR and ^{13}C NMR. The pharmacological evaluation of the synthesized compound, denoted as (KA5), against *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 29213 and *Clostridiums sporogeneses* ATCC 19404, indicate that there is no promising antibacterial activity. However, KA5 shows a competitive anticancer activity (IC50: 8.5µM) upon its evaluation against hepatocellular carcinoma cell line (HepG 2) compared to sorafenib (IC50: 4.51µM). Moreover, human skin fibroblast (HSF) was used to investigate the effect of KA5 on normal cell lines, (IC50: 5.53µM). The presented biological evaluations resulted in better understanding of structure-activity relationship for 1, 3, 4-trisubstituted pyrazoles and revealed a great opportunity for more investigations for novel pyrazole-containing anticancer agents.

Keywords: Azo compound, HepG2, HSF, pyrazole, cytotoxicity, antibacterial, anticancer, sorafenib.

INTRODUCTION

Cancer incidence is being increased from year to year representing the second leading cause of death worldwide (Organization, 2020 and Hulvat, 2020). For instance, in Gulf region hepatocellular carcinoma incidence rate in 2025 is expected to exceed 1 million cases compared to 8000 during the period of 1998 to 2012 (Albarrak and Al-Shamsi, 2023). Among factors responsible about the dramatic increase in cancer mortality rates is multi-drug resistance for the current anticancer agents in addition to lifestyle changes (Alolyan *et al*., 2024; Meci *et al*., 2024). Generally, reasons for drug resistance can be classified into pharmacological and cells related factors. Pharmacological factors such as drug inactivation, alteration of drug target, while cellular factors include cell death inhibition and DNA damage repair (Housman *et al*., 2014; Bukowski *et al*., 2020; Sangwan *et al*., 2024). Based on that, it is concluded that designing and discovering novel anticancer pharmacophores is an urgent need to overcome drug-resistance. Pyrazole containing compounds are considered one of the scaffolds providing diverse pharmacological activities including, antibacterial, analgesic, anti-inflammatory, anticancer, anticonvulsant, antirheumatic and antitubercular (Faria *et al*., 2017; Yerragunta *et al*., 2014; Fayed *et al*., 2023; Kalluraya *et al*., 2001; Ray *et al*., 2023; Isloor *et al*., 2009; El-Gamal *et al*., 2022; Bhatt *et al*., 2018; Alsayari *et al*., 2021; Said *et al*., 2024; Zhang *et al*., 2023). As with any heterocyclic moiety, pyrazole pharmacophore can be linked to many scaffolds to produce various pharmacological activities (Razali and Jamain, 2023).

Azo compounds are compounds containing nitrogen-nitrogen double bond (-N=N-). Chemically, this group of compounds can be classified, according to the number of azo moieties in the molecule, into mono-, dis-, tris- and poly -azo's (Singh *et al*., 2023). Medicinally, compounds contain azo moieties have different biological activities including antimicrobial, anti-inflammatory, antiviral, antifungal and anticancer activities (Thomas *et al*., 2019; Metwally *et al*., 2012; Ali *et al*., 2018).

Condensation of azo (diazinyl) group to heterocyclic functional groups such as pyrazole resulted in biologically active compounds having analgesic, antioxidant, and anticancer activities (Oruç *et al*., 2006; Ibrahim and Khalaf, 2023). Here in this work, diazinyl linked pyrazole compound was synthesized and biologically evaluated for antibacterial activity against four bacterial strains and for anticancer activity against human skin fibroblast, HSF and liver cancer cell line, HepG 2.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co. and used as received without further purification. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker AVANCE and Varian Unity INOVA-500 MHz NMR and 126 MHz for ^{1}H and ^{13}C NMR spectrometer, respectively, in $CDCl_3$ using solvent residual peaks as an internal standard; CDCl3 (δ = 7.27 ppm) for ^{1}H NMR and CDCl3 (δ = 77.23 ppm) for ^{13}C NMR. The signal multiplicities were reported as s (sin-glet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broad) and the J-coupling constants were in Hz. HPLC and LC-MS Analysis: LC-MS analysis of pure compounds was performed using a Nexera Lite HPLC system 2050c 3D connected with an LCMS-2050 mass spectrometer (Shimadzu, Japan). In HPLC analysis, a C8 column Acclaim TM 120 (4.6 mm I.D. × 150 mm, particle size; 5 µm) was used using gradient elution with methanol as a

