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Synthesis and biological evaluation of new unsaturated derivatives of cyclic compounds as potent antioxidant agent

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ABSTRACT

*In our ongoing research efforts to improve and develop new treatment strategies against oxidative stress, we develop simple and efficient methods to synthesis a new derivatives as stable free radical scavenger through conjugation of the different moieties to enone **8a-c** & **16** as starting compounds and containing different active parts as indoline (**9a-c**,**11a-c, 12a-c &13a-c**), quinoline **10a-f**, pyrimidine **15a,b** and pyridine **17**. In addition, the UV absorption by some representative members indicated λ_{max} in the range of 400-320nm (UVA) as in compound **17** and λ_{max} in the range of 320-290nm (UVB) as in **9b.** This finding represents an indication that these compounds might proof themselves as photoprotectors. This might be due to their characteristic structure that can scavenge free radicals and protect the skin against their harmful effects and they can be applied topically. Moreover, they were evaluated pharmacologically for their in vitro antioxidant activity using L-ascorbic acid as positive control according to ABTS antioxidant assay, Erythrocyte hemolysis and Bleomycin depended DNA damage methods and the structures were confirmed by elemental analysis as well as ^{1}H NMR, IR and mass spectral data.*

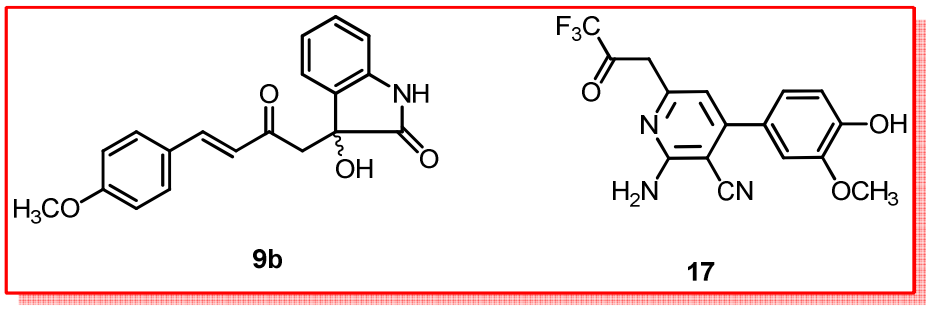

Figure 1. Representative examples of new antioxidant compounds might proof as photoprotectors.

Keywords: Oxidative stress, Synthesis, Indolines, Quinoline, Pyrimidines, Pyridine, photoprotectors and Antioxidant activity

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INTRODUCTION

Free radicals and other reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$) hydrogen peroxide (H_2O_2) are constantly generated by many biological processes and may be considered as a measure of

biological inefficiency [**1**]. Human body uses antioxidant defence system to neutralise the excessive level of ROS, this system consists of enzymes such as superoxide dismutases (SOD), catalases and glutathione peroxidases (GPXs) and numerous non-enzymatic small molecules distributed widely in biological system and capable of scavenging free radicals, these molecules include glutathione (GSH), α-tocopherol (vitamin E), vitamin C, β-carotene and selenium [**2**]. The imbalance between (ROS) production and the available antioxidant defence leads to a widely accepted phenomenon called oxidative stress [**3**]. In oxidative stress the ROS that are produced in excess are responsible for the oxidative damage of biomolecules such as lipids, proteins and DNA within cell. The oxidative damage of these biomolecules may lead to formation of non function molecules which precipitate in the tissue of [**4, 5**] as, Muscles, lead to inflammation and muscle spasm, Vascular system, lead to atherosclerosis, heart, kidney and liver diseases and Nerve cells lead to Alzheimer disease. Moreover, when oxidative stress make damage of the DNA, it will produce non function molecules, which may lead to apoptosis of cells or cancer. All these results may induce "Aging". In the last decade, much effort has been directed toward finding molecules capable of preventing or retarding this oxidative stress challenge. These are, however many naturally occurring substances and synthetically pharmacological agents which function to protect against the potentially harmful effects of prooxidants, these substances are termed antioxidants [**6-10**]. The objectives of this study were to evaluate the antioxidant activity and the efficacy of some new compounds containing pharmacophoric groups as styryl which found in Resveratrol **A** [**11**], in *trans* stilbene template such as the 4-methoxystilbene analogues B [**12**], enones as C and Curcumin which is the principal Curcuminoid D of the popular Indian spice Turmeric and has antioxidative property [**13-16**].

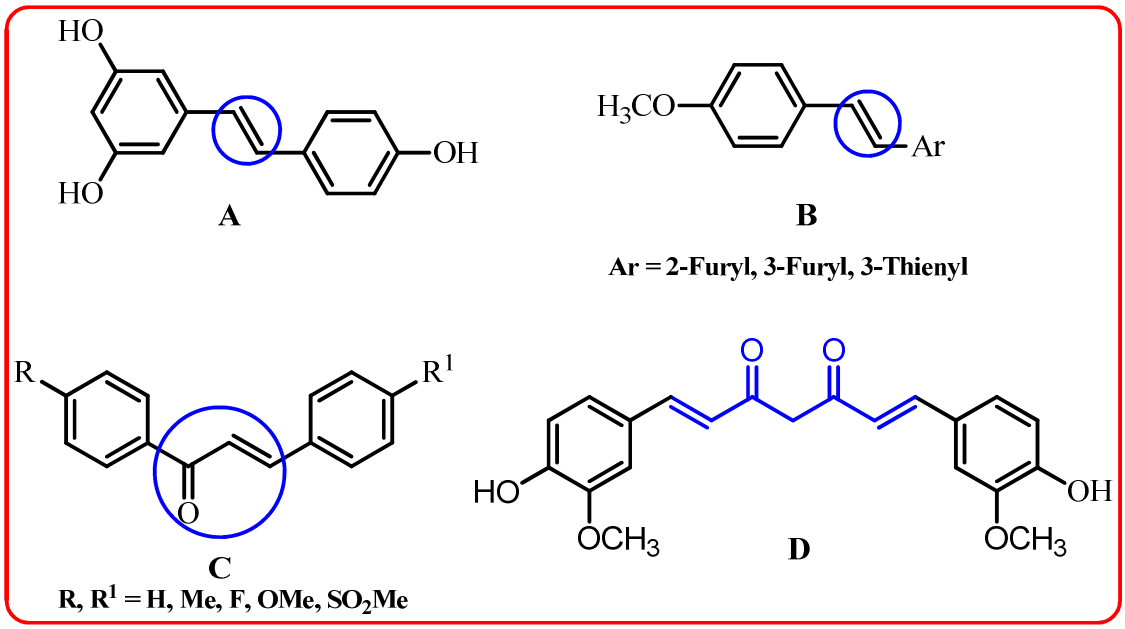


Figure 2. Representative examples of antioxidant compounds containing pharmacophoric groups

