

# Anti-oxidant Activity of Coumarins

\*Tehmeena Ahad, Jasia Nissar and A.H. Rather

\*Division of Food Science and Technology, Skuast-k Shalimar 190025

**Abstract**—Many coumarin derivatives have special ability to scavenge reactive oxygen species (ROS)—free radicals, such as hydroxyl radicals, superoxide radicals or hypochlorous acid, and to influence processes involving free radical-injury. They have also been found to inhibit lipid peroxidation and to possess vasorelaxant, anti-inflammatory and anticoagulant. The coumarins are extremely variable in structure, due to the various types of substitutions in their basic structure, which can influence their biological activity. This research work highlights the current progress in the development of coumarin scaffolds for drug discovery as novel anti-oxidant agents. The major challenges about coumarins include the translation of current knowledge into new potential lead compounds and the repositioning of known compounds for the treatment of oxidative disorders. antioxidant activity was carried out with 4-hydroxy-, 5-chloro-4-hydroxy and 7-hydroxy-4-methyl coumarin derivatives by DPPH free radical scavenging method, Super oxide method and Nitric oxide method. The  $IC_{50}$  value was determined for each compound. From results of DPPH, Super oxide and Nitric oxide methods, it found that compound I and II displayed strong antioxidant ( $P < 0.001$ ) activity compared to the ascorbic acid and it suggested that these compounds could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases. Compound III is also showed good anti oxidant activity.

**Key words:**-coumarin, DPPH, antioxidant, Nitric oxide

## 1. INTRODUCTION

Many natural as well as synthetic coumarins and more complex related derivatives have recently drawn much attention due to its broad pharmacological activities include anti-bacterial, anti-thrombotic and vasodilatory, antimutagenic, lipoxygenase and cyclooxygenase inhibition, scavenging of reactive oxygen species, and anti-tumourigenic, appears to be based on the coumarin nucleus.[2,3] Many coumarin derivatives have special ability to scavenge reactive oxygen species (ROS)—free radicals, such as hydroxyl radicals, superoxide radicals or hypochlorous acid, and to influence processes involving free radical-injury.[1] They have also been found to inhibit lipid peroxidation and to possess vasorelaxant, anti-inflammatory and anticoagulant activities.

The recognition of key structural features within coumarin family is crucial for the design and development of new analogues with improved activity and for the characterization of their mechanism of action and potential side effects. The coumarins are extremely variable in

structure, due to the various types of substitutions in their basic structure, which can influence their biological activity. In view of the considerable importance of the coumarins and its derivatives, the present work is aimed for testing of target compounds for their free radical scavenging activity by using DPPH, super oxide and nitric oxide free radical scavenging methods.

## 2. MATERIALS AND METHODS

Coumarin compounds were procured from local market of Kashmir and prepared various concentrations with DMSO.

## 3. CHEMICALS

1,1-Diphenyl-2-picryl hydrazyl (DPPH), Griess reagent and Dimethyl sulphoxide were obtained from MP Biomedicals Ltd., USA, Nitroblue tetrazolium (NBT), Riboflavin, Ascorbic acid and sodium nitroprusside were obtained from SD fine chemicals Ltd., India. Deoxy ribose was obtained from Merck India. ethylene diamine tetra acetic acid (EDTA), ferrous sulphate, trichloro acetic acid, Hydrogen peroxide ( $H_2O_2$ ), mannitol, potassium dihydrogen phosphate, potassium hydroxide, phenazinemethosulfate were of analytical grade and obtained from Ranbaxy fine chemicals.

## 4. DETERMINATION OF ANTI OXIDANT ACTIVITY DPPH RADICAL SCAVENGING ACTIVITY

The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). When Antioxidants react with DPPH., which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical-scavenging antioxidant) and is reduced to the DPPH and as consequence the absorbance's decreased from the DPPH. Radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured [2]. More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenylpicrylhydrazine; non radical) with the loss of this violet colour (although there would be

expected to be a residual pale yellow colour from the picryl group still present)[3].

