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ANTIOXIDANT EFFECT OF NARINGIN ON NICKEL-INDUCED TOXICITY IN RATS: AN *IN VIVO* AND *IN VITRO* STUDY

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ABSTRACT

The aim of the study was to evaluate the circulating antioxidant such as vitamin C, vitamin E and GSH in nickel (Ni) induced toxicity in rats and in vitro free radical scavenging assay. In this investigation nickel sulfate (20 mg/kg body weight (b.w.) was administered intraperitoneally (i.p.) for 20 days to induce toxicity. Naringin was administered orally (80 mg/kg body weight) for 20 days with i.p. injection of nickel sulfate. The toxic effect of nickel was indicated by significantly decreased activities of non-enzymatic antioxidants like reduced glutathione, vitamin C and vitamin E. Treatment with naringin exhibited a significant ($P < 0.05$) increase in Ni-induced rats. The free radical scavenging properties of naringin were investigated with different *in vitro* methods such as 2, 2'- diphenyl- 1-picrylhydrazyl radical ($DPPH^{\bullet}$), 2, 2'- azinobis (3-ethylbenzo thiazoline- 6- sulfonic acid) radical ($ABTS^{\bullet+}$), hydroxyl radical, superoxide anion scavenging activity and reducing power. In addition to that ascorbic acid, butylated hydroxyl toluene was used as the reference antioxidant radical scavenger compounds. Among the different concentration, 500 µM of naringin had significantly effective compared to other concentration in all *in vitro* assay. Based on these findings naringin possess potent *in vivo* and *in vitro* antioxidant activity and also effective free radical scavenger, augmenting its therapeutic value.

INTRODUCTION: Free radicals have been implicated in the causation of several diseases such as including asthma, cancer, cardiovascular disease, cataract, diabetes, gastrointestinal inflammatory disease, liver disease, muscular degeneration and other inflammatory processes [1] and compounds that can scavenge free radicals have great potential in ameliorating these disease processes. Reactive oxygen species (ROS) are continuously produced during cell metabolism. Under normal conditions, they are scavenged and converted to nonreactive species by different intracellular enzymatic and non-enzymatic antioxidant system [2].

Overproduction or an ineffective elimination of ROS may induce oxidative stress and cause damage to all types of biomolecules, such as proteins, lipids and nucleic acids [3]. A certain amount of oxidative damage takes place even under normal conditions; however, the rate of this damage increases during aging and other pathological events, as the efficiency of antioxidative and repair mechanism decreases, leading to the condition of oxidative stress [4]. Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species [5, 6].

Plants containing flavonoids have been reported to possess strong antioxidant properties [7, 8]. 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 [9, 10]. Most of the antioxidants commercially in use (e.g., butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are synthetic and some of them have been suspected of causing or promoting negative health effects; therefore, some restrictions are placed on their applications and there is a trend to substitute them with naturally occurring antioxidants [11,12].

So, attention has been drawn to the health promoting activity of plant foods and its active components. Flavonoids are a group of polyphenolic compounds diverse in chemical structure and characteristics. They are widely distributed in foods of plant origin such as vegetables, fruits, tea and wine [13]. Scavenging of free radicals seems to play a considerable part in the antioxidant activity of flavonoid compounds. Naringin is a flavonone present in grape fruit and related citrus. Currently, there is much interest in the usefulness of citrus fruits because of their intake appears to be associated with reduced risk of certain chronic diseases and increased survival [14]. Naringin has been reported to have several pharmacological properties such as antimicrobial, antimutagenic, anticancer, anti-inflammatory, cholesterol lowering, free radical scavenging and antioxidant effects [15, 16]. The objective of the present study was to investigate the antioxidant activity of the naringin using different *in vivo* and *in vitro* models.

MATERIALS AND METHODS:

Chemicals: Naringin, nickel sulfate, reduced glutathione (GSH), 2, 2′-dipyridyl, 2, 4-dinitro phenylhydrazine (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) and butylated hydroxytoulene were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from S.D. Fine chemicals Mumbai, India and were of analytical grade.

Animals: Male albino rats of Wistar strain with a body weight ranging from 200 to 210 g, were procured from 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 study was approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg. No.160/1999/CPCSEA, Proposal number: 644), Annamalai University, Annamalainagar.

