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Research Article

THIAZOLIDIN-4-ONE DERIVATIVES ON HUMAN LUNG FIBROBLAST SHOWS OXYGEN FREE RADICAL SCAVENGING ACTIVITY

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ABSTRACT

The antioxidants are proving crucial tools in the exploration of oxidant stress-related diabetic pathologies and despite the noticeable prospective merit of the safety and efficacy of antioxidant supplementation in any future treatment remains to be conventional. The development of innovative methods for the synthesis of five-member heterocyclic compounds is an ever-expanding area in bioorganic and medicinal chemistry. Specifically, those containing the thiazolidinedione ring have been expansively used as key building blocks for synthesizing various drugs. In present study we endeavor to display a more chemically versatile and diverse thiazolidin-4-one derivatives as a suitable pharmacophore for antioxidant activity. Antioxidant activity was evaluated by using both enzymatic and non-enzymatic activities such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) on cell lines and free radical scavenging activity by DPPH (1, 1-diphenyl-2-picryl-hydrazil) assay method and ferric reducing antioxidant power (FRAP) assays. Finally, all tested compounds exhibited a talented antioxidant activity. In addition, all the synthesized derivatives showed non-toxic effects against a diseased human lung fibroblast (COPD), HCC7231 (TACC CCL-96). In prospect study, the movement of the compounds may be manipulated by optimizing a lead molecule by introducing un-saturation or heterocyclic ring at C_5 of thiazolidinediones. The outcomes of such studies may be positive for the clinical applications in humans and may open up a new therapeutic avenue.

Keywords: Thiazolidinediones, antioxidant, ascorbic acid, DPPH, free radical scavenging activity, Catalase (CAT), Superoxide dismutase (SOD), Glutathione peroxidase (GPX).

INTRODUCTION

Antioxidant substances represent one of the most important defense mechanisms against free radicals, but the only endogenous antioxidant molecules cannot be effective enough to counteract the injuries caused by ROS, particularly in the current times, where lifestyles based on smoke, drugs, alcohol, unbalanced diet, pollution, incorrect exposure to solar radiation and so forth can facilitate free radicals formation. For this reason increasing the intake of dietary antioxidant is of great importance to enjoy good health, as evidenced by studies on food characterized by high antioxidants content[1].

In present studies we have established the antioxidant properties of peroxisome proliferator-activated receptor gamma (PPARγ) agonists, in different experimental models. The PPARγ is a nuclear receptor that plays key roles in the regulation of oxidative biochemical reactions. Thiazolidinediones (TZDs) function as high-affinity PPARγ ligands. The thiazolidines-2,4-diones (TZDs) have been extensive researched due to their deep involvement in regulation of different physiological processes like cell proliferation, angiogenesis, inflammation and glucose metabolism as wells as a strong association with the inhibition of T-cell activation and inflammatory disease[2].

Thiazolidinone, a saturated form of thiazole with carbonyl group on fourth carbon, has been considered as a magic moiety (wonder nucleus) because it gives out novel derivatives with different types of biological activities such as antibacterial, antifungal[3], anti-inflammatory, anti tubercular, anti-neoplastic, anti-seizure[5], cardio tonic, besides shows latent insulin-tropic[4] activities. Thus, these classes of drugs are of on the risemagnitude as a therapeutical approach in various diseases.

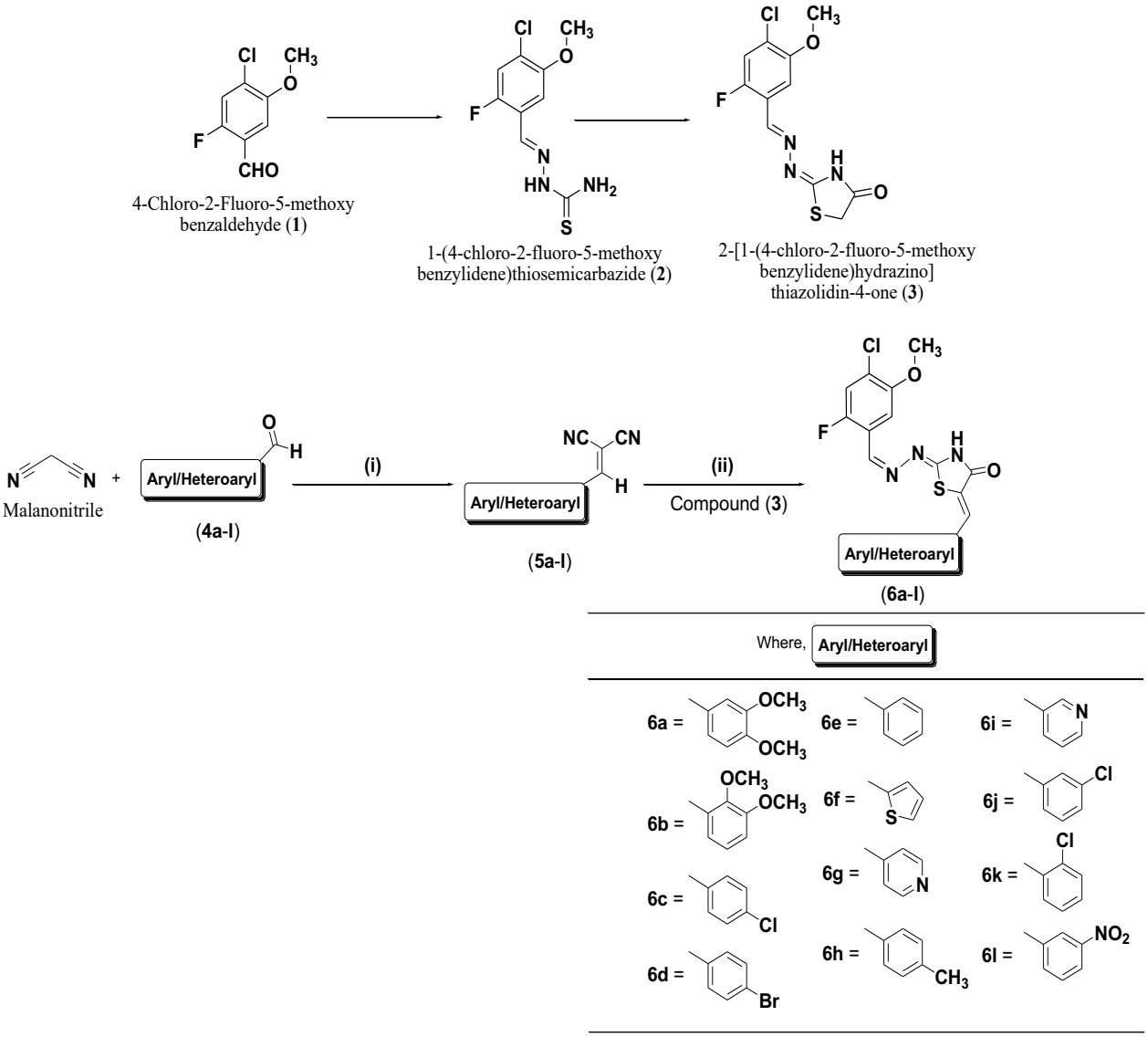
Cell defense themselves against toxic free radicals andother ROS, they develop a variety of antioxidant defenses. These include enzymes such as superoxide dismutase (SOD), which dismutates superoxide, catalase (CAT), which converts hydrogen peroxide into water andoxygen and glutathione peroxidase (GPX), which destroys toxic peroxides. Other molecules that can counteract ROS include glutathione, flavonoids, ubiquinol- 10, glucose and albumin. External sources of antioxidative protection include vitamins C, E, A, and provitamin A, as well as the minerals selenium and zinc.

