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Original Research

Silymarin and naringenin protects nicotine induced oxidative stress in young rats.

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Key Words
Flavonoids; Naringenin; Nicotine toxicity; Oxidative stress; Silymarin

Abstract
Protective efficacy of silymarin, a flavonoids complex known as 'milk thistle' and extracted from the fruit of *Silybum marianum* (L.) Gaertn. (*Carduus marianus* L., Asteraceae), and narigenin, a citrus bioflavanoid commonly found in citrus fruits, were studied against nicotine induced hepatic oxidative stress in young rats. Recent studies have shown that silymarin and naringenin possess antioxidant and free radical scavenging properties. Male wistar rats were injected with nicotine hydrogen tartarate (0.75mg/kg, intraperitoneally) and simultaneously given oral treatment with silymarin and naringenin (50 mg/kg each) for 21days to evaluate hepatic damage and antioxidant potential. Our results demonstrate a significant decrease in hepatic glutathione peroxidase (GPx) and glutathione reductase (GR) activities and an increase in glutathione S-transferase (GST) and thiobarbituric acid reactive substances (TBARS) levels on nicotine exposure. A similar trend was observed in blood and brain biochemical variables. Co-supplementation of silymarin or naringenin with nicotine was beneficial in the recovery of altered TBARS levels and GPx, GR and GST activity. Our results thus indicate the antioxidant potential of these flavonoids, which might be of benefit in the clinical recovery of subject exposed to nicotine. We thus recommend that diet rich in flavonoids may be of help in protecting nicotine-induced cytotoxicity.

INTRODUCTION

Nicotine is a natural alkaloid obtained from the leaves and stems of the *Nicotiana tabacum* and *Nicotiana rustica*. Nicotine content of cigarette varies among different brands of cigarette. Despite the measures to discourage smoking, it has grown more rapidly in the recent past. Nicotine enters the body when inhaled during smoking and orally through ingested tobacco products which gets rapidly absorbed into the circulatory system. Nicotine which is absorbed through the lungs gets rapidly metabolized in the liver [1], leading to major adverse effects on the liver, immune and oncogenic system [2-4]. Smoking also causes genotoxic effect on rat liver [5] and a direct genotoxicity in human target cells *in vitro* [6]. The deleterious toxic effects of nicotine are, at least in part, due to increased production of free radicals and reactive oxygen species (ROS) [7]. The increased generation of ROS by nicotine can produce a condition of oxidative stress which has been suggested to play a major role in the pathogenesis of several smoking-related diseases such as cancer, cardiovascular and oral diseases [8-10].

Flavonoids are a group of natural products including flavones, flavanones, and isoflavones; several beneficial biological activities of flavonoids including antioxidant, antitumor, and anti-inflammation properties have been identified in several previous studies [11-13]. Reports suggest that the number of hydroxyl (-OH) substitutions is a critical factor in the reactive oxygen species (ROS) scavenging activity of flavonoids; flavonoids with more -OH groups exhibited more potent antioxidant activity [14, 15]. The antioxidant nature of flavonoids is defined mainly by the presence of a B-ring catechol group (dihydroxylated B-ring) capable of readily donating hydrogen (electron) to stabilize a radical species. The presence of 2,3 unsaturation in conjugation with a 4-oxo- function in the C-ring and the presence of functional groups capable of binding transition metal ions, such as iron and copper are also responsible for the antioxidant nature of the flavonoids (Fig.1)[16, 17].

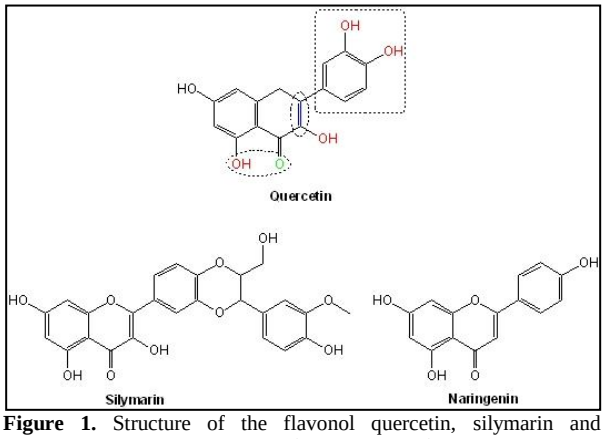

Figure 1. Structure of the flavonol quercetin, silymarin and naringenin showing important features in defining the classical antioxidant potential of flavonoids.

Silymarin is a polyphenolic flavonoid derived from milk thistle (*Silybum marianum*), which has anti-inflammatory, cytoprotective, and anticarcinogenic effects, that suppress the TNF-induced production of ROS and lipid peroxidation [18]. Silymarin has been shown to improve the antioxidant status in blood and liver [19]. Antioxidant potential of quercetin against nicotine induced oxidative stress in rat has already been demonstrated [20]. In another study the possible preventive role of vitamin E and *Hippophae rhamnoides* during nicotine exposure on parameters suggestive of oxidative stress has been explored in blood and tissues while *H.rhamnoides* was unable to provide protection to altered brain biochemical variables [21].

Naringenin is a natural flavonoid, aglycone of naringin and is widely distributed in citrus fruits, tomatoes, cherries, grapefruit and cocoa. Naringenin has also been extensively investigated for its pharmacological activities, including antitumor [22], anti-inflammatory [23], and hepatoprotective effects [24]. Structural similarity of silymarin and naringenin with quercetin (Fig.1) raised the possibility of its use as an antioxidant against nicotine toxicity.

