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Research Article

Synergistic Drug-Drug Interactions (Ddis) Effects on Anti-Inflammatory Activities of N-Phenyl-2,4-Dichlorophenyl Hydrazone and Piroxicam

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Abstract

Drug-drug interactions (DDIs) has become an emerging and powerful strategy in drug designing and optimization. In recent years, there has been an increase in research seeking to increase the therapeutic index of already marketed drugs with new chemical entity serving as actual activity enhancers as well as synergistic relationships. Herein, we report the synergistic drug-drug interactions of compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine with piroxicam as an enhancer of efficacy showcasing faster onset of action with 48.09% inhibition and longer duration of action with 86.48% inhibition. This is compared to piroxicam's moderate onset of action with 35.50% inhibition and shorter duration of action with 60.25% inhibition. Compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine has also demonstrated potential increase in therapeutic index as a combination therapy with superior efficacy to NSAIDs-proton pump inhibitor combination therapy for the treatment of inflammatory diseases.

Key words: drug-drug interactions; pharmacokinetic enhancer; inflammation; piroxicam; n-phenyl-2; 4-dichlorophenyl hydrazone

1. Introduction

Drug-drug interactions (DDIs) is the term that covers the results of combining two or more medications, including both desired and undesired consequences. Drug-drug interaction (DDI) studies are intended to identify how one drug may affect another's metabolism and, vice versa. The results of DDI studies always fall into one of these two categories: (a) when a second drug increases the first drug's catabolism, decreasing the first drug's efficacy; or (b) when a second drug decreases the first drug's catabolism, increasing the first drug's efficacy (or increasing its toxicity) [1]. Drug interactions are occasionally considered to be detrimental and leading to adverse outcomes. However, in some circumstances, some are involved in the enhancement of treatments through combinatorial approach. This action is accomplished through; targeting an identified mechanism of resistance, inhibiting a metabolic pathway to enhance the pharmacokinetic (PK) profile of the primary drug, and enhancing a pharmacological activity. Examples include levodopa (L-Dopa) and dopadecarboxylase inhibitors, β-lactam antibiotics and clavulanic acid, 5-fluorouracil (5-FU) and folinic acid, and penicillin and probenecid.

Recently, kenpaullone, an inhibitor of glycogen synthase kinase GSK3β has been identified to enhance the activity of the alkylating agent, temozolomide in glioblastoma. This was achieved by increasing the apoptosis-inducing activity of temozolomide through attenuation of stem-cell properties of glioblastoma cells [2,3]. Another example is the enhancement of paclitaxel activity in tumoral cells by Carba1, a microtubule-destabilizing carbazole derivative. Although paclitaxel stabilizes the microtubules, Carba1's modification of tubulin dynamics encouraged paclitaxel accumulation in the microtubules, leading to increased efficacy [2,4].

Pharmacodynamic interactions, also known as pharmacodynamic DDIs, are DDIs that result from the combined pharmacodynamic actions of the interacting drugs [5]. The second drug is often chosen based on one of these factors: (a) Both drugs are used to treat the same condition. This is the case with the DDI trial involving the study drug regorafenib and the second drug, irinotecan. When treating colorectal cancer, irinotecan and regorafenib are frequently co-administered. (b) The second drug is for a separate condition, given at the same time as the study drug, such as a contraceptive, statin, acetaminophen, warfarin, or a monoamine oxidase inhibitor. The condition that the second drug is intended to treat in this instance is different from that of the study drug [1].

Pharmacokinetic studies often involve a second drug. This relates to clinical pharmacokinetic studies that are used to predict which classes of drugs are most likely to participate in DDIs with the study drug rather than to assess effectiveness or safety. In this case, the second drug is a recognized inhibitor of a cytochrome P450 enzyme and is typically employed as a model "second drug" in research on drug-drug inhibition [1]. Example is the employment of ketoconazole in clinical investigations of DDIs as a model "second drug." Ketoconazole inhibits cytochrome P450 3A4 when utilized in research on drug-drug interactions (CYP3A4). Although the medicine ketoconazole is technically an antifungal, this sort of clinical pharmacokinetic investigation that employs ketoconazole has no relation to the treatment of fungi [1]. Sometimes the association of an already marketed drug with typically one adjuvant molecule improves both the activity and potency of the drug. More recently, some drug-drug combinations have been included in a modern drug design strategy. These small molecules, also known as enhancers, boosters, or activators, hardly ever or never directly affect health. They can, however, enhance the action of a medicinal drug already in use, leading to greater therapeutic effects [2].

