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IN VIVO AND *IN VITRO* ANTIOXIDANT ACTIVITIES OF COUMARIN ON CHEMICAL INDUCED HYPERGLYCEMIC RATS

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ABSTRACT

The present study was aimed at investigating the antioxidant activities of coumarin in streptozotocin (STZ)-nicotinamide (NA) induced type 2 diabetic rats. Diabetes was induced in experimental rats by a single intraperitoneal (i.p.) injection of STZ (45 mg/kg body weight (b.w.)), 15 min after the i.p. administration of NA (110 mg/kg b.w.). Diabetic rats were administered coumarin intragastrically at 100 mg/kg b.w. for 45 days. The antioxidant activities of coumarin have been evaluated by using a range of *in vivo* assays and *in vitro* assays. In case of *in vivo* studies the levels of plasma thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (HP) and conjugated dienes (CD) were measured. The levels of vitamin C, vitamin E and reduced glutathione (GSH) in the plasma and tissues were assayed. In case of *in vitro* assays 2, 2′-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$), hydroxyl radical, superoxide anion scavenging activity and reducing power were measured. In addition to that ascorbic acid and butylated hydroxyl toluene were used as the reference antioxidant radical scavenger compounds. Among the different concentration (10, 20, 30, 40, 50 µM), 50 µM of coumarin had significantly effective compared to other concentration in all *in vitro* assay. These findings suggest that coumarin possess *in vivo* and *in vitro* antioxidant activity as an effective free radical scavenger augmenting its therapeutic value.

INTRODUCTION: Diabetes mellitus is a serious complex chronic condition that is a major source of ill health worldwide. Large numbers of people around the world are suffering from diabetes especially over the past two decades, from 30 million to 230 million [1]. Oxidation and production of free radicals are an integral part of normal cell metabolism. A free radical is defined as any atom or molecule possessing unpaired electrons. Free radicals such as $O^{-}2$ (superoxide anion), ^{-}OH (hydroxyl radical) and $^{1}O_2$ (singlet oxygen) are formed as a part of the normal metabolic process.

Free radicals can cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans [2]. Thus, free radicals result in the consumption of antioxidant defenses which may lead to disruption of cellular functions and oxidative damage to membranes and enhance susceptibility to lipid peroxidation [3].

Reactive oxygen species (ROS) such as superoxide anions ($O2.^{-}$), hydroxyl radical ($OH^{\cdot}$) and nitric oxide (NO) inactivate enzymes and damage important cellular components causing injury through covalent binding and lipid peroxidation [4]. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by other mechanisms and thus prevent disease [5].

Antioxidants may act as free radical scavengers, reducing agents, chelating agents for transition metals, quenchers of singlet oxygen molecules and/or activators of antioxidative defense enzyme systems to suppress the radical damages in biological systems [6, 7]. The evaluation of the antioxidant properties of specific chemical scavengers is of particular value for their potential use in preventing or limiting the damage induced by free radicals.

Several methods are used to measure the antioxidant activity of a biological material. The most commonly used for their ease, speed and sensitivity are those involving chromogen compounds of a radical nature to stimulate RONS. The presence of the antioxidant leads to the disappearance of these radical chromogens, the two most widely used being the $ABTS^{+\cdot}$ and the DPPH radicals. DPPH is a free radical that is acquired directly without preparation while $ABTS^{+\cdot}$ must be generated by enzymatic or chemical reactions [8].

A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases. There are some synthetic antioxidant compounds such as butylated hydroxytoluene, butylated hydroxyanisole tertiary butylhydroquinone which are commonly used in processed foods. However, it has been suggested that these compounds have shown toxic effects like liver damage mutagenesis.

Many phenolic compounds have been found to be effective antioxidants in biological systems [9]. Plant phenolics are multifunctional and can act as reducing agents (free radical terminators), metal chelators and singlet oxygen quenchers. Studies have shown that consumption of foods and beverages rich in phenolic content is correlated with reduced incidence of heart disease [10]. The most common plant phenolic antioxidants include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols and polyfunctional organic acids [11].

