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Enhanced therapeutic potential of naringenin–phospholipid complex in rats

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Abstract

Naringenin is a naturally occurring flavanone, possessing a variety of biological activity. Due to its rapid elimination, naringenin needs frequent administration to maintain an effective plasma concentration. We have evaluated the therapeutic potential of naringenin–phospholipid complex under oxidative stress conditions compared with free naringenin. Naringenin–phospholipid complex was prepared and assessed for antioxidant activity in carbon tetrachloride intoxicated rats at a dose level of 100 mg kg^{-1} (p.o.). Liver function tests were studied by assessing serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, serum alkaline phosphatase and total bilirubin. Marker enzymes of liver, namely glutathione peroxidase, superoxide dismutase, catalase and thiobarbituric acid reactive substances, were measured to evaluate the antioxidant potential at the same dose level. The plasma concentration of naringenin was also measured. It was observed that the naringenin–phospholipid complex enhanced the antioxidant activity of the biomolecule and protected the liver significantly for a longer time as compared with free naringenin at the same dose level. Phospholipid complex of naringenin produced better antioxidant activity than the free compound with a prolonged duration of action, which may be helpful in reducing the fast elimination of the molecule from body.

Introduction

Naringenin (4′,5,7-trihydroxyflavanone, Figure 1) is the aglycone of naringin, a flavonoid specific to citrus fruits and the predominant flavanone in grapefruit (*Citrus paradisi*) (up to 10% of the dry weight) responsible for the bitterness of grapefruit juices (Ortuño et al 1995). This bioflavonoid possesses anti-inflammatory, anticarcinogenic, and antitumour effects (Middleton & Kandaswami 1994; Benavente-Garcia et al 1997; Montanari et al 1998). It is also found to exert anti-estrogenic activity (Ruh et al 1995; Miksicek 1993; Kuiper et al 1998). Furthermore, naringenin seems to affect different oxidative processes associated with chronic degenerative diseases. It partially deactivates the Fenton reaction (Cheng & Breen 2000) and restores glutathione-dependent protection against lipid peroxidation in α-tocopherol-deficient liver microsomes (Van Acker et al 2000). It produces inhibitory activity in malonaldehyde production (Lee et al 2003; Saija et al 1995) and inhibits cytochrome P450 enzymes (Ueng et al 1999).

Despite this wide range of therapeutic activity, its unfavourable pharmacokinetics associated with a lower half-life and rapid clearance from the body restricts its use as a potent phyto molecule. Erlund et al (2001) reported that the elimination half-lives of naringenin from orange juice and grapefruit juice were 1.3 and 2.2 h, respectively. Therefore, to maintain steady plasma concentration so as to exert the desired therapeutic activity, frequent administration of the molecule is required, which necessitates the need for development of a dosage form that can maintain the concentration of naringenin in blood for a longer period.

Phospholipids play a major role in drug delivery technology. It is an important carrier for those drug molecules which require sustained/controlled release in-vivo due to faster elimination from the body. Thus we have evaluated the antioxidant activity of naringenin–phospholipid complex in comparison with pure naringenin in carbon tetrachloride(CCl_4)-treated rats. Moreover, the influence of complexation on the plasma concentration of naringenin over different time periods was investigated.

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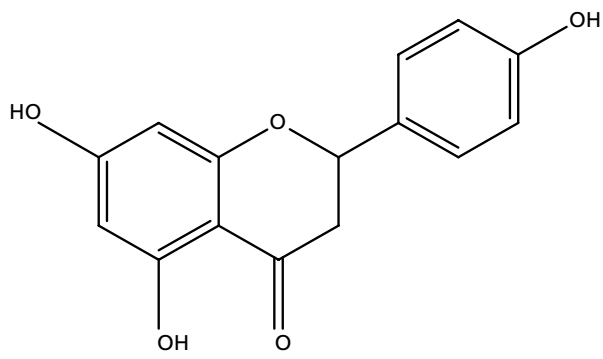

Figure 1 Structure of naringenin.

Materials and Methods

Test samples and standards

Naringenin (Sigma Chemical, St Louis, MO) was suspended in distilled water with Tween 20 (1%, v/v). Naringenin–phospholipid complex was prepared by the method of Bombardelli & Patri (1991) and will be described later. Naringenin suspension and formulation (100 mg kg^{-1} body weight) were administered orally as test samples. The normal group received vehicle alone.