In the present study, the antioxidative actions of 5-(substituted) thiazolidine-4-one were evaluated by their abilities to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, to protect cell viability and to inhibit the formation of lipid peroxides. The effect of TZD derivatives on the activity of antioxidant enzymes such as SOD, CAT and GPX was also investigated[6].

MATERIALS AND METHODS

The chemicals worn in the present project work were purchased from Rankem, Merck and Spectrochem. The melting point of the synthesized compound was determined by open capillary with Thiel's melting point tube (capillary tube method). TLC plates

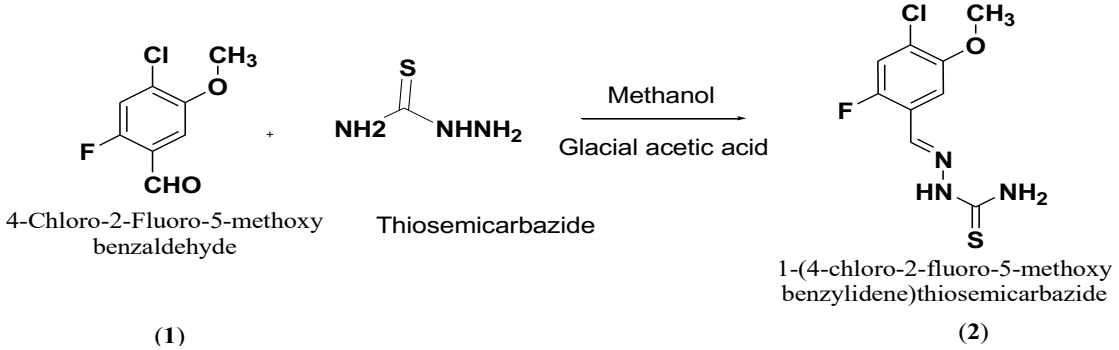
were prepared by using Merck Silica Gel 60 GF 254. Visualization was done in UV light chamber at 254 nm, iodine chamber. The IR spectra of the synthesized compounds were recorded on a Fourier Transform Infra Red spectrometer (model Shimadzu 8400 S) in the range of 400-4000 cm^{-1} as KBr pellets. (^1H NMR and C^{13} NMR) data of the compound was carried out in Bruker 200 spectrospin NMR at Astra Zeneca Pharma India Limited, Bangalore and Bruker 400 spectrospin NMR at Indian Institute of Science, Bangalore, Inida. The solvent used for NMR was $CDCl_3$.



Scheme of Chemical synthesis

Experimental procedure

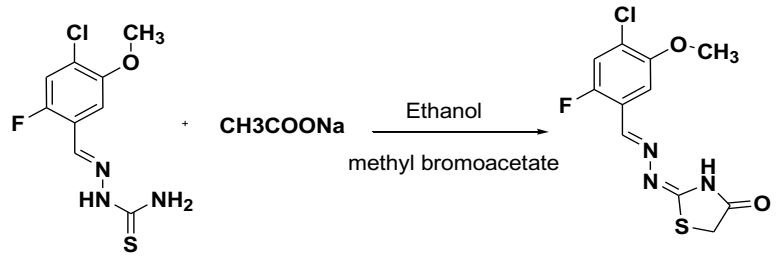
Synthesis of 1-(4-chloro-2-fluoro-5-methoxybenzylidene)thiosemicarbazide



Procedure

To a constantly stirred solution of compound 1 (4.0 g, 0.0191 mol, 1 equiv.) and thiosemicarbazide (1.91 g, 0.021 mol, 1.1 equiv.) in anhydrous methanol (40 mL), a catalytic amount of glacial acetic acid (0.15 equiv.) was added. The reaction mixture was refluxed for 4 h. After cooling to room temperature, the solid separated was filtered and washed with cold methanol to afford off white crystalline solid of compound (2)