Coumarin (1,2- benzopyrone), is a naturally occurring compound being present in tonka beans, cassia, lavender, yellow sweet clover, fruits (e.g. bilberry, cloudberry) green tea and chicory [12]. Biological effects of coumarin include anti-bacterial, antiviral, antimutagenic, antioxidant, lipoxygenase and polyoxygenase inhibition, lipid

peroxidation inhibition and scavenge superoxide hydroxyl radicals [13, 14]. Various studies have demonstrated that coumarin is a potential antioxidant and its antioxidant activity is due to its ability to scavenge free radicals and to chelate metal ions [15].

In our previous studies, we have reported the effect of coumarin on hepatic key enzymes of glucose metabolism in the liver of diabetic rats [16] and also its protective effect of glycoprotein components in streptozotocin-nicotinamide induced hyperglycemic rats [17]. This study was aimed to investigate the antioxidant and radical scavenging properties of coumarin with different analytical methodology. The antioxidant potential of coumarin was explained on the basis of *in vivo* and *in vitro* methods.

MATERIALS AND METHODS:

Chemicals: Streptozotocin, 2, 2′-dipyridyl 2, 4-dinitro phenyl hydrazine (DNPH), 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB), 2, 2′-diphenyl-1-picrylhydrazyl radical (DPPH), 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS), butylated hydroxytoulene (BHT) and coumarin were obtained from Sigma Aldrich Fine Chemicals (St. Louis, MO, USA). The entire chemicals were of analytical grade and were obtained from Hi Media and SD fine chemicals, Mumbai, India.

In vivo antioxidant activity:

Animals: Male Wistar-albino rats weighing 200–220 g body weight were procured from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and were maintained in an air conditioned room (25±1°C) with a 12 h light/12 h dark cycle. Feed and water were provided *ad libitum* to all the animals. The experimental protocol was approved by the Institutional Ethical committee of Annamalai University (Reg.No. 160/1999/CPCSEA; Vide No. 565, 2008). The animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India.

Induction of type 2 diabetes: Non-insulin dependent diabetes mellitus was induced in overnight fasted rats by a single i.p. injection of 45 mg/kg STZ, 15min after the i.p. administration of 110mg/kg NA [18]. Streptozotocin was dissolved in citrate buffer (0.1 M, pH 4.5) and nicotinamide was dissolved in normal physiological saline. Hyperglycemia was confirmed by the elevated levels of glucose in plasma, determined at 72h. The blood glucose levels above 250 mg/dl were considered to be diabetic and were used in the experiment.

Experimental design: The rats were divided into four groups comprising of six animals in each group. The groups were treated as follows:

Group 1:	Normal control (vehicle treated)
Group 2:	Normal rats received coumarin (100 mg/kg b.w.) dissolved in corn oil using intragastric tube for 45 days [16]
Group 3:	Diabetic control
Group 4:	Diabetic rats received coumarin (100 mg/kg b.w.) dissolved in corn oil using intragastric tube for 45 days [16]

At the end of 45 days, the animals were deprived of food overnight and sacrificed by decapitation. Plasma was separated for the estimation of lipid peroxides and non enzymic antioxidants. For the determinations of vitamin E level in the liver and kidney, tissues were weighed and lipids were extracted by the method of Folch *et al*. [19] using chloroform- methanol mixture ($CHCl_3$: MeOH) (2:1; v/v). For the estimation of non-enzymic

antioxidants, tissues were minced and homogenized (10%w/v) in 0.1M phosphate buffer (pH 7.0) and centrifuged for 10 min and resulting supernatant was used for antioxidant assays.

Estimation of lipid peroxidation: Lipid peroxidation byproducts of TBARS and HP in plasma (0.5 ml) were estimated colorimetrically using the methods of Fraga *et al*. [20] and Jiang *et al*. [21], respectively. In brief, 0.1 ml of plasma was treated with 2 ml of TCA- trichloroacetic acid- HCl reagent (0.37%TBA, 0.25M HCl and 15%TCA, 1:1:1 ratio), placed for 15 min in a water bath and then cooled and centrifuged at 3500 ×g for 10 min at room temperature, the absorbance of clear supernatant was measured at 535 nm against a reference blank.

