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MEDICINAL CHEMISTRY | RESEARCH ARTICLE

Synthesis of the 3,5-diphenyl-1*H*-pyrazole and cytogenetic and oxidative alterations after exposure of cultured human whole blood cells

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Abstract: The 3,5-diphenyl-1*H*-pyrazole was obtained by condensation reaction of dibenzoylmethane and thiosemicarbazide in acetic acid under conventional heating and microwave irradiation method. The structure of the 3,5-diphenyl-1*H*-pyrazole confirmed by IR, ¹H, and ¹³C NMR and X-ray diffraction and the geometry optimization was carried out using density functional theory (DFT) methods at B3LYP/6-31G, 6-31G(d), 6-31G(d, p), 6-311G(d, p), 6-311G(2d, 2p), 6-31+G(d, p), 6-311++G(d, p) levels. In addition, cytotoxic and oxidative effects were investigated in cultured human peripheral blood cells.

Subjects: Biochemistry; Pharmacology; Chemistry; Medicine

Keywords: pyrazole; thiosemicarbazide; cytotoxic

1. Introduction

The pyrazole compounds are the important class of nitrogen containing heterocyclic compounds. Recently, substitute pyrazole derivatives are significant interest due to their roles in the medicinal and agriculture industries (1–5).

ABOUT THE AUTHORS

Our research group has been working on the synthesis of pyrazole, pyrimidine and pyridazinone compounds from the class of heterocyclic compounds, the investigation of advanced step reactions, and the biological activities of these compounds.

We are wondering what the consequences will be if the research reported in this manuscript is to be studied *in vivo* in the future. If these *in vivo* tests performed at the advanced stage of the current study are positive, more comprehensive projects and articles can be made accordingly.

PUBLIC INTEREST STATEMENT

We have been working on heterocyclic chemistry for the last 20 years. We concentrated more on pyrazole-derived compounds due to interesting activity of analgesic, antipyretic, antihypertensive, anti-inflammatory, pesticides, herbicides, plant growth regulators, and calcium ion modulators.

In the study, we obtained pyrazole compound with conventional heating and microwave irradiation method and characterized on the basis of ¹H NMR, ¹³C NMR, FT-IR, and elemental analysis. Additionally, the structure of 3,5-diphenyl-1*H*-pyrazole was illuminated with X-ray diffraction and pyrazole compound have been optimized geometrically with density functional theory (DFT) in Gaussian in order to obtain information about the 3D geometries and electronic structures.

The 3,5-diphenyl-1*H*-pyrazole tested *in vitro* in order to assess their cytotoxic and pro-oxidant potentials. On the biological properties including tests 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide MTT and Lactate dehydrogenase (LDH) assay, Total antioxidant capacity (TAC) and Total oxidative stress (TOS) levels were performed.

The pyrazole derivatives are found in the structures of a variety of drugs for example, Zometapine, Viagra, Celebrex (6–8), and some pesticides like Cyenopyrafen, Fenpyroximate, and Tebufenpyrad (9).

The various methods have been developed in order to the synthesis of the substituted pyrazole derivatives. Hydrazines and 1,3-diketones are most commonly used in the synthesis of substituted pyrazole compounds, but these reactions lead to the formation of undesired isomers (10, 11, 12). In this study, we synthesized the 3,5-diphenyl-1H-pyrazole using thiosemicarbazide and dibenzoylmethane with catalyzed by HCl under conventional heating and microwave irradiation. In both methods, products of excellent purity and high efficiency were obtained.

Also, some structural data for the 3,5-diphenyl-1H-pyrazole (CAS) were determined by DFT at B3LYP with using basis sets 6-31G, 6-31G(d), 6-31G(d, p), 6-311G(d, p), 6-311G(2d, 2p), 6-31 + G(d, p), and 6-311++G(d, p), respectively. The computational data were done with Gaussian09 (13).