Experimental design: The animals were randomly divided into four groups of six rats in each group.

Group 1	Control rats treated i.p. with isotonic saline for 20 days.
Group 2	Normal rats received naringin (80mg/kg body weight) dissolved in water and administered orally using an intragastric tube for 20 days.
Group 3	Rats received Ni as nickel sulfate (20mg/kg body weight) i.p. in isotonic saline for 20 days.
Group 4	Rats received Ni i.p. (20mg/kg b.w.) with oral administration of naringin 80 mg/kg body weight) for 20 days

At the end of the experimental period, animals in different groups were sacrificed by cervical decapitation. Blood samples were collected in heparinised tubes, for plasma. Plasma separated by centrifugation was used for various biochemical estimations.

Biochemical assays:

Determination of plasma non-enzymatic antioxidants: Ascorbic acid (vitamin C) concentration was measured by Omaye *et al,* [17]. To 0.5 mL of plasma, 1.5 mL of 6% TCA was added and centrifuged (3500 ×g, 20 min). To 0.5 mL of supernatant, 0.5 mL of DNPH reagent (2% DNPH and 4% thiourea in 9N sulfuric acid) was added and incubated for 3 h at room temperature. After incubation, 2.5 mL of 85% sulfuric acid was added and colour developed was read at 530 nm after 30 min.

Vitamin E was estimated by the method of Desai [18]. Vitamin E was extracted from plasma by addition of 1.6 mL ethanol and 2.0 mL petroleum ether to 0.5 mL plasma and centrifuged. The supernatant was separated and evaporated on air. To the residue, 0.2 mL of 0.2% 2, 2-dipyridyl, 0.2 mL of 0.5% ferric chloride was added and kept in dark for 5 min. An intense red colored layer obtained on addition of 4 mL butanol was read at 520 nm. Reduced glutathione (GSH) was determined by the method of Ellman [19]. 1 mL of supernatant was treated with 0.5 mL of Ellman's reagent (19.8 mg of 5, 5-dithiobisnitro benzoic acid in 100 mL of 0.1% sodium citrate) and 3.0 mL of phosphate buffer (0.2 M, pH 8.0) was added and the absorbance was read at 412 nm in spectrophotometer.

Free radical scavenging activity: The ability to scavenging the stable free radical, DPPH was measured as a decrease in absorbance at 517 nm by the method of Mensor *et al,* [20]. To a methanolic solution of DPPH (90.25 mmol), an equal volume of naringin (100-500 µmol) dissolved in distilled water was added and made up to 1.0 mL with methanolic DPPH. An equal amount of methanol was added to the control. After 20 min, the absorbance was recorded at 517 nm in a Systronics UV-visible Spectrophotometer.

Total antioxidant activity assay: Total antioxidant potential of naringin was determined by the ABTS assay, as described by Miller et al. [21]. The reaction mixture contained ABTS (0.002 M), naringin (100-500 µmol) and buffer in a total volume of 3.5 mL. The absorbance was measured at 734 nm in a Systronics UV- visible Spectrophotometer.

Superoxide anion scavenging activity: Superoxide anion scavenging activity of naringin was determined by the method of Nishmiki *et al,* [22] with modification. 1 mL of NBT (100 µmol of NBT in 100mM phosphate buffer, pH 7.4), 1 mL of NADH solution (14.68 µmol of NADH in 100 mmol phosphate buffer, pH 7.4) and varying volumes of naringin (100-500 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 naringin was used as blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging.

Hydroxyl radical scavenging assay: The hydroxyl radical scavenging activity was determined by the method of Halliwell *et al*, [23]. The following reagents were added in the order stated below. The incubation mixture in a total volume of 1 mL contained 0.1 mL of 100 mmol of potassium dihydrogen phosphate-KOH buffer, varying volumes of naringin (100-500 µmol), 0.2 mL of 500 mmol of ferric chloride, 0.1 mL 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 naringin. 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 [24]. Different concentrations of naringin (100-500 µmol) were prepared in methanol mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K_3Fe (CN) (2.5 mL, 1%). The mixture was incubated at 50^oC for 20 min and 2.5 mL of TCA (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min.