Plasma (0.5ml) was treated with 0.9ml of Fox reagent (88 mg of butylated hydroxy toluene (BHT), 7.6mg of xylenol orange and 0.8mg of ammonium iron sulphate were added to 90ml of methanol and 10ml of 250 mM sulphuric acid and incubated at 37°C for 30 min. Then the absorbance was read at 560nm.

The levels of CD were assessed by the method of Rao and Racknagel [22]. In brief, 1.0ml of plasma, 5.0ml chloroform- methanol reagent (2:1v/v) was added, mixed thoroughly and centrifuged for 5 minutes. To this, 1.5ml of cyclohexane was added and the absorbance was read at 233nm against a cyclohexane blank. The amount of CD formed was calculated using a molar extinction coefficient of 2.52x104 cm^{-1}. In plasma CD was measured the ratio of absorbance at 240 and 214nm.

Estimation of vitamin C: Vitamin C was estimated by the method of Omaye *et al* [23]. 0.5ml of plasma (or) tissue homogenate was mixed thoroughly with 1.5ml of 6% TCA and centrifuged for 10 min at 3500 ×g. After centrifugation, 0.5ml of the supernatant was mixed with 0.5ml of DNPH reagent and allowed to stand at room temperature for an additional 3 hrs, then added 2.5ml of 85% sulphuric acid and allowed to stand for 30 min. Then the absorbance was read at 530 nm. A set of standards containing 10-50µg of ascorbic acid were taken and processed similarly along with a blank. Ascorbic acid values were expressed as mg/dl in plasma and µM/mg in tissue.

Estimation of vitamin E (α-tocopherol): Vitamin E was determined by the method of Baker et al. [24]. To 0.1 ml of plasma (or) lipid extract, 1.5 ml of ethanol and 2 ml of petroleum ether were added, mixed and centrifuged at 3000 ×g for 10 min. The supernatant was evaporated to dryness at 80 °C then 0.2 ml of 2, 2−1-dipyridyl solution and 0.2 ml of ferric chloride solution was added and mixed well. This was kept in dark for 5 min and added 2 ml of butanol. Then the absorbance was read at 520 nm. Standards of α-tocopherol in the range of 10–100 µg were taken and treated similarly along with blank containing only the reagent. The values were expressed as mg/dl in plasma and µM/mg in tissue. Protein was determined by the method of Lowry et al. [25].

Estimation of reduced glutathione: GSH was determined by the method of Ellman [26]. A known weight of tissue was homogenized in phosphate buffer. From this 0.5ml was pipetted out and precipitated with 2ml of 5% TCA, then centrifuged at 3200 ×g for 20 min. 1 ml of plasma (or) supernatant was taken and added to it 0.5 ml of Ellman's reagent and 3ml of phosphate buffer (0.2M, pH 8.0). Then the absorbance was read at 412 nm. A series of standards were treated in a similar manner along with a blank containing 3.5 ml of buffer. The values were expressed as mg/dl in plasma and mg/100 g-tissue**.**

Estimation of tissue protein: Protein in the tissues was determined by the method of Lowry *et al*. [25]

using bovine serum albumin as the standard. Values were expressed as mg/g of tissue.

Statistical analysis: Values are given as mean±S.D. for six rats in each group. Data were analyzed by one-way analysis of variance followed by Duncan's Multiple Range Test (DMRT) using SPSS version 13.0 (SPSS, Chicago, USA). The limit of statistical significance was set at P<0.05 and the values sharing a common superscript did not differ significantly [27].

***In vitro* antioxidant activity:**

Free radical scavenging activity: The ability to scavenging the stable free radical, DPPH was measured as a decrease in absorbance at 517nm by the method of Mensor *et al* [28]. To a methanolic solution of DPPH (90.25 mmol), an equal volume of coumarin (10-50 µmol) was added and made up to 1.0mL with methanolic DPPH. An equal amount of methanol was added to the control. After 20 min, the absorbance was recorded at 517nm in a Systronics UV-visible Spectrophotometer. The inhibition of free radicals by DPPH in percentage terms (%) was calculated by using the following equation.

