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Synthesis and Biological Evaluation of Substituted Pyrazoline Derivatives Bearing 3,4,5-Trimethoxyphenyl Moiety as Anticancer, Anti-inflammatory and Antioxidant Agents

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ABSTRACT

In search for a potent agent, 3-(4-nitrophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole 3a with the chalcone scaffold (E)-1-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one 2a was synthesized to design 3-(4-nitrophenyl)-1-substituted phenyl-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole 4a-e and 3-(4-nitrophenyl)-N-substituted phenyl-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide 5a-e. IR, ^{1}H NMR and mass spectral analysis, confirmed the structure of compounds. All compounds evaluated for in vitro anticancer, anti-inflammatory and antioxidant activity. Among the series, compound 3a and 5b found to possess potent anticancer activity against breast carcinoma (MCF7). The data for antioxidant activity revealed that, compound 4b and 5c exhibited significant 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity as compared to the standard drug ascorbic acid. Compounds 2a, 4d and 5b showed excellent nitric oxide radical scavenging activity. While, compounds 2a, 4e, 5a, 5b and 5c showed pronounced superoxide radical scavenging activity, However all the compounds tested for H_2O_2 radical scavenging activity were devoid of activity. Additionally, compounds 4b, 4d, 4e and 5b showed good anti-inflammatory activity.

Keywords: Pyrazoline, Carbothioamide, Anticancer, Antioxidant, Anti-inflammatory

INTRODUCTION

Certainly, cancer is a serious and dreadful disease, difficult to alleviate. Chemotherapy is still one way for the treatment of cancer. However, currently available anticancer agents manifested undesirable side effects such as low bioavailability, toxicity and drug-resistance [1]. Indeed, this has warned scientist across the world to procure anticancer agent with novel chemical entity and mode of action. Recent literatures suggest, those drugs bind to the colchicines, are extensively explored as vascular-disrupting agents for cancer therapy. Combretastatin-A4 (a, CA-4, Figure 1), derived from the African willow tree *Combretum caffrum*, proved to be excellent for such an approach which binds to tubulin within the colchicine binding site, obstructing normal mitotic spindle formation. Therefore, disruption of tubulin polymerization, also arrest formation of tumoral vasculature [2,3]. Concomitantly, combretastatin-A4 provides a simple structural template for the design of related compounds and large number of combretastatin-type analogues bearing 3,4,5-trimethoxy phenyl moiety, requisite for potential anticancer activity have been synthesized, some of which are recently reviewed [4,5].

Inflammation is body's way of dealing with infection and tissue damage, but there is a fine balance between the beneficial effects of inflammation cascades and their potential for long-term tissue destruction. If they are not controlled, inflammation cascades can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis. Within many inflammation pathways, there are often pivotal molecular targets that, when antagonized block the output of the pathway. In an attempt, few number of pivotal target were identified which have yielded many successful anti-inflammatory drugs. These target include the enzyme Cyclooxygenase (COX-1 and COX-2), cytokines Tumor Necrosis Factor-α (TNF-α), Interleukin-6 and interleukin-2 (IL), receptor for the Cysteinyl leukotrienes C4 and D4 (CysLTs) and nuclear membrane receptors (corticosteroids) [6,7]. Therefore, inhibition of these target have become a major focus of current drug discovery and development, and an important *in vitro* method for evaluating the bioactivity of drugs [8,9].

The literature survey reports that, pyrazoline and N-substituted pyrazoline derivative find wide variety of application in pharmaceutical field. Particularly, pyrazoline derivative demonstrated COX-2 inhibitors [10], anti-inflammatory, analgesic, antimicrobial [11,12], antitumor [13], Monoamine oxidase (MAO-B) inhibitors [14] and antioxidant activity [15]. On the other hand, pyrazoline bearing 3,4,5-trimethoxy phenyl moiety and dimethoxy, trimethoxychalcone derivatives also reported as effective antitumor [13], anti-inflammatory agent [16,17].

In particular, pyrazolines and their derivatives have found application as Nonsteroidal Anti-inflammatory Drugs (NSAIDs) such as antipyrene, celecoxib, (b,c; Figure 1). Although, antipyrine is the first pyrazoline derivative used as an analgesic and antipyretic agent but its use is constrained owing to GI side effect therefore; development of alternative to NSAIDs is indeed a need of time.