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mobile phase. For LC-MS, the following parameters were used: the nebulizing gas flow was 0.3 L/min, the flow of drying gas was set to 6.0 L/min, the flow of heating gas was set to 7.0 L/min and the desolvation temperature was set to 400°C. The m/z range of 100-500 was selected with a scan time of 0.5 s; the data were collected in the positive ion mode at a detector voltage of 2.0 kV. Thin layer chromatography (TLC) was performed for reaction monitoring on pre-coated silica gel 60 F254 (Merck) sheets and visualized by ultraviolet light (254nm). Purification of synthesized compounds was via recrystallization from methanol. Biological Assays were performed according to the standard methods.

General method for synthesis of KA5 and structure characterization

Synthesis of diazonium salt was achieved by slow addition of 2.5ml of conc. HCl to 10mmol of *p*-bromoaniline, before addition of 5mL of 15% $NaNO_2$ and the mixture was left for 15min. in 0°C. On the other flask, 10mmol of benzoyl acetone was dissolved in 20mL EtOH and left for 10min. The obtained diazonium salt was added to the second flask and ran for 10min. After that, the crude extract was crystallized by methanol and the purity was checked with thin layer chromatography and characterization was carried out by mass spectrometer.

To obtain the tri substituted pyrazole, KA5, the purified intermediate from the previous reaction was refluxed with phenyl hydrazine in ethanol at 80°C for 1hr. to result in precipitate that was filtered and crystallized in methanol and the structure was characterized using mass spectrometry, ^{1}H NMR and ^{13}C NMR.

KA5 4-((4-bromophenyl) diazinyl)-5-methyl-1,3-diphenyl-pyrazole)

^{1}H NMR (500 MHz, Chloroform-d) δ 7.62-7.60 (m, 2H), 7.55 (d, J=1.7 Hz, 2H), 7.40 (d, J=2.7 Hz, 1H), 7.38 (d, J = 4.7 Hz, 3H), 7.37 (s, 1H), 7.35 (d, J = 2.1 Hz, 1H), 7.33 (d, J = 4.3 Hz, 3H), 7.32-7.31 (m, 1H), 2.65 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-d) δ 152.39, 144.09, 141.90, 139.45, 136.14, 132.22, 130.89, 129.14, 129.08, 128.49, 128.24, 127.89, 125.26, 123.99, 123.78, 15.23.

MS calculated for $C_{22}H_{17}N_2Br$ [M+H] and [M+Na] yield 417, 419 and 439, 441 as reported (fig. 1 and 2)

Biological evaluation

Bacteriostatic and bactericidal tests

Inoculum preparation

A disc from each of *E.coli ATCC 8739, P. aeruginosa ATCC 9027, S.aureus ATCC 29213* and *C. sporogenes ATCC 19404* was inoculated into 100 ml of TSB, tryptic soy broth, media and incubated at 37ºC for 24 hr. noting that *C. sporogenes ATCC 19404* was grow under anaerobic conditions. In order to prepare the solid media, a loopful from the incubated broth was streaked in tryptic soy agar medium and incubated in 37ºC for 24 hr. Normal saline was used after sterilization and inoculated by 3-4 colonies (from organism plate), and adjusted to reach a turbidity equivalent of 0.5 McFarland standard for each strain using Densi CHEK coptical device. That adjustment results in a suspension containing approximately 1-2 X 108 CFU/mL followed by dilution of 1:100 by addition of 200µl of suspension of into 19.8 ml sterile Muller Hinton Broth to obtain 20ml inoculum containing 1 x 106 CFU/ml.