We recently reported that chronic administration of nicotine results in the imbalance of prooxidant/antioxidant status in the circulation of experimental rats [25]. These harmful species are known to cause oxidative damage to a number of molecules in the cells, including membrane lipids, proteins and nucleic acids [25, 26]. The potential harmful effects of these species are controlled by the cellular antioxidant defense system, including enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants (reduced glutathione, Vitamins A, E, C, *etc*) [28]. Although antioxidant properties of silymarin and naringenin are well defined, there are no reports which suggest protective efficacy of these flavanoids against nicotine induced oxidative stress. Thus, the present study was planned to elucidate if concomitant administration of silymarin or naringenin with nicotine can ameliorate toxic effects of nicotine in terms of biochemical and hematological variables indicative of oxidative stress.

MATERIALS AND METHODS

Chemicals

Nicotine hydrogen tartarate (Sigma-Aldrich, St. Louis, MO, USA), and all other chemicals were of analytical grade or the highest purity available and were purchased from Merck (Darmstadt, Germany), BDH Chemical (Mumbai, India) or Sigma (St. Louis, MO, USA). Triple distilled water prepared by Millipore (New Delhi, India) was used through-out the experiment for the preparation of reagents and buffers used for various biochemical assays in our study.

Animals, experimental design and treatment

All animals were obtained from the Animal House Facility of Defence Research and Development Establishment (DRDE), Gwalior, India. The Animal Ethical Committee of DRDE approved the experimental protocol. All experiments were performed on male Wistar rats weighing 60 ± 10 g housed in stainless steel cages in an air conditioned room with temperature maintained at 25 ± 2^{o}C and 12 h alternating day and night cycles. Rats were allowed to feed on a standard rat chow diet (Ashirwad Feeds, Chandigarh, India; metal content of diet in ppm dry weight: Cu 10, Zn 45, Mn 55, Co 5, Fe 75) and water *ad libitum*,

Twenty rats were equally divided into four groups and treated as below for 3 weeks (21 days):

Group 1; normal drinking water

Group 2; nicotine as nicotine hydrogen tartarate (0.75 mg/kg, intraperitoneally)

Group 3; nicotine as in group 2 + silymarin (50 mg/kg, orally, once daily)

Group 4; nicotine as in group 2 + naringenin (50 mg/kg, orally, once daily)

After 21days of exposure animals were sacrificed under light ether anesthesia. Blood was collected and liver and brain were rinsed in cold saline, blotted, weighted and used for various biochemical variables as well as metal estimation.

Reactive oxygen species

Amount of ROS in blood was measured using 2′,7′-dichlorofluorescin diacetate (DCFDA) that gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci *et al* [29]. Briefly, whole blood was diluted to 1.5% with ice-cold 40 mM

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Tris-HCl buffer (pH 7.4) and placed on ice. 40 µl of 1.25 mM DCFDA in methanol was added for ROS estimation. All samples were incubated for 15 min in a water bath at 37°C after addition of DCFDA. Fluorescence was determined at 488 nm excitation and 525 nm emission wavelength using a fluorescence plate reader (Perkin Elmer LS-55, Waltham, MA, USA).

Blood glutathione (GSH)

Analysis of blood GSH concentration was performed by slightly modifying the method of Jollow *et al* [30]. 0.2 ml of whole blood was added to 1.8 ml of distilled water and incubated for 10 minutes at 37°C for complete hemolysis. After adding 3 ml of 4% sulphosalicylic acid, tubes were centrifuged at 2500 rpm for 15 minutes. To the supernatant 0.2 ml of 10 mM solution of 5,5'dithiobis(2 nitrobenzoic acid) (DTNB) was added in presence of phosphate buffer (0.1 M, pH 7.4). Absorbance recorded at 412 nm was used for calculation of GSH concentration.

Thiobarbituric acid reactive substances (TBARS)

Measurement of lipid peroxidation was done by the method described by Ohkawa *et al* [31] in blood and tissue. 0.1 ml of 5% RBC hemolysate was added to 0.2 ml of 8.1% SDS (w/v) and incubated for 10 min. Then 1.5ml of 20% acetic acid (pH 3.5) was added followed by adding 1.5 ml of 0.8% thiobarbituric acid (w/v) and 0.7 ml distilled water and incubation for 1 h in boiling water bath. One millilitre of distilled water was added to the solution after cooling and centrifuged at 6000 rpm for 15 min. The malondialdehyde formation was determined by reading absorbance at 535 nm.

Tissue lipid peroxidation was measured in whole-brain and liver homogenate [5% brain and 10% liver homogenate (w/v) in 150 mM KCl] for 30 min at 37°C. The incubation was interrupted by adding 1 ml of 10% trichloroacetic acid. After centrifugation (1 ml) supernatant was then mixed with 1 ml of 0.65% thiobarbituric acid. The mixture was then kept in a boiling water bath for 15 min. The amount of TBARS was calculated using a molar extinction coefficient of 1.56×10^5/M/cm. The absorbance of supernatant was read at 535 nm and the values were expressed as nmoles of MDA/ml blood or µg/gm tissue weight in tissue samples.

Tissue glutathione

Tissue GSH content was measured as described by Hissin and Hilf [32]. For this reason, 0.25 g of tissue sample was homogenized on ice with 3.75 ml of 0.1M phosphate-0.005M EDTA buffer (pH 8.0) and 1 ml of 25% HPO_3, which was used as a protein precipitant. The total homogenate was centrifuged at 100,000g for 30 min at 4°C.