Synthesis of 2-[1-(4-chloro-2-fluoro-5-methoxybenzylidene) hydrazino]thiazolidin-4-one



1-(4-chloro-2-fluoro-5-methoxy benzylidene)thiosemicarbazide

(2)

2-[1-(4-chloro-2-fluoro-5-methoxy benzylidene)hydrazino] thiazolidin-4-one

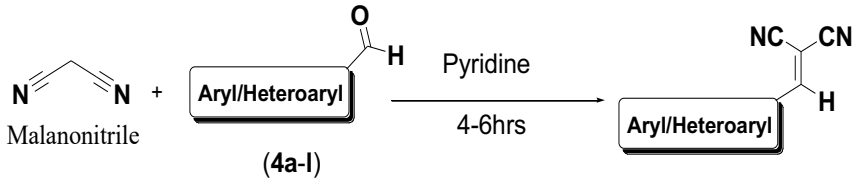
(3)

Procedure

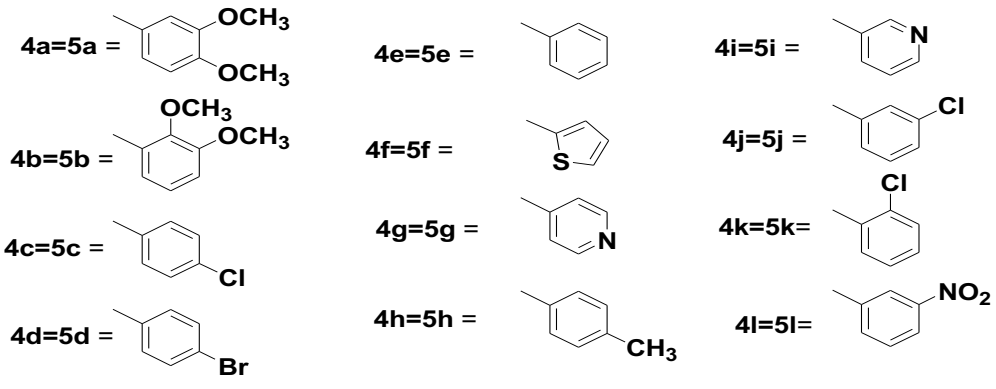
To a constantly stirred solution of compound 2 (4.0 g, 0.0191 mol, 1 equiv.) and ethyl acetate (1.91 g, 0.021 mol, 1.1 equiv.) in anhydrous ethanol (40 mL), a catalytic amount of methyl bromo acetate (0.15 equiv.) was added. The reaction mixture was refluxed for 4 h. After cooling to room temperature, the solid separated was filtered and washed with cold methanol to afford off white crystalline solid of compound (3)

General procedure for synthesis of substituted arylidinemalanonitriles (5a-l)

To a constantly stirred solution of malononitrile (0.5 g, 0.00757 mol.) in 10.0 mL of ethanol, an appropriately substituted aromatic/heteroaromatic aldehyde (4a-l; 0.00757 mol) and 2-4 drops of pyridine was slowly added. The reaction mixture was then either refluxed for 3-5 h (for substituted benzaldehydes) or was stirred at room temperature for 4-6 h (for substituted heteroaromatic aldehydes). The precipitate formed after cooling was filtered to get respective arylidinemalanonitriles (5a-l). The compounds so obtained were fairly pure to carry out the next step.