The scavenging reaction between (DPPH.) and an antioxidant (H-A) was shown in figure 2. 4.3 mg of DPPH (1, 1-Diphenyl -2-picrylhydrazyl) was dissolved in 3.3 ml methanol; it was protected from light by covering the test tubes with aluminum foil. 150 µl DPPH solution was added to 3ml methanol and absorbance was taken immediately at 517nm for control reading. 50 µl of various concentrations of coumarin compounds as well as standard compound (Ascorbic acid) were taken and the volume was made uniformly to 150 µl using methanol. Each of the samples was then further diluted with methanol up to 3ml and to each 150 µl DPPH was added. Absorbance was taken after 15 min. at 517nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. The IC<sub>50</sub> values for each drug compounds as well as standard preparation were calculated. The DPPH free radical scavenging activity was calculated using the following formula:

$$\% \text{ scavenging} = [\text{Absorbance of control} - \text{Absorbance of test}$$

$$\text{sample}/\text{Absorbance of control}] \times 100$$

The effective concentration of sample required to scavenge DPPH radical by 50% (IC<sub>50</sub> value) was obtained by linear regression analysis of dose-response curve plotting between %inhibition and concentrations [12].

#### Super oxide free radical scavenging activity

Super oxide is biologically important as it can form singlet oxygen and hydroxyl radical. Overproduction of super oxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. Super oxide anion are generated in PMS-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan.

100 µl of Riboflavin solution [20 µg], 200 µl EDTA solution [12mM], 200 µl methanol and 100 µl NBT (Nitro-blue tetrazolium) solution [0.1mg] were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer [50mM]. The absorbance of solution was measured at 590nm using phosphate buffer as blank after illumination for 5 min. This is taken as control. 50 µl of different concentrations of coumarin compounds as well as standard preparation were taken and diluted up to 100 µl with methanol. To each of these, 100 µl Riboflavin, 200 µl EDTA, 200 µl methanol and 100 µl NBT was mixed in test tubes and further diluted up to 3 ml with phosphate buffer. Absorbance was measured after illumination for 5 min. at 590 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. The IC<sub>50</sub> value for each compound as well as standard preparation were calculated. [5-6, 8, 13-14, 15-18]

$$\% \text{ scavenging}/\text{Inhibition} = [\text{Absorbance of control} - \text{Absorbance of test}$$

$$\text{sample}/\text{Absorbance of control}] \times 100$$

#### 5. NITRIC OXIDE FREE RADICAL SCAVENGING ACTIVITY

Nitric oxide (NO<sup>•</sup>) has also been involved in a variety of biological functions, including neurotransmission, vascular homeostasis, antimicrobial, and antitumor activities. Despite the possible beneficial effects of NO<sup>•</sup>, its contribution to oxidative damage is also reported. This is due to the fact that NO<sup>•</sup> can react with superoxide to form the peroxyxynitrite anion, which is a potential oxidant that can decompose to produce OH<sup>•</sup> and NO<sup>•</sup>. The procedure is based on the principle that, sodium nitro-prusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Large amounts of NO<sup>•</sup> may lead to tissue damage.

50 µl of each of the concentrations of coumarin compounds previously dissolved in DMSO, as well as ascorbic acid (standard compound) were taken in separate tubes and the volume was uniformly made up to 150 µl with methanol. To each tube 2.0 ml of sodium nitroprusside (10 mM) in phosphate buffer saline was added. The solutions were incubated at room temperature for 150 minutes. The similar procedure was repeated with methanol as blank which served as control. After the incubation, 5 ml of griess reagent was added to each tube including control. The absorbance of chromophore formed was measured at 546 nm on UV-visible spectrometer Shimadzu, UV-1601, Japan. Ascorbic acid was used as positive control. The IC<sub>50</sub> value for each test compounds as well as standard preparation were calculated [6-8, 19-23].