Chemicals

Hydrogenated soy phosphatidyl choline (HSPC) was procured from Lipoid, Ludwigshafen, Germany. Thiobarbituric acid, trichloroacetic acid, carmellose sodium (sodium carboxymethylcellulose), n-hexane and other chemicals were obtained from Loba Chemie, Mumbai, India and S.D. Fine Chemicals, Kolkata, India. Dichloromethane was obtained from Qualigen Fine Chemicals, Mumbai, India. Glutathione, bovine serum albumin, Tris base, nitroblue tetrazolium, and 5, 5-dithiobis (2-nitrobenzoic acid) reagent were purchased from SRL Chemicals, Mumbai, India.

Preparation of naringenin–phospholipid complex

Complex of naringenin with phospholipid was prepared by the method of Bombardelli & Patri (1991). In brief, 1 mol naringenin was refluxed with 1 mol HSPC in 20 mL dichloromethane until all the naringenin had dissolved. The volume of this resulting solution was reduced to 2–3 mL and 10 mL n-hexane was added to obtain the complex as a precipitate. The complex was filtered, dried under vacuum and stored in an air-tight container until further use.

Animals

Male albino rats (Wistar strain; 180–200 g) were obtained from a local supplier. Animals were housed in groups of seven or eight in colony cages at an ambient temperature of 20–25°C and 45–55% relative humidity under a 12-h light/dark cycle. They had free access to pellet chow (Brook Bond, Lipton India) and water was freely available. The experiment was performed under the ethical guidelines as provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Treatment schedule

Adult rats were divided into five groups of 15 animals each. Group 1 received distilled water with Tween 20 (1%, v/v) orally for seven days only and served as vehicle control. Group 2 received a single dose of an equal mixture of CCl_4 and olive oil (50%, v/v; 5 mL kg^{-1}, i.p.) on the seventh day. Group 3 was treated with naringenin suspension in distilled water with Tween 20 (1%, v/v) at a dose of 100 mg kg^{-1}/day orally for seven days. Group 4 animals received 100 mg kg^{-1} naringenin on days 1, 3, 5 and 7. Group 5 was treated with naringenin–phospholipid complex equivalent to 100 mg kg^{-1} naringenin on alternate days (1, 3, 5 and 7) like group 4 animals up to day 7. On day 7, a single dose of an equal mixture of CCl_4 and olive oil was administered (50%, v/v; 5 mL kg^{-1}, i.p.) to the animals of groups 3, 4 and 5 1 h after naringenin treatment.

Liver function test and antioxidant activity

Animals were fasted and 24 h after intoxication were anaesthetized with ether to collect blood from the retro orbital plexus on day 8. Serum was separated by centrifugation. Liver function tests were performed by measuring serum glutamate oxaloacetae transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) (Reitman & Frankel 1957), serum alkaline phosphatase (SALP) (Kind & King 1954) and total bilirubin (Malloy & Erelyn 1937). After blood collection, five animals from each group were chosen randomly and killed by cervical decapitation under light ether anaesthesia. Immediately after killing, livers were dissected out for biochemical estimation. After washing with ice-cold saline, liver homogenate was prepared in 0.1 M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged and supernatant was used for the estimation of glutathione peroxidase (GPX) (Mohandas et al 1984), superoxide dismutase (SOD) (Kakkar et al 1984; Raja et al 2005), catalase (CAT) (Rigo & Rotilio 1977) and thiobarbituric acid reactive substances (TBARS) (Ohkawa et al 1979). Protein concentrations were determined using purified bovine serum albumin as standard (Lowry et al 1951). The same experimental protocol was repeated on day 9 and day 10. For the liver function test, 15 animals were used on day 8, which was further reduced to 10 and 5 animals on day 9 and day 10, respectively, as five animals on each of days 8 and 9 were killed for the enzyme assay. For antioxidant activity on days 8, 9 and 10, five animals were used for all the groups. There was no drug treatment on days 8, 9 and 10.

Estimation of naringenin plasma concentration

Rats were divided into three groups containing five animals each. Group 1 animals received naringenin suspension in distilled water with Tween 20 (1%, v/v) at a dose level of 100 mg kg^{-1}/day orally, for seven days. Group 2 animals received 100 mg kg^{-1} naringenin on days 1, 3, 5 and 7. Group

3 animals were treated with naringenin–phospholipid complex, equivalent to 100 mg kg^{-1} naringenin on alternate days like group 2 animals up to day 7. From day 8, blood samples were collected from the retro orbital plexus of the rats at different time points and the concentration of naringenin in rat plasma was determined as reported by Felgines et al (2000). In brief, blood samples were collected from the retro orbital plexus of the rats at different time points and kept in heparinized tubes. Plasma was acidified with 10 mM acetic acid to prevent loss of flavonoids and stored at –20°C until analysis. For HPLC analysis, plasma was mixed with daidzein as an internal standard and acidified to pH 4.9 with 0.1 volume 0.58 M acetic acid solution. All plasma samples were treated by adding 2.8 volume acetone and resulting mixtures were centrifuged for 5 min at 12 000 *g* at room temperature. Supernatant was evaporated to the initial volume of plasma. A 20-μL portion of each plasma sample was injected into a Hypersil BDS C-18, 5-μm column. Water:H_3PO_4 (99.5:0.5) was used as solvent A and acetonitrile as solvent B for elution, with a flow rate of 1.5 mL min^{-1}. Absorbance was monitored at 320 nm.