The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 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: The concentration of non-enzymatic antioxidants (vitamin C, vitamin E and GSH) in plasma of normal and experimental rats is depicted in **Table 1**. In nickel sulfate treated rats, the nonenzymatic antioxidants were found to be significantly ($P < 0.05$) decreased when compared to control rats. Toxic rats when treated with naringin lead to a significant ($P < 0.05$) increase in nonenzymatic antioxidants.

TABLE 1: CHANGES IN THE ACTIVITIES OF VITAMIN- C, VITAMIN- E AND REDUCED GLUTATHIONE (GSH) IN PLASMA OF CONTROL AND EXPERIMENTAL RATS

Parameters	Control	Naringin (80 mg/kg)	Nickel sulfate (20 mg/kg)	Nickel sulfate + Naringin (80 mg/kg)
Vitamin- C (mg/dl)	1.76 ± 0.09[a]	1.87 ± 0.12[a]	1.49 ± 0.07[b]	1.64 ± 0.08[c]
Vitamin- E (mg/dl)	1.31 ± 0.08[a]	1.42 ± 0.13[a]	0.74 ± 0.06 [b]	1.17 ± 0.11[c]
GSH (mg/dl)	19.74 ± 1.32[a]	20.26 ± 1.71[a]	14.89 ±1.32[b]	17.78 ± 1.11[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 shows the percentage scavenging action of naringin on free radical generation. Naringin scavenges DPPH radical in a dose-dependent manner (100-500 µM). The DPPH radical scavenging activity was detected and compared with ascorbic acid. However, the highest percentage (39.2%) scavenging activity of naringin was observed at 500 µM.

TABLE 2: DPPH-FREE RADICAL SCAVENGING ASSAY

Concentration (µg/ml)	Ascorbic acid (%)	Naringin (%)
100	12.4	8.5
200	21.5	16.3
300	27.6	23.7
400	34.5	29.4
500	45.4	39.2

Table 3 shows the total antioxidant activity was measured using the ABTS assay. Inhibition of the ABTS radical showed dose-dependent (100-500 µM) scavenging activity. The percentage scavenging activity of naringin increases with increasing concentration. However, the highest percentage (32.5%) scavenging activity was observed at 500 µM and compared with butylated hydroxytoulene.

TABLE 3: ABTS- TOTAL ANTIOXIDANT SCAVENGING ASSAY

Concentration (µg/ml)	Ascorbic acid (%)	Naringin (%)
100	12.5	8.3
200	17.5	12.7
300	25.3	21.6
400	33.6	29.4
500	36.2	32.5

Table 4 shows the percentage in vitro scavenging effects of naringin on superoxide radical. As seen in table 4, naringin scavenges the above mentioned radicals in vitro in a dose-dependent manner. The percentage scavenging activity of naringin increases with increasing concentration. The highest percentage (73.8%) scavenging activity was observed at 500 µM and compared with ascorbic acid.

TABLE 4: SUPEROXIDE RADICAL SCAVENGING ASSAY

Concentration (µg/ml)	Ascorbic acid (%)	Naringin (%)
100	20.2	15.5
200	34.3	30.4
300	49.2	44.6
400	65.7	59.4
500	77.6	73.8

Table 5 shows the percentage *in vitro* scavenging effects of naringin on hydroxyl radical. Naringin scavenges the above mentioned radicals in vitro in a dose-dependent manner. The percentage scavenging activity of naringin increases with increasing concentration. The highest percentage (38.3%) scavenging activity was observed at 500 µM and compared with ascorbic acid.

TABLE 5: HYDROXYL RADICAL SCAVENGING ASSAY

Concentration (µg/ml)	Ascorbic acid (%)	Naringin (%)
100	12.5	8.7
200	21.4	16.2
300	28.7	23.4
400	37.2	33.5
500	45.5	38.3

Table 6 shows the reducing power of naringin and the reference compound, ascorbic acid increased steadily with increasing concentration. The reducing powers of naringin increases with increasing concentration. However, the highest percentage (0.046%) scavenging activity was observed at 500 µM.