For the GSH assay, 0.5 ml supernatant and 4.5 ml of phosphate buffer (pH 8.0) were mixed. The final assay mixture (2.0 ml) contained 100 µl of supernatant, 1.8 ml of phosphate-EDTA buffer and 100 µl *o*-phthaldehyde (OPT; 1,000 µL/ml in absolute methanol, prepared fresh). Samples were incubated at room temperature for 15 min and fluorescence was measured at 350 nm (Ex)/420 nm (Em) with Perkin-Elmer LS-55 (Waltham, MA, USA).

Glutathione peroxidase (GPx) and glutathione-S-transferase (GST)

Glutathione peroxidase was determined by the literature method Flohe and Gunzler [33] at 37ºC. Supernatant was obtained after centrifuging 5% liver homogenate at 1,500g for 10 min followed by 11,000 rpm for 30 min at 4°C was used for GPx assay. One ml of reaction mixture was prepared which contained 0.3 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml of GSH (2 mM), 0.1 ml of sodium azide (10 mM), 0.1 ml of H_2O_2 (1 mM) and 0.3 ml of liver homogenate. After incubation at 37°C for 15 min, reaction was terminated by addition of 0.5 ml of 5% TCA. Tubes were centrifuged at 1,500g for 5 min and supernatant was collected. 0.2 ml phosphate buffer (0.1 M, pH 7.4) and 0.7 ml of DTNB (0.4 mg/ml) was added to 0.1 ml of reaction supernatant. After mixing, absorbance was recorded at 420 nm. A molar extinction coefficient of 6.22×10^3 Mcm^{-1} was used to determine the activity.

GST activity was determined by the literature protocol of Habig *et al* [34]. The reaction mixture contained 0.02 ml of 1-chloro-2,4-dinitro benzene (1 mM) and 2.9 ml of GSH (0.3 mg GSH/ml in 0.2 M phosphate buffer, pH 7.4) and 30 µl of liver supernatant and change in color was monitored by recording absorbance (340 nm) at 30 s intervals for 3 min. The enzyme activity was expressed in nmole conjugate/min/mg protein.

Glutathione reductase (GR) activity

Glutathione reductase was determined by the literature method of Worthington *et a*l [35] at 37ºC. Supernatant was obtained after centrifuging 5% tissue homogenate at 1,500g for 10 min, followed by 10,000 rpm for 30 min at 4ºC was used for GR assay. To measure the GR activity, 60mM EDTA was mixed with 0.1 M phosphate buffer (pH 7.4), 2 mM NADPH and 60 mM glutathione disulfide (GSSG) was added, just before the enzymatic determination supernatant was added to start the reaction. The assay was run at 340 nm for 5 min with absorbance readings taken every 60 seconds.

Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and lactate dehydrogenase (LDH)

Serum glutamic oxaloacetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT) and lactate dehydrogenase (LDH) activities were measured in serum using Merck (Darmstadt, Germany) kits.

Statistical analysis

Experimental results are expressed as the mean ± SEM and are accompanied by a number of observations. Data are assessed by the method of one-way analysis of variance (ANOVA). If this analysis indicated significant difference among the group mean, then unexposed and exposed group (with or without treatment) was compared by Dunnett's multiple comparision test. Values with matching symbol notation in each column are not significant at 5% level of probability.

RESULTS

Effect on blood oxidative stress variables

Figure 2 shows the effect of silymarin and naringenin on blood ROS, GSH and TBARS levels during nicotine exposure. The blood ROS and TBARS levels increased significantly and GSH level decreased (not significant) following nicotine exposure. Administration of silymarin during nicotine exposure was beneficial in recovering both blood ROS and TBARS levels significantly.

Figure 3 shows the effect of silymarin and naringenin on blood GPx, GR and GST activity during nicotine exposure. The blood GPx, GR and GST activity decreased on nicotine exposure. Administration of silymarin and nicotine during nicotine exposure was beneficial in improving blood GR and GST activity significantly, while no effect was noted in GPx activity.

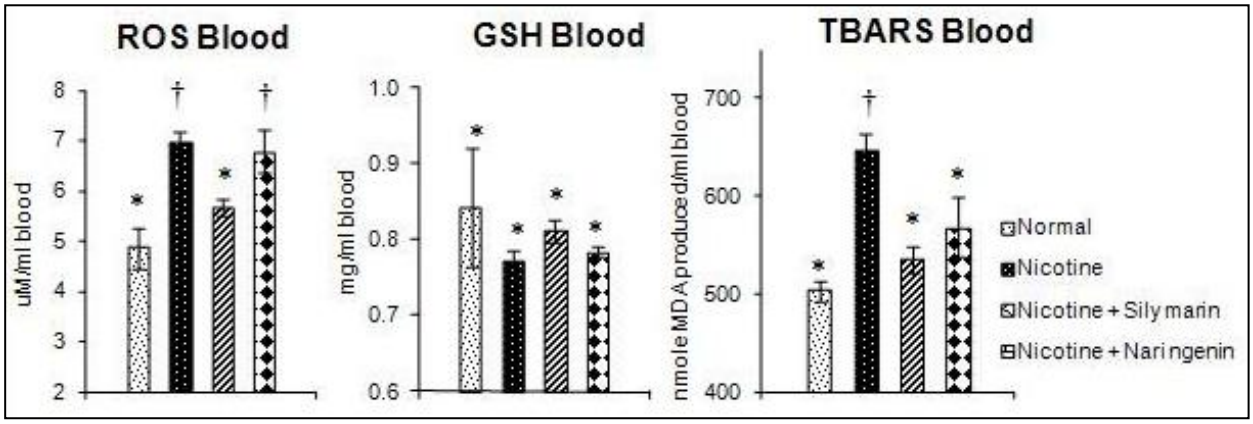

Figure 2. Nicotine-induced change in reactive oxygen species (ROS), reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) levels in blood and their response to administration of silymarin and naringenin in young rats. Values are mean ± SEM; n = 5; values with matching symbol notation (* or †) in each column are not significant at 5% level of significance.; nicotine exposed animals are compared to normal animals; treatment (silymarin or naringenin) animals are compared to nicotine exposed animals.