Statistics

Data were expressed as mean ± s.e.m. For liver function test and antioxidant activity, the statistical analyses were carried out using one-way analysis of variance followed by Dunnett's test. For the plasma concentration study, the statistical analysis was carried out using analysis of variance followed by Newman–Keuls Multiple Comparison Test. $P < 0.05$ was considered significant.

Results

Liver function test

Hepatic damage induced by CCl_4 caused a significant rise in marker enzymes SGPT, SGOT, SALP and in serum bilirubin. Pretreatment with pure naringenin (100 mg kg^{-1}) on alternate days could not lower the elevated enzyme levels significantly, whereas animals treated with the same dose of pure naringenin for seven consecutive days produced an ameliorative effect on day 8 ($P < 0.05$). On days 9 and 10 it failed to exhibit significant protection of the liver. Pretreatment with the naringenin–phospholipid complex on alternate days significantly protected the rat liver against the toxic effects of CCl_4 on days 8, 9 ($P < 0.01$) and 10 ($P < 0.05$) (Table 1).

Antioxidant activity

Antioxidant activity of the naringenin–phospholipid complex on CCl_4-intoxicated rats is shown in Table 2. GPX, SOD and CAT levels in liver homogenates were significantly ($P < 0.01$) reduced in CCl_4-treated animals when compared with normal rats. Pretreatment with pure naringenin on alternate days failed to produce a significant increase in the GPX, SOD and CAT level when compared with CCl_4-treated animals. However, naringenin–phospholipid complex at the same treatment schedule showed a significant increase in these enzyme levels ($P < 0.01$) in liver homogenate as compared with CCl_4-treated animals on days 8 and 9. It also produced a significant increase in enzymatic levels on the tenth day ($P < 0.05$). Even naringenin pretreatment for seven consecutive days failed to increase the reduced level of these enzymes significantly. Level of TBARS in liver homogenates of CCl_4-challenged rats significantly increased ($P < 0.05$) when compared with vehicle control rats. Pretreatment with naringenin–phospholipid complex (equivalent to 100 mg kg^{-1} naringenin) showed a significant ($P < 0.05$) decrease in TBARS when compared with CCl_4-treated animals, whereas free naringenin upon daily or alternate day administration failed to bring down the increased level of TBARS.

Naringenin plasma concentration

Figure 2 shows the plasma concentration of naringenin at different time points for different groups of animals treated with naringenin or the complex. It was observed that complexation of naringenin with phospholipid led to an enhanced plasma level of this biomolecule for a longer time.

Discussion

With the therapeutic potential of natural health products, botanicals have a major role to play in the management of varied diseases. To maximize the healing properties of these herbal drugs, it is necessary to develop value added delivery systems from natural resources (Mukherjee 2001, 2002a). Various active constituents obtained from different plants and herbs are gaining considerable interest because of their potential antioxidant and free radical scavenging activity. Continuing research is essential to elucidate the therapeutic efficacy of those natural molecules, as well as to develop delivery systems for those herbs to enhance the utilization of the phytomolecules to achieve better therapeutic activity (Mukherjee 2003).

In CCl_4 hepatotoxicity, free radicals are generated. Cytochrome P450 plays an important role in metabolizing CCl_4 to produce the trichloromethyl radical, which initiates a cascade of free radical reactions resulting in an increase in lipid peroxidation and a reduction in some enzyme activities (Recknagel et al 1989). Many compounds with antioxidant potential have been tested for protective activity against CCl_4 toxicity (Mukherjee 2002b; Rai et al 2006). Naringenin possesses inhibitory activity against cytochrome P450 enzymes, which leads to inhibition of free radical reactions governed by these enzymes, and thus prevents CCl_4-induced hepatotoxicity in rat liver. According to the data reported in various literature, the best protection against liver intoxicating agents is obtained in the animal when the compounds are administered rapidly, either intraperitoneally or intravenously, to obtain high concentrations in the bloodstream and the target organ. But in most cases it fails due to poor absorption of the compounds. In some cases though it is absorbed rapidly, but the needed therapeutic activity fails to be achieved due to fast elimination of the compound. Naringenin, though having rapid absorption into the blood after oral administration (Erlund et al 2001), has a fast elimination rate necessitating