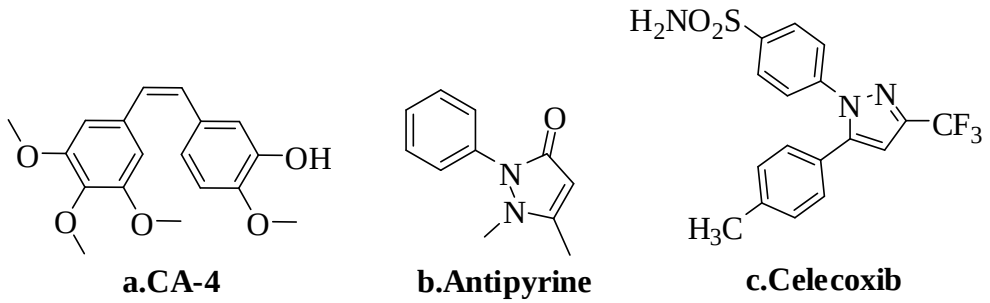


Figure 1: Biologically active synthetic and natural compounds

During respiration, Reactive Oxygen Species (ROS) produce as natural byproduct of oxygen metabolism and play a vital role in human health and development provided they are under control. Although, cells are able to defend against ROS through the use of enzymes such as superoxide dismutase and catalase [18,19]. Nevertheless, a balance between formation of ROS and their detoxification is essential for normal cellular function. However, imbalance can cause damage to cell components such as proteins, lipids, sugars and nucleotides [20] leading to oxidative stress with induction of various disease such as cancer [21], atherosclerosis [22], cardiovascular [23], inflammation [24]. Many studies have revealed that agents, synthetic or naturally occurring having ability to defend against ROS are therapeutically effective in the treatment of these diseases.

Prompted by the afore-mentioned literature and in persistence of our earlier work on different heterocyclic derivatives [25,26]. We envisage the synthesis of structurally diverse series of pyrazoline derivatives and examining their anti-inflammatory, anticancer and antioxidant activity. Therefore, a molecule bearing more than one pharmacophore each with different mode of action could be advantageous for curing more than one disease.

MATERIALS AND METHODS

All the chemicals and solvents used were of analytical grade and used as supplied unless otherwise stated. All reactions monitored by Thin Layer Chromatography (TLC) on 0.2 mm Merck silica gel F_{254} plates. Melting points determined with digital thermometer and were uncorrected. Infrared (IR) spectra recorded on Fourier Transform Infrared (FT-IR) spectrometer (Perkin Elmer, Maharashtra, India). Proton Nuclear Magnetic Resonance (^{1}HNMR) spectra recorded on ^{1}HNMR Bruker DRX FT NMR at 400 MHz spectrometer in Deuterated chloroform ($CDCl_3$) solvent. All chemical shifts (d) are cited in parts per million downfield from TMS and coupling constant (J) are referred in hertz (Hz). Abbreviations used in the splitting pattern were as follows: s=singlet, d=doublet, t=triplet q=quintet and m=multiplet. The mass spectra obtained on Shimadzu LCMS-2010 EV (Maharashtra, India).

EXPERIMENTAL SECTION

General procedure for the synthesis of (E)-1-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (2a)

To a mixture of 4-nitroacetophenone (1 mmol) and 3,4,5-trimethoxybenzaldehyde (1 mmol) in ethanol (5 ml), sodium hydroxide (5%, 2 ml) was added slowly. The reaction mixture was stirred at room temperature. After completion of the reaction (monitored by TLC), the reaction mixture was poured into crushed ice. The solid separated was filtered, washed with water several times and recrystallized from ethanol to afford compound 2a.

General procedure for the synthesis of compounds (4a-e)

To a suspension of 3a (1 mmol) in 5 ml absolute ethanol, substituted phenyl hydrazine (1.0 mmol) was added and the mixture stirred at reflux for 4 h. After completion of the reaction (monitored by TLC), the reaction mixture was allowed to cool to room temperature. Upon cooling, the precipitated solid then filtered, washed with hot ethanol (2 × 3 ml), and dried under vacuum to obtain title compounds (4a-e).