% = [(A control-A sample)/A blank] x 100

Where A control is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound.

Total Antioxidant activity assay: Total antioxidant potential of coumarin was determined by the ABTS assay, as described by Miller *et al* [29]. The reaction mixture contained ABTS (0.002 M), coumarin (10-50 µmol) and buffer in a total volume of 3.5mL. The absorbance was measured at 734nm in a Systronics UV- visible Spectrophotometer. The percentage of inhibition was calculated.

Super oxide anion scavenging activity: Superoxide anion scavenging activity of coumarin was determined by the method of Nishmiki *et al.,* [30] with modification. 1mL of NBT (100 µmol of NBT in 100mM phosphate buffer, pH 7.4), 1mL of NADH solution (14.68 µmol of NADH in 100 mmol phosphate buffer, pH 7.4) and varying volumes of coumarin (10-50 µmol) were mixed well. The reaction was started by the addition of 100 µmol of PMS (60 µmol/100 mmol of phosphate buffer pH 7.4). The reaction mixture was incubated at 30ºC for 15 min. The absorbance was measured at 560 nm in a spectrophotometer. Incubation without coumarin was used as blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging. The % of inhibition was calculated.

Hydroxyl radical-scavenging activity: The hydroxyl radical scavenging activity was determined by the method of Halliwell *et al* [31]. The following reagents were added in the order stated below. The incubation mixture in a total volume of 1mL contained 0.1mL of 100 mmol of potassium dihydrogen phosphate-KOH buffer, varying volumes of coumarin (10-50 µmol), 0.2mL of 500 mmol of ferric chloride, 0.1mL of 1 mmol of ascorbic acid, 0.1mL of 10 mmol of H_2O_2 and 0.2 mL of 2-deoxy ribose.

The contents were mixed thoroughly and incubated at room temperature for 60 min. Then 1 mL of 1% TBA (1 gm in 100 mL of 0.05 N NaOH) and 1 mL of 28% TCA were added. All the tubes were kept in a boiling water bath for 30 min. The absorbance was read in a spectrophotometer at 532 nm with reagent blank containing distilled water in a place of coumarin. The percentage scavenging activity was determined. Decreased absorbance of the reaction mixture indicated increased hydroxyl radical scavenging activity. **Reducing power:** The reducing power was determined according to the method of Oyaizu [32].

Different concentrations of coumarin (10-50 µmol) were prepared in methanol mixed with phosphate buffer (2.5mL, 0.2 M, pH 6.6) and potassium ferricyanide [K_3Fe (CN) (2.5mL, 1%). The mixture was incubated at 50^oC for 20 min and 2.5mL of TCA (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5mL) was mixed with distilled water (2.5mL) and $FeCl_3$ (0.5mL, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a standard.

RESULTS:

Effects of plasma lipid peroxidation: Table 1 shows the levels of TBARS, HP and CD in plasma of control and experimental rats. Diabetic rats had elevated levels of TBARS, HP and CD in the plasma, when compared with normal control rats. Rats treated with coumarin significantly decreased the lipid peroxidation markers in diabetic rats.

Effect of coumarin on plasma antioxidants: Table 2 depicts the changes in vitamin C, vitamin E and GSH in plasma of normal control and experimental animals. Vitamin C, vitamin E and GSH levels were significantly lower in diabetic rats than in normal control rats. In contrast diabetic rats treated with coumarin led to significant increase in the plasma antioxidant levels of vitamin C, vitamin E and GSH.

Effect of coumarin on non enzymic antioxidants: Table 3 shows the influence of coumarin on content of vitamin C, vitamin E, and GSH in liver and kidney of normal and experimental groups. There was a significant decrease in the levels of vitamin C, vitamin E and GSH in liver and kidney of diabetic control rats when compared with normal control rats. Oral administration of coumarin to diabetic rats led to a significant increase in the levels of vitamin C, vitamin E, and GSH when compared with diabetic control rats.