Pharmacokinetic (PK) interactions (PK DDIs) sometimes called dispositional interactions is the modification of one drug's PK or disposition (ADME) by another, which is the hallmark of these interactions. If these changes are severe enough, the exposure to the affected drug may change significantly, eventually modifying how the drug works [6]. In terms of PK, exposure is measured by the region enclosed by a drug's plasma concentration versus time curve. The area under the curve, or AUC, is used to describe that region. The pharmacological benefits which may be enhanced efficacy and/or undesirable side effects may manifest if the exposure (i.e., AUC) is greatly elevated. The therapeutic effects might not manifest if the exposure is sufficiently reduced, and therapeutic failure can result. Changes in the peak plasma concentration (C_{max}) of one drug brought on by another drug may occasionally be sufficient to have clinically relevant adverse effects [6].

The "victim" drug in a DDI is the drug whose disposition is altered, and the "perpetrator" drug is the one that alters the victim's disposition [6]. A perpetrator might be expected to increase the AUC of a victim drug by slowing its metabolism in the liver or the intestine, blocking the efflux of the victim drug from hepatocytes into the bile, or increasing its oral absorption. This could happen if the perpetrator decreased protein or tissue binding of the victim drug, reduced its renal secretion into the urine, inhibited the transporter-mediated efflux of the victim drug back from intestinal epithelial cells into the lumen of the intestine, or slowed its intestinal metabolism [6].

On the other hand, a perpetrator may be anticipated to reduce the AUC of a victim drug by limiting its absorption or bioavailability, speeding up its metabolism in the liver or intestine, or by increasing its efflux from intestinal cells into the intestine's lumen or from hepatocytes into bile. Theoretically, a perpetrator drug's involvement with any aspect of the ADME of a victim drug might change the victim drug's AUC. This broad mechanistic view of DDIs allowed for the long-term prediction of several two-drug DDIs [5]. The vast majority of metabolic DDIs involve the inhibitors, inducers, and substrates of the cytochrome P450 enzymes (CYPs). Although, theoretically any inhibitor or inducer of any drug-metabolizing enzyme could act as a perpetrator of DDIs and any substrate of any drug-metabolizing enzyme could become a victim. This is largely because CYPs predominate over all other types of enzymes in the metabolism of drugs [5].

Elvitegravir is a dramatic and unusual case since it is one of the few times when the package label calls for simultaneous administration of a CYP enzyme inhibitor. The CYP enzyme inhibitor was used to boost the study drug's exposure. Another pharmacokinetic enhancer, cobicistat, is used to inhibit CYP3A and stop the degradation of certain drugs by CYP3A. For inhibiting CYP3A-mediated drug catabolism, ritonavir or cobicistat are referred to as helpful "pharmacokinetic boosters or enhancers" [1].

P-gp controls how well pharmaceuticals are absorbed through the intestines, therefore medicine that can stimulate or inhibit P-gp activity can lead to the development of DDI. The bioavailability of drugs that are ineffectively absorbed can be greatly increased by P-gp inhibition. It is known that sildenafil decreases Pgp's transporter function, providing a potential method to increase the availability and perhaps even the effectiveness of anticancer drugs [7].

Drug interactions that affect distribution may be caused by interactions with drug transporters and drug displacement from plasma proteins, most notably serum albumin [8]. Displacement of one drug or substance by another drug or substance from its binding site or sites, which results in a rise in the concentration of the free drug, is an example of a drug interaction of widespread clinical concern but unusual clinical importance. Higher distribution and clearance take place as a result of the increased free drug concentration, and the pharmacodynamic action is also improved [8]. In this situation, drugs that have a high affinity for plasma proteins, a small volume of distribution (Vd 1 L/kg), a prolonged half-life, and a low therapeutic index might have negative clinical consequences.

The pharmacological displacement seen when warfarin and diclofenac are co-administered is a classic example. Because diclofenac and warfarin have a similar affinity for albumin, when it is given to a patient who is taking warfarin on a long-term basis, the warfarin is dislodged from its binding site. Serious hemorrhagic responses emerge as free warfarin concentrations in the plasma rise [9].

The drug's elimination from the body might experience a variety of interactions in the organ from which it is expelled [10]. When two or more medications employ the same transport route, DDI may result from a process of competition at the level of active tubular secretion. One example is how NSAIDs affect the amount of methotrexate toxicity when the anti-proliferative drug's renal excretion is inhibited [11].

The interactions between the drugs can, however, be used therapeutically. Probenecid, for example, can raise the serum levels of cephalosporins and penicillins, delaying their renal elimination and reducing dose requirements. Probenecid works by interfering with an organic anion transporter in the renal tubules by competitive inhibition, which raises the

plasma concentrations of other transporter substrates while decreasing excretion [12].