Broth macrodilution method

In 12 well plates, 2ml of bacterial suspension was inoculated in first well and 1ml from MHB was inoculated in the remaining wells. 1ml from the first well was transferred to the next well in addition to the previously added 1 ml of MHB to result in 1:2 dilution, followed by addition of 1 ml from the 1:2 dilution well to next to make 1:4 dilution. 9 further dilutions for were prepared by the same method to make the final concentration of 5.0 X 10 5 CFU/ml. Another 1ml from each one of the four bacterial suspension was diluted and cultured in order to measure density. A control well, contain broth only, was added to each sample/plate and the negative control well which contain the broth only was added to each plate. All plates were incubated at 37°C for 24 hr. *C. sporogenes* was incubated under anaerobic condition as indicated before. Upon incubation, a loopful was taken from MIC concentration and higher concentrations then streaked into TSB agar plate and incubated at 37°C for 24 hr. with *C. sporogenes ATCC 19404* was incubated under anaerobic condition.

HepG2 and HSF cytotoxicity Assays

Cell culture source

HSF and HepG 2 cells, from Nawah Scientific Inc., (Cairo, Egypt) and maintained in DMEM media supplemented with 100units/mL of penicillin, 100mg/mL of streptomycin and 10% of inactivated fetal bovine serum in humidified, 5% (v/v) CO_2 atmosphere at 37°C.

Cytotoxicity assay

Cell viability was evaluated using SRB assay. 100µL of cells suspension (containing 5x10^3 cells) were incubated in 96 well plates for 24 hr. 100µL media containing the tested compounds were added at various concentrations as indicated. After incubation with the drug, cells fixation was carried out by replacing media with 150µL of 10% of TCA and left at 4ºC. After 1hr., TCA solution was removed and cells were washed 5 times with sterile distilled water. After that, 70µL of SRB solution (0.4% w/v) was added and the plates were incubated for 10 min. at room temperature in a dark place at room temperature for 10 min, before they were washed 3 times with 1% acetic acid and air-dried for 12hr. 150µL of TRIS (10 mM) was then added to dissolve protein- bound to SRB stain; followed by measuring the absorbance at 540 nm using an Infinite F50 microplate reader (TECAN, Switzerland) (Allam *et al*., 2018, Skehan *et al*., 1990).

RESULTS

As indicated in scheme 1, KA5 was synthesized by reacting *para* bromobenzene diazonium chloride with benzoyl acetone followed by condensation with phenyl hydrazine to obtain 4-((4-bromophenyl) diazinyl)-5-methyl-1,3-diphenyl-pyrazole, KA5. The product purity was tested by TLC and HPLC. (fig. 1) and characterized using LRMS, ^{1}H NMR and ^{13}C NMR (fig. 1&2).

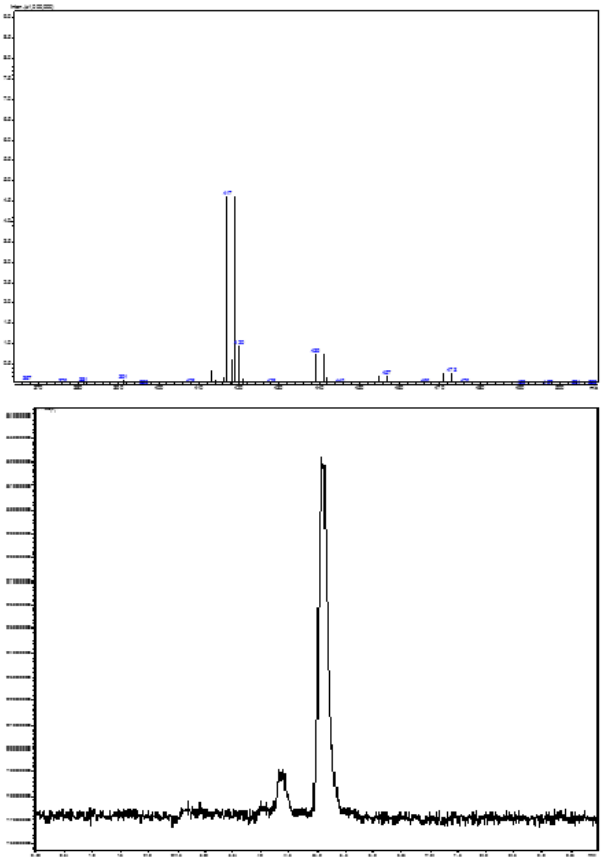


Fig. 1: Chromatogram and Mass spectrum of KA5, [M+H] and [M+Na] with the two isotopes for each.