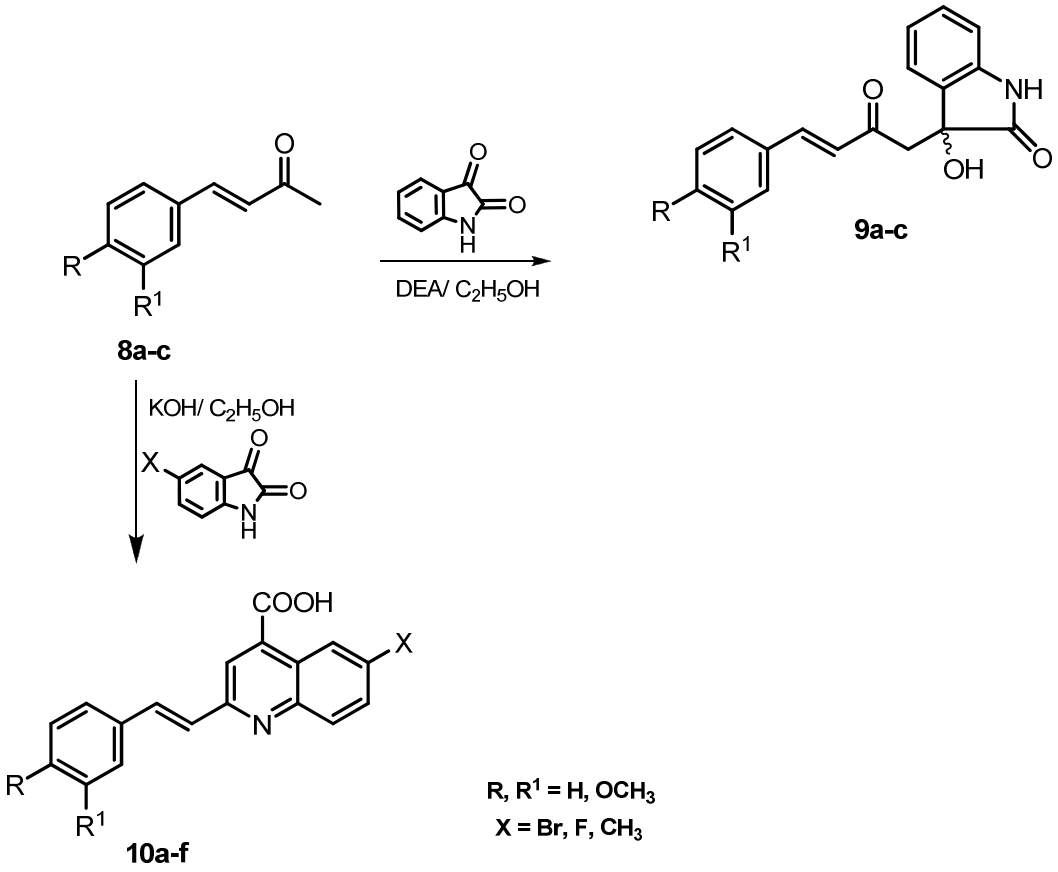
RESULTS AND DISCUSSION

2.1. Chemistry

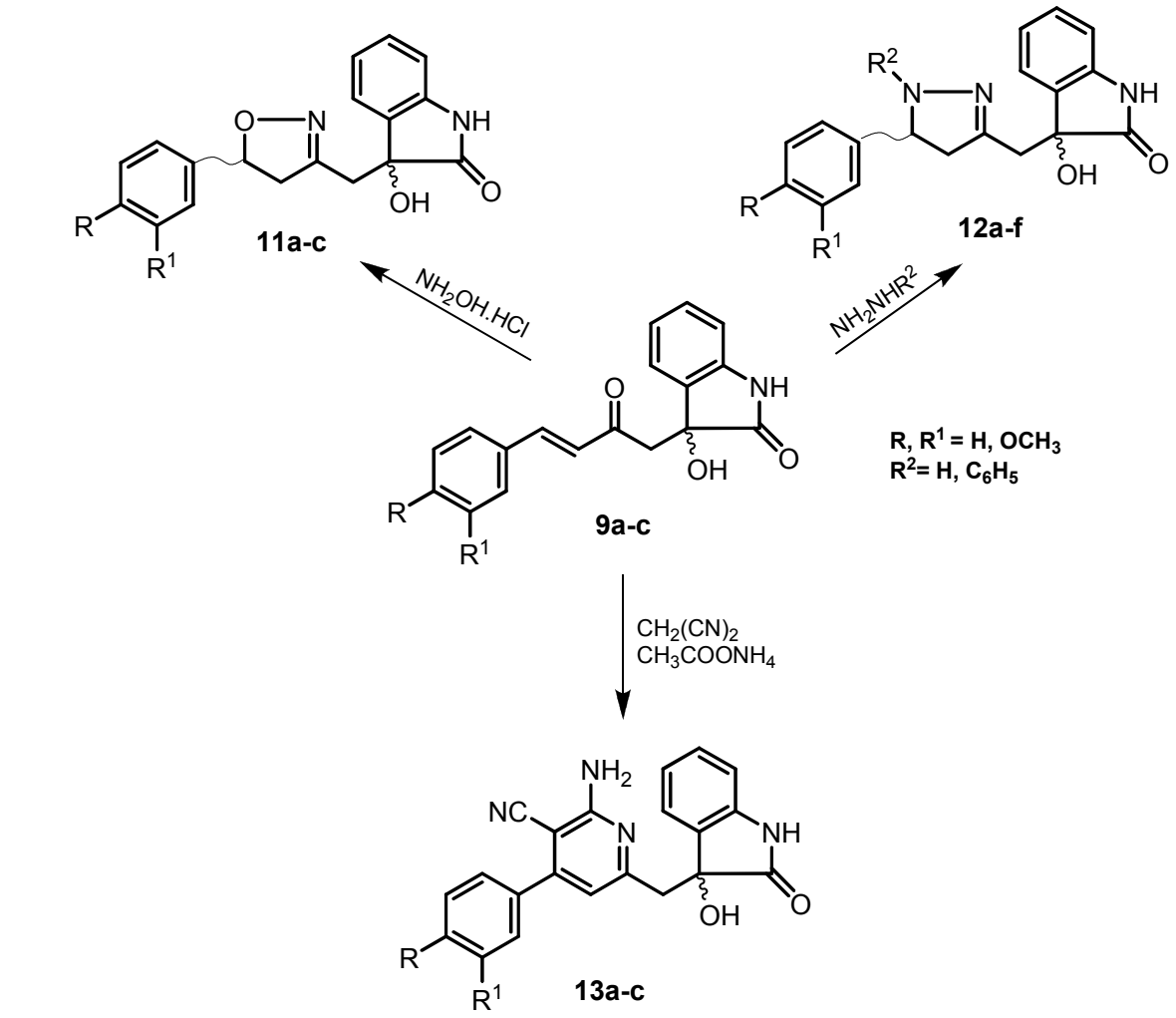
The synthetic routs of the proposed compounds are outlined in schemes **1-3.** Our initial goal was to prepare 3-hydroxy-3-[(*E*)-4-(3,4-disubstituted phenyl)-2-oxobut-3-enyl]indolin-2-ones **9a-c**. This was synthesised analogues to Popp method [**17**, **18**] through addition reaction of 4-(substituted phenyl)but-3-en-2-ones **8a-c** with isatin in absolute ethanol in presence of few drops of *N*, *N*-diethylamine (DEA) as a catalyst, the final product were easily separated in good yield **Scheme 1**. Compounds **9b, c** showed ^{1}H NMR spectra indicated that the products exist as the E-steroissomers ($J_{CH=CH}$= 16.3-16.5 Hz rang). Whereas, compounds **8a-c** were reacted according to "Pftzinger reaction [**19**] to prepare 6-substituted-2-(4-substitutedstyryl) quinoline-4-carboxylic acid **10a-f** through the reaction with isatins in alcoholic KOH Scheme ***1***. The mass spectra of compounds **10a, b** and **10f** showed molecular ion peaks confirmed their molecular weights, and generate ionic fragments that verified their structure. (M^+-44 or -45) is the common fragment ion peak eliminated from compounds **10a, b** due to decarboxylation. Cyclocondensation of 3-hydroxy-3-[(*E*)-4-(substituted phenyl)-2-oxobut-3-enyl]indolin-2-ones **9a-c** with hydroxylamine hydrochloride in alcoholic potassium hydroxide, hydrazine hydrate, phenyl hydrazine, or malononitrile in the presence of excess ammonium acetate in ethanol gave 3-[4,5-dihydro-5-((un) substituted phenyl)-1,2-oxazol-3-ylmethyl]-3-hydroxyindolin-2-ones **11a-c**, 3-(4,5-dihydro-1,3,5-trisubstituted pyrazol-3-ylmethyl)-3-hydroxyindolin-2-ones **12a-f** and 3-[6-amino-5-cyano-4-((un)substituted phenyl)pyridin-2-ylmethyl]-3-hydroxyindolin-2-ones **13a-c**,

respectively ***Scheme 2***. On the other hand, 4-(4-Methoxy-3-substituted phenyl)-6-methylpyrimidin-2-amines **14, 15** were synthesised according to the procedure described by Pandeye *et al* [**20**] through the reaction of 4-(substituted phenyl)but-3-en-2-ones **8b,c** with guanidine hydrochloride in the presence of alcoholic sodium hydroxide provided the requisite compounds in good overall yield. The IR data of compounds **14** exhibited absorption bands at 3280 (NH_2) and 1625-1613 (C=C, C=N of ring) and no absorption band new at 1655 (C=O, butenone). Moreover, Schiff's base condensation reaction of 2-aminopyrimidine derivatives **14a**,**b** with isatin in absolute ethanol containing few drops of glacial acetic acid gave the corresponding imines **15a**,**b** ***Scheme 3***. The IR data of compound **15a** exhibited absorption bands at 3385 (NH) and 1734 (C=O), in addition to characteristic bands at 1635-1613 (C=C, C=N of ring).