Inflammation is a biological defense mechanism that occurs when the body's tissue homeostasis is disrupted by the presence of biological, chemical, or physical agents. The immune system produces a number of pro-inflammatory mediators, but when these mediators are overproduced, as it happens in chronic inflammation, it can result in the development of a number of chronic diseases. As a result, it becomes crucial to slow down the inflammation process, and for this reason, non-steroid anti-inflammatory medicines are typically employed with the risk of negative side effects [13].

Non-steroidal anti-inflammatory drugs (NSAIDs) have become widely used as analgesics, antipyretics, and anti-inflammatory therapies across the world. The market size for NSAIDs in the United States was estimated at $5.78 billion in 2021, and it was expected to reach $7.10 billion by 2025. In 2019, the worldwide NSAIDs market was worth $15.58 billion, with a forecast of $24.35 billion by 2027 [14,15]. One of the major reasons driving the NSAIDs market is the rising prevalence of pain and inflammation-causing disorders [14]. In this study, we examine the anti-inflammatory activity of compound N-phenyl-2,4-dichlorophenyl hydrazone and it effect as efficacy enhancer in inflammation therapy.

2. Materials and Methods

2.1 Chemistry

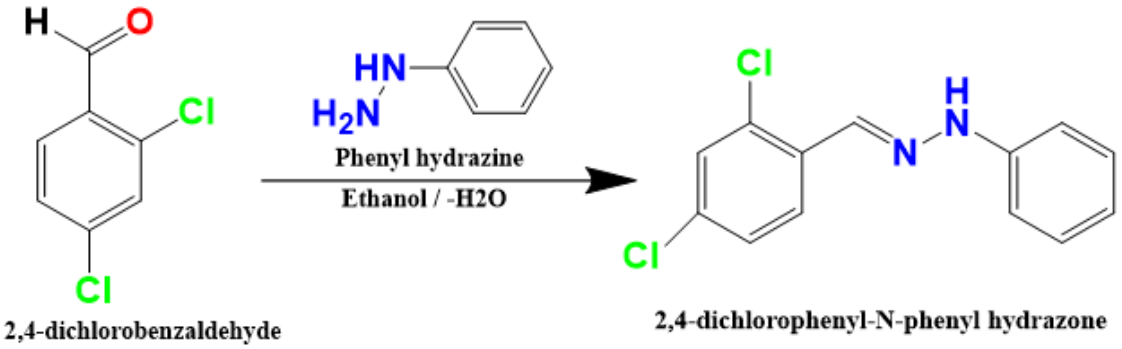
All the reagents used were purchased from Sigma Aldrich, and they were used with no further purification. Electrothermal Engineering LTD 9100 apparatus was employed in the melting points determination of the synthesized compounds. The FTIR spectra were recorded on Agilent technologies spectrometer model 543, and the 1H and 13C NMR spectra were obtained using a Brucker AMX 400 MHz spectrometer operating at 400 MHz and 101 MHz respectively with dimethyl sulfoxide (DMSO) used as the solvent. Chemical shifts (δ) are reported in parts per million and are referenced to the NMR solvent peak.

2.1.1 In-silico pharmacokinetics (ADME) and toxicity studies

The drug-likeness studies, *in-silico* pharmacokinetics, and toxicity studies were evaluated on ADMETlab 2.0 (https://admetmesh.scbdd.com) and Protox-II web (https://tox-new.charite.de). The ADMETlab 2.0 was used to evaluate detailed parameters of drug-likeness, absorption, distribution, metabolism, and excretion. The toxicity studies were conducted on Protox-II and ADMETlab 2.0 web servers.

2.1.2 Synthesis

Equimolar quantities of 2,4-dichloro benzaldehyde (20mmol) and phenylhydrazine (20mmol) were mixed in 30ml of ethanol at room temperature. The mixture was continuously stirred for 3hrs and the progress of the reaction was monitored by TLC. The white crystalline solid formed was filtered off, dried, and then recrystallized from pet ether.



Scheme 1: Synthesis of 1-(2,4-dichloro benzylidene)-2,4-phenyl hydrazine.

2.2 Pharmacology Study

2.2.1 Experimental Animals.

Twenty-eight Swiss albino mice (15-34 g) were used in the experiments. The animals were procured within the Zaria community, Kaduna State, Nigeria. The mice were housed in single-sex cages under a 12-hour light:12 hour dark cycle (lights on at 6 am) in a controlled-temperature room (22 ± 2 °C with 50 ± 10% humidity) at the animal house. The mice were kept for 2 weeks to stabilize, habituate (acclimatize) and become more adult. After 7 weeks, all the animals were adults at the age of 7 weeks and weights of 15–34 g. Availability of standard diet and water was ad libitum. All animal experiments were performed as per the requirement of the bio-ethical committee protocols of the ABU Committee on Animal Use and Care in compliance with the guidelines for the care and use of laboratory animals provided by the National Institute of Health (NIH publication no. 85–23, revised 1985). Ethical approval was sought and obtained from ABU Committee on Animal Use and Care.