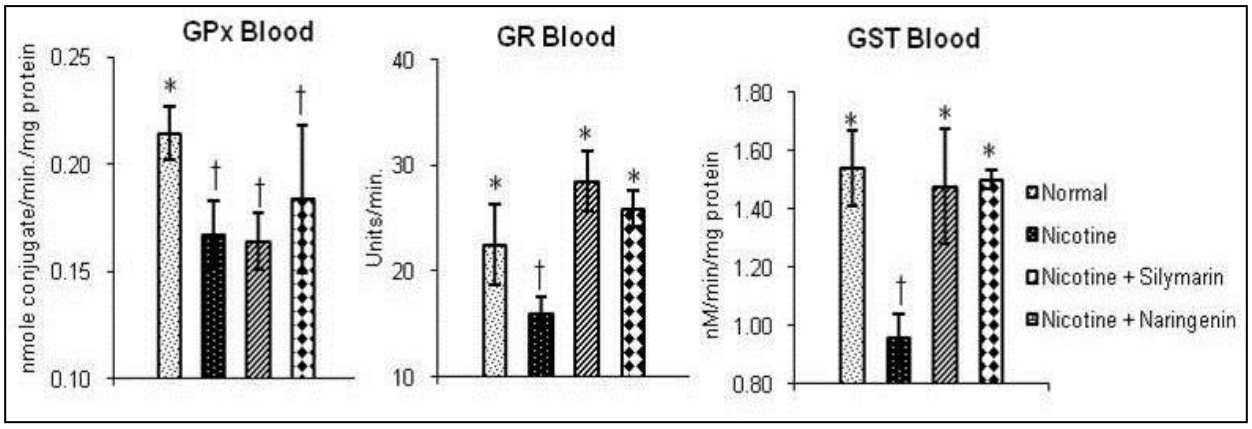

Fig. 3 Nicotine-induced change in glutathione peroxidise (GPx),glutathione reductase (GR) and glutathione-S-transferase (GST) activities in blood and their response to administration of silymarin and naringenin young rats. Values are mean ± SEM; n = 5; values with matching symbol notation (* or †) in each column are not significant at 5% level of significance.; nicotine exposed animals are compared to normal animals; treatment (silymarin or naringenin) animals are compared to nicotine exposed animals.

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Effect on liver biochemical variables and membrane damage enzyme

Table 1 shows the effect of nicotine on serum clinical variables of hepatic damage as SGOT and SGPT activity in exposed rats. Concomitant administration of silymarin and naringenin with nicotine provided significantly protection to these altered serum clinical variables.

Effect on hepatic oxidative stress and lipid peroxidation

Table 2 indicate toxic effects on some hepatic oxidative stress variables upon nicotine exposure and subsequent treatment. We found a significant increase in liver TBARS after nicotine exposure. Supplementation of the two flavanoids silymarin and naringenin resulted in a significant recovery in the lipid peroxidation.

Nicotine intoxication imposes deleterious effects on the glutathione dependent enzyme in liver as evidenced by a significant depletion of glutathione peroxidase and glutathione reductase activity suggesting impaired antioxidant defence system and the inhibited liver GPx and GR activity showed significant protection following treatment with silymarin and naringenin.

Interestingly, we observed a reverse trend as GST level showed an increase on nicotine exposure and a decline after the treatment with flavanoids. The increased GST activity following nicotine exposure suggests a counteracting mechanism adopted by system to metabolize nicotine. The reduced liver GST activity in silymarin and naringenin treated animals also signifies the reduced body burden of nicotine. The reason behind this unusual trend needs to be evaluated further.

Effect on brain oxidative stress and lipid peroxidation

Table 3 indicates toxic effects on some brain oxidative stress variables upon nicotine exposure and subsequent treatment. Nicotine intoxication imposes deleterious effects on the glutathione dependent enzyme in brain as evidenced by a significant depletion in glutathione peroxidase and glutathione S-transferase activity suggesting impaired antioxidant defence system and this inhibited brain GST activity showed significant protection following treatment with silymarin and naringenin.

Table 1. Effect of silymarin and naringenin administration on some clinical variables in young rats exposed to nicotine

	Normal	Nicotine	Nicotine + Silymarin	Nicotine + Naringenin
SGOT (U/l)	$47.1 \pm 5.8^{*}$	$76.3 \pm 7.9^{\dagger}$	$69.3 \pm 5.1^{\dagger}$	$45 \pm 5.9^{*}$
SGPT (U/l)	$20.7 \pm 1.8^{*}$	$30.2 \pm 1.7^{\dagger}$	$25.4 \pm 1.1^{*}$	$33.9 \pm 1.9^{\dagger}$
LDH (U/l)	$42.7 \pm 3.4^{*}$	$52.1 \pm 3.7^{\dagger}$	$52.4 \pm 6^{\dagger}$	$56 \pm 5.9^{\dagger}$

Values are mean ± SEM; n = 5; values with matching symbol notation (* or †) in each column are not significant at 5% level of significance.; nicotine exposed animals are compared to normal animals; treatment (silymarin or naringenin) animals are compared to nicotine exposed animals.