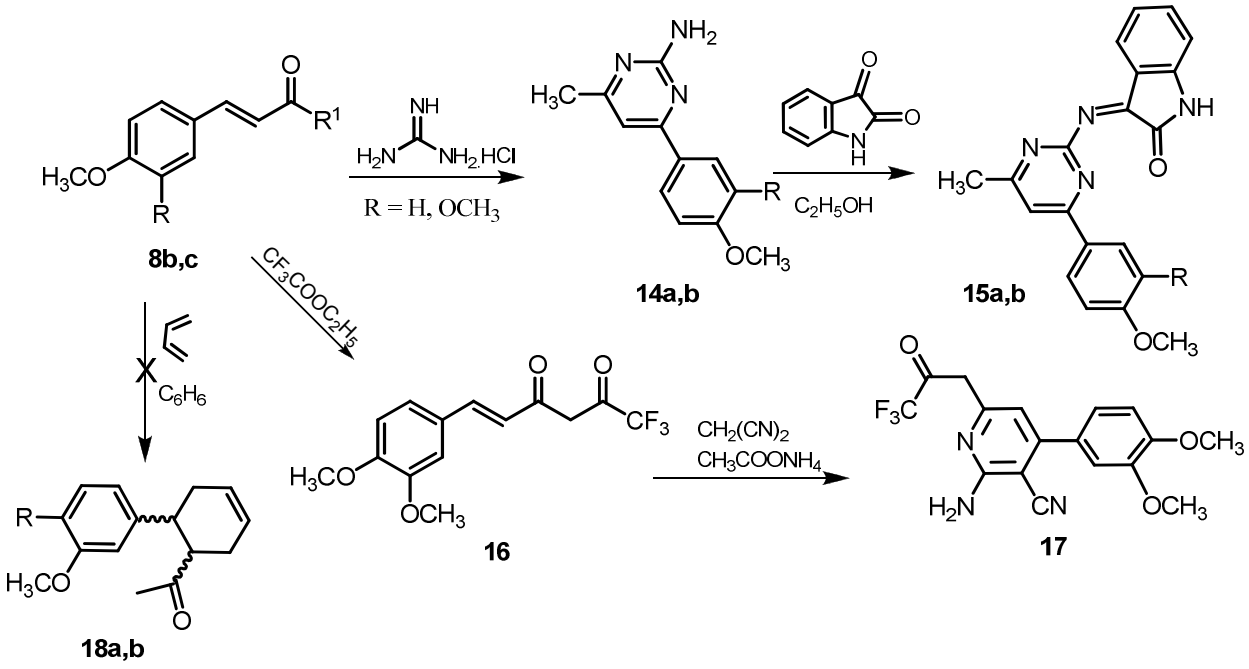
The second starting compound 1, 1, 1-trifluoro-6-(4-hydroxyphenyl) hex-5-ene-2, 4-diones **16** *via* base-catalyzed condensation of ethyl trifluoroacetate with butenone analogues **8c** in methanolic solution of sodium methoxide and (MTBE). Reaction of compound **16** with malononitrile and excess ammonium acetate was achieved to afford (2-amino-4-(3, 3, 3-trifluoro-2-oxopropyl)-6-(4-hydroxyphenyl) pyridine-3-carbonitrile **17 Scheme *3.*** The IR spectral data of compounds **17** exhibited absorption bands at 1633-1630 (C=O), 2215-2213 (CN) and 3389-3346 (NH_2), and no absorption bands at ~1611-1599 (C=C, vinyl). Generally, Diels-Alder reaction is carried out by reaction a double bond to a conjugated diene and formation of a six-membered ring [**21**]. Formation of **18a,b** through Diels-Alder reaction of 1,3-butadiene and butenone analogues **18b,c** in benzene at -5^{o} was failed .



Scheme 1

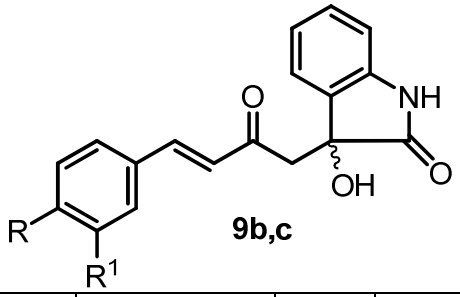


Scheme 2



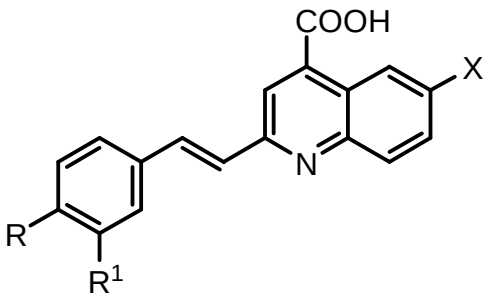
Scheme 3

Table 1: Physicochemical data of compounds 9b,c.



Comp. No.	R	R^1	Mol. Form. (M.W)	M.p°C	Yield %	Analysis	
						Calc.	Found
9b	OCH_3	H	$C_{19}H_{17}NO_4$ (323.34)	160-162	63	C :70.58 H : 5.30 N : 4.33	C :70.59 H : 5.32 N : 4.31
9c	OCH_3	OCH_3	$C_{20}H_{19}NO_5$ (353.37)	170-172	42	C :67.98 H : 5.42 N : 3.96	C :67.97 H : 5.42 N : 3.98

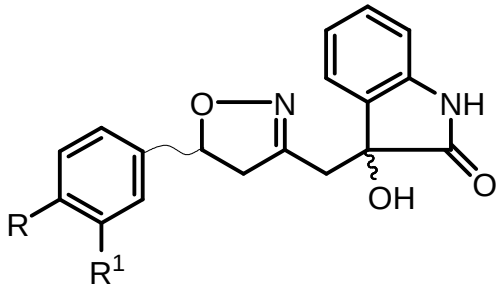
Table 2: Physicochemical data of compounds 10a-f.