$$\% \text{ scavenging}/\text{Reduction} = [\text{Absorbance of control} - \text{Absorbance of test}$$

$$\text{sample}/\text{Absorbance of control}] \times 100$$

#### 6. RESULTS AND DISCUSSION:

##### DPPH free radical scavenging activity

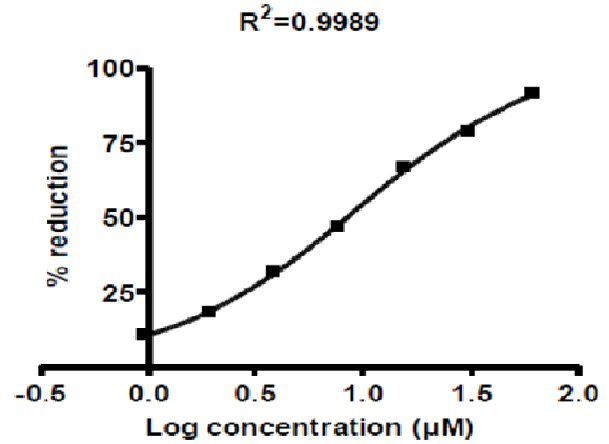
One of the quick methods to evaluate antioxidant activity is the scavenging activity on DPPH, a stable free radical and widely used index. In the DPPH Free radical scavenging activity, three coumarin compounds I, II and III were evaluated for their free radical scavenging activity with ascorbic acid as standard compound. The IC<sub>50</sub> was calculated for each coumarin compounds as well as ascorbic acid as standard and summarized in table 1 and graphically represented in figure 3-6. The scavenging effect increased with the increasing concentrations of test compounds. The IC<sub>50</sub> value for coumarin compounds were 799.83 µM, 712.85

$\mu\text{M}$  and  $872.97 \mu\text{M}$  for compounds I, II and III respectively which were comparatively lower than the  $\text{IC}_{50}$  ( $829.85 \mu\text{M}$ ) of ascorbic acid except compound III. From the results of DPPH, It showed that all three coumarin compounds are equally effective as antioxidant compared to ascorbic acid.

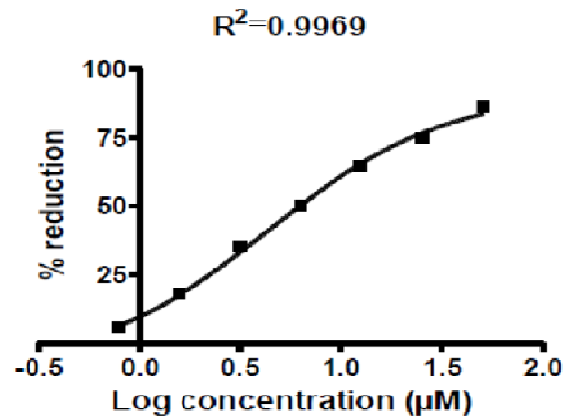
**Table 1. DPPH free radical scavenging activity of coumarin compounds**

Compound no.	Conc. ( $\mu\text{M}$ )	Log Conc. ( $\mu\text{M}$ )	% Reduction	$\text{IC}_{50}$ ( $\mu\text{M}$ )
	61.6595	1.790	91.58	
	30.8319	1.489	78.86	
	15.4170	1.188	66.7	
	77.090	0.887	46.87	
	38.548	0.586	31.89	
	19.275	0.285	18.09	
<b>I</b>				8.172
	0.9638	-0.0160	10.61	
	50.8159	1.706	86.09	
	0.9638	-0.0160	10.61	
	50.8159	1.706	86.09	
	25.4097	1.405	74.6	
	12.7057	1.104	64.45	
	63.533	0.803	50.23	
	31.769	0.502	35.12	
	15.885	0.201	17.79	
<b>II</b>				6.975***
	0.7943	-0.100	5.78	
	567544	1.754	85.4	
	283792	1.453	76.06	
<b>III 8.728</b>				
	141906	1.152	61.76	
	70958	0.851	47.98	
	35481	0.550	29.09	
	17742	0.249	15.06	
	567544	1.754	85.4	
	283792	1.453	78.06	
<b>Ascorbic acid (standard)</b>				8.21
	141906	1.152	64.76	
	70958	0.851	47.98	
	35481	0.550	30.08	
	17742	0.249	16.06	