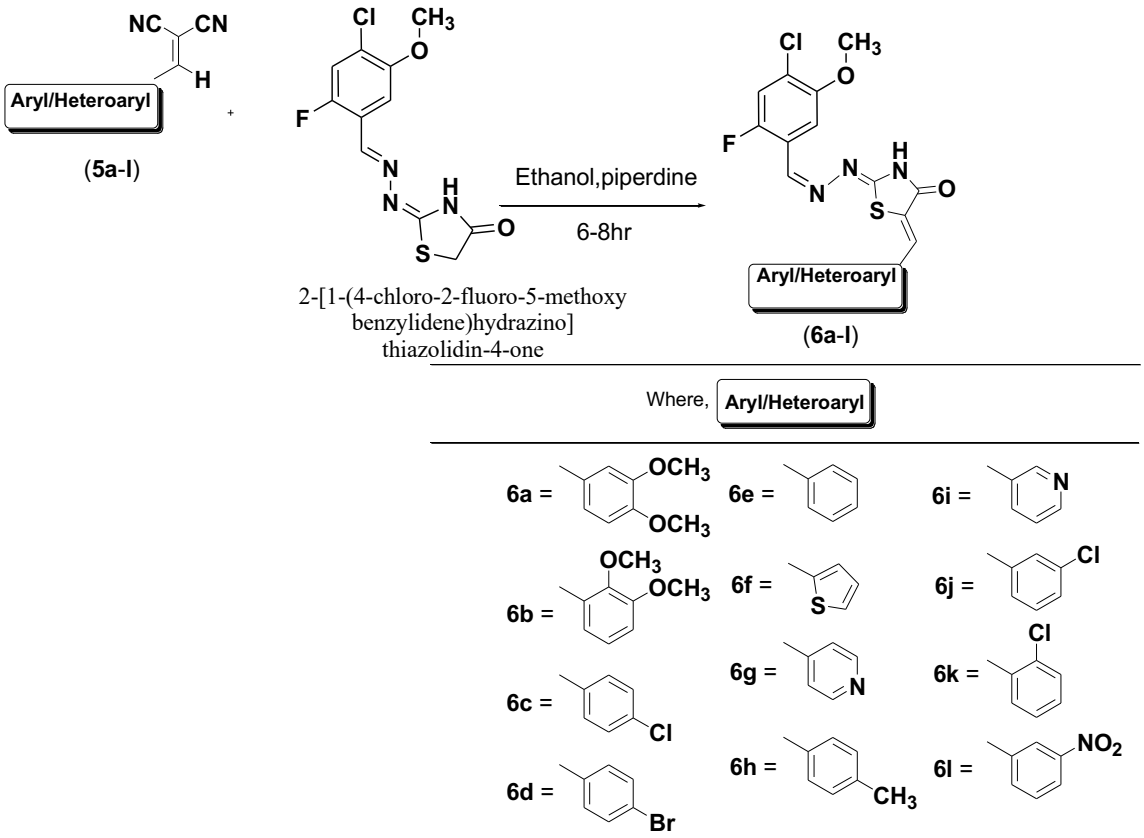


Where, Aryl/Heteroaryl



General procedure for synthesis of substituted 5-(substituted benzylidene)-2-(4-chloro-2-fluoro-5-methoxybenzylidene)hydrazono) thiazolidin-4-one (6a-l)

To a continuously stirred mixture of compound 2 (0.35 g, 0.00110 mol) and appropriate arylidinemalanonitriles (5a-l; 0.00110 mol) in ethanol (8 mL), few drops of piperidine were added. The reaction mass was refluxed for 6-8 h. The progress of the reaction was constantly monitored by TLC. After cooling, the separated solid or residue was filtered, washed with hot ethanol. All the compounds were further purified by recrystallized in ethanol in order to get the desired title compounds (6a-l).



***In vitro* screening for antioxidant activity**

Cell culture

Human lung fibroblast (COPD), HCC7231 (TACC CCL-96) cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2 / 95% O_2. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, sigma aldrich, India) containing 5% fetal bovine serum (FBS, Sigma aldrich, India), 100 mg/ml of kanamycin, 100 unit/ml of penicillin G (Sigma aldrich, India) and 2 mM L-glutamine (Sigma aldrich, India).[7]

Free radical scavenging activity by DPPH assays method

DPPH (1, 1-diphenyl-2-picryl-hydrazil) is stable free radical. Methanol solution of DPPH is used to appraise the antioxidant activity of numerous synthetic compounds. Antioxidant on interface with DPPH, transfer electron or hydrogen atom to DPPH, thus neutralizing its free radical character and convert it to1, 1-diphenyl-2-picryl hydrazine. The extent of discoloration indicates the scavenging action of the drug. The change in absorbance produced at 517 nm has been used as measure of its antioxidant activity.[8]

Chemicals used

1, 1-diphenyl-2-picryl-hydrazil (DPPH)-Sigma Ltd., Ascorbic Acid-Qualigens, Methanol-Qualigens

Preparation of DPPH solution

It was prepared by dissolving 33 mg of DPPH in 1 lit. Of methanol just before use and kept in dark amber colored bottle to protect from sunlight.

Sample preparation

Preparation of stock solution of derivatives

It was prepared by dissolving 50 mg of TZD derivatives in 100 ml of methanol.

Standard preparation

Preparation of Ascorbic Acid solution

It was prepared by dissolving 50 mg of ascorbic acid in 100 ml of methanol.

Procedure

A 10, 20, 30, 40, 50 µg/ml concentrations of derivatives and ascorbic acid were prepared. From this stock solution 1 ml has been pipette out and 5 ml methanol solution of DPPH was added, traumatized well and the mixture was incubated at 37^0C for 30 minute absorbance of all samples were measured against blank at 517 nm. The absorbance of DPPH reagent alone was taken as control.

The % radical scavenging activity can be calculated following formula

$$\% \text{ free radical Scavenging activity} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control \& calculated } IC_{50} \text{ value}} \times 100$$

Ferric ion reduction method

FRAP activity was precise according to the method postulated by Benzie and Strain. Briefly, acetate buffer (300 mM, pH 3.6), TPTZ (2, 4, 6-tripyridyl-s-triazine) 10 mM in 40 mM HCl and $FeCl_3 \cdot 6H_2O$ (20 mM) were assorted in the ratio of 10: 1: 1 to acquire the working FRAP reagent. TZD derivatives (0.5 mL) was mixed with 3 mL of effective FRAP reagent and absorbance was measured at 593 nm after vortexing. Methanol solutions of $FeSO_4 \cdot 7H_2O$ ranging from 100 to 2000 µM were arranged and used for the preparation of the calibration curve of known Fe^{2+} concentration. The parameter correspondent concentration was defined as the concentration of antioxidant having a Ferric-TPTZ reducing ability equivalent to that of 1 mM $FeSO_4 \cdot 7H_2O$.[8]

Antioxidant activity by ferric ion reduction method can be calculated by the following formula:

$$\textbf{\% Activity} = [At / As] \times 100$$

Where, **As** = absorbance by standard drug solution at 510 nm.
At = absorbance by the sample solution at 510 nm.

Assays for antioxidant enzymes

The cells were treated with 100 mg/ml of synthesized derivatives. The cells were then lysed in a lysis buffer appropriate for the requirements of each assay, as described below. Results are expressed as relative percentage of enzyme activity per mg protein compared with corresponding control cultures.[9]

Cell viability

Cell viability is often defined as the number of healthy cells in a sample. Cell viability was conventional by the MTT assay, which is based on tetrazolium dye reduction is dependent on NADPH-dependent oxido-reductase enzymes largely in the cytosolic compartment of the cell. V79-4 cells were seeded in a 96 well plate at a concentration of 1.2 ´105 cells/ml. Sixteen h after plating, cells were treated with concentrations of 100 mg/ml and 1 h later 1 mM H_2O_2 was added to the culture. Cells were incubated for an additional 24 h at 37ºC. During the last 4 h, cells were incubated with 20 ml of MTT stock solution (5 mg/ml) in 200 ml medium at 37ºC. The water insoluble formazan so formed can be solubilized using isopropanol or other solvents and the dissolved material is measured spectrophotometrically yielding absorbance as a function of concentration of converted dyeat 570 nm. The results were worn to build a graph of percentage cell viability against concentration of fractions. The percentage of cell viability was calculated using formula.[10]

$$\text{Percentage cell viability} = 100 - \{(At-Ab)/(Ac-Ab)\} \times 100$$

Where At is the absorbance of test sample or positive controls, Ab is the absorbance of blank and Ac is the absorbance of negative control.