Table 2. Effect of silymarin and naringenin administration on some hepatic variables in young rats exposed to nicotine

	Normal	Nicotine	Nicotine + Silymarin	Nicotine + Naringenin
GSH	$4.4 \pm 0.15^{*}$	$4.29 \pm 0.13^{*}$	$3.80 \pm 0.11^{*}$	$4.46 \pm 0.22^{*}$
TBARS	$2.81 \pm 0.27^{*}$	$5.16 \pm 0.73^{\dagger}$	$2.66 \pm 0.2^{*}$	$3.25 \pm 0.41^{*}$
GPx	$0.246 \pm 0.011^{*}$	$0.187 \pm 0.016^{\dagger}$	$0.235 \pm 0.008^{*}$	$0.216 \pm 0.009^{*}$
GR	$20.9 \pm 0.75^{*}$	$16.1 \pm 0.91^{\dagger}$	$18.0 \pm 1.06^{*}$	$18.8 \pm 0.9^{*}$
GST	$9.04 \pm 0.28^{*}$	$10.2 \pm 0.28^{\dagger}$	$8.16 \pm 0.27^{*}$	$7.24 \pm 0.11^{\ddagger}$

Values are mean ± SEM; n = 5; values with matching symbol notation (* or †) in each column are not significant at 5% level of significance.; nicotine exposed animals are compared to normal animals; treatment (silymarin or naringenin) animals are compared to nicotine exposed animals. (GSH – mg/g; TBARS – µg/g; GPx – nmole conjugate produced/min/mg protein; GR – units/l; and GST – nmole/min./mg protein)

Table 3. Effect of silymarin and naringenin administration on some brain biochemical variables in young rats exposed to nicotine

	Normal	Nicotine	Nicotine + Silymarin	Nicotine + Naringenin
GSH	$6.59 \pm 0.21^{*}$	7.70 ± 0.338	$8.20 \pm 0.13^{*}$	$7.85 \pm 0.29^{*}$
TBARS	$4.75 \pm 0.45^{*}$	$5.47 \pm 0.3^{*}$	$5.2 \pm 0.3^{*}$	$3.87 \pm 0.37^{*}$
GPx	$0.124 \pm 0.004^{*}$	$0.105 \pm 0.001^{\dagger}$	$0.099 \pm 0.003^{\dagger}$	$0.107 \pm 0.001^{\dagger}$
GR	$14.9 \pm 0.85^{*}$	$11.4 \pm 0.84^{*}$	$12.1 \pm 0.74^{*}$	$12.4 \pm 0.39^{*}$
GST	$0.966 \pm 0.028^{*}$	$0.628 \pm 0.019^{\dagger}$	$0.944 \pm 0.036^{*}$	$1.009 \pm 0.029^{*}$

Values are mean ± SEM; n = 5; values with matching symbol notation (* or †) in each column are not significant at 5% level of significance.; nicotine exposed animals are compared to normal animals; treatment (silymarin or naringenin) animals are compared to nicotine exposed animals. (GSH – mg/g; TBARS – µg/g; GPx – nmole conjugate produced/min/mg protein; GR – units/l; and GST – nmole/min./mg protein)

DISCUSSION

The effect of nicotine on different organs has not been completely elucidated to date. It was illustrated that iminium metabolite of nicotine via electron transfer with redox cycling might be involved in the production of radical entities. Free radicals are widely involved in the intracellular processes low levels of free radicals appear to participate in the basic metabolic processes whereas high concentrations are associated with oxidative insult [36]. Wetscher *et al* [37] showed that nicotine is chemotactic for polymorphonuclear (PMN) leucocytes and enhances the responsiveness of PMN leucocytes to activate complement C5a, leading to the generation of oxygen free radicals. Thus, it can be assumed that nicotine exerts its toxic effects through generation of ROS [7]; it disrupts the mitochondrial respiratory chain leading to an increased generation of superoxide anions and hydrogen peroxide [38]. Superoxide anion and hydrogen peroxide are the major free radicals responsible for cellular damage [3]. Increased lipid peroxidation and conjugated dienes in liver and kidney are other characteristic observations in nicotine exposed rats [39], while being a potent carcinogen it also plays an important role in the pathogenesis of liver, lung and kidney [40]. Glutathione-related enzymes, such as GPx and GR function either directly or indirectly as antioxidant whereas GST plays an important role in metabolic detoxification. Considering this, it is expected that use of antioxidants provides a viable and novel option to the treatment of nicotine. The aim of the present investigation thus was to evaluate the efficacy of flavonoids, silymarin and naringenin in protecting oral nicotine-exposed oxidative damage to blood, brain and liver. Although, inhalation is the major route of nicotine exposure (cigarette smoke), there are recent report that cigarette smokers will switch to alternative oral nicotine delivery products to reduce their health risks [41].

Flavonoids are naturally occurring substances that exhibits various pharmacological and therapeutic applications [42, 44]. Due to their phenolic structures, they have antioxidant effect and also inhibit free radical-mediated processes. Hepatoprotective effects are achieved via several mechanisms like antioxidant effects [44-46] including inhibition of lipid peroxidation [47]; stimulation of ribosomal RNA polymerase and subsequent protein synthesis leading to enhanced hepatocyte regeneration [48]; enhanced liver detoxification via inhibition of phase I detoxification [49, 50], enhanced glucuronidation and protection from GSH depletion [51]. Silymarin may also show neuroprotection since besides being an antioxidant it penetrates the blood-brain barrier (BBB) to reach CNS [52]. Antioxidant, metal chelating and free radical scavenging properties of naringenin have also been reported earlier [53-56].