General procedure for the synthesis of 3-(4-nitrophenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole (3a)

To a suspension of chalcone (2a) (1 mmol) in 5 ml absolute ethanol, hydrazine hydrate (80%) (2.0 mmol) was added and the mixture stirred at reflux for 4 h. After completion of the reaction (monitored by TLC), the reaction mixture was allowed to cool to room temperature. Upon cooling, the precipitated solid then filtered, washed with hot ethanol (2 × 3 ml), and dried under vacuum to obtain title compound 3a.

General procedure for the synthesis of compounds (5a-e)

To a suspension of 3a (1 mmol) in 5 ml absolute ethanol, substituted phenyl isothiocynate (1.0 mmol) was added and the mixture stirred at reflux for 1h. After completion of the reaction (monitored by TLC), the reaction mixture was allowed to cool to room temperature. Upon cooling, the precipitated solid then filtered, washed with hot ethanol (2×3 ml), and dried under vacuum to obtain title compound (5a-e).

In vitro anti-inflammatory activity by protein denaturation method

The reaction mixture (10 ml) consists of 0.4 ml of egg albumin (from fresh hen's egg), 5.6 ml of Phosphate Buffered Saline (PBS, pH 6.4) and 4 ml of synthetic derivatives (1 mM) [27]. Similar volume of double-distilled water served as control. Then the mixtures were incubated at (37 ± 2°C) in an incubator for 15 min, and heated at 70°C for 5 min. After cooling, the absorbance measured at 660 nm using vehicle as blank. Diclofenac sodium at 1 mM was used as reference standard, and treated similarly for the determination of absorbance.

The percentage inhibition of protein denaturation calculated by using the following formula:

$$\text{Percentage Inhibition}=100 \times (V_t/V_c-1)$$

Where, V_t=Absorbance of test sample, V_c=Absorbance of control.

Cytotoxic assay of compound on MCF-7 (breast cancer cell line)

The assay relies on the ability of SRB [28], a bright-pink amino xanthene dye with two sulfonic groups that bind protein components of the cells, that have been fixed to the tissue culture plates by Trichloroacetic acid (TCA) to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude. The MCF-7 cell line was maintained in Dulbecco's Modified Eagle's (DMEM) medium, supplemented with 10% fetal bovine serum. The cells were plated at a density of 1×10^4 cells/well in a 96-well plate, and cultured for 24 h at 37°C and 5% CO_2 in CO_2 incubator (Nu Air, USA). Control sample were prepared without drug sample and synthesized compound in 0.2% PBS. Dimethyl sulfoxide (DMSO) was prepared in two fold serial dilutions with the concentration of 1 mM and exposed to cells. Next, without removing the cell culture supernatant, 100 µl of cold TCA (50%) was added into each well and plates were incubated at 1 h for 4°C followed by the washing step using slow-running tap water. Plates were then allowed to dry at room temperature. For the staining step, 100 µl 0.4% SRB in 1% glacial acetic acid was added into each well and left at room temperature for 30 min. immediately afterwards, plates were rinsed four times with 1% (v/v) acetic acid to remove unbound dye. Then, 200 µl of 10 mM Tris base solution was added into each well to solublize protein-bound dye and plates were then placed on a shaker for 10 min. Optical density (OD) was measured for plate at 550 nm wavelength. The results were compared with the standard drug inhibitor 5-flurouracil (20 µg/ml). The percentage growth inhibition was calculated using following formula:

Percentage Cell Inhibition=100-{(At-Ab)/(Ac-Ab)} × 100

Where, At=Absorbance value of test compound, Ab=Absorbance value of blank, Ac=Absorbance value of control.

Antioxidant activity

DPPH radical scavenging activity

The ability of compounds to scavenge DPPH radical was assessed using literature method with slight modification. Concisely, 1 ml of synthesized compounds as 1 mM was mixed with 3.0 ml DPPH (0.5 mmol/l in methanol), the resultant absorbance was recorded at 517 nm after 30 min. incubation at 37°C. The percentage scavenging activities were derived using the following formula:

$$\text{Percentage of Inhibition (\%)}=[(\text{A control}-\text{A sample})/\text{A control}] \times 100$$

Where, $A_{control}$=Absorbance of DPPH, A_{sample}=Absorbance of reaction mixture (DPPH with Sample).