Superoxide dismutase (SOD) activity

The principle of SOD activity assay was based on the inhibition of nitroblue tetrazolium (NBT) reduction. NBT is reduced to blue formazan by O_2-, which has a strong absorbance at 560 nm. The presence of SOD inhibits this reaction. The cells were homogenized in 0.05 M sodium carbonate buffer, pH 10.2. The reaction mixture contained, 1.9 ml of phosphate buffer [pH 7.8], 1x 10-2 M methionine, 16.8 x 10-5 M NBT and 1.17 x 10-6 M riboflavin, with suitable cell lysates in a total volume of 3 ml. Illumination of the solution taken in 10 ml beaker was carried out in an aluminum foil lined box, with a 15W fluorescent lamp for 10 minutes. The reaction was initiated by the addition of 50 ml of xanthine oxidase (0.1 mg/ml) and incubated for 30 min at room temperature. The reaction was stopped by adding 6 mM copper (II) chloride and centrifuged at 1500 rpm for 10 min. Control without the enzyme source was always included. The absorbance was measured at 560 nm.[11]

Catalase (CAT) activity

The assay is based on the disappearance of H_2O_2 in the presence of the enzyme source at 26^0C.

The reaction mixture contained 1.2 mM of 3% (v/v) H_2O_2 and 0.2 ml of cell lysates in 0.05 M phosphate buffer (pH 7.0) at a final volume of 1.0 ml. Samples allowed to stand for 25 minutes and the absorbance of the samples were monitored at 505 nm. The change in absorbance is proportional to the breakdown of H_2O_2.[12]

Glutathione peroxidase (GPX) activity

Cell lysates was washed in phosphate buffer, pH 7.4 which consisted of 50 mM tris-HCl, pH 7.5, 1 mM EDTA, 10 mM glutathione (GSH), 1 mM NaN3 (Sodium azide), 1 unit of glutathione reductase, 1.5 mM NADPH and 0.1 ml of cell lysates. After incubation for 10 min at 37ºC, H_2O_2 was added to each sample at a final concentration of 1 mM. GPX activity was measured as the rate of NADPH oxidation at 340 nm.[13]

Table 1: Physical Properties of Synthesized Compounds

S. No.	C.C.*	State	% yield	MP
1.	**6a**	Yellow solid	45%	232°C
2	**6b**	Yellow solid	46%	215 °C
3	**6c**	Yellow solid	52%	225-227°C
4	**6d**	Yellow solid	42%	245-247 °C
5	**6e**	Yellow solid	61%	202-204°C
6	**6f**	Yellow orange solid	57%	212-214°C
7	**6g**	Yellow solid	49%	238-240 °C
8	**6h**	Yellow solid	50%	213-215°C
9	**6i**	Yellow solid	55%	220-223 °C
10	**6j**	Yellow solid	48%	227-229 °C
12	6l	Yellow solid	62%	239-242°C

Table 2: Elemental and Spectral analysis data of Synthesized Compounds

Comp. Code	Elemental Analysis (Calculated)	IR. values (cm^{-1})
6a	C= 30.76, H= 2.58, N= 11.96, O= 27.32, S= 27.38	3115.99 (N-H Str.), 3001.46 (Ar-H Str.), 2957.65 (CH Str. of CH_3), 1687.41 (C=O Str.), 1624.30 (C=C Str.), 1591.71 (C=N Str.)
6b	C= 53.24; H=3.78; N= 4.78; O=27.28; S=10.93	3109.88 (N-H Str.), 3040.73 (Ar-H Str.), 2948.43 (C-H Str. of CH_3), 1696.80 (C=O Str.), 1623.69 (C=C Str.), 1595.01 (C=N Str.)
6c	C= 47.31; H=3.25; N=15.05; O=22.92; S, 11.48	3077.23 (N-H Str.), 3052.86 (Ar-H Str.), 2932.41 (C-H Str. of CH_3), 1729.20 (C=O Str.), 1629.90 (C=C Str.), 1518.99 (C=N Str.)
6d	C= 64.14, H= 7.00, N= 7.48, O= 12.82, S= 8.56	3049.65 (N-H Str.), 3020.56 (Ar-H Str.), 2930.54 (C-H Str. of CH_3), 1728.47 (C=O Str.), 1628.72 (C=C Str.), 1518.65 (C=N Str.)
6e	C= 60.78, H= 6.71, N= 11.19, O= 12.78, S= 8.54	3112.43 (N-H Str.), 3013.70 (Ar-H Str.), 2959.85 (C-H Str. of CH_3), 1693.90 (C=O Str.), 1590.71 (C=C Str.), 1510.87 (C=N Str.)
6f	C= 63.31, H= 6.71, N= 7.77, O= 13.32, S= 8.90	3115.27 (N-H Str.), 3060.27 (Ar-H Str.), 2963.89 (C-H Str. of CH_3), 1694.20 (C=O Str.), 1590.52 (C=C Str.), 1514.47 (C=N Str.)
6g	C= 59.65, H= 6.12, N= 7.73, O= 17.66, S= 8.85	3011.90 (N-H Str.), 2935.45 (Ar-H Str.), 2837.61 (C-H Str. of CH_3), 1719.49 (C=O Str.), 1633.77 (C=C Str.), 1598.56 (C=N Str.)
6h	C= 61.31, H= 5.71, N= 6.77, O= 11.32, S= 7.90	3118.74 (N-H Str.), 3019.85 (Ar-H Str.), 2964.49 (C-H Str. of CH_3), 1697.55 (C=O Str.), 1592.59 (C=C Str.), 1514.23 (C=N Str.)
6i	C= 61.31, H= 6.31, N= 7.17, O= 13.12, S= 7.90	2999.21 (N-H Str.), 2929.09 (Ar-H Str.), 2833.81 (C-H Str. of CH_3), 1713.41 (C=O Str.), 1625.05 (C=C Str.), 1514.55 (C=N Str.)
6j	C= 59.31, H= 7.71, N= 6.77, O= 11.32, S= 7.90	3063.62 (N-H Str.), 2917.80 (Ar-H Str.), 2831.79 (C-H Str. of CH_3), 1720.58 (C=O Str.), 1628.09 (C=C Str.), 1599.95 (C=N Str.)
6l	C= 60.31, H= 6.01, N= 7.07, O= 13.12, S= 8.60	3106.33 (N-H Str.), 3039.40 (Ar-H Str.), 2957.11 (C-H Str. of CH_3), 1693.10 (C=O Str.), 1619.19 (C=C Str.), 1598.60 (C=N Str.)