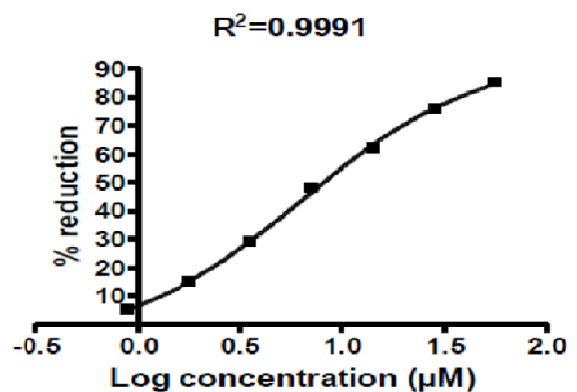
Table 1 showed the  $\text{IC}_{50}$  values of coumarin compounds compared with ascorbic acid, \*\*\* $p < 0.001$  compared with  $\text{IC}_{50}$  value of ascorbic acid



**Fig. 1 Log concentration ( $\mu\text{M}/\text{ml}$ ) vs % reduction for compound I by DPPH free radical scavenging assay method**



**Fig. 2 Log concentration ( $\mu\text{M}/\text{ml}$ ) Vs % reduction for compound II by DPPH free radical scavenging assay method**



**Fig. 3 Log concentration ( $\mu\text{M}/\text{ml}$ ) Vs % reduction for compound III by DPPH free radical scavenging assay method**

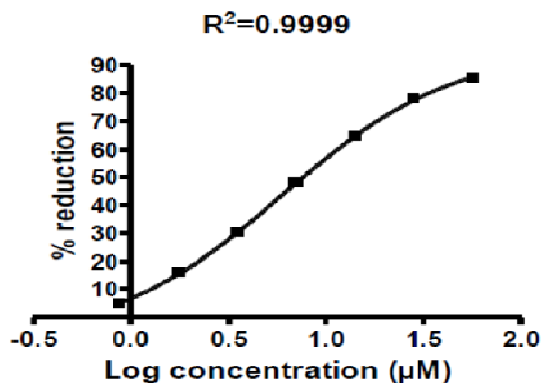


Fig. 4 Log concentration ( $\mu\text{M/ml}$ ) Vs % reduction for ascorbic acid by DPPH free radical scavenging assay method

Coumarins recognized as possessing potent anti oxidant activity are also strong scavengers of DPPH. DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine. The solution therefore loses colour stoichiometrically depending on the number of electrons taken up. Substances capable of donating electrons/hydrogen atoms are able to convert DPPH (Purple) into their nonradical form 1, 1-diphenyl-2-picrylhydrazine (Yellow), a reaction which can be followed spectrophotometrically. Free radical scavenging activity of the coumarin compounds is concentration dependent, as the concentration of the test compounds increases, the radical scavenging activity increases and lower  $\text{IC}_{50}$  value reflects better protective action. From results, it may be postulated that coumarin compounds I, II and III were able to reduce the stable free radical DPPH to the yellow-colored 1,1-diphenylpicrylhydrazine exhibiting better free radical scavenging activity than the standard antioxidant Ascorbic acid. Structure activity relationship study showed that the anti oxidant activity of these coumarin derivatives could be attributed to electron donating nature of the substituents like  $-\text{OH}$ ,  $-\text{CH}_3$  and  $-\text{Cl}$  on coumarin scaffold, reduce free radical DPPH and prevent the damage of cell. The more hydrogen donors, the stronger is the anti oxidant activity. These anti oxidants should display anti oxidant activity if one or more the groups like  $-\text{OH}$ ,  $-\text{CH}_3$  are free, since they are known to be good hydrogen donors [24, 25].