NO radical scavenging activity

NO radical scavenging activity carried out as per the reported method [29]. Nitric oxide radicals generated from sodium nitroprusside solution. 1 ml of 10 mM sodium nitroprusside mixed with 1 ml of 1 mM synthesized compounds in phosphate buffer (0.2 M pH 7.4). The mixture incubated at 25°C for 150 min. After incubation, the reaction mixture mixed with 1.0 ml of prepared Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dichloride and 2% phosphoric acid). The absorbance was measured at 546 nm and percentage of inhibition was calculated using the same formula as above. The decreasing absorbance indicates a high nitric oxide scavenging activity.

$$\text{Percentage of inhibition (\%)}=(A_{Control}-A_{sample})/A_{control}) \times 100$$

Where, $A_{control=}$absorbance of nitric oxide, $A_{sample=}$absorbance reaction mixture (nitric oxide with sample).

H_2O_2 scavenging activity

The hydrogen peroxide scavenging assay carried out by the reported method [30]. A solution of hydrogen peroxide (40 mM) prepared in phosphate buffer (pH 7.4). The 1 mM concentrations of various synthetic compounds added to a hydrogen peroxide solution (0.6 ml, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min. against a blank solution containing phosphate buffer without drug. The percentage scavenging of hydrogen peroxide of synthetic compounds and standard compounds calculated by using the following formula:

$$\text{Percentage scavenged } (H_2O_2)=(A_0-A_1)/A_0 \times 100$$

Where, $A_{0=}$The absorbance of control, $A_{1=}$The absorbance in presence of the sample of MO and standards.

SOR scavenging assay

The superoxide radical scavenging activity was performed by the reported method [31]. The superoxide radicals generated in 3.0 ml of Tris–HCl buffer (16 mM, pH 8.0), containing 0.5 ml of nitroblue tetrazolium (NBT) (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml of synthetic compound (1 mM) solution and 0.5 ml Tris–HCl buffer (16 mM, pH 8.0). The reaction initiated by adding 0.5 ml Phenazine Methosulfate (PMS) solution (0.12 mM) to the mixture, incubated at 25°C for 5 min and then the absorbance measured at 560 nm against a blank sample. Decreased absorbance of reaction mixture, indicate increased superoxide anion scavenging activity.

$$\text{Percentage of inhibition (\%)}=(A_{Control}-A_{sample})/A_{control}) \times 100$$

Where, $a_{control=}$Absorbance of control, $a_{sampl=}$Absorbance reaction mixture (superoxide with sample).