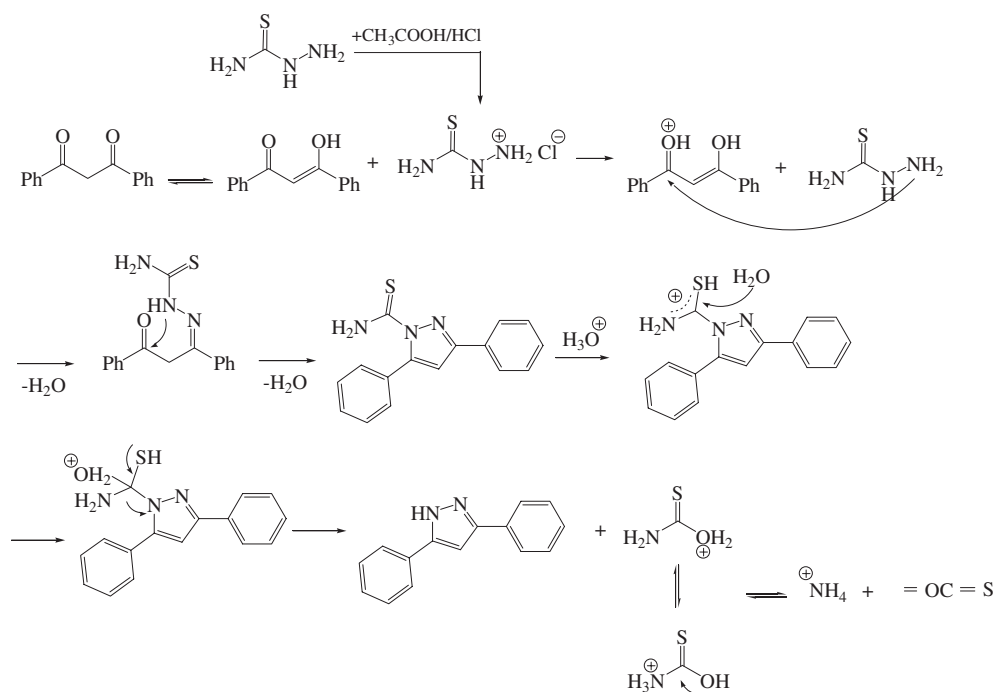
The pyrazole compounds have an ability to support cytotoxic properties (14, 15). In this study the antiproliferative action of 3,5-diphenyl-1H-pyrazole was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays; oxidative effects by measuring total antioxidant capacity (TAC) and total oxidant status (TOS) levels on cultured human whole blood cells.

2. Synthesis

The 3,5-diphenyl-1H-pyrazole prepared reaction of dibenzoylmethane and thiosemicarbazide in acetic acid (Scheme 1).