## 7. SUPER OXIDE FREE RADICAL SCAVENGING ACTIVITY

Super oxide free radical scavenging activity was performed with three coumarin compounds and ascorbic acid (standard compound). The  $\text{IC}_{50}$  was measured for each compounds and standard compound. The  $\text{IC}_{50}$  for Compound I, II and III were  $641.21\mu\text{M}$ ,  $722.77\mu\text{M}$  and  $2079.69\mu\text{M}$  respectively and for ascorbic acid, it was  $2051.16\mu\text{M}$ . Compounds I and II have

potent anti oxidant activity than compound III comparable to ascorbic acid at low  $\text{IC}_{50}$ . Results are summarized in table 2 and graphically represented in figure 7 to 10. From the graph, it was observed that as concentration increases, the % scavenging is increasing linearly for all three compounds and ascorbic acid (standard preparation), revealed by the regression analysis.

*In-vitro* super oxide radical scavenging activity is measured by riboflavin/light/NBT (Nitro blue tetrazolium) reduction. The method is based on generation of super oxide radical by auto oxidation of riboflavin in presence of light. Super oxide is biologically important as it can form singlet oxygen and hydroxyl radical. Overproduction of super oxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. The super oxide radical reduces NBT to a blue colored formazan that can be measured at 560 nm. From results, it was found that the compounds I, II and III showed potent free radical scavenging activity compared to the ascorbic acid (standard) at low  $\text{IC}_{50}$ . These coumarin compounds are electron donors due to the presence of electron donating substituents groups like  $-\text{OH}$ ,  $-\text{Cl}$  and  $-\text{CH}_3$  at different positions of coumarin scaffold. This compound donated their electrons to the superoxide and scavenges them to prevent their further interaction with NBT followed by inhibition of formation of blue colored formazan product [26].

## 8. NITRIC OXIDE SCAVENGING ACTIVITY

Nitric oxide scavenging activity was performed with three coumarin compounds and ascorbic acid as standard compound and the reductive potential of all three compounds and standard preparations exhibited dose dependent activity. The  $\text{IC}_{50}$  was calculated for coumarin compounds found more significant than ascorbic acid. The  $\text{IC}_{50}$  of compounds I, II and III were 743.02, 716.14 and 648.63 respectively which were found lower than  $\text{IC}_{50}$  of ascorbic acid ( $3083.18\mu\text{M}$ ). Results are tabulated in table 3 and graphically represented in figures. From the graph, it was observed that as concentration increases, the % scavenging is increasing linearly for all three compounds and ascorbic acid (standard preparation), which revealed by the regression analysis. From results, it confirmed that the compound III showed potent anti oxidant activity than I and II. All three coumarin compounds showed good anti oxidant activity than ascorbic acid.

Table 2. Super oxide free radical scavenging activity for

Table 2. Super oxide free radical scavenging activity for coumarin compounds and ascorbic acid

Compound no.	Conc. (µM)	Log conc. (µM)	% Reduction	IC <sub>50</sub> (µM)
I	61.6595	1.790	84.28	6.227*
	30.8319	1.489	76.65	
	15.4170	1.188	66.76	
	7.7090	0.887	55.65	
	3.8548	0.586	40.67	
	1.9275	0.285	30.45	
	0.9638	-0.0160	16.67	
II	5081.59	1.706	81.61	7.21***
	2540.97	1.405	74.43	
	1270.57	1.104	62.18	
	635.33	0.803	47.82	
	317.69	0.502	34.62	
	158.85	0.201	21.43	
	79.43	-0.100	12.25	
III	56.7544	1.754	63.38	21.01
	28.3792	1.453	54.67	
	14.1906	1.152	43.93	
	70.958	0.851	34.79	
	35.481	0.550	28.5	
	17.742	0.249	18.69	
	0.8851	-0.053	8.86	
Ascorbic acid (standard)	56.7544	1.754	64.38	20.72
	28.3792	1.453	54.29	
	14.1906	1.152	42.68	
	70.958	0.851	35.80	
	35.481	0.550	29.5	
	17.742	0.249	20.54	