Our results show that nicotine exposure causes a significant increase in blood ROS levels suggesting that free radicals were involved in oxidative stress. Alteration in blood and tissue GSH level upon nicotine exposure was not significant. The treatment with silymarin was able to reverse the trend. However, naringenin is not able to reduce the increased ROS levels. This requires further exploration.

Lipid is considered as an index to monitor the function of membrane integrity, while TBARS (the end product of lipid peroxidation) is measured as the index of lipid peroxidation [57]. In the present investigation, there was a marked increase in blood and liver TBARS levels on nicotine exposure which was significantly reduced by silymarin and naringenin administration. The preventive effects of silymarin and naringenin have been related to the inhibition of lipid peroxides formation or free radicals scavenging property as evident from the decreased TBARS level.

Antioxidants enzymes which prevent biological macromolecules from oxidative injury and remove peroxides, free radicals and superoxide anion generated within the cell are considered to be the body's primary defense. Glutathione peroxidase is the enzyme that removes hydrogen peroxide generated by superoxide dismutase in cytosol and mitochondria by oxidizing GSH to GSSG [58]. On the other hand, GR is an enzyme that reduces GSSG to GSH, a sulfhydryl form which is an important cellular antioxidant to scavenge reactive oxygen species in cell. We observed a significant depletion of GPx and GR activities in blood, liver and brain following nicotine exposure. Nicotine induced depression in GPx activity in liver and kidney of exposed rats may be attributed to the perturbation in normal oxidative mechanism or enhanced utilization during detoxification of nicotine [39]. In the present study, administration of silymarin and naringenin significantly protected GPx activities by directly scavenging ROS as well as by inhibiting lipid peroxidation, suggesting antioxidant properties of flavonoids. Nicotine inhibited glutathione reductase activity in the liver, lungs, heart, stomach, kidney, and testicular tissues *in vivo* compared with the control group which may be due to competitive inhibition by binding the active site of GR [59]. It is also possible that this may lead to non-competitive inhibition by binding other sites affecting three dimensional structure of the enzyme [60]. The reason for the increased GR activity in the brain may be a compensatory mechanism to remove the increased reactive oxygen species due to nicotine administration in brain [21]. In our present study, we observed an unchanged brain GR activity on nicotine exposure. Glutathione-S-transferase is

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believed to participate in the detoxification process by catalyzing the conjugation of electrophilic xenobiotics to GSH, by binding various ligands covalently and non-covalently and by expressing GPx activity towards lipid hydroperoxides. Nicotine-induced decrease in brain GST activity in rats has been reported earlier and it was suggested that the decrease in activity may be due to an decrease in mRNA level encoding different GST isoenzymes [21]. In the present study, we also found a decreased GST activity in blood and brain of rats while a moderate increase in liver GST activity was observed which might be due to the extensive metabolism of nicotine in the liver. An increased liver GST has been reported earlier in the mice exposed to nicotine [61]. Co-supplementation of silymarin or naringenin along with nicotine restored the elevated activity of GST towards normal. The inhibitory effects of plant polyphenols (quercetin, purpurogallin, alizarin, and ellagic acid) on various GST isoenzymes have been shown to be very effective in reducing these effects [62]. Since the polyphenols are known to inhibit the GST activity, there may be a competition between the two for the active site of the enzyme.

Nicotine ingestion also resulted in the elevation of serum markers such as SGOT, SGPT and LDH which may be attributed to membrane damage [20]. Naringenin in particular was able to reverse these changes, however, the same was not observed with silymarin. Thus, it is possible that a dose higher than the one used in the present study may offer significant protection against the systemic damage induced by nicotine.

The results suggest ingestion of nicotine may lead to alteration in various biochemical variables, particularly suggestive of oxidative stress affecting blood and liver. However, supplementation of silymarin or naringenin might be beneficial in reducing some of these altered biochemical variables. Thus, these flavonoids may be easily incorporated into the diet or could be co-supplemented and thus may afford a protective effect against nicotine-induced cytotoxicity.

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REFERENCES

1. Hukkanen J, Jacob P, Benowitz NL. Metabolism and disposition kinetics of nicotine. Pharmacol Rev 2005; 57:79-115.

2. El-Zayadi AR. Heavy smoking and liver. World J Gastroenterol 2006; 12:6098-101.

3. Helen A, Krishnakumar K, Vijayammal PL, Augusti KT. Antioxidant effect of onion oil (*Allium cepa* Linn) on the damages induced by nicotine in rats as compared to alphatocopherol. Toxicol Lett 2000; 116:61-8.

4. Demina DV, Toporkova LB, Kozlov VA, Khaldoyanidi SK, Orlovskaya IA. Formation of immunodeficiency in newborn mice exposed to nicotine during intrauterine development. Bull Exp Biol Med 2005; 139:692-4.

5. Pool-Zobel BL, Klein RG, Liegibel UM, Kuchenmeister F, Weber S, Schmezer P. Systemic genotoxic effects of tobacco-related nitrosamines following oral and inhalational administration to Sprague-Dawley rats. Clin Invest 1992; 70:299-306.

6. Kleinsasser NH, Sassen AW, Semmler MP, Harréus UA, Richter E. The tobacco alkaloid nicotine demonstrates genotoxicity in human tonsillar tissue and lymphocytes. Toxicol Sci 2005; 86:309-17.