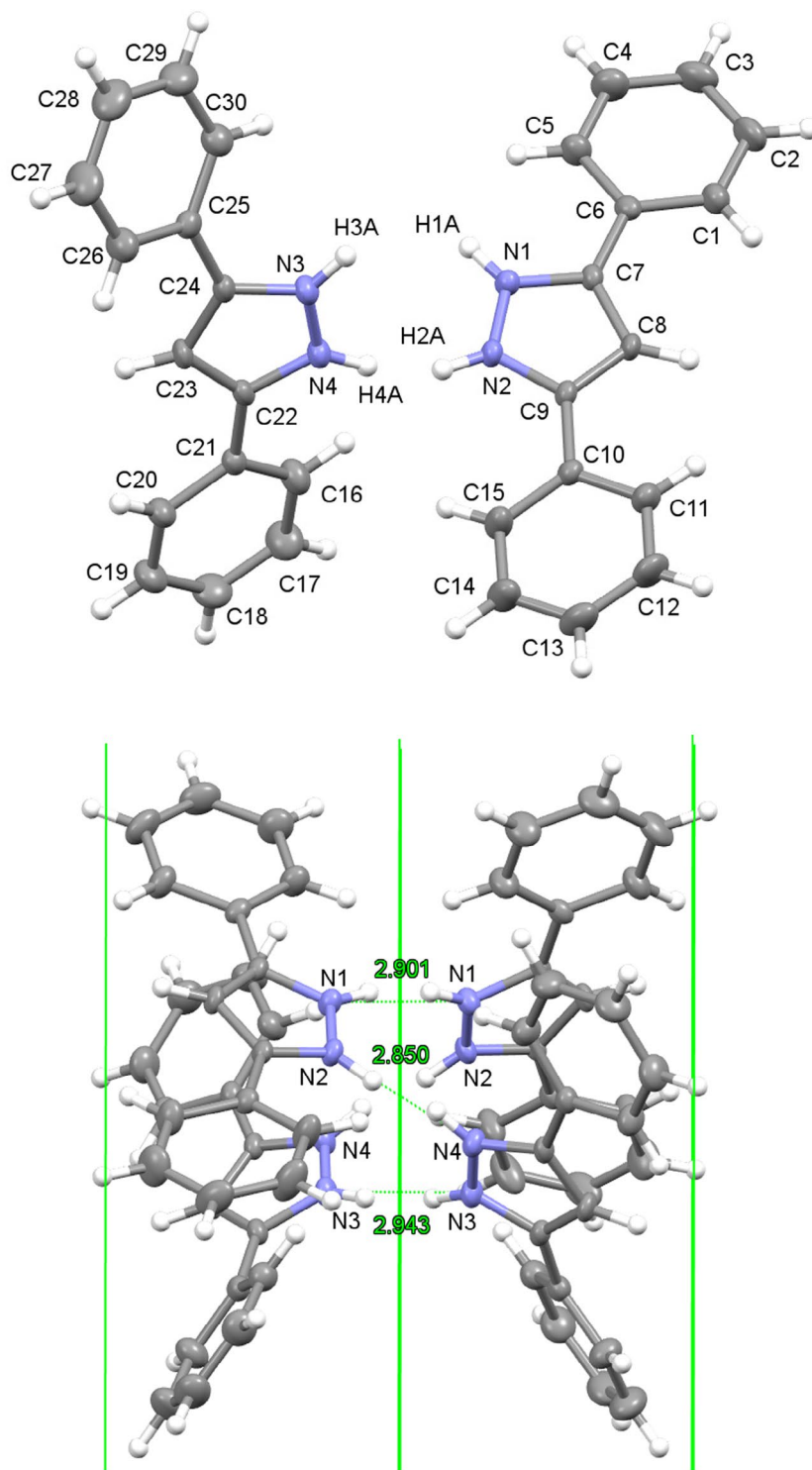
This reaction was catalyzed by HCl and resulted in good yield. The heating was carried out by both conventional and microwave irradiation. The rates and percentage yields were compared by both methods. The results show that the microwave irradiation is rapid and purity more than conventional heating.

Scheme 1. Synthesized of 3,5-diphenyl-1H-pyrazole.



The data of the 3,5-diphenyl-1*H*-pyrazole were agreed with the one reported in the literature (16, 17). The IR, ¹H, and ¹³C-NMR data are in accordance with the proposed structure. Also molecular structure of the compound has been successfully determined using single crystal X-ray diffraction analysis (Figure 1).

Figure 1. The single crystal X-ray diffraction.



X-ray diffraction of the molecule showed the tetramer structure is the asymmetric unit (Figure 1). The pyrazoles form cyclic tetramer subject to quadruple proton transfers (18, 19). Green lines show 2-fold proper rotation axis.

3. Calculation analysis

The 3,5-diphenyl-1*H*-pyrazole was studied in theoretical analysis in order to obtain some structural and physicochemical data. The geometry optimizations were performed within the framework of density functional theory (DFT) at B3LYP with using basis sets 6-31G, 6-31G(d), 6-31G(d, p), 6-311G(d, p), 6-311G(2d, 2p), 6-31+G(d, p), and 6-311++G(d, p), respectively. The calculated total energies (zero point corrected), frontier molecular orbital energies (HOMO and LUMO), and inter-frontier energy gap data were presented in Table 1. The lowest gap energy was obtained in 6-311G(2d, 2p) base set. For this reason, the most suitable base set could be the 6-311G(2d, 2p) than other.

The representative optimized geometry structure and the frontier orbitals are shown in Figure 2. The electron density of HOMO is localized on pyrazole ring moiety, and the electron density of LUMO is distributed at the main molecular skeleton.