Table 2 represents the IC<sub>50</sub> value found for various coumarin compounds by super oxide free radical scavenging activity, \*\*\*p < 0.001 compared with IC<sub>50</sub> value of ascorbic acid

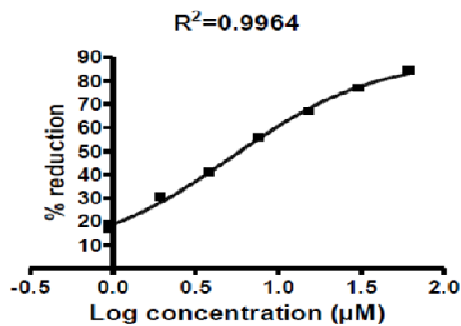


Fig. 5 Log concentration (µM/ml) vs % reduction for compound I by super oxide free radical scavenging assay method

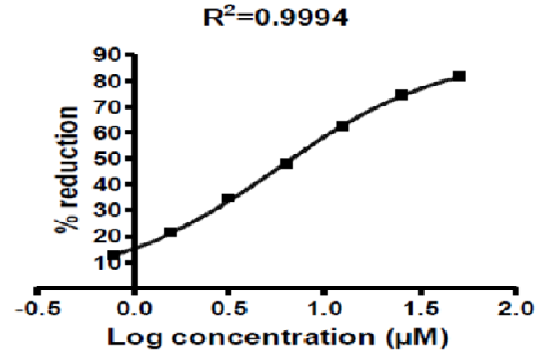


Fig. 6 Log concentration (µM/ml) vs % reduction for compound II by super oxide free radical scavenging assay method

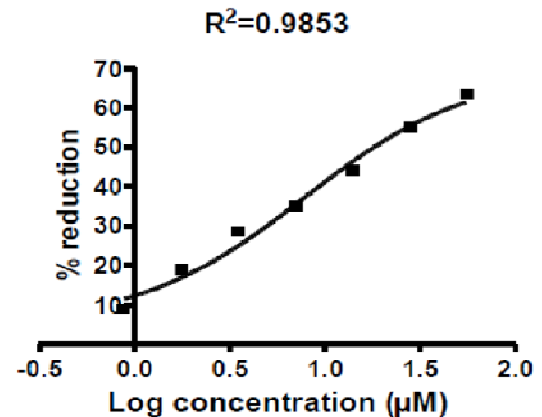


Fig. 7 Log concentration (µM/ml) vs % reduction for compound III by super oxide free radical scavenging assay method

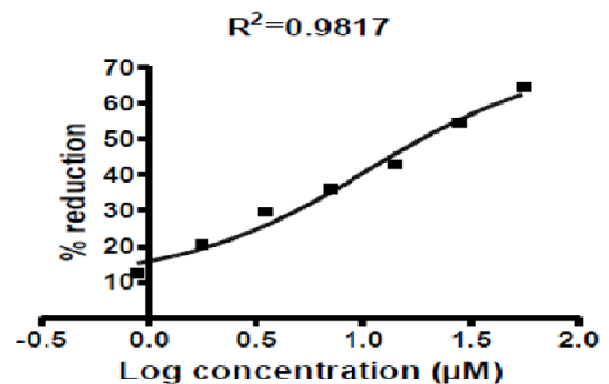


Fig. 8 Log concentration (µM/ml) vs % reduction for ascorbic acid by super oxide free radical scavenging assay method

Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Excess concentration of nitric oxide is implicated in the cytotoxic