7. Yildiz D, Ercal N, Armstrong DW. Nicotine enantiomers and oxidative stress. Toxicology 1998; 130:155-65.

8. Therriault MJ, Proulx LI, Castonguay A, Bissonnette EY. Immunomodulatory effects of the tobacco-speciWc carcinogen, NNK, on alveolar macrophages. Clin Exp Immunology 2003; 132:232-8.

9. Benowitz NL, Gourlay SG. Cardiovascular toxicity of nicotine: implications for nicotine replacement therapy. J Am Coll Cardiol 1997; 29: 1422–31.

10. Reibel J. Tobacco and oral diseases: update on the evidence, with recommendations. Medicinal Principles and Practice 2003; 11:22-32.

11. Chen Y, Yang L, Lee TJ. Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-kappa B activation. Biochem Pharmacol 2000; 59:1445-57.

12. Chen YC, Shen SC, Lee WR, Hou WC, Yang LL, Lee TJF. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin and quercetin pentaacetate in RAW264.7 macrophages. J Cell Biochem 2001; 82:537-48.

13. Chen YC, Shen SC, Lee WR, Lin HY, Ko CH, Shih CM, Yang LL. Wogonin and fiset in induction of apoptosis through activation of caspase3 cascade and alternative expression of p21 protein in hepatocellular carcinomacells SK-HEP-1. Arch Toxicol 2002; 76:351-9.

14. Areias FM, Rego AC, Oliveira CR, Seabra RM. Antioxidant effect of flavonoids after ascorbate/Fe(2+)-induced oxidative stress in cultured retinal cells. Biochem Pharmacol 2001; 62:111-8.

15. Hendriks JJ, de Vries HE, van der Pol SM, van den Berg TK, van Tol EA, Dijkstra CD. Flavonoids inhibit myelin phagocytosis by macrophages; a structure-activity relationship study. Biochem Pharmacol 2003; 65:877-85.

16. Rice-Evans CA, Miller N, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996; 20:933-56.

17. Bors W, Michel C, Stettmaier K. Structure-activity relationships governing antioxidant capacities of plant polyphenols. Methods Enzymol 2001; 335:166-80.

18. Manna SK, Mukhopadhyay A, Van NT, Aggarwal BB. Silymarin suppresses TNF-induced activation of NF-kappaB, c-Jun N-terminalkinase, and apoptosis. J Immunol 1999; 163:6800-9.

19. Skottova N, Kazdova L, Oliyarnyk O, Vecera R, Sobolova L, Ulrichova J. Phenolics-rich extracts from Silybum marianum and Prunella vulgaris reduce a high-sucrose diet induced oxidative stress in hereditary hypertriglyceridemic rats. Pharmacol Res 2004; 50:123-30.

20. Muthukumaran S, Sudheer AR, Menon VP, Nalini N. Protective effect of quercetin on nicotine-induced prooxidant and antioxidant imbalance and DNA damage in Wistar rats. Toxicology 2008; 243:207-15.

21. Gumustekin K, Altinkaynak K, Timur H, Taysi S, Oztasan N, Polat MF, Akcay F, Suleyman H, Dane S, Gul M. Vitamin E but not *Hippophea rhamnoides* L. prevented nicotine-induced oxidative stress in rat brain. Hum Exp Toxicol 2003; 22:425-31.

22. Kanno S, Tomizawa A, Hiura T, Osanai Y, Shouji A, Ujibe M, Ohtake T, Kimura K, Ishikawa M. Inhibitory effects of naringenin on tumor growth in human cancer cell lines and sarcoma S-180-implanted mice. Biol Pharm Bull 2005; 28:527-30.

23. Hirai S, Kim YI, Goto T, Kang MS, Yoshimura M, Obata A, Yu R, Kawada T. Inhibitory effect of naringenin chalcone on inflammatory changes in the interaction between adipocytes and macrophages. Life Sci 2007; 81:1272-9.

24. Lee E, Oh E, Joohyun L, Donggeun S, Juneyoung L. Use of the tail moment of the lymphocytes to evaluate DNA damage in human biomonitoring studies. Toxicol Sci 2004; 81:121-32.

25. Jain A, Flora SJS. Dose related effects of nicotine on oxidative injury in young, adult and old rats. J Environmental Biology 2012; 33:233-8.

26. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993; 90:7915-22.

27. McCord JM. Human disease, free radicals, and the oxidant/antioxidant balance. Clin Biochem 1993; 26:351-7.

28. Bondy SC, Orozco J. Effects of ethanol treatment upon sources of reactive oxygen species in brain and liver. Alcohol Alcohol 1994; 29:375-83.

29. Socci DJ, Bjugstad KB, Jones HC, Pattisapu JV, Arendash GW. Evidence that oxidative stress is associated with the pathophysiology of inherited hydrocephalus in the H-Tx rat model. Exp Neurol 1999; 155:109-17.

30. Jollow DJ, Mitchell JR, Zamppaglione Z, Gillette JR. Bromobenzene induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolites. Pharmacology 1974; 11:151-5.

31. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thioharbituric acid reaction. Anal Biochem 1979; 93:351-8.

32. Hissin PJ, Hilf R. Fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976; 74:214-26.

33. Flohe L, Gunzler WA. Assays of glutathione-peroxidase. Methods Enzymol 1984; 105:114-21.

34. Habig WH, Pabst MJ, Jakohy WB. Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. J Biol Chem 1974; 249:7130-39.

35. Worthington DJ, Rose MM Human glutathione reductase, purification of crystalline enzyme from erythrocytes. Eur J Biochem 1974; 48:167-77.