KA5 was evaluated for antibacterial effect against four strains of bacteria: *Staphylococcus aureus* ATCC 29213, and *Closterdium sporogenes* ATCC 19404 as representatives for Gram positive bacteria and *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027, using ciprofloxacin as positive control. Antibacterial results, represented in (table 1), indicate that KA5 did not show neither bacteriostatic nor bactericidal activities. Due to presence of 1, 3, 4-trisubstituted pyrazole group, which was proofed previously to have anticancer activity (Bekhit *et al*., 2019, Bekhit *et al*., 2005), further investigations were performed to evaluate the anticancer activity of KA5. KA5 cytotoxicity was tested on human normal cells (HSF) and hepatocellular carcinoma cells (HepG 2) using sorafenib as positive control. *In-vitro* cytotoxicity results (fig. 3), indicate that KA5 has a promising anticancer activity against HepG 2 cell line, (IC50: 8.5μM) compared to sorafenib (IC50: 4.51 μM) as indicated in (table 2).

DISCUSSION

Pyrazole derivatives have been studied for their bacteriostatic and bactericidal activities, and pyrazole containing compounds have shown promising results (Kumar *et al*., 2020). Although some a 1,3,4-trisubstituted pyrazoles linked to azo group showed antibacterial activity (Kumar *et al*., 2012), KA5 upon did not showed any antibacterial activity against Gram positive nor Gram negative bacteria. Upon searching for the biological activities for 1,3,4-trisubstituted pyrazole derivatives, it was found that majority of these studies are focused on testing antibacterial, anti-inflammatory and anticancer activities (Bekhit *et al*., 2019; Bekhit *et al*., 2005).

For instance, Abadi *et al*. (2003) investigated a similar scaffold containing 1, 3, 4-trisubstituted pyrazole (fig. 4). Based on their investigations, the essential requirements for anticancer activity of the tested scaffold can be summarized in the following points:

- Presence of azo moiety at position 4, the replacement with imine will lead to complete loss of activity.
- Phenyl and *para* nitro phenyl groups attached to C3 and N1, respectively.
- Halo substituted phenyl group linked to azo group.

However, similar criteria were found in KA5 with some differences such as absence of spacing carbon between azo and pyrazole group.

Comparing the obtained anticancer activity results of KA5 on HepG 2 and HSF cell lines with the mentioned scaffold, can lead to a better understanding of structure activity relationship. Azo group can be linked to pyrazole directly or can be separated by one carbon space in addition to halo substitution in phenyl group can be at *ortho* or *para* positions. Moreover, unsubstitution of phenyl groups at N1 and C3 will not affect the anticancer activity. These results can be used as a guide to synthesize more 1, 3, 4-trisubistituted pyrazoles as promising anticancer agents to be tested against various cancer cell lines.

KA5 4-((4-bromophenyl) diazinyl)-5-methyl-1,3-diphenyl-pyrazole)

^{1}H NMR (500 MHz, Chloroform-d) δ 7.62-7.60 (m, 2H), 7.55 (d, J=1.7 Hz, 2H), 7.40 (d, J=2.7 Hz, 1H), 7.38 (d, J

CONCLUSION

1, 3, 4- trisubstituted azo pyrazole derivative was synthesized and evaluated against Gram-positive and negative bacteria as well as for its anticancer activity on normal and hepatocellular carcinoma cell line. *In-vitro* evaluation results emphasize that presence of azo group at 4 position is important for activity regardless of the space from pyrazole ring.