RESULT AND DISCUSSION

Chemistry

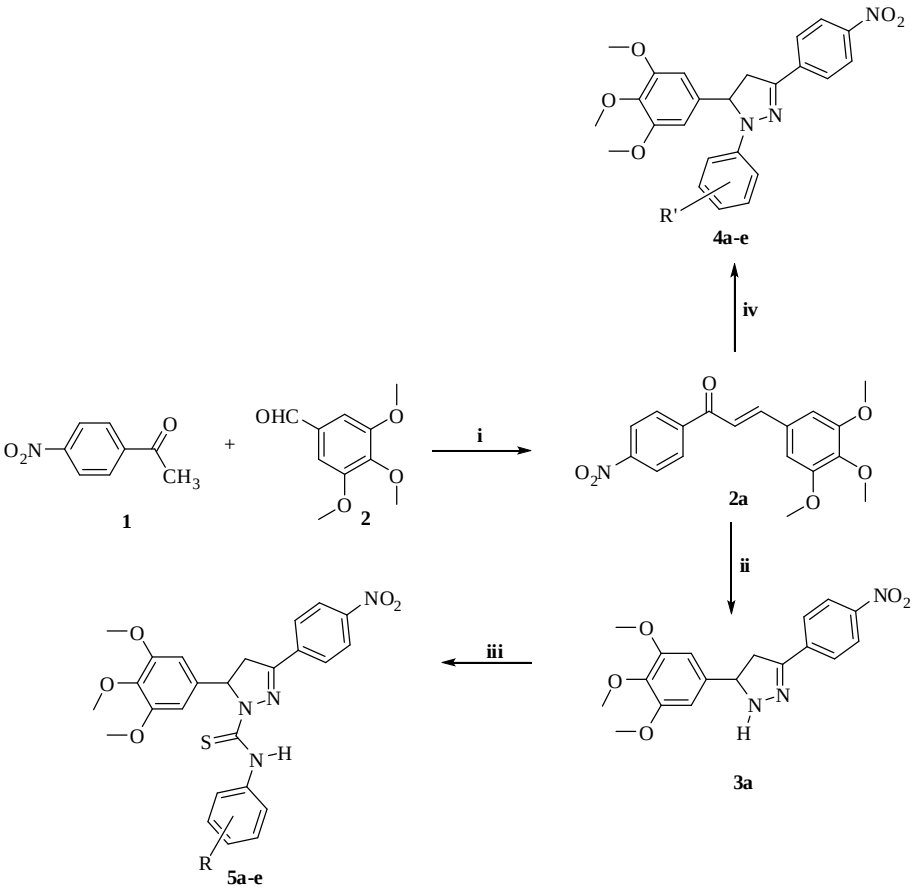
Various research groups across the world are working on anticancer agent based on chalcone derivatives. Due to their abundance in plants and easy to synthesis-base promoted Claisen-Schmidt condensation, have encouraged researcher to design, synthesize wide variety of chalcone, a scaffold constituting α, β-unsaturated enone into various heterocyclic moiety.

In the present investigation, compounds 4a-e and 5a-e were synthesized from (E)-1-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one 2a, as depicted in Scheme 1. 2a was prepared by the Claisen-Schmidt condensation of 1-(4-nitrophenyl) ethanone 1 and 3,4,5-trimethoxybenzaldehyde 2, which on condensation with 80% hydrazine hydrate in ethanol furnished 3a and compounds 4a-e were obtained by condensing substituted phenyl hydrazine in ethanol. Compounds 5a-e obtained by the reaction of pyrazoline derivative 2a with substituted phenyl isothiocynates. All the synthesized compounds confirmed by IR, ^{1}H NMR and Mass spectral data.

Biological evaluation

Anticancer activity

The synthesized compounds evaluated for *in vitro* cytotoxic activity against human breast cancer cell line, MCF7. 5-flurouracil, one of the most effective anticancer agents, was used reference drug in this study. The percent inhibition of the synthesized compounds comparison with reference drug has showed in Table 1. The result revealed that compound 3a, the pyrazoline derivative of 2a, showed excellent anticancer activity comparable with that of standard drug, 5-flurouracil. However, substitution of halo aryl at the N1 position of pyrazoline 3a exhibited very poor anticancer activity. In contrast, presence of halo aryl thiocarbamoyl at the N1 position of the pyrazoline 3a, especially 5b with fluro group exhibited good anticancer activity. However, other compound 5c, though substituted with thiocarbamoyl, showed moderate activity.



Scheme 1: Reagents and conditions: (i) NaOH, Ethanol, rt, 4h; (ii) $H_2NNH_2.H_2O$, Ethanol, 70-80°C, 6 h; (iii) Phenyl isothiocynate, Ethanol, 70-80°C, 30 min; (iv) Phenyl hydrazine, Ethanol, 70-80°C, 4 h

In vitro anti-inflammatory activity

All the synthesized compounds evaluated for anti-inflammatory activity by protein denaturation method (Table 1). The result reveals that compounds 4b, 4d and 4e showed significant inhibition (73.12-76.87%) as compared with standard diclofenac sodium (90.21%), whereas compounds 4a, 4c, 5b, and 5c displayed moderate inhibition (56.87-64.37%). While rest of the compounds 2a, 3a, 5a, 5d and 5e were devoid of activity. Despite the fact that, inhibition of all the synthesized compounds are not notable. Nevertheless, an interesting conclusion can be drawn that compounds 4a-e, having halo aryl at the N1 position of the pyrazoline 3a, exhibited remarkable anti-inflammatory activity in the order of 2-Cl>3-Cl>H>2,4-Cl_2>2-F compare to their precursor 3a. Interestingly, introduction of substituted thiocarbamoyl (5b and 5c) at the N1 position of the pyrazoline 3a also showed enhanced anti-inflammatory activity compare to their precursor 3a.