Table 3: H^1NMR and C^{13}NMR data of Synthesized Compounds

Comp. Code	H^1NMR (400 MHz, DMSO-d_6, δ, ppm)	C^{13}NMR (100 MHz, DMSO, δ ppm)
6a	12.43 (s, 1H, NH), 7.55 (s, 1H, C=C-H), 7.44 (s, 1H, C=C-H),7.28 (s, 1H, Ar-H), 7.25 (s, 1H, Ar-H), 6.97-6.95 (d, 1H, *J* = 8.40 Hz),3.83 (s, 3H, -OCH_3), 3.83 (s, 3H, -OCH_3), 3.82 (s, 3H, -OCH_3)	167.25 (C=O, thiazolidin-4-one), 163.38 (C=N, 2-ylidene carbon), 156.96 (C=N of thiazolidone), 151.39, 149.69, 148.95, 136.31, 129.24, 128.88 (C-H,benzylidene carbon), 126.36, 122.55, 120.46, 114.14, 112.03, 55.63 (-OCH_3), 55.51 (-OCH_3), 55.47 (-OCH_3)
6b	12.55 (s, 1H, NH), 7.75 (s, 1H, C=C-H), 7.45 (s, 1H, C=C-H),7.34 (s, 1H, Ar-H),7.27 (s, 1H, Ar-H), 7.25-7.23 (d, 1H, *J* = 7.88 Hz), 7.20-7.15 (m, 4H), 3.84 (s, 3H, -OCH_3), 3.83 (s, 3H, -OCH_3), 3.78 (s, 3H, -OCH_3)	167.18 (C=O, thiazolidin-4-one), 163.68 (C=N, 2-ylidene carbon), 156.78 (C=N of thiazolidone), 152.72, 149.73, 147.86, 136.49, 128.87, 126.26, 124.41 (C-5 of thiazolidone), 123.08 (C-H, benzylidene carbon), 119.66, 114.69, 60.97, 55.84 (-OCH_3), 55.54 (-OCH_3), 55.48 (-OCH_3)
6c	12.59 (s, 1H, NH), 7.75 (s, 1H, C=C-H), 7.67-7.65 (d, 2H, *J* = 8.60 Hz), 7.45 (s, 1H, C=C-H),7.27 (s, 1H, Ar-H), 6.98-6.96 (d, 1H, *J* = 8.40 Hz), 3.83 (s, 3H, -OCH_3)	166.99 (C=O, thiazolidin-4-one), 163.84 (C=N, 2-ylidene carbon), 156.38 (C=N of thiazolidone), 149.76, 136.60, 134.17, 132.58, 129.24, 128.84, 127.41 (C-H, benzylidene carbon), 124.11 (C-5 of thiazolidone), 55.49 (-OCH_3)
6d	12.38 (s, 1H, NH), 7.70 (s, 1H, C=C-H), 7.58-7.56 (d, 2H, *J* = 8.34 Hz), 7.54 (s, 1H, C=C-H), 7.25 (s, 1H, Ar-H), 6.98-6.97 (d, 1H, *J* = 8.28 Hz), 3.80 (s, 3H, -OCH_3)	167.42 (C=O, thiazolidin-4-one), 164.13 (C=N, 2-ylidene carbon), 156.62 (C=N of thiazolidone), 150.57, 136.82, 133.56, 132.62, 129.65, 127.94 (C-H, benzylidene carbon), 126.97, 125.04 (C-5 of thiazolidone), 123.41, 79.64, 79.41, 79.19, 56.34 (-OCH_3)
6e	12.56 (s, 1H, NH), 7.66-7.64 (d, 2H, *J* = 7.56 Hz), 7.59 (s, 1H, C=C-H), 7.56-7.52 (t, 2H, *J* = 7.58 Hz), 7.47-7.43 (m, 1H), 7.29 (s, 1H, Ar-H), 6.98-6.96 (d, 1H, *J* = 6.88 Hz), 3.84 (s, 3H, -OCH_3)	167.13 (C=O, thiazolidin-4-one), 163.72 (C=N, 2-ylidene carbon), 156.70 (C=N of thiazolidone), 149.73, 148.96, 136.54, 133.65, 129.79, 129.71 (C-H, benzylidene carbon), 129.19, 128.58, 126.25, 123.29 (C-5 of thiazolidone), 55.54 (-OCH_3)
6f	12.51 (s, 1H, NH), 7.97-7.96 (d, 1H, *J* = 5.00 Hz), 7.86 (s, 1H, C=C-H), 7.62-7.61 (d, 1H, *J* = 3.44 Hz), 7.30 (s, 1H, Ar-H), 7.22-7.16 (m, 2H), 6.97-6.92 (m, 2H), 3.84 (s, 3H, -OCH_3), 3.78 (s, 3H, -OCH_3)	166.91 (C=O, thiazolidin-4-one), 163.70 (C=N, 2-ylidene carbon), 156.30 (C=N of thiazolidone), 149.75, 148.99, 137.82, 136.59, 133.23, 131.82, 128.87, 126.24, 122.18 (C-H, benzylidene carbon), 120.98 (C-5 of thiazolidone), 55.54 (-OCH_3)
6g	12.61 (s, 1H, NH), 8.72-8.71 (d, 2H, *J* = 5.04 Hz), 7.57-7.56 (d, 2H, *J* = 5.10 Hz), 7.53 (s, 1H, C=C-H), 7.27 (s, 1H, Ar-H), 7.18-7.17 (d, 1H, *J* = 8.28 Hz), 6.99-6.97 (d, 1H, *J* = 8.22 Hz), 3.84 (s, 3H, -OCH_3)	167.20 (C=O, thiazolidin-4-one), 164.59 (C=N, 2-ylidene carbon), 156.53 (C=N of thiazolidone), 150.97, 149.71, 141.31, 137.19, 129.51, 129.18 (C-5 of thiazolidone), 126.18 (C-H, benzylidene carbon), 123.70, 65.31, 56.18 (-OCH_3)