2.2.2 Oral Acute Toxicity Studies and lethality (LD_{50}) test.

The acute toxicity and lethality (LD50) of the titled compounds were estimated in mice using the Fixed Dose Procedure (FDP) — OECD TG 420. This OECD guideline method does not use the death of animals as a clear sign of toxicity. Instead, any changes such as changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic, and central nervous systems, somatomotor activity, and behavior patterns. Attentions are particularly directed at observations of increased motor activity, anesthesia, tremors, arching and rolling, clonic convulsions, tonic extension, lacrimation, Straub reaction, salivation, muscle spasm, writhing, hyperesthesia, loss of righting reflex, depression, ataxia, stimulation, sedation, blanching, hypnosis, cyanosis and analgesia, diarrhea, lethargy, sleep, and coma were taken as a sign of toxicity (Deora *et al*., 2010) [16].

The limit test was employed in the experiment, a group (n = 3) was used for the test compound. The test animals were male Swiss albino mice with weights ranging between 20 – 25 g. The test dose of the titled compound was prepared by dissolving 150mg of each titled compound in 3 ml of deionized water to form an aqueous suspension of 50mg/ml concentration which is equivalent to 2000mg/kg per body weight for mice weighing 25g. The compound was administered orally in the form of deionized water suspension (1% w/v) at an appropriate volume equivalent to a dose of 2000 mg/kg (n = 3). Animals were observed continuously for the first one hour for any toxic symptoms after administration and then for the next 24 hours, 48 hours, 7 days, and 14 days.

2.2.3 Anti-inflammatory activity evaluation.

The anti-inflammatory activity of the titled compound was evaluated using Carrageenan-induced hind paw edema in mice model. The method of Kasahara *et al*., 1985 [17] as described in our previous study [14].
The measurement of the hind paw was carried out using a thickness gauge vernier caliper before each treatment (Vo) and in each interval (Vt) after the administration of the test compound, dosing vehicle, and the reference drug. The data were expressed as mean ± standard error of the mean (n=5). The data were analyzed statistically using a Two-way analysis of variance (ANOVA) with replication, residual error test, and Tukey's Multiple Comparison Test. The percentage of swelling inhibition was calculated using the following equation:

$$\text{Inhibition } (\%) = \{[(Vt - Vo)_{control} - (Vt - Vo)_{treated}]/(Vt - Vo)_{control}\} \times 100 \text{Eq. (4)}$$

Where Vt and Vo relate to the average volume in the hind paw of the mice after carrageenan injection and before carrageenan injection respectively. All the results were expressed as Mean ± Standard Error of Mean (S.E.M.) and percentage of inhibition.

3. Results

3.1 In-silico pharmacokinetics (ADME) and toxicity studies

Compounds	Caco-2	MDCK	Pgp-inhi.	Pgp-subs.	HIA	F30%	F20%	F10%
Hydrazone	-4.28	12.0×10^{-6}	0.004	0.032	0.003	0.976	0.062	0.55
Piroxicam	-6.05	18.0×10^{-6}	0.082	0.002	0.010	0.003	0.002	0.56

Table 1: Absorption

Compounds	Plasma Protein Binding	Volume Distribution	BBB	Fraction Unbound
Hydrazone	100.52%	2.731 L/kg	0.198	0.82%
Piroxicam	73.60%	0.340 L/kg	0.967	27.15%

Table 2: Distribution

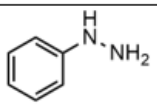
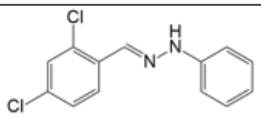
Compounds	CYP1A2		CYP2C19		CYP2C9		CYP2D6		CYP3A4	
	Inhib.	Subs.	Inhib.	Subs	Inhib.	Subs	Inhib.	Subs	Inhib.	Subs
Hydrazone	0.99	0.47	0.95	0.12	0.81	0.84	0.55	0.76	0.20	0.26
Piroxicam	0.17	0.62	0.13	0.20	0.10	0.95	0.22	0.14	0.20	0.58

Table 3: Metabolism.