TABLE 1: CHANGES IN THE LEVELS OF TBARS, HP, CD IN THE PLASMA OF NORMAL CONTROL AND EXPERIMENTAL RATS

Groups	Normal control	Normal + Coumarin (100mg/kg)	Diabetic control	Diabetic + Coumarin (100mg/kg)
TBARS (mM/dl)	2.3 ± 0.19^a	2.42 ± 0.21^a	7.28 ± 0.57^b	3.1 ± 0.26^c
HP (10^{-5}mM/dl)	12.53 ± 0.93^a	11.95 ± 0.96^a	23.47 ± 1.54^b	14.72 ± 1.06^c
CD (mM/mg)	0.74 ± 0.06^a	0.69 ± 0.05^a	1.03 ± 0.13^b	0.85 ± 0.07^c

Values are given as mean ± S.D. from six rats in each group. Values not sharing a common letter (a-c) differ significantly at P<0.05 (DMRT)

TABLE 2: CHANGES IN LEVELS OF VIT. C, VIT. E AND GSH IN THE PLASMA OF NORMAL CONTROL AND EXPERIMENTAL RATS

Groups	Normal control	Normal + Coumarin (100mg/kg)	Diabetic control	Diabetic + Coumarin (100mg/kg)
Vitamin - C (mg/dl)	1.62 ± 0.13^a	1.64 ± 0.13^a	0.59 ± 0.05^b	1.48 ± 0.12^c
Vitamin - E (mg/dl)	1.45 ± 0.13^a	1.49 ± 0.13^a	0.53 ± 0.04 ^{b}	1.26 ± 0.10^c
GSH (mg/dl)	23.76 ± 1.37^a	24.53 ± 1.40^a	11.34 ± 0.83^b	19.48 ± 1.26^c

Values are given as mean ± S.D. from six rats in each group. Values not sharing a common letter (a-c) differ significantly at P<0.05 (DMRT)

TABLE 3: INFLUENCE OF COUMARIN ON CONTENT OF VIT. C, VIT. E AND GSH IN EXPERIMENTAL RATS LIVER AND KIDNEY

Groups	Normal control	Normal + Coumarin (100mg/kg)	Diabetic control	Diabetic + Coumarin (100mg/kg)
Vit. C (µM/mg of tissue)				
Liver	1.36 ± 0.06^a	1.34 ± 0.07^a	0.73 ± 0.04^b	1.24 ± 0.06^c
Kidney	1.18 ± 0.05^a	1.17 ± 0.05^a	0.46 ± 0.02^b	0.85 ± 0.03^c
Vit. E (µM/mg of tissue)				
Liver	0.62 ± 0.03^a	0.65 ± 0.03^a	0.27 ± 0.01^b	0.54 ± 0.02^c
Kidney	0.39 ± 0.02^a	0.41 ± 0.02^a	0.14 ± 0.01^b	0.32 ± 0.02^c
GSH (mg/100 g of tissue)				
Liver	39.43 ± 3.55^a	43.17 ± 4.23^a	18.52 ± 1.92^b	33.82 ± 3.47^c
Kidney	31.77 ± 3.13^a	32.48 ± 3.25^a	16.65 ± 1.26^b	27.37 ± 2.32^c

Values are mean ± SD for six rats in each group. Values not sharing a common superscript letter differ significantly at p< 0.05 (DMRT)

***In vitro* antioxidant activity:** Several concentrations ranging from 10-50 µm/mL of the coumarin was tested for their antioxidant activity in different in vitro models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner in all the models.

Table 4 shows the percentage scavenging action of coumarin on free radical generation. Coumarin scavenges DPPH radical in a dose-dependent manner (10-50 µM). The DPPH radical scavenging activity was detected and compared with ascorbic acid. However, the highest percentage (37.68%) scavenging activity of coumarin was observed at 50 µM.

TABLE 4: DPPH-FREE RADICAL SCAVENGING ASSAY

Concentration (µg/ml)	Ascorbic acid (%)	Coumarin (%)
10	10.84	7.72
20	19.4	13.24
30	34.92	22.24
40	43.52	29.77
50	52.92	37.68

Table 5 shows the total antioxidant activity was measured using the ABTS assay. Inhibition of the ABTS radical showed dose-dependent (10-50 µM) scavenging activity. The percentage scavenging activity of coumarin increases with increasing concentration. However, the highest percentage (31.72%) scavenging activity was observed at 50 µM and compared with butylated hydroxytoulene.