Table 1. Frontier molecular orbital energies (HOMO and LUMO) and inter-frontier energy gap

Basis set	Total energy (au)	HOMO (eV)	LUMO (eV)	E (eV)
6-31G	-683.64333774	-8.10	2.85	5.25
6-31G(d)	-683.90327128	-8.00	3.02	4.98
6-31G(d, p)	-683.92719176	-8.01	3.01	5.00
6-311G(d, p)	-683.92719176	-8.01	3.01	5.00
6-311G(2d, 2p)	-683.94713410	-7.95	3.07	4.88
6-31+G(d, p)	-683.65921988	-8.09	0.61	7.48
6-311++G(d, p)	-683.76898475	-8.11	0.58	7.53

Figure 2. Frontier orbitals Ball Bond Type (a), HOMO (b), LUMO (c) (6-311G(2d,2p) level).

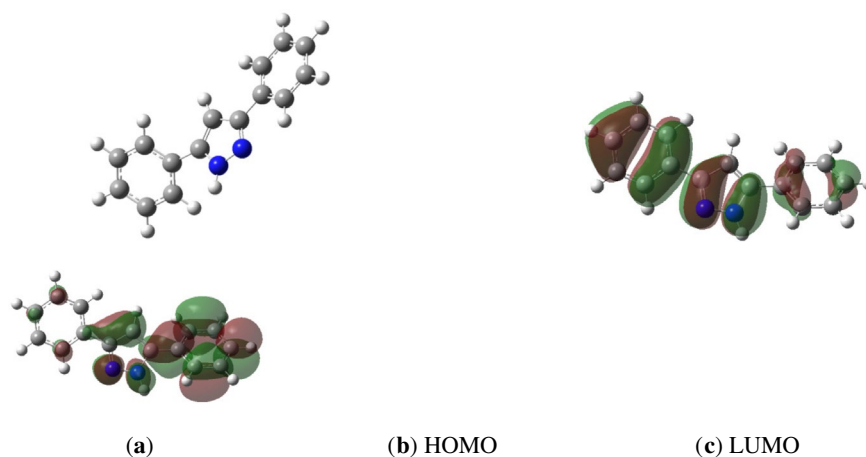
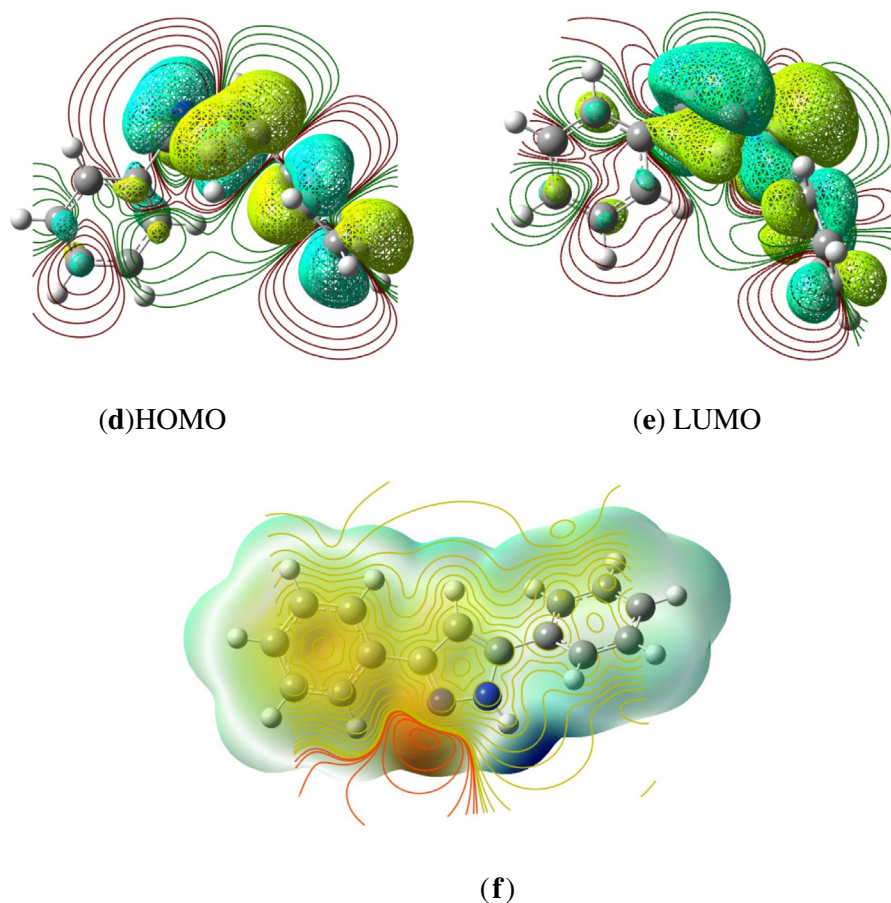