TABLE 6: REDUCING POWER

Concentration (µg/ml)	Ascorbic acid	Naringin
	Absorbance at 700 nm	
100	0.015	0.009
200	0.024	0.017
300	0.034	0.03
400	0.044	0.037
500	0.056	0.046

DISCUSSION: Free radicals and other reactive species are thought to play an important role in many human diseases. Establishing their precise role requires the ability to measure them and the oxidative damage that they cause [25]. Vitamin C is a primary antioxidant, water-soluble vitamin that can directly scavenge singlet oxygen, superoxide and hydroxyl radicals. Numerous reports have shown the positive effect of vitamin C as an antioxidant and scavenge of free radicals [26]. Vitamin E (α-tocopherol) is a well-known

antioxidant: it acts as a free radical scavenger, more specifically within cell membranes by preventing the oxidation of polyunsaturated lipids by free radicals such as the hydroxyl radical. The antioxidant potential of vitamin E is no longer disputed. Most in vivo studies have shown that vitamin E improves various parameters of oxidative stress in both animals [27] and humans [28].

GSH is a tripeptide (L-γ-glutamylcysteinyl glycine), an antioxidant and a powerful nucleophile, critical for cellular protection such as detoxification of ROS, conjugation and excretion of toxic molecules and control of inflammatory cytokine cascade [29]. Depletion of GSH in tissues leads to impairment of the cellular defense against ROS and may result in peroxidative injury. In our study we observed decreased concentration of vitamins C, E and GSH in plasma in Ni-induced rats. Near normal levels of these antioxidants were observed in naringin administrated rats. It might be due to excellent antioxidant activity of naringin with mechanism involving both free radical scavenging and metal chelation [30].

Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. In this study, free radical scavenging activity of naringin was determined using $DPPH^{\bullet}$ method. Research reports have shown that $DPPH^{\bullet}$ is widely used to evaluate the free radical scavenging effects of various antioxidant substances and polyhydroxy aromatic compounds [31]. $DPPH^{\bullet}$ is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical $DPPH^{\bullet}$. $DPPH^{\bullet}$ radical reacts with suitable reducing agent. The electrons become paired off and solution loses color stoichiometrically depending on the number of electrons taken up. In this study, naringin in vitro scavenges $DPPH^{\bullet}$ in dose dependently. The highest percentage scavenging effect of naringin on $DPPH^{\bullet}$ at the concentration of 500 µM was 39.2%.

The ABTS assay is based on the inhibition of the absorbance of the radical cation ABTS + which has a characteristic long wavelength absorption spectrum [32]. The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. It is an excellent tool for determining the antioxidant activity of hydrogen donating antioxidants and of chain breaking antioxidants. The total antioxidant activity was measured using the ABTS assay. Inhibition of the ABTS radical shows dose-dependent scavenging activity. The highest percentage scavenging effect of naringin on ABTS at the concentration of 500 µM was 32.5%.

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals [33]. From the investigations on the superoxide radical scavenging capacities, it was found that the naringin inhibits superoxide radicals in a dose dependent manner. In the PMS/NADH-NBT system, superoxide anions derived from dissolved oxygen by PMS/NADH coupling reaction reduce NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anions in the reaction mixture. In our study we have used different concentration of naringin (100-500 µM). The highest percentage scavenging effect of naringin on superoxide at the concentration of 500 µM was 73.8%.

Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of deoxyribose by the free radicals generated by the Fenton reaction. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which

produces a pink chromogen with thiobarbituric acid. The scavenging activity of naringin was showed dose-dependent (100-500 µM) scavenging activity. However, the highest percentage scavenging activity was observed at 500 µM and the percentage was 38.3%.

The reducing power of the compound may serve as a significant indicator of its potential antioxidant activity [34]. The reducing power (absorbance at 700 nm) of naringin was 0.046% at a dose of 500 µM showing that naringin can act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reactions. The reducing power of naringin might be due to the hydroxyl substitutions in the aromatic ring.

Thus, the present findings clearly demonstrated that naringin was an effective antioxidant in various *in vivo* studies like vitamin C, vitamin E and GSH in vitro antioxidant assays including $DPPH^{\bullet}$, total antioxidant activity by ABTS, superoxide anion radical scavenging, hydroxyl radical scavenging and reducing power.

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