TABLE 5: ABTS- TOTAL ANTIOXIDANT SCAVENGING ASSAY

Concentration (µg/ml)	Butylated Hydroxytoulene (%)	Coumarin (%)
10	8.99	6.34
20	17.46	13.16
30	23.62	16.92
40	33.74	22.36
50	42.71	31.72

Table 6 shows the percentage in vitro scavenging effects of coumarin on superoxide radical. Coumarin scavenges the above mentioned radicals *in vitro* in a dose-dependent manner. The percentage scavenging activity of coumarin increases with increasing concentration. The highest percentage (31.52%) scavenging activity was observed at 50 µM and compared with ascorbic acid.

TABLE 6: SUPEROXIDE RADICAL SCAVENGING ASSAY

Concentration (µg/ml)	Ascorbic acid (%)	Coumarin (%)
10	9.67	6.15
20	17.24	11.43
30	28.92	17.96
40	37.13	25.75
50	44.78	31.52

Table 7 shows the percentage in vitro scavenging effects of coumarin on hydroxyl radical. Coumarin scavenges the above mentioned radicals in vitro in a dose-dependent manner. The percentage scavenging activity of coumarin increases with increasing concentration. The highest percentage (43.66%) scavenging activity was observed at 50 µM and compared with ascorbic acid.

TABLE 7: HYDROXYL RADICAL SCAVENGING ASSAY

Concentration (µg/ml)	Ascorbic acid (%)	Coumarin (%)
10	12.35	9.85
20	23.42	17.6
30	37.24	26.05
40	47.53	35.21
50	59.87	43.66

Table 8 shows the reducing power of coumarin and the reference compound, ascorbic acid increased steadily with increasing concentration. Increased absorbance with the increased concentrations of the reaction mixture indicated the increased reducing power. However, the highest (0.034) scavenging activity was observed at 50 µM.

TABLE 8: REDUCING POWER

Concentration	Ascorbic acid	Coumarin (µg/ml)
	Absorbance at 700 nm	
10	0.01	0.007
20	0.018	0.013
30	0.028	0.023
40	0.034	0.027
50	0.046	0.034

DISCUSSION: The effective antioxidants from natural sources are the only alternatives to synthetic antioxidants in counteracting the free radicals mediated diseases. Recently, various phytochemicals and their effect on health, especially the suppression of free radicals have been studied. There is increasing evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural antioxidants contained in spices, herbs and medicinal plants [33].

The plasma lipid peroxide levels in STZ - induced diabetes is generally thought to be due to pathological changes to tissues that increase the production and liberation of lipid peroxides in to the circulation [34]. Coumarin has been reported to inhibit lipoxygenase activity, lipid peroxidation and scavenges superoxide and hydroxyl radicals [35]. Treatment with coumarin brought back lipid peroxidative markers to near normal levels, which could be as a result of improved glycemic control and antioxidants status.

The non-enzymic antioxidants such as vitamin C, vitamin E and reduced glutathione play a vital role in quenching of hyperglycemia mediated free radicals. GSH, being the most important biomolecule against chemically induced toxicity can participate peroxides in the presence of GPx. GSH also functions as free radical scavenger and in the repair of free radical caused biological damage [36]. Reduced glutathione, a direct free radical scavenger, also reported to protect the cellular system against the noxious effects of lipid peroxidation [37].

Moreover, reduced glutathione acts as a co-substrate for the activity of glutathione peroxidase and as a cofactor for many enzymes [38]. The decreased levels of plasma GSH in diabetes could be due to its increased utilization in trapping the oxyradicals [34]. In our study, diabetic rats exhibited a decreased level of GSH, which might be due to increased utilization for scavenging free radicals and increased consumption by GPx and GST. Treatment with coumarin can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH and detoxifies the free radicals generated. The decrease of GSH may hence be responsible for low GPx activity in diabetic tissues. It has been proposed that antioxidants that maintain the concentration of GSH may restore the cellular defense mechanisms, block lipid peroxidation and thus protect the tissue against oxidative damage [39].