Figure 3. The three-dimensional molecular orbital HOMO and LUMO electrostatic potential maps and total density SCF/ESP at the planar grid map.



The three-dimensional molecular orbital HOMO (d) and LUMO (e) electrostatic potential maps with contour and total density SCF/ESP at the planar grid map (f) were given in Figure 3. If the molecular orbital electrostatic potential map is examined, positive charge is indicated by blue, negative charge is indicated by yellow and red.

4. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT and Lactate dehydrogenase (LDH) assay

The MTT colorimetric assay was used to measure cytotoxicity and cell proliferation. The 3,5-diphenyl-1*H*-pyrazole was evaluated for its cytotoxicity on cultured human whole blood cells. Cultured human whole blood samples were treated with various concentrations of the 3,5-diphenyl-1*H*-pyrazole for 48 h. The obtained results showed that the 3,5-diphenyl-1*H*-pyrazole has cytotoxic potentials at all tested concentrations (Table 2). In addition, in order to determine cytotoxic effects of the 3,5-diphenyl-1*H*-pyrazole on cultured human whole blood cells, LDH release assay was also used. Pyrazole derivative increased the release of LDH enzyme in a dose-dependent manner (Table 2). These results are in accordance with results of some recent studies. In a recent study, dichloridobis[1-[amino(thio)oxymethyl]-5-hydroxy-3-phenyl-1*H*-pyrazole- κ N2]copper(II) was found as cytotoxic on human umbilical vein endothelial cells (HUVECs) (20). Similarly it was examined cytotoxic properties of some nickel complexes with pyrazole ligands and reported that the treatments with a range of drug concentrations (10^{-7} to 10^{-3} M) for 48 h caused increases of cell death on HUVECs by MTT assay (21). In another study, it was determined that some pyrano[2,3-*c*]pyrazoles showed low toxic effects on MDCK cell line (22). In addition, Reddy et al. (22) reported that 1,3-diphenyl-1*H*-pyrazole derivatives have cytotoxic effect on several cancer cell lines (22). Moreover, some *N*-arylpyrazole derivatives showed cytotoxic effects against various human tumor cell lines (23, 24).

Table 2. The cytotoxic and oxidative effects of 3,5-diphenyl-1H-pyrazole *in vitro*

Concentration (mg/L)	MTT (cell viability %)	LDH release (%)	TAC (Trolox Equiv/mmol L ⁻¹)	TOS (H ₂ O ₂ Equiv/ μ mol L ⁻¹)
Control ⁻	100 ^e	100 ^a	6.2 \pm 0.7 ^d	11.6 \pm 2.3 ^a
Control ⁺	40.3 \pm 5.9 ^a	245.4 \pm 25.8 ^e	12.5 \pm 1.5 ^e	39.1 \pm 3.2 ^d
10 mg/L	80.8 \pm 7.2 ^d	145.5 \pm 18.2 ^b	5.9 \pm 0.7 ^d	12.1 \pm 2.8 ^a
25 mg/L	70.4 \pm 6.8 ^c	151.3 \pm 16.5 ^b	5.2 \pm 0.8 ^c	13.6 \pm 2.4 ^b
50 mg/L	70.9 \pm 7.0 ^c	164.8 \pm 17.0 ^c	5.0 \pm 0.5 ^c	14.7 \pm 2.8 ^b
100 mg/L	68.4 \pm 6.2 ^c	174.5 \pm 19.2 ^{cd}	4.6 \pm 0.7 ^b	16.3 \pm 3.1 ^b
200 mg/L	55.8 \pm 6.4 ^b	189.2 \pm 17.6 ^d	4.1 \pm 0.8 ^{ab}	19.4 \pm 2.9 ^{bc}
400 mg/L	39.8 \pm 4.9 ^a	193.1 \pm 16.3 ^d	3.8 \pm 0.8 ^a	26.1 \pm 3.0 ^c

Notes: Each individual whole blood culture without the compound 1 was studied as a negative control group (Control⁻). Different letters (e.g. a, b, c, d) denote a significant difference ($p < 0.05$) between treatments based on the Duncan's *post hoc* analysis.