6h	12.50 (s, 1H, NH), 7.55 (s, 1H, Ar-H), 7.52 (s, 1H, C=C-H), 7.36-7.34 (d, 2H, *J* = 8.04 Hz), 7.29 (s, 1H, Ar-H), 7.21-7.15 (m, 2H), 6.98-6.92 (m, 2H), 3.84 (s, 3H, -OCH_3), 2.26 (s, 3H, -CH_3)	167.22 (C=O, thiazolidin-4-one), 163.59 (C=N, 2-ylidene carbon), 156.80 (C=N of thiazolidone), 149.71, 139.84, 136.46, 135.41, 130.88, 129.84 (C-H, benzylidene carbon), 129.80, 128.75, 126.29, 122.06 (C-5 of thiazolidone), 55.47 (-OCH_3), 21.04 (-CH_3)
6i	12.66 (s, 1H, NH), 8.85 (s, 1H, C=C-H), 8.60-8.59 (d, 1H, *J* = 4.76 Hz), 8.02-8.01 (d, 1H, *J* = 8.00 Hz), 7.62 (s, 1H, C=C-H), 7.58-7.55 (dd, 1H, *J* = 7.89, 4.24 Hz), 7.28 (s, 1H, Ar-H), 3.83 (s, 3H, -OCH_3)	166.85 (C=O, thiazolidin-4-one), 163.93 (C=N, 2-ylidene carbon), 156.39 (C=N of thiazolidone), 151.05, 149.76, 148.96, 136.69, 135.79, 129.81, 128.82, 126.16, 125.70 (C-5 of thiazolidone), 125.30 (C-H, benzylidene carbon), 124.09, 55.47 (-OCH_3)
6j	12.54 (s, 1H, NH), 7.68 (s, 1H, C=C-H), 7.56-7.48 (m, 5H, Ar-H), 7.14 (s, 1H, C=C-H), 7.02-7.00 (d, 1H, *J* = 8.76 Hz), 3.80 (s, 3H, -OCH_3)	167.06 (C=O, thiazolidin-4-one), 160.99 (C=N, 2-ylidene carbon), 155.76 (C=N of thiazolidone), 150.12, 148.95, 137.97, 135.87, 133.77, 131.01, 129.50, 129.23, 128.71, 127.01 (C-H, benzylidene carbon), 125.14 (C-5 of thiazolidone), 117.39, 55.43 (-OCH_3)
6l	12.70 (s, 1H, NH), 8.45 (s, 1H, Ar-H), 8.26-8.24 (d, 1H, *J* = 8.20 Hz), 8.06-8.04 (d, 1H, *J* = 7.92 Hz), 7.72 (s, 1H, C=C-H), 7.27 (s, 1H), 7.22-7.15 (m, 2H), 6.97-6.95 (d, 1H, *J* = 8.36 Hz), 3.83 (s, 3H, -OCH_3)	166.74 (C=O, thiazolidin-4-one), 164.18 (C=N, 2-ylidene carbon), 155.86 (C=N of thiazolidone), 149.78, 148.95, 136.83, 135.38, 135.25, 130.71, 128.79, 126.40 (C-5 of thiazolidone), 126.35 (C-H, benzylidene carbon), 126.05, 124.06, 123.76, 55.52 (-OCH_3)

Figure 1: DPPH Radical scavenging assay of Synthesized Compounds and Ascorbic acid

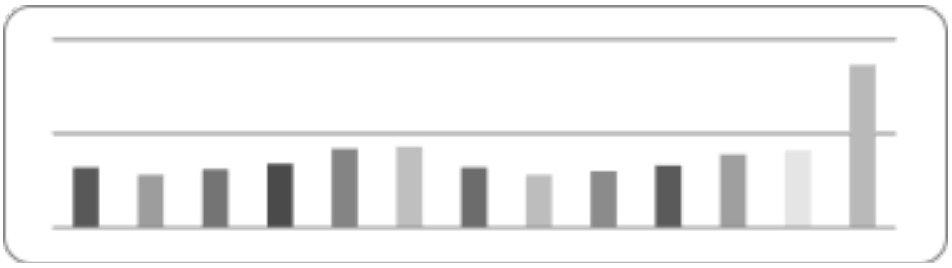


Figure 2: Ferric reducing activity of Synthesized Compounds and Ascorbic acid

Table 4: Enzymatic antioxidant activity of *TZD* derivatives on SOD (A), GPX (B) and CAT (C) activity on human fibroblast cells