Compounds	Clearance (mL/min/kg)	Half-life ($T_{1/2}$)
Hydrazone	5.697	0.152
Piroxicam	1.033	0.561

Table 4: Excretion

3.2 Synthesis

Entry	Hydrazine	Product	Time (hrs)	Yield (%)	Mp (ºC)
1			3	68.80	123-124

1-(2,4-dichlorobenzylidene)-2-phenylhydrazine. Yield 68.80%, Crystalline white solid, mp 123-124 ºC. FTIR (KBr, cm-1): 3302 (N-H), 3030 (C-H_{imine}), 1572 (C=N), 1517 (C=$C_{aromatic}$), 1252 (C-N), 1047 (C-Cl). H^1 NMR spectrum (400 MHz, DMSO-*d*6) δ, ppm: H^1 NMR spectrum (400 MHz, DMSO-*d*6) δ, ppm: 7.01 d (1H_{arom}, *J* = 7.1 Hz), 7.16 d (2H_{arom}, 8.2 Hz), 7.22 d (2H_{arom}, 7.8 Hz), 7.47 (H_{arom}, 8.5 Hz), 7.63 (H_{arom}, 1.7 Hz), 8.09 (H_{arom}, 8.4), 8.19 (H_{imine}), 11.23 (1H, NH). ^{13}C NMR spectrum (101 MHz, DMSO-*d*6), δ, ppm: 112.30, 126.11, 126.54, 127.94, 128.62, 129.56, 131.89, 133.18, 134.34, 136.91, 139.89.

Table 5: Synthesis of N-phenyl-2,4-dichlorophenyl hydrazone.

3.3 Pharmacology

3.3.1 Oral Acute Toxicity Studies and Lethality

Compounds	Dose(mg/kg)	No. of Mice	Sign of toxicity	No. of dead mice
Hydrazone	2000	3	Lithargy	None

Table 6: Oral Acute Toxicity Studies and Lethality (LD_{50}) results.

3.3.2 Anti-inflammatory activity evaluation results.

Test sample	0hr	1hr 30min.	3hrs	4hrs 30min.	6hrs
*10 mg/kg	1.30 ± 0.02	1.58 ± 0.02	1.61 ± 0.04	1.54 ± 0.04	1.37 ± 0.03
30 mg/kg	1.26 ± 0.04	1.69 ± 0.06	1.63 ± 0.02	1.54 ± 0.02	1.39 ± 0.01
50 mg/kg	1.29 ± 0.05	1.68 ± 0.06	1.67 ± 0.09	1.60 ± 0.03	1.54 ± 0.01
Piroxicam	1.32 ± 0.03	1.66 ± 0.04	1.67 ± 0.04	1.53 ± 0.01	1.52 ± 0.02
Celecoxib	1.27 ± 0.01	1.62 ± 0.04	1.66 ± 0.05	1.56 ± 0.02	1.52 ± 0.02
Normal Saline	1.27 ± 0.03	1.80 ± 0.05	1.87 ± 0.07	1.81 ± 0.04	1.76 ± 0.03

* = Co-administration of 10 mg/kg each of the hydrazone and piroxicam. Mean ± S.E.M of hind paw thickness, n=5, p-value = 0.00064 < α = 0.05, F = 5.31 > F Critical = 2.46

Table 7: Mean ± S.E.M of hind paw thickness at 10mg/kg dose. n=5

Test sample	0hr	1hr 30min.	3hrs	4hrs 30min.	6hrs.
10 mg/kg*	0.00%	48.09%	47.97%	56.41%	86.48%
30 mg/kg	0.00%	17.94%	37.84%	47.58%	72.54%
50 mg/kg	0.00%	26.72%	36.82%	43.49%	50.00%
Piroxicam	0.00%	35.50%	42.06%	55.41%	60.25%
Celecoxib	0.00%	33.21%	34.80%	46.84%	49.18%
Normal Saline	0.00%	0.00%	0.00%	0.00%	0.00%

* = Co-administration of 10 mg/kg each of the hydrazone and piroxicam.

Table 8: Percentage inhibitions of hind paw edema at 10mg/kg dose.

4. Discussion

4.1 Chemistry

Twenty minutes after the start of the reaction white crystalline solids were produced with 68.80% yield after three hours according to results in **Table 5**. The melting point was deduced to be 123-124 ºC. Spectroscopic analysis of the purified product indicated N-H stretching, imine C-H

stretching, and C=N stretching are features of the FTIR absorption signals at 3302 cm^{-1}, 3030 cm^{-1}, and 1572 cm^{-1}. The hydrazone functional group's synthesis in the molecule was verified by the singlet proton peaks' locations at 8.19 ppm and 11.23 ppm as well as by the C-13 peak's location at 136.91 ppm [18].

4.2 Pharmacology

4.2.1 Oral acute toxicity and lethality studies

The oral acute toxicity studies and LD_{50} of compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine was evaluated at 2000 mg/kg dose in mice according to OECD guidelines. There was observed motor activity reduction and slight sleepiness which summed up as lethargy as a sign of toxicity within the first hour of administration. This symptom wears off after the first our oral administration with no further sign of toxicity over the fourteen days of study. This examination implies a remote risk of acute intoxication and indicates a high degree of relative safety for the oral administration of these compounds according to results in **Table 6**. Furthermore, there was no mice death count observed during the fourteen days study, implying that the lethality dose of compound N-phenyl-2,4-dichlorophenyl hydrazone is greater than 2000 mg/kg dose. By this, the LD_{50} of the compound 1-(2,4-dichloro phenyl)-2-phenyl hydrazine can be said to fall into Class V of the globally harmonized system (GHS) (2000 mg/kg < LD50 ≤ 5000 mg/kg).