Comp. No.	**R**	**R^1**	**X**	**Mol. Form. (M.W)**	**M.p (°C) (R.S*)**	**Yield %**	**Analysis**	
							Calc.	Found
10a	OCH_3	H	Br	$C_{19}H_{14}BrNO_3$ (384.22)	263 (AW)	60	C :59.39 H : 3.67 N : 3.65	C :59.36 H : 3.69 N : 3.62
10b	OCH_3	H	F	$C_{19}H_{14}FNO_3$ (323.32)	199-200 (AW)	60	C :70.58 H : 4.36 N : 4.33	C :70.52 H : 4.39 N : 4.32
10c	OCH_3	H	CH_3	$C_{20}H_{17}NO_3$ (319.35)	250 (AW)	55	C :75.22 H : 5.37 N : 4.39	C :75.25 H : 5.36 N : 4.33
10d	OCH_3	OCH_3	Br	$C_{20}H_{16}BrNO_4$ (414.25)	280 (EW)	72	C :57.99 H : 3.89 N : 3.38	C :57.96 H : 3.85 N : 3.84
10e	OCH_3	OCH_3	F	$C_{20}H_{16}FNO_4$ (353.34)	158-160 (EW)	52	C :67.98 H : 4.56 N : 3.96	C :67.96 H : 4.55 N : 3.98
10f	OCH_3	OCH_3	CH_3	$C_{21}H_{19}NO_4$ (349.38)	294 (AW)	65	C :72.19 H : 5.48 N : 4.01	C :72.16 H : 5.49 N : 4.04

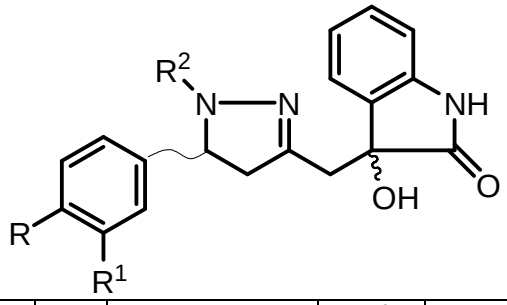
***R.S**: Recrystallisation solvent, AW: acetic acid/water, DW: DMF/water, EAA: ethanol/acetic acid, EE: ethanol/ethyl acetate.*

Table 3: Physicochemical data of compounds 11a-c.



Comp. No.	R	R^1	Mol. Form. (M.W)	(M.p °C)	Yield %	Analysis	
						Calc.	Found
11a	H	H	$C_{18}H_{16}N_2O_3$ (308.33)	197-200	60	C : 70.12 H : 5.23 N : 9.09	C : 70.15 H : 5.21 N : 9.10
11b	OCH_3	H	$C_{19}H_{18}N_2O_4$ (338.36)	199-201	62	C :67.44 H : 5.36 N : 8.28	C :67.45 H : 5.39 N : 8.30
11c	OCH_3	OCH_3	$C_{20}H_{20}N_2O_5$ (368.38)	180-182	53	C :65.21 H : 5.47 N : 7.60	C :65.23 H : 5.49 N : 7.58

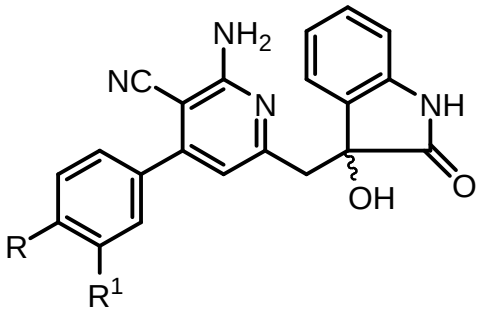
Table 4: Physicochemical data of compounds 12a-f.



Comp.No.	R	R^1	R_2	Mol. Form. (M.W)	(M.p °C) (R.S*)	Yield % Method	Analysis	
							Calc.	Found
12a	H	H	H	$C_{18}H_{17}N_3O_2$ (307.35)	180-182 (AA)	55 (A)	C : 70.34 H : 5.58 N : 13.67	C: 70.33 H: 5.59 N: 13.65
12b	OCH_3	H	H	$C_{19}H_{19}N_3O_3$ (337.37)	139-140 (M)	60 (A)	C : 67.64 H : 5.68 N : 12.46	C: 67.63 H : 5.69 N: 12.45
12c	OCH_3	OCH_3	H	$C_{20}H_{21}N_3O_4$ (367.40)	120-122 (E)	45 (A)	C : 65.38 H : 5.76 N : 11.44	C: 65.33 H : 5.79 N: 11.45
12d	H	H	C_6H_5	$C_{24}H_{21}N_3O_2$ (383.44)	180-182 (AA)	53 (B)	C : 75.18 H : 5.52 N : 10.96	C : 75.19 H : 5.55 N: 10.70
12e	OCH_3	H	C_6H_5	$C_{25}H_{23}N_3O_3$ (413.47)	139-140 (E)	60 (B)	C :72.62 H : 5.61 N : 10.16	C :72.65 H : 5.14 N : 10.14
12f	OCH_3	OCH_3	C_6H_5	$C_{26}H_{25}N_3O_4$ (443.49)	120-122 (EA)	42 (B)	C :70.41 H : 5.68 N : 9.47	C :70.43 H : 5.69 N : 9.45

**R.S: Recrystallisation solvent, AA: acetic acid, E: ethanol, EA: ethyl acetate, M: methanol*

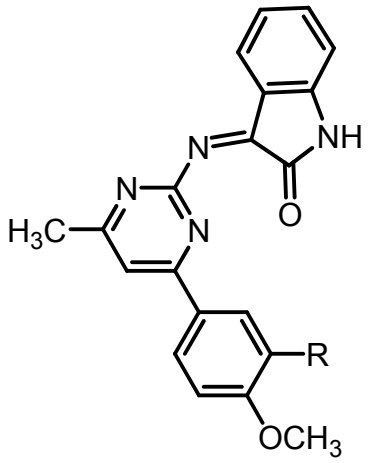
Table 5 : **Physicochemical data of compounds 13a-c.**



Comp. No.	R	R^1	Mol. Form. (M.W)	((M.p°C) R.S*)	Yield %	Analysis	
						Calc.	Found
13a	H	H	$C_{21}H_{16}N_4O_2$ (356.38)	173-175 (DW)	53	C : 70.77 H : 4.53 N : 15.72	C : 70.78 H : 4.52 N : 15.72
13b	OCH_3	H	$C_{22}H_{18}N_4O_3$ (386.40)	200-201 (E)	60	C :68.38 H : 4.70 N : 14.50	C :68.39 H : 4.71 N : 14.48
13c	OCH_3	OCH_3	$C_{23}H_{20}N_4O_4$ (416.43)	210-213 (E)	42	C :66.34 H : 4.84 N : 13.45	C :66.38 H : 4.86 N : 13.41

**R.S: Recrystallisation solvent, DW: DMF/water, E: ethanol.*

Table 6: Physicochemical data of compounds 15a,b



Comp. No.	R	Mol. Form. (M.W)	(M.p °C)	Yield %	Analysis	
					Calc.	Found
15a	H	$C_{20}H_{16}N_4O_2$(344.37)	190-192	56	C :69.76 H : 4.68 N: 16.27	C :69.79 H : 4.64 N : 16.29
15b	OCH_3	$C_{21}H_{18}N_4O_3$ (374.39)	215-217	53	C :67.37 H : 4.85 N : 14.96	C :67.38 H : 4.90 N : 14.92

2.2. Biological screening

2. 2.1 Antioxidant screening

The newly synthesized compounds have been subjected for preliminary screening of their *in vitro* antioxidant activity using L-ascorbic acid as positive control according to the following methods.

2. 2. 1. 1. ABTS antioxidant assay [22, 23].

$ABTS^+$, 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation decolourization test is a photometric method widely used for the assessment of antioxidant activity. $ABTS^+$ was generated by oxidation of ABTS with potassium persulfate**.** The absorbance is bleached by antioxidants due to their capacity to reduce the preformed radical. The results of the preliminary qualitative antioxidant screening (scavenger activity) of the tested compounds indicated that compounds **11b, 12e,f** highly active while, **11a**, **12c,d**, **14b,c** were moderately active and slightly active respectively, in comparison with the reference compound (**Table 8**). **11b, 12e,f** are highly active, while compounds

2. 2. 1. 2. Erythrocyte hemolysis assay [24-26].

There is evidence that ROS can induce the process of cell death so erythrocyte hemolysis assay was performed mediated by peroxy radicals. A suspension of erythrocytes in phosphate buffer saline (PBS) was added to the same volume of 2,2'-azobis[2-amidinopropane]dihydrchloride (AAPH) solution in PBS containing samples to be tested and The percentage hemolysis was calculated by the equation (A / B) x 100 % .. The results indicated that hemolysis than the reference L-ascorbic acid. While, compounds **12a**, **b, e** were either inactive or slightly active. (**Table 9**).

2. 2 .1 .3. Bleomycin dependant DNA damage assay [27-29].