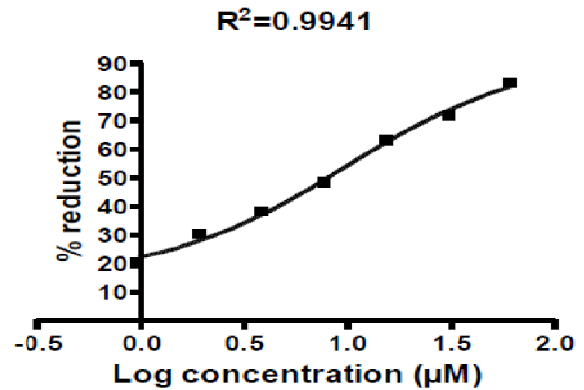


effects observed in various disorders like AIDS, cancer, alzheimer’s and arthritis. Oxygen reacts with the excess NO to generate nitrite and peroxy nitrite anions, which act as free radicals. From results of Nitric oxide method, it proved that coumarin compounds I, II and III are equally effective as anti oxidant compared to the ascorbic acid. These compounds compete with oxygen to react with NO and thus inhibit the generation of the nitrite and peroxy nitrite anions. This attribute of these compounds may be due to the electron donating nature of the substituents groups like –OH at the 4<sup>th</sup> position (compound I), –CL and –OH at the 5<sup>th</sup> and 7<sup>th</sup> position(compound II), –CH<sub>3</sub> at 7<sup>th</sup> position(compound III) of 1,2-benzopyrone nucleus [24, 27].

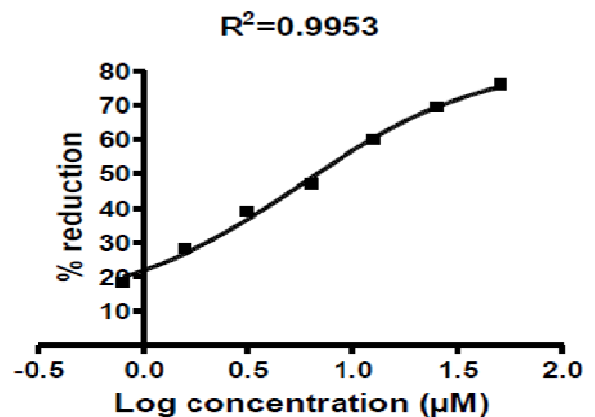
**Table 3. Nitric oxide free radical scavenging activity for coumarin compounds**

Compound no. (µM)	Concentration	Log conc. (µM)	% Reduction	IC <sub>50</sub> (µM)
I	61.6595	1.790	83.04	743.02
	30.8319	1.489	71.52	
	15.4170	1.188	62.96	
	77.090	0.887	48.16	
II	19.275	0.285	30.24	716.14
	0.9638	-0.0160	20.04	
	50.8159	1.706	76.08	
	25.4097	1.405	69.64	
	12.7057	1.104	60.04	
	6.3533	0.803	47.06	
	3.1769	0.502	38.88	
	1.5885	0.201	28.24	
	0.7943	-0.100	18.4	
	III	56.7544	1.754	
28.3792		1.453	75.84	
14.1906		1.152	65.2	
70.958		0.851	49.68	
35.481		0.550	38.02	
17.742		0.249	28.92	
0.8851	-0.053	18.8		
Ascorbic acid (standard)	56.7544	1.754	62.64	3083.18
	28.3792	1.453	45.44	
	14.1906	1.152	36.08	
	70.958	0.851	32.32	
	35.481	0.550	26.67	
	17.742	0.249	18.86	