Comp code	**100 µgm/ml**
6e	0.21 ± 0.15*
6g	0.20 ± 0.45*
6j	0.23 ± 0.40**
Untreated	0.33 ± 0.15
Ascorbic acid	0.33 ± 8.15

Comp code	**100 µgm/ml**	**Comp code**	**100 µgm/ml**
6e	7.23 ± 2.55*	**6e**	2.13 ± 2.25*
6g	8.30 ± 2.25*	**6g**	3.21 ± 1.05*
6j	9.29 ± 1.40**	**6j**	4.09 ± 1.10**
Untreated	11.10 ± 1.48	**Untreated**	6.98 ± 4.48
Ascorbic acid	10.90 ± 6.48	**Ascorbic acid**	7.28 ± 1.48

Values are expressed as mean ± S.E.M., ANOVA and Tukey-kramer multiple comparisons carried out $^{***}p < 0.001$ highly significant
Each experiment was performed at least 3 times and data are expressed as average enzyme unit per mg protein from control ± S.D

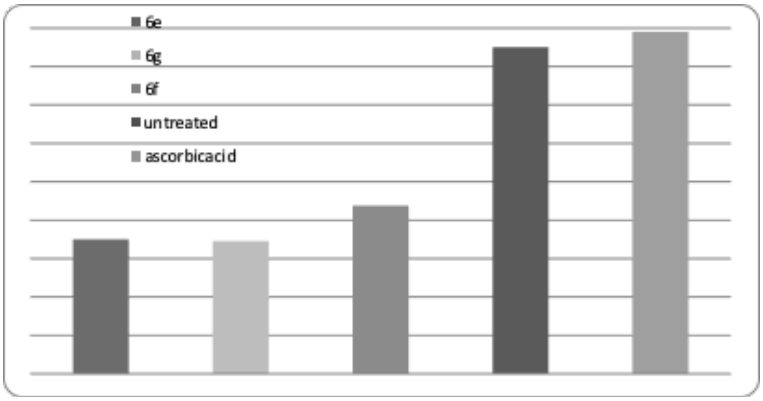


Figure 3: MTT assay/Percentage cell viability of Synthesized Compounds and Ascorbic acid

RESULT AND DISCUSSION

The structure of new compounds prepared during present investigation has been authentically established by their UV, FTIR, H^1NMR, and C^{13} NMR. In following reaction, the spectral studies of some selected compounds have been dealt.

The synthesis of substituted 5-(substituted benzylidene)-2-(4-chloro-2-fluoro-5-methoxybenzylidene) hydrazono) thiazolidin-4-one was done by refluxing appropriate arylidinemalanonitriles in ethanol few drops of piperidine were added. The reaction mass was refluxed for 6-8 h. It was proved by the following peaks of IR 3115.99 (N-H Str.), 3001.46 (Ar-H Str.), 2957.65 (C-H Str. of CH_3), 1687.41 (C=O Str.), 1624.30 (C=C Str.), 1591.71 (C=N Str.) as shown in Table 2. Further proof was obtained from H^1NMR spectrum which clearly shows these prominent peaks at 12.43, 7.55, 7.28 and 3.83 Indicating the presence of –NH, C=CH, Ar-H and OCH_3. ^{13}C-NMR (100 MHz, DMSO, δ ppm): 167.25 (C=O, thiazolidin-4-one), 163.38 (C=N, 2-ylidene carbon), 156.96 (C=N of thiazolidone), 151.39, 149.69, 148.95, 136.31, 129.24, 128.88 (C-H, benzylidene carbon) as shown in Table 3, Further substitution reaction with appropriate arylidinemalanonitriles leads to substituted 5-(substituted benzylidene)-2-(4-chloro-2-fluoro-5-methoxybenzylidene) hydrazono) thiazolidin-4-one 6a-l. The formation of the product was determined from TLC by comparing R*f* values of starting materials and the product.

The compounds 6a-6l was screened for enzymatic and non-enzymatic antioxidant activity by using SOT, GPX and CAT enzymes and free radical scavenging activity by DPPH (1, 1-diphenyl-2-picryl-hydrazil) assay method and Ferric ion reduction method, ascorbic acid was used as reference standard as shown in Figure 1-3. When derivatives were screened for non-enzymatic activity 6e, 6g and 6j showed promising activity, so these derivatives were future subjected to enzymatic antioxidant activity[14]. Based on all the evidences it is suggested that TZD derivatives have moderate levels of DPPH radical scavenging activity, inhibit lipid per oxidation, promote cell viability, protect H_2O_2-induced apoptosis and enhance the effects of various antioxidant enzymes. It could be great significance as therapeutic agents in preventing or slowing the oxidative stress associated with degenerative diseases such as cancer and various other human ailments[15].

CONCLUSION

Both enzymatic and non-enzymatic experiments in the present study are measured to be preliminary and more sophisticate research is obligatory to reach a concrete conclusion about the findings of the present study. 2-(substituted benzylidene) thiazolidin-4-one is an excellent molecule and in future specific antioxidant derivatives can be synthesized by introducing un-saturation or heterocyclic ring at C_5 of thiazolidinediones. Therefore, in depth extensive preclinical and clinical studies should be an exigency to class out bioactive compounds.

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