5. Total antioxidant capacity (TAC) and Total oxidative stress (TOS) levels

The balance of reactive oxygen species (ROS) formation and antioxidant defense system is crucial to cell survival (25). On the contrary; imbalance of oxidant/antioxidant levels can cause oxidative stress. This state of oxidative stress may result in injury to proteins, DNA and membrane lipids which can cause cell death (26). And, the increases of TOS levels or decreases of TAC levels cause accumulation of free radicals such as ROS and NOS. This situation creates an environment of oxidative stress (OS), where ROS can cause damage to cytotoxicity and genotoxicity (27–29). Therefore, we studied the combination of cytotoxicity (MTT) and oxidative (TAC and TOS) parameters for evaluating biological effects of the 3,5-diphenyl-1H-pyrazole *in vitro*.

The antioxidant and oxidant properties of the 3,5-diphenyl-1H-pyrazole have not been investigated on human peripheral blood cells. To determine antioxidant/oxidant potentials, TAC and TOS assays performed using automated colorimetric measurement methods. The obtained results showed that the 3,5-diphenyl-1H-pyrazole did not change the TAC levels in cultured human whole blood cells in any concentrations. Besides, it increased TOS levels in a dose-dependent manner compared with the controls as shown in Table 2. In accordance with our study, Reddy et al. (22) exhibited that the 3,5-diphenyl-1H-pyrazole derivatives increased the production of reactive oxygen species (22). Besides, it has been reported that the pyrazole derivatives causes oxidative stress by induction CYP2A5 and CYP2E1 (30–32) and inhibits the effective antioxidant enzyme; catalase activity (33). Likewise, Lu et al. (34) found that the pyrazole derivatives treatment alone can induce oxidant stress in rats as shown by increased protein carbonyl formation, malondialdehyde levels, and *in situ* superoxide production.

6. Conclusion

As a conclusion, we synthesized known compound using different method and tested *in vitro* in order to assess cytotoxic and pro-oxidant potentials. The 3,5-diphenyl-1H-pyrazole showed cytotoxic and pro-oxidant potentials on cultured human whole blood cells. But it requires further studies before using in medical and other industrial areas.

7. Experimental

Melting point was measured with an electrothermal apparatus. Routine FT-IR spectra were recorded on a Thermo Scientific FT-IR (ATR sampling accessory). ¹H-NMR spectra were recorded on a Bruker 400 MHz spectrometer with tetramethylsilane as internal standard. Microwave irradiation was carried out in Anton Paar microwave reactor (300 W). All experiments were followed by TLC using DC Alufolien Kieselgel 60 F 254 Merck and Camag TLC lamp (254/366 nm).

7.1. Syntheses of the 3,5-diphenyl-1H-pyrazole

7.1.1. The classical heating method

An equimolar mixture of dibenzoylmethane (1 mmol) and thiosemicarbazide (1 mmol) was refluxed in 10 ml acetic acid with catalyzed HCl for approximately 8 h. After evaporation, the oily residue obtained was treated with dry ether. The crude product formed was recrystallized from toluene to give 0.166 g (75%).

7.1.2. The microwave irradiation method

An equimolar mixture of dibenzoylmethane (1 mmol) and thiosemicarbazide (1 mmol) in 10 ml acetic acid with catalyzed HCl was introduced into a glasses cylindrical flask placed in an Anton Paar microwave reactor and irradiated (300 W) for 30 min at 120°C under pressure. After cooling, the solvent was evaporated under reduced pressure; the oily residue obtained was treated with dry ether. The crude product formed was recrystallized from toluene to give 0.178 g (80%).