Vitamin C is a hydrophilic antioxidant in plasma because it disappears faster than other antioxidant when plasma is exposed to reactive oxygen species [40]. A significant decrease in the level of plasma vitamin C in diabetic rats could be due to the increased utilization of vitamin C as an antioxidant defense against ROS or to decrease in the GSH level, as GSH is required for the recycling of vitamin C [41], if vitamin C is present, vitamin E levels are preserved because of vitamin C regenerates vitamin E from its oxidised form.

Vitamin E is a membrane stabilizer [42] that interrupts the chain reaction of lipid peroxidation by reacting with lipid peroxy radicals, thus protecting the cell structure against damage [43]. Vitamin E supplementation in streptozotocin-induced diabetic rats resulted in a decrease of plasma lipid peroxidation in comparison with untreated animals, associated with higher cell glutathione content and SOD activity, thus suggesting that vitamin E supplementation could in part reduce the imbalance between oxidants and antioxidants [44]. Oral administration of coumarin to diabetic rats restored the level of vitamin E to near normal levels. Both the vitamin C and vitamin E significantly decreased in the liver and kidney of

diabetic rats. Administration of coumarin to diabetic rats increased the levels of vitamin C and vitamin E.

The DPPH radical was widely used as the model system to investigate the scavenging activities on several natural compounds such as phenolic and anthocyanins. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm.

Radical scavenging activity increased with increasing percentage of the free radical inhibition. DPPH is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. These radicals react with suitable reducing agents, the electrons become paired off and the solution loses colour depending on the number of electrons taken up [45]. Results indicated definite scavenging activity of the coumarin towards DPPH radicals in comparison with ascorbic acid.

The decolorization of $ABTS^{\bullet+}$ cation radical is an unambiguous way to measure the antioxidant activity of phenolic compounds. The ability of a compound to scavenge $ABTS^{\bullet+}$ radical can demonstrate oxygen radical absorbance capacity. Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734nm which decreases with the scavenging of the proton radicals [46]. The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$, which involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. It is a decolourization assay, thus the radical cation is performed prior to addition of antioxidant test system, rather than the generation of the radical taking place continually in the presence of antioxidant.

Superoxide dismutase is an antioxidant enzyme that neutralizes the free radicals in the cell; it dismutates superoxide anion (O^{-}_{2}), into H_2O_2 and protects the cells from damage by cleaning up O^{-}_{2}. The level of SOD activity represents the intracellular antioxidation ability. Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated [2]. Superoxides are produced from molecular oxygen due to oxidative enzyme of body as well as via non-enzymatic reaction such as autooxidation by catecholamines. In the present study, superoxide radical reduces NBT to a blue colored formazan that is measured at 560 nm.

Hydroxyl radical is the most reactive free radical and it can be formed from superoxide anion and hydrogen peroxide in the presence of metal–ions, such as copper or iron. When a hydroxyl radical reacts with aromatic compounds, it can attach across a double bond, resulting in a hydroxyl cyclohexadienyl radical. The highly reactive ·OH can cause oxidative damage to DNA, lipids and proteins [47]. As is the case for many other free radicals, ·OH can be neutralized if it is provided with a hydrogen atom. The sample exhibited hydroxyl radical scavenging activity in a dose dependent manner in the range of 10-50 µm/mL in the reaction mixture (Figure 3). Overall, the scavenging activities of phenolic substances might be due to the active hydrogen donating ability of hydroxyl substitutions.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the antioxidant activity of antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention

of continued hydrogen abstraction, reductive capacity and radical scavenging antioxidant activity.

CONCLUSION: According to data obtained from the present study, coumarin was found to be an effective antioxidant in different *in vivo* and *in vitro* assay including DPPH, ABTS radical, superoxide anion radical, hydroxyl radical scavenging and reducing power. Further understand of the mechanism of coumarin antioxidant action may ultimately be useful in the design of drugs that improve hyperglycemia.

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