Bleomycin is a cytotoxic antibiotic derived from *Streptomyces verticellus* mostly of the tested compounds has high ability to scavenger the peroxy radical with decreasing the ability to erythrocyte. It has antitumor, antibacterial and antiviral properties. Several investigations have shown that bleomycin induces apoptosis in mammalian cells. The observed data indicated that compound **10** has distinctive protecting activity to DNA. In addition, compound **7** have dominant activity similar to L-ascorbic acid (**Table 10**).

3. Chemistry

3. 1. Expirmental

Melting points (^{0}C, uncorrected) were determined on a *Fisher-John*s apparatus (Fischer-Scientific, Pittsburgh,PA, USA). UV spectra were recorded on Shimadzuc (Model 1601 PC) UV-Visible spectrophotometer. IR spectra (KBr) were recorded on a Pye-Unicam SP 1000 spectrometer (υ in cm^{-1}) (Pye-Unicam Ltd., Cambridge, UK). ^{1}H NMR

spectra were recorded on a Bruker Ac 250 FT NMR spectrometer using tetramethylsilane (TMS) as internal reference (chemical shifts in ppm, δ units). Mass spectra data analyses were recorded on Varian Mat. 112 A spectrophotometer. Analytical thin layer chromatography (TLC) was applied to monitor the reactions using precoated plates (silica gel 60 f-265, Merk, Darmstadt, Germany) and spots were visualized with UV light. The result of elemental analysis (C, H, N) were within ± 0.4 % of the theoretical values. Thin layer chromatography (TLC) was preformed on silica gel GLF plates, 250 microns. (*E*)-4-phenylbut-3-en-2-one and (*E*)-4-(4 -methoxyphenyl)-but-3-en-2-one, (*E*)-4-(3,4 -dimethoxyphenyl)-but-3-en-2-one , **(8a-c**) respectively, were synthesized as described [**30- 35**] and **9a** & **14a** were prepared as described [**36, 37**] respectivelly.

General procedure for preparation of 3-hydroxy-3-[(*E*)-4-(3,4-disubstituted phenyl)-2-oxobut-3-enyl]indolin-2-ones (9b,c).

A mixture of isatin (1.4 g, 0.01 mol), butenone derivatives **8a-c** (0.01 mol) and DEA (4 drops) in absolute ethanol (50 ml) was heated under reflux for 30 min. After standing for two days at room temperature, the separated solid was filtered, dried and recrystallised from MeOH. Melting points, yield percentages and microanalytical data are shown in(**Table 1**).

3-Hydroxy-3- [(*E*)-4-(4-methoxyphenyl)-2-oxobut-3-enyl] indolin-2-one (**9b**).

IR: 3430 (OH), 3150 (NH), 1725, 1675 (C=O), 1624 (C=C). **^{1}H NMR** ($CDCl_3$)**:** δ 10.2 (s, 1H, NH, D_2O exchangeable), δ 7.1-8.0 (m, 9H, H-4 & Ar-H), δ 6.8 (d, 1H, J=16, H-3), δ 3.6 (2d, 2H, CH_2), δ 5.9 (s, 1H, OH, D_2O-exchang.), δ 3.8 (s, 3H, OCH_3). **MS** (m/z %): 323 (3.7, M^+), 294 (94.4), 280 (28.98), 251 (27.42), 161 (100.00), 147 (24.3), 146 (58.5), 133 (37.1), 119 (30.1) 77 (30.4).

3-Hydroxy-3-[(*E*)-4-(3, 4-dimethoxyphenyl)-2-oxobut-3-enyl]indolin-2-one (9c).

IR: 3435 (OH), 3145 (NH), 1720, 1675 (C=O), 1635 (C=C). **^{1}H NMR** ($CDCl_3$)**:** δ 10.4 (s, 1H, NH, D_2O exchangeable), δ 7.3-8.1 (m, 9H, H-4 & Ar-H), δ 6.8 (d, 1H, J=16, H-3), δ 3.6 (2d, 2H, CH_2), δ 5.8 (s, 1H, OH, D_2O-exchang.), δ 3.9 (s, 6H, OCH_3).

General procedure for preparation of 2-(3, 4-disubstituted styryl)-6-(un) substituted quinoline-4-carbox- ylic acids (10a-f).

A mixture of the appropriate butenone derivatives **8a-c** (0.005 mol), isatin (0.78 g, 0.005 mol) or 5-substituted isatin (0.005 mol) and potassium hydroxide (1.3 g, 0.023 mol) in 50 % aqueous ethanol (20 ml) was heated under reflux for 20 h. Then, the reaction mixture was diluted with 30 % aqueous ethanol, filtered and neutralized with 50% acetic acid. The precipitated solid was filtered, dried and recrystallised from the suitable solvent. Melting points, recrystallisation solvents, yield percentages and microanalytical data are shown in (**Table 2**).

6-Bromo-2-(4-methoxystyryl)quinoline-4-carboxylic acid (10a).

IR: 3450 (COOH), 1734 (C=O), 1699 (C=C). **^{1}H NMR** ($CDCl_3$)**:** 11.0 (s, 1H, COOH), 7.1-8.2 (m, 9H, styryl H-2 & Ar-H), 6.8 (d, 1H, J=16, styryl H-1), 3.7 (s, 3H, OCH_3). **MS** (m/z %): 383/385 (16.7/26.3, M^+/M^++2), 382 (25.5), 371 (9.6), 368 (11.3),334 (20.5), 339 (24.30), 295 (27.2), 216 (32.2), 171 (37.2), 161 (46.4), 121 (70.1), 108 (50.6), 89 (100.0), 79/81 (38.5/37.2), 77 (80.0) 62 (37.2).

6-Fluoro-2-(4-methoxystyryl)quinoline-4-carboxylic acid (10b).

IR: 3455 (COOH), 1730 (C=O), 1685 (C=C). **^{1}H NMR** ($CDCl_3$)**:** 11.2 (s, 1H, COOH), 7.1-8.4 (m, 9H, styryl H-2 & Ar-H), 6.7 (d, 1H, J=16, styryl H-1), 3.8 (s, 3H, OCH_3). **MS** (m/z %): 323 (71.4, M^+), 322 (75.5, M^+-1), 279 (27.1), 278 (40.8), 238 (100.0), 161 (42.3), 121 (82.0), 108 (11.5), 83 (30.4), 77 (13.4).

2-(4-Methoxystyryl)-6-methylquinoline-4-carboxylic acid (10c).

IR: 3455 (COOH), 1730 (C=O), 1685 (C=C). **^{1}H NMR** ($CDCl_3$)**:** 11.0 (s, 1H, COOH), 7.2-8.2 (m, 9H, styryl H-2 & Ar-H), 6.9 (d, 1H, J=16, styryl H-1) 3.7 (s, 3H, OCH_3), 2.3 (s, 3H, CH_3). **MS** (m/z %): 318 (3.7, M^+-1), 308 (3.4), 300 (4.3), 294 (9.4), 288 (3.1), 223 (5.9), 161 (51.8), 133 (34.2), 121 (100.0), 91 (46.4), 77 (53.5).

6-Bromo-2-(3,4-dimethoxystyryl)quinoline-4-carboxylic acid (10d).

IR: 3455 (COOH), 1730 (C=O), 1685 (C=C). **^{1}H NMR** ($CDCl_3$)**:** 11.2 (s, 1H, COOH), 7.3-8.2 (m, 8H, styryl H-2 & Ar-H), 6.8 (d, 1H, J=16, styryl H-1), 3.9 (s, 6H, OCH_3).

6-Fluoro-2-(3,4-dimethoxystyryl)quinoline-4-carboxylic acid (10e).

IR: 3455 (COOH), 1730 (C=O), 1685 (C=C). **^{1}H NMR** ($CDCl_3$)**:** 10.9 (s, 1H, COOH), 7.2-8.2 (m, 8H, styryl H-2 & Ar-H), 6.8 (d, 1H, J=16, styryl H-1), 3.9 (s, 6H, OCH_3). **MS** (m/z %): 353 (2.6, M^+), 269 (6.0), 268 (24.0), 237 (7.7), 151 (100.0), 137(8.7), 83 (9.1), 77 (4.2).