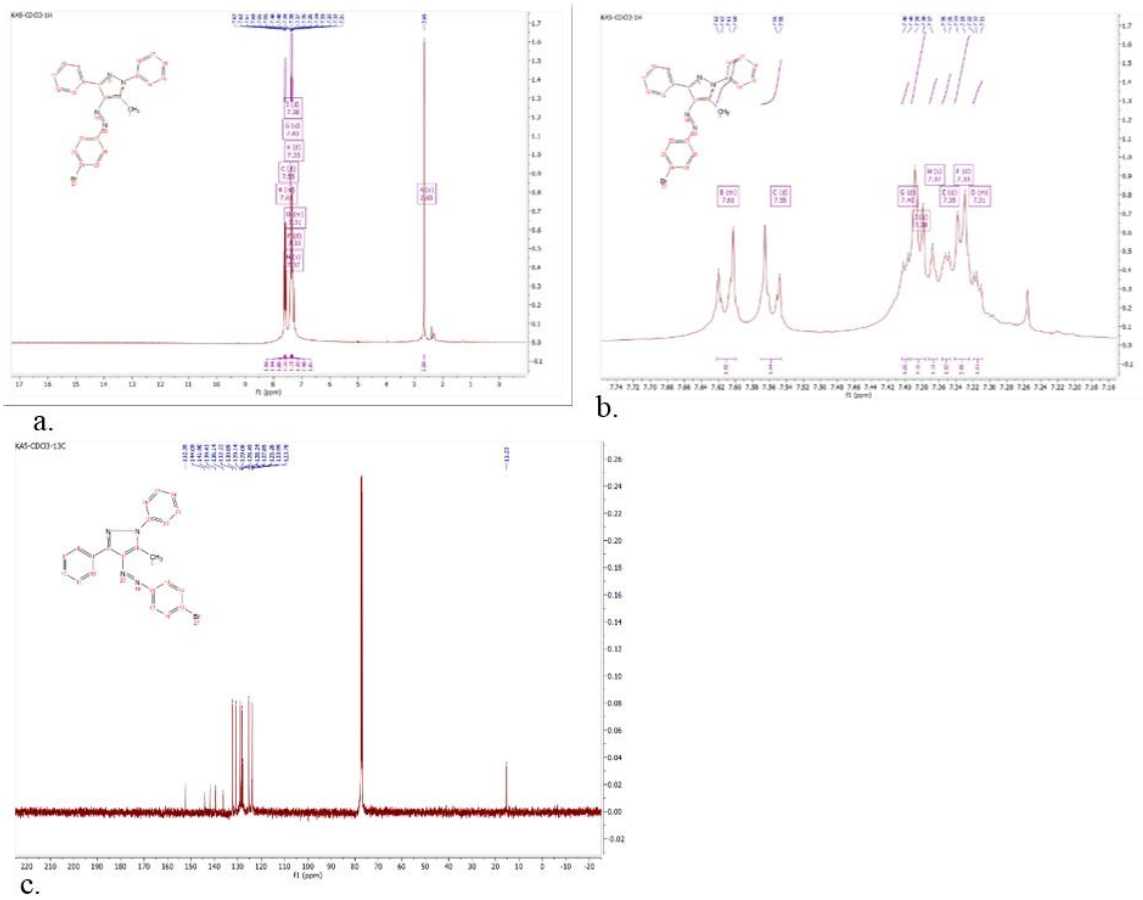


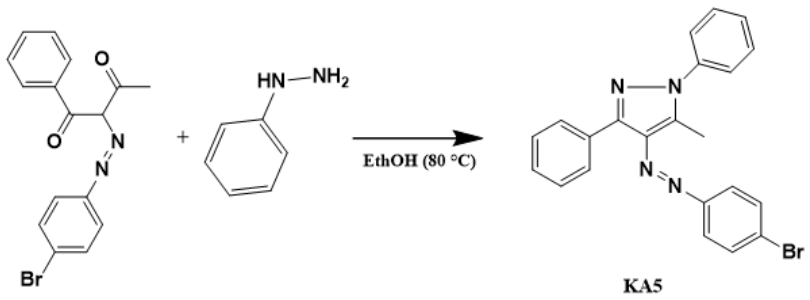
Fig. 2: ^{1}H NMR and ^{13}C NMR of KA5, a and c, respectively, proofing the structure of KA5

Table 1: Antibacterial Activity for KA5 against *E. Coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 29213 and *C. sporogenes* ATCC 19404

	E. Coli ATCC 8739		*P. aeruginosa* ATCC 9027		*S. aureus* ATCC 29213		*C. sporogenes* ATCC 19404	
Compound	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
KA5	>100	> 100	>100	> 100	>100	> 100	>100	> 100
Ciprofloxacin	≤ 9.7	≤ 9.7	≤ 9.7	≤ 9.7	≤ 9.7	≤ 9.7	≤ 9.7	≤ 9.7

Table 2: IC50 results of KA5 and sorafenib against human skin fibroblasts, HSF and hepatocellular carcinoma cell line, HepG2.