4.2.2 Anti-inflammatory activity

Compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine demonstrated statistically significant (p-value < 0.005) anti-inflammatory activity at all the tested doses in the carrageenan-induced paw edema in mice according to the results in **Table 7**.

The test compound was observed to exhibit slow onset of action at 30 mg/kg and 50 mg/kg doses with percentage inhibitions of 17.94% and 26.72% after first one and half hour of inflammation induction. These are compared to the 35.50% and 33.21% inhibitions of paw edema of the reference drugs piroxicam and celecoxib respectively. The slow onset of action of compound 1-(2,4-dichloro phenyl)-2-phenyl hydrazine may be due to its pharmacokinetic profile [19] as observed in the results in **Table 1-4**. Though compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine exhibited good absorption in both human colon adenocarcinoma cell lines (Caco-2) , Madin−Darby Canine Kidney cells (MDCK), and human intestinal absorption (HIA) with -4.28 cm/s, 12.0×10^{-6} cm/s, 0.003 respectively in *in-silico* models which is better than that of piroxicam with -6.05 cm/s, 18.0×10^{-6} cm/s, and 0.010 respectively as demonstrated in **Table 1**. It high plasma protein binding (PPB) of 100.52% leads to its high distribution within tissues (Vd = 2.731 L/kg) with consequent low fraction unbound (F_u = 0.82%) thereby causing it low bioavailability, as 30% of the test compound is not bioavailable (F30% = 0.976) compared to piroxicam with proper PPB of 73.60%, volume distribution of 0.340 L/kg, F_u of 27.15%, and consequent high bioavailability (F30% = 0.003) **Table 2**. This explains why compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine exhibited slow onset of action and while piroxicam exhibited rapid onset of action accordingly.

However, there is observed increase in onset of action as the dose was increased from 30mg/kg to 50mg/kg dose of compound N-phenyl-2,4-dichlorophenyl hydrazone suggesting possible increase in bioavailability. Also, compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine exhibited progressive increase in activity over the hours of inflammation induction in carrageenan-induced paw edema in mice for each dose as indicated in **Table 8**. Conversely, when the activity of compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine was compared at these two doses, there was steady decrease in activity after the first three hours of inflammation induction as the dose increased from 30mg/kg to 50mg/kg. This observed decrease in activity has been attributed to the high clearance rate (CL = 5.697 mL/min/kg) of compound 1-(2,4-dichloro phenyl)-2-phenyl hydrazine, resulting in increased in clearance and reduced F_u as dose increases. This high clearance of compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine compared to the rate of clearance of piroxicam (CL = 1.033 mL/min/kg) in **Table 4** can also be traced to it possible metabolism by CYP enzymes [19] in **Table 3**. This can be accounted for by its possible early metabolism and contributing first-pass effect which is a consequence of its high lipophilicity [19], and generally limiting it bioavailability.

Co-administration of 10 mg/kg dose of compound N-phenyl-2,4-dichlorophenyl hydrazone and 10 mg/kg of piroxicam resulted in marked increase in onset of action of 48.09% compared to either of the two agents' independent onset of action. Furthermore, the co-administered

dose was superior in activity to piroxicam and celecoxib used as reference drugs with steady increase in activity over the hours of inflammation induction. More importantly, the co-administered dose demonstrated significant longer duration of action with 86.48% inhibition of paw edema compared 60.25% and 49.18% for piroxicam and celecoxib respectively. Though the mechanism associated with the enhanced efficacy has not been fully elucidation, however, the in-silico studies revealed that compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine is a stronger inhibitor of P-glycoprotein compared to piroxicam with 0.004 and 0.082 respectively as illustrated in **Table 1.** Also, piroxicam is an inhibitor of cytochrome P450 metabolizing enzymes CYP1A2, CYP2C19, and CYP3A4 that are responsible for the metabolism of compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine. Therefore, the efficacy enhancements of the combinatorial approach maybe primarily due to pharmacokinetic drug-drug interactions (DDI). Furthermore, the longer half-life of compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine ($T_{1/2}$ = 0.152) compared to piroxicam ($T_{1/2}$ = 0.561) as illustrated in **Table 4** suggests that compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine will remain in circulation eliciting activity longer after piroxicam may have been excreted contributing to the longer duration of action of the combined dose. Therefore, the enhanced activity of the co-administered dose maybe due to increased inhibition of P-glycoprotein transporter and cytochrome P450 metabolizing enzymes leading to increased bioavailability and delayed metabolism and a consequent increased exposure to the target site. However, since the two agents are active against particular disease state, the observed efficacy is due to contributing activities of both compound N-phenyl-2,4-dichlorophenyl hydrazone and piroxicam which explains the synergistic pharmacokinetic-pharmacodynamics DDIs.