2-(3,4-Dimethoxystyryl)-6-methylquinoline-4-carboxylic acid (10f).
IR: 3455 (COOH), 1730 (C=O), 1685 (C=C). **^{1}H NMR** ($CDCl_3$)**:** 11.1(s, 1H, COOH), 7.3-8.2 (m, 8H, styryl H-2 & Ar-H), 6.9 (d, 1H, J=16, styryl H-1), 3.9 (s, 6H, OCH_3), 2.3 (s, 3H, CH_3).

General procedure for preparation of 3-[4, 5-dihydro-5-((un)substituted phenyl)-1, 2-oxazol-3-ylmethyl]-3-hydroxyindolin-2-ones (11a-c)
A suspension of **9a-c** (0.003 mol) in absolute ethanol (15 ml), was added to a solution of hydroxylamine hydrochloride (0.2 g, 0.003 mol) and potassium hydroxide (0.2 g, 0.004 mol) in absolute ethanol (10 ml). The resulting mixture was heated under reflux for 10 h. On cooling, the precipitated solid was filtered, dried and recrystallised from ethanol. Melting points, yield percentages and microanalytical data are shown in (**Table 3**).

3-(4,5-Dihydro-5-phenyl-1,2-oxazol-3-ylmethyl)-3-hydroxyindolin-2-one (11a).
^{1}H NMR (DMSO-d_6): 11.7 (s, 1H, NH, D_2O exchangeable), 8.2 (s, 1H, OH, D_2O exchangeable), 6.8-8.0 (m, 9H, Ar-H), 5.7-5.9 (t, 1H, oxazolyl, CH-5), 4.8 (2d, 2H, CH_2), 3.4-3.5 (d, 2H, oxazolyl, CH-4).

3-[4,5-Dihydro-5-(4-methoxyphenyl)-1,2-oxazol-3-ylmethyl]-3- hydroxyindolin-2-one (11b).
^{1}H NMR (DMSO-d_6): 11.7 (s, 1H, NH, D_2O exchangeable), 8.2 (s, 1H, OH, D_2O exchangeable), 6.8-8.0 (m, 8H, Ar-H), 5.7-5.9 (t, 1H, oxazolyl, CH-5), 4.8 (2d, 2H, CH_2), 3.8 (s, 3H, OCH_3), 3.4-3.5 (d, 2H, oxazolyl, CH-4).

3-[4,5-Dihydro-5-(3,4-dimethoxyphenyl-1,2-oxazol-3-ylmethyl]-3-hydroxyindolin-2-one (11c).
^{1}H NMR (DMSO-d_6): 11.7 (s, 1H, NH, D_2O exchangeable), 8.2 (s, 1H, OH, D_2O exchangeable), 6.8-8.0 (m, 7H, Ar-H), 5.7-5.9 (t, 1H, oxazolyl, CH-5), 4.8 (2d, 2H, CH_2), 3.9 (s, 6H, OCH_3), 3.4-3.5 (d, 2H, oxazolyl, CH-4). **MS** (m/z %): 367 (2.3, M^+-1), 363 (3.0), 338 (1.9), 308 (8.8), 305 (100.0), 290 (16.3), 275 (5.8), 263 (3.5), 142 (1.0), 92 (2.0), 74 (22.9).

General procedure for preparation of 3-(4, 5-dihydro-1,3,5-trisubstituted pyrazol-3-ylmethyl)hydroxyindolin-2-ones (12a-f).
Method A:
Hydrazine hydrate (98 %) (1.3 g, 0.04 mol) was added to a solution of **9a-c** (0.01 mol) in absolute ethanol (30 ml) and the mixture was heated under reflux for 6 h. The solvent was concentrated *in vacuo*. On cooling the separated solid was filtered, dried and recrystallised from the appropriate solvent.

Method B:
Phenyl hydrazine (0.9 g, 0.01 mol), was added to a solution of **9a-c** (0.01 mol) in glacial acetic acid (20 ml). The mixture was heated under reflux for 20 h. On cooling, the separated solid was filtered, dried and recrystallised from the appropriate solvent.
Melting points, recrystallisation solvents, yield percentages and microanalytical data are shown in (**Table 4**).

3-(4,5-Dihydro-5-phenyl-1*H*-pyrazol-3-ylmethyl)-3-hydroxy indolin-2-one (12a).
^{1}H NMR (DMSO-d_6): 9.9 (s, 2H, NH, D_2O exchangeable), 8.4 (s, 1H, OH, D_2O exchangeable), 7.0-8.0 (m, 9H, Ar-H), 5.1-5.3 (t, 1H, pyrazolyl, CH-5), 4.9 (2d, 2H, CH_2), 3.4 (d, 2H, pyrazolyl, CH-4). **MS** (m/z %): 307 (7.2, M^+), 296 (3.3), 243 (2.3), 116 (13.0),74 (100.0).

3-[4,5-Dihydro-5-(4-methoxyphenyl)-1*H*-pyrazol-3-ylmethyl]-3-hyd- roxyindolin-2-one (12b).
^{1}H NMR (DMSO-d_6): 9.9 (s, 2H, NH, D_2O exchangeable), 8.3 (s, 1H, OH, D_2O-exchangeable), 6.8-8.0 (m, 8H, Ar-H), 5.1-5.2 (t, 1H, pyrazolyl, CH-5), 4.9 (2d, 2H, CH_2), 3.7 (s, 3H, OCH_3), 3.4 (d, 2H, pyrazolyl, CH-4).

3-[4,5-Dihydro-5-(3,4-dimethoxyphenyl)-1*H*-pyrazol-3-ylmethyl]-3-hydroxyindolin-2-one (12c).
^{1}H NMR (DMSO-d_6): 9.9 (s, 2H, NH, D_2O exchangeable), 8.3 (s, 1H, OH, D_2O-exchangeable), 6.8-8.0 (m, 7H, Ar-H), 5.1-5.2 (t, 1H, pyrazolyl, CH-5), 4.9 (2d, 2H, CH_2), 3.9 (s, 6H, OCH_3), 3.4 (d, 2H, pyrazolyl, CH-4).

3-(4,5-Dihydro-1,5-diphenyl-1*H*-pyrazol-3-ylmethyl)-3-hydroxyindo-lin-2-one (12d).
^{1}H NMR (DMSO-d_6):10.1 (s, 1H, NH, D_2O exchangeable), 8.3 (s, 1H, OH, D_2O-exchangeable), 6.8-8.0 (m, 14H, Ar-H), 5.0-5.2 (t, 1H, pyrazolyl, CH-5), 4.5 (2d, 2H, CH_2), 3.1-3.5 (d, 2H, pyrazolyl, CH-4).

3-[4,5-Dihydro-5-(4-methoxyphenyl)-1-phenyl-1*H*-pyrazol-3-ylmethyl]-3-hydroxyindolin-2-one (12e).
^{1}H NMR (DMSO-d_6):10.1 (s, 1H, NH, D_2O exchangeable), 8.3 (s, 1H, OH, D_2O-exchangeable), 6.8-8.0 (m, 13H, Ar-H), 5.0-5.2 (t, 1H, pyrazolyl, CH-5), 4.5 (2d, 2H, CH_2), 3.7 (s, 3H, OCH_3) 3.1-3.5 (d, 2H, pyrazolyl, CH-4). **MS** (m/z %): 413 (6.1 M^+), 383 (1.7), 266 (19.6), 265 (100.0), 251 (1.0), 235 (0.3), 162 (1.0), 91 (1.8), 77 (0.1), 66 (0.4).