***In vitro* antioxidant activity**

Reactive oxygen species and nitrogen species contribute to the pathophysiology of anti-inflammatory conditions [32]. Taking into the account of multifactorial character of oxidative stress, which is involved in many pathological states. We have evaluated antioxidant activity of synthesized compounds against reactive oxygen species such as (DPPH), Nitric oxide (NO), hydrogen peroxide (H_2O_2), and super oxide radical (SOR) (Table 1). Among the series, compounds 4a, 5a, and 5d (49.41-64.70%) showed excellent activity against DPPH radical, compared to standard drug ascorbic acid (44.18%). While, the compounds 2a, 3a, 4c and 5a (41.17-43.52%) displayed good activity. However, rest of the compounds exhibited poor activity.

In case of NO radical scavenging activity, compounds 2a, 4d, and 5b showed excellent activity (79.50-62.29%) as compared to standard drug ascorbic acid (42.63%). Compounds 3a, 4b, 4c and 5c demonstrated good activity (41.80-34.42%), while; the remaining compounds were devoid of activity.

The SOR scavenging activity results revealed that compounds 2a, 4a, 5a, 5b and 5c found to possess excellent activity (95.93-86.17%) as compared to standard ascorbic acid (74.07%). While, other compounds were found showing poor activity, except 4d, which showed moderate activity (59.34%)? On the contrary, all compounds evaluated against H_2O_2 demonstrated poor activity. The wide variation in the free radical scavenging potential for the tested compounds may be due to the variation in the proton–electron transfer, owing to differences in their structure.

Table 1: Anticancer, anti-inflammatory, and antioxidant activities of pyrazoline derivatives

Entry	R	% inhibition (1 mM)					
		Anti-inflammatory	Anti-cancer	Antioxidant activity			
		Egg albumin	MCF-7	DPPH	NO	SOR	H_2O_2
2a	-	40.00	36.95	43.52	79.50	93.49	22.02
3a	-	41.87	84.78	41.17	40.16	21.95	13.25
4a	2,4-Cl	58.12	11.95	64.70	26.22	10.56	10.52
4b	3-Cl	75.62	ND	17.64	41.80	26.82	10.72
4c	2-F	56.87	ND	43.52	40.16	32.52	26.61
4d	2-Cl	76.87	ND	23.52	62.29	59.34	20.27
4e	H	73.12	9.78	35.29	29.50	95.93	26.31
5a	2,4-Cl	31.20	ND	5.88	26.00	86.17	10.52
5b	4-F	64.37	78.26	43.52	70.49	95.12	20.27
5c	4-OCH_3	62.50	43.47	57.05	34.42	90.24	23.97
5d	4-NO_2	16.66	ND	49.41	31.96	18.69	8.18
5e	H	46.25	ND	17.64	1.63	42.27	14.23
Control	-	-	-	-	-	-	-
AA	-			44.18	42.63	74.07	47.17
DS	-	90.21	-	-	-	-	-
5-FU	-	-	84.78	-	-	-	-

ND=Not determined; AA=Ascorbic acid; DS=Diclofenac sodium; 5-FU=5-Fluorouracil

CONCLUSION

In summary, we have synthesized structurally diverse series of 1-*N*-substituted phenyl pyrazoline 3a, 4a-e and N-substituted phenyl-pyrazole-1-carbothioamide 5a-e from the precursor (E)-1-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one 2a. All the compounds tested for their *in vitro* anticancer, anti-inflammatory and antioxidant (H_2O_2, DPPH, SOR and NO) activity. Compound 5b exhibited promising anticancer, anti-inflammatory and antioxidant activities. Moreover, compound 3a showed remarkable anticancer activity similar with the standard anticancer agent, 5-flurouracil. All other compounds also showed the potential to demonstrate anti-inflammatory and antioxidant activities.

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