Furthermore, compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine have demonstrated significant antimicrobial activity in *in-vitro* assay [18] against wide range of bacterial and fungi strains including *Helicobacter pylori* which causes excessive secretion of gastro-intestinal acid. It has also been demonstrated that compound 1-(2,4-dichloro benzylidene)-2-phenyl hydrazine efficiently inhibited proton pump enzyme H^+/K^+ ATPase showcasing plausible interaction with key amino acid CYS822 [19]. Therefore, the use of compound N-phenyl-2,4-dichlorophenyl hydrazone in combination therapy as a new design strategy in the treatment of inflammatory diseases will lead to improved therapeutic index as a result of enhanced efficacy and diminished gastro-intestinal toxicity associated with piroxicam and other NSAID therapy. The enhanced efficacy is an advantage over the conventional NSAIDs-proton pump inhibitor combination therapy and, therefore, resultant increase in therapeutic index.

Authors' contributions

Sodeeq Babalola, Abdullahi Y. Idris, Sanni Y. M., and Asmau N. Hamza contributed in the concept development, design and method development.
Sodeeq Babalola, Abdulrahman Bashir, Umar Yakubu contributed in the laboratory experiment.
Sodeeq Babalola and AbdulNafiu Bushirah Alaro contributed in results analysis.
Sodeeq Babalola, Nosakhare Igie, Isaiah Odeyemi, Salimat Sofela, Thompson Odion Igunma, and Oladimeji S. Olaluwoye contributed in manuscript writing and review.

Conflict of interest

All authors declared no conflict of interest.