3-[4,5-Dihydro-5-(3,4-dimethoxyphenyl)-1-phenyl-1*H*-pyrazol-3-yl-methyl]-3-hydroxyindolin-2-one (12f).
^{1}H NMR (DMSO-d_6):10.4 (s, 1H, NH, D_2O exchangeable), δ 8.6 (s, 1H, OH, D_2O-exchangeable), 6.8-8.0 (m, 12H, Ar-H), 5.2-5.4 (t, 1H, pyrazolyl, CH-5), 4.8 (2d, 2H, CH_2), 3.8 (s, 6H, OCH_3), 3.4 (d, 2H, pyrazolyl, CH-4).

General procedure for preparation of 3-[6-amino-5-cyano-4-((un)substituted phenyl) pyridine-2-ylmethyl]-3-hydroxyindolin-2-ones (13a-c).
A mixture of **9a-c** (0.005 mol), malononitrile (0.3 g, 0.005 mol) and ammonium acetate (3.1 g, 0.04 mol) in absolute ethanol (30 ml) was heated under reflux for 6 h. The reaction mixture was concentrated *in vacuo*. On cooling, the precipitated solid was filtered, dried and recrystallised. Melting points, recrystallisation solvents, yield percentages and microanalytical data are shown in (**Table 5**).

3-(6-Amino-5-cyano-4-phenylpyridin-2-ylmethyl)-3-hydroxyindolin-2- one (13a).
IR: 3450 (OH), 3389 (NH_2), 2225 (CN), 1715 (C=O), 1610 (C=N). **^{1}H NMR** (DMSO-d_6): 10.2 (s, 1H, NH, D_2O exchangeable), 8.6 (s, 1H, OH, D_2O exchangeable), 6.8-8.1 (m, 10H, Ar-H), 6.2 (s, 2H, NH_2, D_2O exchangeable), 4.1 (2d, 2H, CH_2).

3-[6-Amino-5-cyano-4-(4-methoxyphenyl)pyridin-2-ylmethyl]-3-hydroxyindolin-2-one (13b).
IR: 3445 (OH), 3385 (NH_2), 2215 (CN), 1720 (C=O), 1615 (C=N). **^{1}H NMR** (DMSO-d_6): 10.3 (s, 1H, NH, D_2O exchangeable), 8.6 (s, 1H, OH, D_2O exchangeable), 6.8-8.1 (m, 9H, Ar-H), 6.2 (s, 2H, NH_2, D_2O exchangeable), 4.1 (2d, 2H, CH_2), 3.7 (s, 3H, OCH_3). **MS** (m/z %): 386 (5.5, M^+), 372 (25.6) , 332 (35.5), 264 (16.8), 238 (50.3), 211 (18.5), 135 (43.2), 121 (76.9), 93 (22.6) 57 (100.0), 55 (84.9).

3-[6-Amino-5-cyano-4-(3,4-dimethoxyphenyl)pyridin-2-ylmethyl]-3-hydroxyindolin-2-one (13c).
IR: 3455 (OH), 3380 (NH_2), 2220 (CN), 1715 (C=O), 1620 (C=N). **^{1}H NMR** (DMSO-d_6): 10.3 (s, 1H, NH, D_2O exchangeable), 8.7 (s, 1H, OH, D_2O exchangeable), 6.8-8.1 (m, 8H, Ar-H), 6.2 (s, 2H, NH_2, D_2O exchangeable), 4.1 (2d, 2H, CH_2), 3.9 (s, 6H, OCH_3).

4-(3,4-Dimethoxyphenyl)-6-methyl pyrimidin-2-amines (14b).
A mixture of the appropriate butenone analogues **3c** (0.01 mol) and guanidine hydrochloride (1.4 g, 0.015 mol) was added to a solution of sodium hydroxide (1.8 g, 0.045 mol) in ethanol (50 ml). The reaction mixture was heated under reflux for 6 h, concentrated *in vacuo* and cooled. The resulting solid was collected, dried and recrystallised from ethanol to yield 1.35 g (53 %) of compound **9** m.p 115-118 ^oC. Analysis (%) Calc.(Found) for compound **14b**: $C_{13}H_{15}N_3O_2$ (245.28): C, 63.66 (63.68); H, 6.16 (6.19); N, 17.13.(17.15). **UV** (C_2H_5OH): λ_{max} 267, ε = 19828.00. **IR**: 3280 (NH_2), 1625, 1613 (C=C, C=N ring).

General procedure for preparation of 3-[4-(substituted phenyl)-6-methylpyrimidin-2-ylimino] indolin-2-one (15a, b):
Isatin (0.4 g, 0.003 mol) and the appropriate pyrimidines (**14a,b**) (0.003 mol) were dissolved in warm absolute ethanol (75 ml) containing glacial acetic acid (5 ml). The reaction mixture was heated under reflux for 18 h and kept at room temperature overnight. The solid precipitated was washed with ethanol, dried and recrystallised from ethanol. Melting points, , yield percentages and microanalytical data are shown in (**Table 6**).

3-[4-(4-Methoxyphenyl)-6-methylpyrimidin-2-ylimino] indolin-2-one (15a).
UV (C_2H_5OH): λ_{max} 272, ε = 8517.24, λ_{max} 224, ε = 26931.03. **IR**: 3385 (NH), 1734 (C=O), 1635, 1613 (C=C, C=N ring). **^{1}H NMR** (DMSO-d_6): 9.1 (s, 1H, NH), 7.1-8.0 (m, 9H, Ar-H), 3.8 (s, 3H, OCH_3), 2.3 (s, 3H, CH_3) .

3-[4-(3, 4-Dimethoxyphenyl)-6-methylpyrimidin-2-ylimino]indolin-2-one (15b).
IR: 3380 (NH), 1734 (C=O), 1635, 1613 (C=C, C=N ring). **^{1}H NMR** (DMSO-d_6): 9.1 (s, 1H, NH), 7.1-8.0 (m, 8H, Ar-H), 3.9 (s, 6H, OCH_3), 2.3 (s, 3H, CH_3) . **MS** (m/z %): 373 (10.9, M^+-1), 372 (24.8), 359 (11.10), 346 (7.32), 333 (15.4), 238 (37.5), 233 (20.4), 226 (12.9), 147 (8.3), 121 (100.0), 115(13.6), 108 (8.8), 89 (9.5), 77 (17.5), 69 (12.2).

1, 1, 1-Trifluoro-6-(3,4-dimethoxyphenyl)hex-5-ene-2,4-diones (16).
A solution of ethyl trifluoroacetate (23.5 g, 0.16 mol) in methyl *tert*-butyl ether (MTBE) (75 ml) was added dropwise to an ice-cooled solution of sodium methoxide (9.5 g, 0.18 mol) in methanol (40 ml), followed by addition of a suspension of appropriate butenone derivatives **8c** (0.15 mol) in MTBE (20 ml) dropwise over 5 min. The mixture was stirred at room temperature for 16 h and 3N HCl (70 ml) was added. The organic layer was collected, washed with brine (75 ml), dried over $MgSO_4$, filtered and concentrated *in vacuo*, the residue was triturated with pet. ether (60-80). The resulting solid was collected by filtration, dried and recrystallised from ethanol to afford g

(42%) of compound **16, mp** 93-95^{O}C. Analysis (%) Calc.(Found) for compound **16**: $C_{14}H_{13}F_3O_4$ (302.25): C, 55.63 (55.60); H, 4.34 (4.39); F, 18.86 (18.80). **UV** (C_2H_5OH**):** λ_{max} 370, ε = 20636.36.

IR: 3346 (NH_2), 2213 (CN), 1633 (C=O)$^{\cdot}$. . **IR**: 3450 (br, OH), 1675, 1639 (C=O), 1583 (C=C). **^{1}H NMR** ($CDCl_3$**):** 6.9-7.5 (m, 3H, H-4 & Ar-H), δ 6.8 (d, 1H, J=16, H-3), 5.1 (s, 1H, OH, D_2O exchangeable), 4.1 (s, 2H, CH_2), 3.9 (s, 6H, OCH_3). **MS** (m/z %): 302 (21.8, M^+), 256 (8.7), 233 (24.0), 205 (28.2), 191 (31.2), 163 (8.2), 130 (15.0), 90 (27.1.3), 68 (100.0), 52 (21.4).