En: 199–200°C, IR (cm⁻¹): 3137, 3085, 2987, 2841, 1888, 1606, 1486, 1308, 1087, 971, 828, 769. ¹H-NMR (DMSO-*d*₆): δ (ppm) 7.01 (s, 1H, =CH), 7.37–7.70 (m, 10H, H_{arom}), 13.38 (s, 1H, NH). ¹³C-NMR (DMSO-*d*₆): δ (ppm) 99.60, 125.07, 127.77, 128.81, 129.40, 133.52, 144.2. Anal. Calcd for C₁₅H₁₂N₂: C, 81.79; H, 5.49; N, 12.72. Found: C, 81.80; H, 5.45; N, 12.70.

7.2. Biological assay

7.2.1. Cell cultures

The human whole blood cultures were set up according to the protocol described by Evans and O'Riordan (23) with minor modification. The blood samples were obtained from four healthy, non-smoking, non-alcoholic, not under drug therapy and with no recent history of exposure to mutagens; males aged 26–28 years. The heparinized blood (0.4 mL) was cultured in 6.0 mL of culture medium (PB-MAX Karyotyping Medium, Gibco, Spain) with 5.0 mg/mL of phytohemagglutinin (Sigma Aldrich, Steinheim, Germany) (35). The 3,5-diphenyl-1H-pyrazole was dissolved in ethanol (40 μL) and a range of its different concentrations (10, 25, 50, 100, 200 and 400 μg/mL) was added to the cultures just before the incubation. The concentrations were selected according to the previous report (36). The negative control was set up with only solvent and without tested compound. Triton-X (containing 20 μL of 0.1% Triton X-100 in 0.85% saline, Sigma-Aldrich) was used as the positive control in the MTT and LDH assays. Ascorbic acid (final concentration of 10 μM, Sigma-Aldrich) and hydrogen peroxide (final concentration of 25 μM, Sigma-Aldrich) were also used as the positive controls in TAC and TOS analysis, respectively.

7.2.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay

The viability of the cells assessed by measuring the formation of a formazan from MTT spectrophotometrically (MTT cell proliferation kit Cayman Chemical Company, USA). The human whole peripheral blood samples were seeded in 96-well plates. Cells were incubated at 37°C in a humidified 5% CO₂/95% air mixture and treated with the 3,5-diphenyl-1H-pyrazole at different concentrations for 48 h. Briefly, MTT was added to the cell cultures for 3 h and formazan crystals formed were dissolved in dimethyl sulfoxide (Sigma-Aldrich). Then the plates were analyzed using Elisa reader (Sigma-Aldrich, USA) at 570 nm wavelength (37–39).

7.2.3. Lactate dehydrogenase (LDH) assay

LDH activity was detected in the culture medium as an index of cytotoxicity, using an LDH kit (Cayman Chemical, USA). In brief, 104–105 μL⁻¹ cells/well were seeded in 96-well plates and exposed to different concentrations (0–400 μg mL⁻¹) of the 3,5-diphenyl-1H-pyrazole for 24 h. At the end of the exposure, 96-well plate was centrifuged at 400 g for 5 min to settle down the 3,5-diphenyl-1H-pyrazole present in the solution. Then, a 100 μL⁻¹ supernatant was transferred to a fresh well of 96-well plate that contained 100 μL of reaction mixture from the kit and incubated for 30 min at room temperature. After incubation, the absorbance of solution was measured at 490 nm using a

microplate reader (Elisa reader Bio-Tek, USA). LDH levels in the media vs. the cells were quantified and compared with the control values according to the instruction of kit (40,41).

7.2.4. TAC and TOS analysis

The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter ($\mu\text{mol H}_2\text{O}_2$ Equiv/L). The automated TAC and TOS assays were carried out by commercially available kits (Rel Assay Diagnostics(r), Gaziantep, Turkey) on plasma samples of the 3,5-diphenyl-1H-pyrazole treated cultures for 2 h (42).

7.2.5. Statistical analysis

Statistical analysis was performed using SPSS software (version 22.0, SPSS, Chicago, IL, USA). The Duncan's test was used to determine whether any treatment significantly differed from the controls or each other. Statistical decisions were made with a significance level of 0.05.

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