36. Kovacic P, Cooksy A. Iminium metabolite mechanism for nicotine toxicity and addiction: Oxidative stress and electron transfer. Med Hypotheses 2005; 64:104-11.

37. Wetscher GI, Bagchi M, Bagchi D, Perdikis G, Hinder PR, Glaser K, Hunder RA. Free radical production in nicotine treated pancreatic tissue. Free Radic Biol Med 1995; 18:877-82.

38. Yildiz D, Liu YS, Ercal N, Armstrong DW. Comparison of pure nicotine and smokeless tobacco extract-induced toxicities and oxidative stress. Arch Environ Contam Toxicol 1999; 37:434-39.

39. Dey SK, Roy S. Role of reduced glutathione in the amelioration of nicotine-induced oxidative stress. Bull Environ Contam Toxicol 2010; 84:385-9.

40. Husain K, Scott RB, Reddy KS, Somani SM. Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system. Alcohol 2001; 25:89-97.

41. O'Connor RJ, Norton KJ, Bansal-Travers M, Mahoney MC, Cummings KM, Borland R. US smokers' reactions to a brief trial of oral nicotine products. Harm Red 2011; 8:1-10.

42. Wickens A. PDR(R) for herbal medicines. Libr J 1999; 124:74.

43. Fleming T (ed). PDR for Herbal Medicines. Medical Economics Company, Montvale, NJ, USA, 2000.

44. Miguez MP, Anundi I, Sainz-Pardo LA, Lindros KO. Hepatoprotective mechanism ofsilymarin: no evidence for involvement of cytochrome P-450 2E1. Chem Biol Interact 1994; 91:51-63.

45. Wagner H. Plant constituents with anti hepatotoxic activity. In: Beal JL, Reinhard E. (eds), Natural Products as Medicinal Agents. Hippokrates-Verlag, Stuttgart, Germany, 1981.

46. Hikino H, Kiso Y, Wagner H, Feibig M. Anti hepatotoxic actions of flavonolignans from silybum marianum fruits. Planta Med 1984; 50:248-50.

47. Bosisio E, Benelli C, Pirola O. Effect of the flavanolignans of silybum–marianum L on lipid-peroxidation in rat-liver microsomes and freshly isolated hepatocytes. Pharmacol Res 1992; 25:147-54.

48. Sonnenbichler J, Zetl I. Biochemical effects of the flavonolignane silibinin on RNA, protein and DNA synthesis in rat livers. Prog Clin Biol Res 1986; 213:319-31.

49. Baer-Dubowska W, Szaefer H, Krajka- Kuzniak V. Inhibition of murine hepatic cytochrome P450 activities by natural and synthetic phenolic compounds. Xenobiotica 1998; 28:735-43.

50. Halim AB, El-Ahmady C, Hassab-Allah S, Abdel- Galil F, Hafez Y, Darwish A. Biochemical effect of antioxidants on lipids and liver function in experimentally induced liver damage. Ann Clin Biochem 1997; 34:656-63.

51. Campos R, Garrido A, Guerra R, Valenzuela A. Silybin di hemisuccinate protects against glutathione depletion and lipid peroxidation induced by acetaminophen on rat liver. Planta Med 1989; 55:417-9.

52. Nencini C, Giorgi G, Micheli L. Protective effect of silymarin on oxidative stress in rat brain. Phytomedicine 2007; 14:129-35.

53. Jeon SM, Bok SH, Jang MK, Lee MK, Nam KT, Park YB, Rhee SJ, Choi MS. Antioxidative activity of naringin and lovastatin in high cholesterol-fed rabbits. Life Sci 2001; 69:2855-66.

54. Jeon SM, Park YB, Choi MS. Anti hypercholesterolaemic property of naringin alters plasma and tissue lipids, cholesterol-regulating enzymes, fecal sterol and tissue morphology in rabbits. Clin Nutr 2004; 23:1025-34.

55. Jung UJ, Kim HJ, Lee JS, Lee MK, Kim HO, Park EJ, Kim HK, Jeong TS, Choi MS. Naringin supplementation lowers plasma lipids and enhances erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects. Clin Nutr 2003;22:561-8.

 DOI 10.5455/oams.130412.or.004

56. Chen YT, Zheng RL, Jia ZJ, Ju Y. Flavonoids as superoxide scavengers and antioxidants. Free Radic Biol Med 1990; 9:19-21.

57. Nishigaki I, Hagihara M, Tsunekawa H, Maseki M, Yagi K. Lipid peroxide levels of serum lipoprotein fractions of diabetic patients. Biochem Med 1981; 25:373-8.

58. Park CS, Li L, Lau BHS. Thymic peptide modulates glutathione redox cycle and antioxidant enzymes in macrophages. J Leukocyte Biol 1994; 55:496-500.

59. Erat M, Ciftci M, Gumustekin K, Gul M. Effects of nicotine and vitamin E on glutathione reductase activity in some rat tissues in vivo and in vitro. Eur J Pharmacol 2007; 554:92-7.

60. Lehninger AL, Nelson DL, Cox MM. Principles of Biochemistry. Second edition, Worth Publishers, New York, NY, USA, 1993.

61. Vijayan V, Helen A. Protective Activity of *Bacopa monniera* Linn. on nicotine-induced toxicity in mice. Phytother Res 2007; 21:378-81.

62. Das M, Singh SV, Mukhtar H, Awasthi YC. Differential inhibition of rat and human glutathione S-transferase isoenzymes by plant phenols. Biochem Biophys Res Commun 1986; 141:1170-76.