2-Amino-6-(3,3,3-trifluoro-2-oxopropyl)-4-(3,4-dimethoxyphenyl) pyridine-3-carbonitrile (17).
A mixture of 1,1,1-trifluoro-6-(3,4-dimethoxyphenyl)hex-5-ene-2,4-diones **16** (0.005 mol), malononitrile (0.3 g, 0.005 mol) in absolute ethanol (30 ml) and ammonium acetate (3.1 g, 0.04 mol) was heated under reflux for 6 h. The reaction mixture was concentrated *in vacuo*, the precipitated solid was filtered, dried and recrystallised from ethanol. to afford g (41%) of compound **17, mp** 153-138^{O}C. Analysis (%) Calc.(Found) for compound **16**: $C_{17}H_{14}F_3N_3O_3$ (365.31): C, 55.89 (55.83); H, 3.86 (3.81); F, 15.50 (15.50), N: 11.50 (11.53). **IR**: 3346 (NH_2), 2213 (CN), 1633 (C=O)$^{\cdot}$. **^{1}H NMR** ($CDCl_3$**):** 6.8-7.4 (m, 4H, Ar-H), 5.5 (s, 1H, OH, D_2O exchangeable), 4.9 (s, 2H, NH_2, D_2O exchangeable), 3.7 (s, 3H, OCH_3), 2.5 (s, 2H, CH_2). **MS** (m/z %): 365 (8.1, M^+), 349 (9.7), 293 (15.3), 269 (8.9), 191 (18.0), 151 (100.0), 91 (7.8) and 77 (8.5).

3.2. Biological screening
3.2.1. Antioxidant screening
3. 2. 1. 1. ABTS antioxidant assay:
3. 2. 1. 1. 1. Preparation of reagents and extracts
a) Preparation of control: ABTS solution prepared as 0.1g /100 ml, MnO_2 solution 25mg/ml (used instead of potassium persulfate). All reagents were prepared in phosphate buffer (pH 7, 0.1M). Mix the two reagents ABTS/MnO_2 (2:3), shake, centrifuge, and the supernatant is green-blue solution ($ABTS^+$ radical solution). This colour remains stable more than 1h. Adjust absorbance at 0.2 at 734 nm. **b)** Preparation of standard antioxidant L-ascorbic acid L-Ascorbic acid solution prepared as 2% solution: 1g /50 ml distilled water. **c)** Preparation of test samples: Each test sample was used in concentration of 0.01mg/ml in methanol/phosphate buffer (1:1).

3. 3. 1. 1.2. Assay:
a) Add 900 ul of (ABTS/MnO_2) to cuvette of spectrophotometer (SPEKOL 11), measure absorbance at 734 nm and measure against blank (methanol/phosphate buffer (1:1); reading.0.2.

b) Add 900 ul of mixture to 100ul standard ascorbic acid and measure against blank (methanol/phosphate buffer (1:1) +100 ul of ascorbic acid).
c) Add 900 ul of mixture to 100 ul of sample and measure against blank (methanol/phosphate buffer (1:1) + 100 ul of sample). The antioxidant activity was expressed in term of percentage of inhibition of superoxide production % Inhibition of superoxide production = (control absorbance - test absorbance) / (control absorbance) x 100.

3. 2. 1. 2. Erythrocyte hemolysis assay
3. 2. 1. 2.1. Preparation of reagents and extracts
Blood was obtained from rats by retro orbital plexus method and collected in heparinised tubes. Erythrocytes were separated from plasma and the buffy coat and washed three times with 10 volumes of 0.15 M NaCl. During the last wash, the erythrocytes were centrifuged at 2,500 rpm for 10 min to obtain a constantly packed cell preparation.

3. 2. 1. 2. 2 Assay:
A 10 % suspension of erythrocytes in pH 7.4 phosphate buffer saline (PBS) was added to the same volume of (200 mM) 2,2'-azobis[2-amidinopropane]dihydrchloride (AAPH) solution in PBS containing samples to be tested in a concentration of 0.2 mg/ml. The reaction mixture was shaken gently while being incubated at 37 ^{0}C for 2h. Then, it was removed, diluted with 8 volumes of PBS and centrifuged at 2.500 rpm for 10 min. The absorbance A of the supernatant was read at 540 nm. Similarly, the reaction mixture was treated with 8 volumes of distilled water to achieve complete hemolysis and absorbance B of the supernatant after centrifugation was measured at 540 nm. The percentage hemolysis was calculated by the equation (A / B) x 100 %.

3. 2 .1 .3. Bleomycin dependant DNA damage assay.
3. 2.1 . 3. 1. Assay
The reaction mixture contained DNA (0.5 mg/ml), bleomycin sulfate (0.05 mg/ml), $MgCl_2$ (0.5 mg/ml), $FeCl_3$ (50 uM) and samples to be tested in a concentration of 0.1 mg/ml. L-ascorbic acid was used as positive control. The mixture was incubated at 37 ^{o}C for 1 h. The reaction was terminated by addition of 0.05 ml EDTA (0.1 M) .The colour was developed by adding 0.5 ml thiobarbituric acid (TBA) (1% w/v) and 0.5 ml HCl (25% v/v) followed by

heating at 80 ^{o}C for 10 min. After centrifugation the extent of DNA damage was measured by increase in absorbance at 532 nm.

Table 6: ABTS antioxidant assay method

Method	ABTS				
Comp. No.	**Absorbance A**	**Inhibition %**	**Comp. No.**	**Absorbance A**	**Inhibition %**
9b	0.37	17.70	**12b**	0.38	15.50
9c	0.35	22.20	**12c**	0.14	68.80
10a	0.3	33.30	**12d**	0.19	57.70
10b	0.34	24.40	**12e**	0.08	82.20
10c	0.25	44.40	**12f**	0.08	82.20
10d	0.25	44.40	**13a**	0.3	33.30
10e	0.27	40.00	**13b**	0.1	77.70
10f	0.39	13.30	**13c**	0.18	60.00
11a	0.2	55.50	**15a**	0.38	15.50
11b	0.05	88.80	**17**	0.3	33.30
11c	0.29	35.50	**L-ascorbic acid**	0.05	88.80
12a	0.26	42.20			

Table 7: Erythrocyte hemolysis antioxidant assay method.

Method	Erythrocyte Hemolysis				
Comp. No.	**Absorbance A**	**Hemolysis %**	**Comp. No.**	**Absorbance A**	**Hemolysis %**
9b	0.014	1.60	**12b**	0.45	52.50
9c	0.009	1.05	**12c**	0.014	1.63
10a	0.02	2.33	**12d**	0.007	0.81
10b	0.036	4.20	**12e**	0.45	52.50
10c	0.006	0.70	**12f**	0.017	1.98
10d	0.02	2.33	**13a**	0.029	3.38
10e	0.02	2.33	**13b**	0.031	3.61
10f	0.025	2.91	**13c**	0.047	5.48
11a	0.033	3.85	**15a**	0.003	0.35
11b	0.006	0.70	**17**	0.032	3.73
11c	0.028	3.26	**L-ascorbic acid**	0.054	6.30
12a	0.281	32.70			

Table 8 : Bleomycin depended DNA damage antioxidant assay method.

Method	Bleomycin depended DNA damage		
Comp. No.	**Absorbance A**	**Comp. No.**	**Absorbance A**
122b	0.04	**12b**	0.009
9c	0.023	**12c**	0.002
10a	0.041	**12d**	0.004
10b	0.033	**12e**	0.01
10c	0.033	**12f**	0.02
10d	0.043	**13a**	0.03
10e	0.132	**13b**	0.016
10f	0.015	**13c**	0.006
11a	0.02	**15a**	0.021
11b	0.036	**17**	0.045
11c	0.085	**L-ascorbic acid**	0.023
12a	0.048		

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