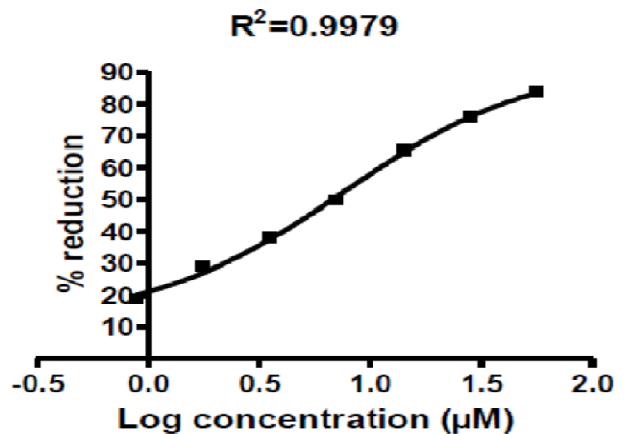
Table 3 represents the IC<sub>50</sub> value found for various coumarin compounds by nitric oxide free radical scavenging activity,\*\*\*p <0.001 compared with IC50 value of ascorbic acid



**Fig. 9 Log concentration (µM/ml) Vs % Reduction for compound I by nitric oxide free radical scavenging assay method**



**Fig. 10 Log concentration (µM/ml) Vs % Reduction for compound II by nitric oxide free radical scavenging assay method**



**Fig. 11 Log concentration (µM/ml) Vs % Reduction for compound III by nitric oxide free radical scavenging assay method**

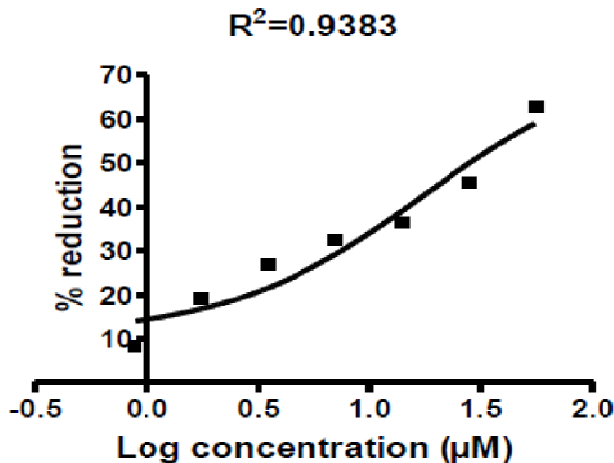


Fig. 12 Log Concentration ( $\mu\text{M/ml}$ ) Vs % Reduction for ascorbic acid by Nitric oxide free radical scavenging assay method

Table 4. Inhibitory concentrations ( $\text{IC}_{50}$   $\mu\text{M/ml}$ ) of procured coumarin compounds by DPPH, super oxide and nitric oxide method

Compound no.	Inhibitory concentration ( $\text{IC}_{50}$ $\mu\text{M/ml}$ )		
	DPPH	SO	NO
I	8.172 $\pm$ 0.1123	6.227 $\pm$ 0.1223***	7.496 $\pm$ 0.1449***
II	6.975 $\pm$ 0.76***	7.21 $\pm$ 0.987***	7.143 $\pm$ 0.1138***
III	8.72 $\pm$ 0.123	21.01 $\pm$ 0.2123	6.475 $\pm$ 0.997***
Ascorbic acid (standard)	8.21 $\pm$ 0.1002	20.72 $\pm$ 0.1135	30.01 $\pm$ 0.3376

Table 4 showed  $\text{IC}_{50}$  of coumarin compounds compared with ascorbic acid, \*\*\* $P < 0.001$  vs. ascorbic acid

## 9. CONCLUSION

In vitro antioxidant activity was carried out with 4-hydroxy-, 5-chloro-4-hydroxy and 7hydroxy-4-methyl coumarin derivatives by DPPH free radical scavenging method, Super oxide method and Nitric oxide method. The  $\text{IC}_{50}$  value was determined for each compound. From results of DPPH, Super oxide and Nitric oxide methods, it found that compound I and II displayed strong antioxidant activity compared to the ascorbic acid and it suggested that these compounds could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases. Compound III is also showed good anti oxidant activity.

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