Compound	IC50 (µM) against HSF	IC50 (µM) against HepG2
KA5	5.53	8.5
Sorafenib	-	4.51



Scheme 1: Synthetic scheme of KA5 starting from azo benzoyl acetone derivative.

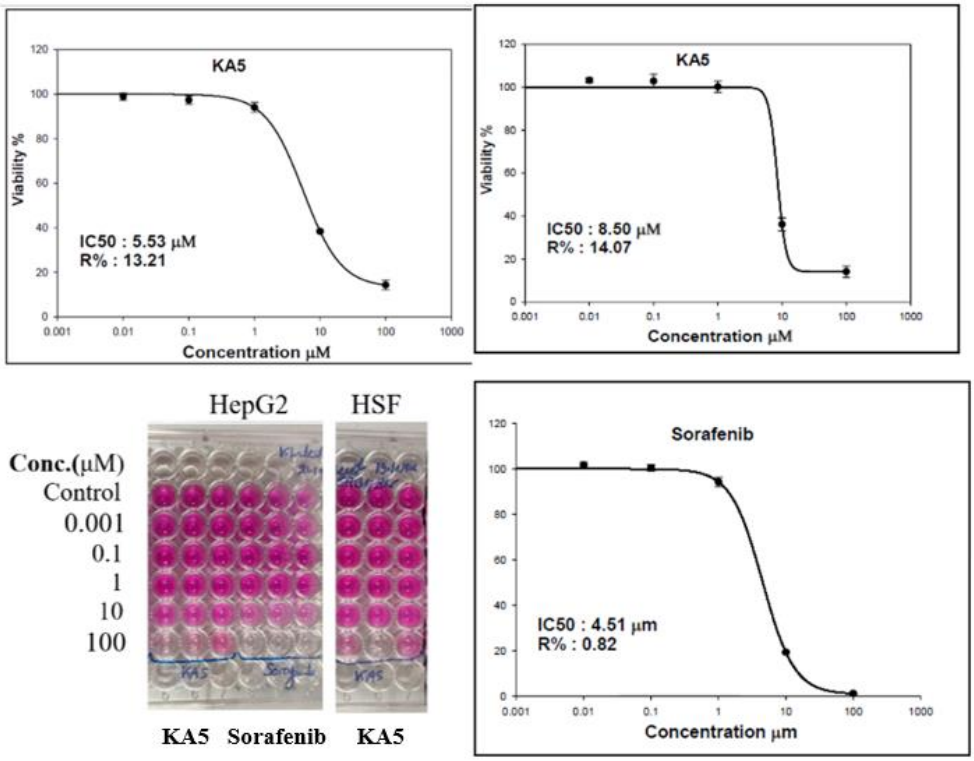


Fig. 3: Cytotoxicity assays of KA5 and sorafenib against hepatocellular carcinoma cell line, HepG2 and human skin fibroblast, HSF, demonstrated by dose-response curves.

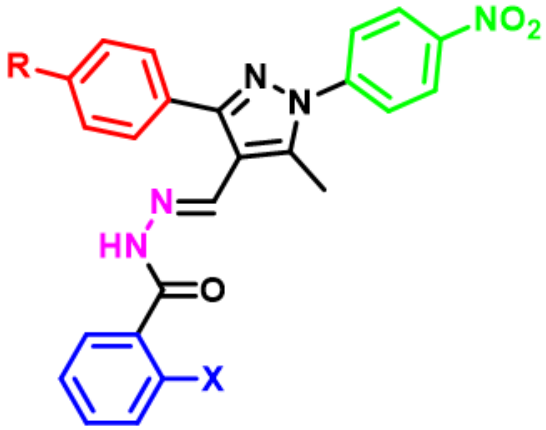


Fig. 4: Similar 1, 3, 4-trisubstituted pyrazole as KA5 showed anticancer activity.

In addition to that, it is concluded that the substitution of phenyl groups at 1 and 3 positions will not abolish the anticancer activity. Current findings need to be further investigated in depth by synthesizing more derivatives and evaluating their anticancer activity to understand their mechanism of action at the molecular level.

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