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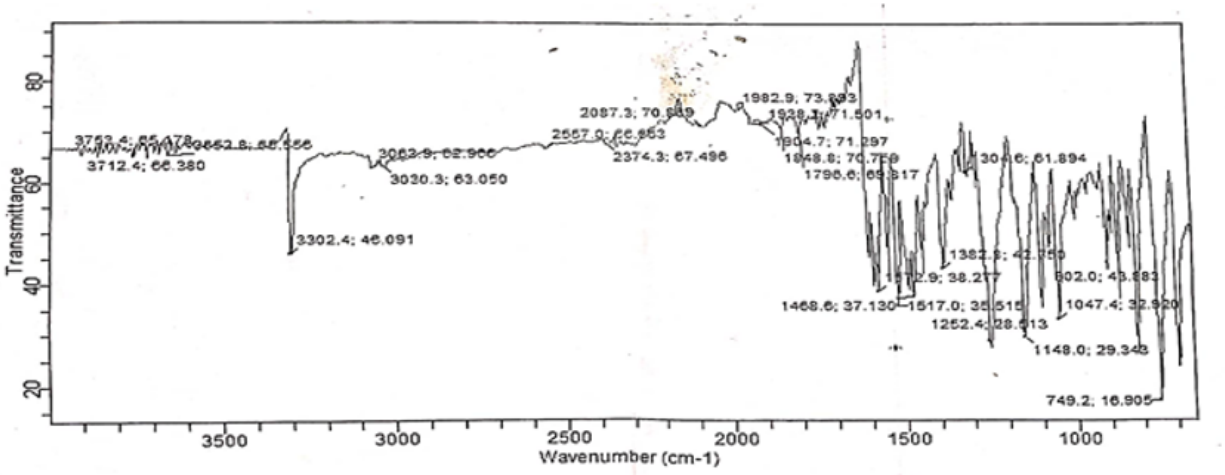
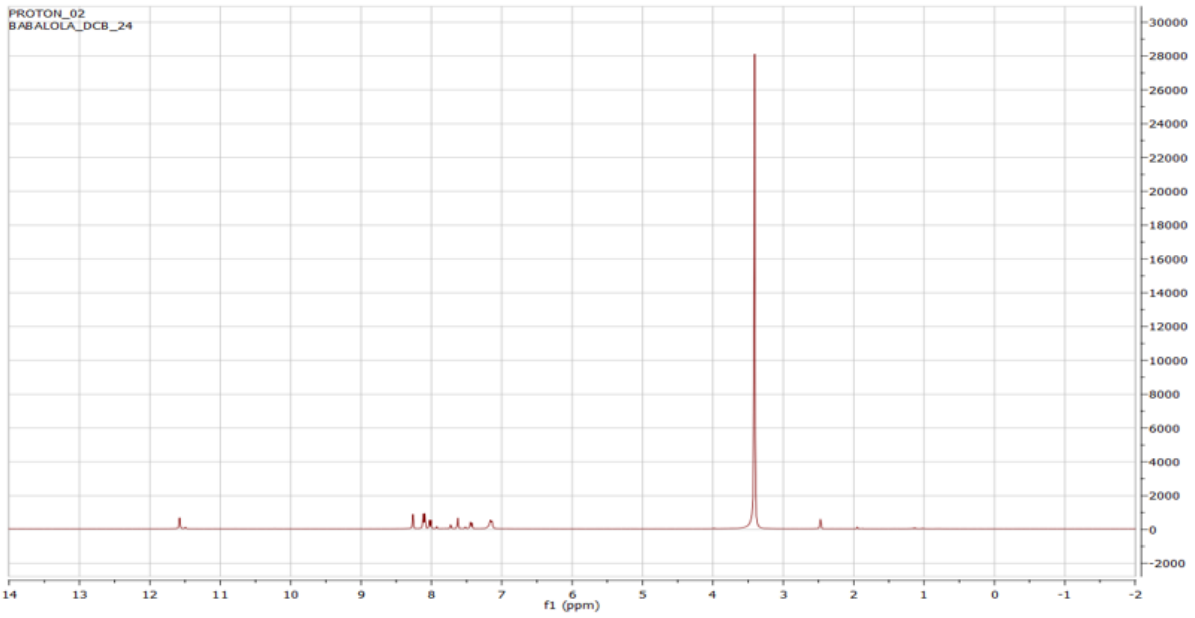
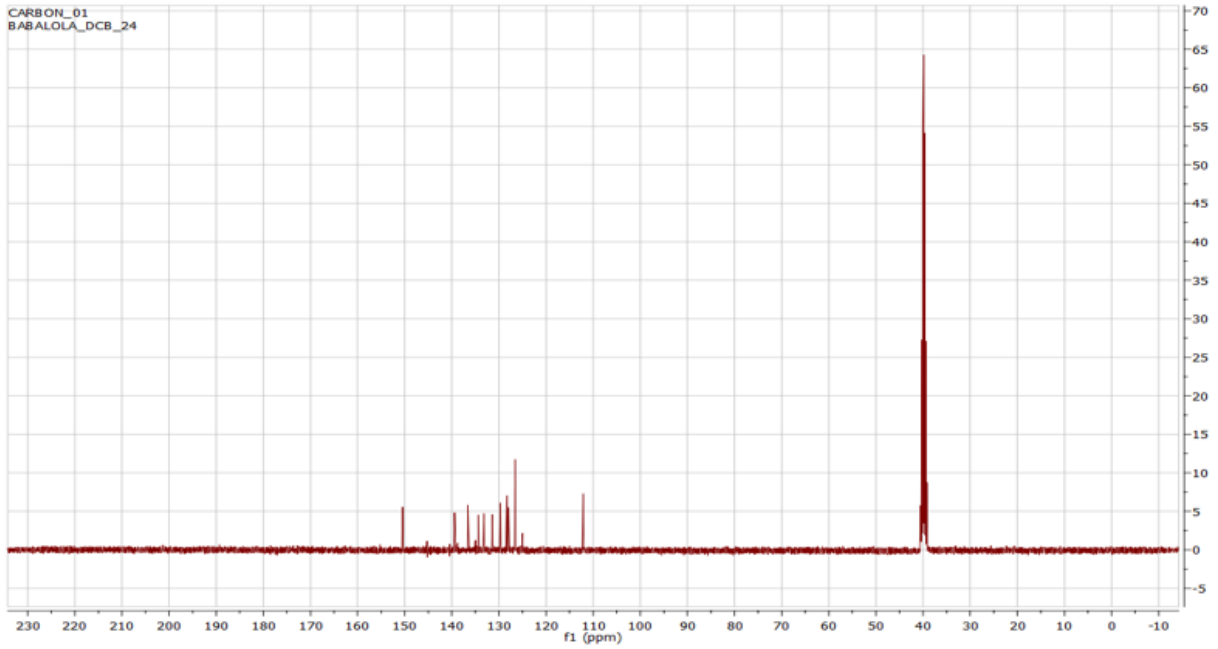
SUPPORTING MATERIALS



S1: FTIR Spectrum of compound 1-(2,4-dichloro benzylideen)-2-phenyl hydrazine



S2: Proton NMR spectrum of compound 1-(2,4-dichloro benzylideen)-2-phenyl hydrazone



S3: C-13 spectrum of compound 1-(2,4-dichloro benzylideen)-1-phenyl hydrazine

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