Table 1 Liver function test after CCl_4 treatment

Treatment	SGPT			SGOT			SALP			Total bilirubin		
	Day 8[a]	Day 9[b]	Day 10[c]	Day 8[a]	Day 9[b]	Day 10[c]	Day 8[a]	Day 9[b]	Day 10[c]	Day 8[a]	Day 9[b]	Day 10[c]
Normal (group 1)	41.38±0.98**	42.23±0.76**	41.68±0.89**	48.08±1.77**	45.68±1.58**	47.64±3.26**	126.60±2.71**	127.90±3.43**	127.30±2.68**	0.67±0.01**	0.69±0.01**	0.68±0.01**
CCl_4 treated (group 2)	83.34±2.39	84.35±1.95	84.30±2.51	112.80±4.79	114.30±5.26	113.80±5.69	193.50±3.07	194.50±4.183	194.20±5.47	1.12±0.01	1.13±0.01	1.13±0.02
CCl_4+naringenin for 7 days (100 mg kg^{-1}) (group 3)	71.72±4.44*	78.54±4.86	78.20±4.82	90.25±5.61*	96.54±5.66	95.00±5.19	178.90±3.35*	184.60±4.36	186.0±8.38	0.95±0.06*	1.01±0.07	1.05±0.08
CCl_4+naringenin on alternate days (100 mgkg^{-1}) (group 4)	76.79±3.56	81.51±3.63	81.10±6.03	101.0±6.26	107.30±5.44	105.30±4.33	186.50±4.08	190.40±5.29	189.70±10.30	1.01±0.04	1.04±0.05	1.07±0.01
CCl_4+naringenin–phospholipid complex on alternate days (equiv to 100 mg kg^{-1} naringenin) (group 5)	44.93±0.96**	52.61±3.41**	61.74±7.35*	62.37±5.09**	74.52±5.65**	83.20±8.88*	150.30±3.55**	159.20±3.42**	165.70±3.06*	0.76±0.02**	0.82±0.04**	0.91±0.06*

*$P < 0.05$, **$P < 0.01$, compared with CCl_4-treated group. [a]Values are mean ± s.e.m. of 15 animals, [b]values are mean ± s.e.m. of 10 animals, [c]values are mean ± s.e.m. of five animals. SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate pyruvate transaminase; SALP, serum alkaline phosphatase, CCl_4, carbon tetrachloride.

Table 2 Effect of naringenin–phospholipid complex on the level of rat liver enzymes

Treatment	GPX			SOD			CAT			TBARS		
	Day 8	Day 9	Day 10	Day 8	Day 9	Day 10	Day 8	Day 9	Day 10	Day 8	Day 9	Day 10
Vehicle control (group 1)	313.80±5.25**	311.00±6.35**	313.30±6.65**	6.27±0.28**	6.26±0.39**	6.32±0.54**	202.60±3.34**	205.70±4.33**	207.10±6.34**	5.48±0.48**	5.73±0.50**	5.88±0.79**
CCl_4 treated (group 2)	179.80±5.46	181.10±6.08	178.80±10.51	3.59±0.09	3.63±0.11	3.75±0.07	123.20±6.76	117.60±10.04	124.00±11.34	13.55±1.13	13.69±1.17	13.39±1.11
CCl_4+naringenin for 7 days (100 mg kg^{-1}) (group 3)	195.30±3.42	198.30±4.36	199.50±8.24	4.05±0.18	4.18±0.23	4.20±0.35	153.30±4.16	150.90±5.04	147.50±6.77	10.72±1.04	10.21±1.31	9.99±1.27
CCl_4+naringenin on alternate days (100 mg kg^{-1}) (group 4)	188.80±2.86	192.50±3.60	192.60±4.18	3.86±0.14	3.96±0.19	4.06±0.16	145.00±11.81	143.20±8.27	141.40±12.39	11.38±0.81	10.82±0.77	10.54±0.83
CCl_4+naringenin–phospholipid complex on alternate days (equiv to 100 mg kg^{-1} naringenin) (group 5)	279.60±4.50**	281.20±6.28**	222.60±13.37*	5.56±0.10**	5.36±0.18**	5.10±0.29*	189.60±6.11**	190.00±6.59**	184.50±7.91*	9.59±0.89*	9.29±0.93*	8.68±1.19*

Values are mean ± s.e.m. of five animals. *$P < 0.05$, **$P < 0.01$, compared with CCl_4-treated group. GPX, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; TBARS, thiobarbituric acid reactive substances. GPX, SOD and catalase activity were expressed as U (mg protein)$^{-1}$ and TBARS was expressed as nmol malonaldehyde (MDA) (mg protein)$^{-1}$.

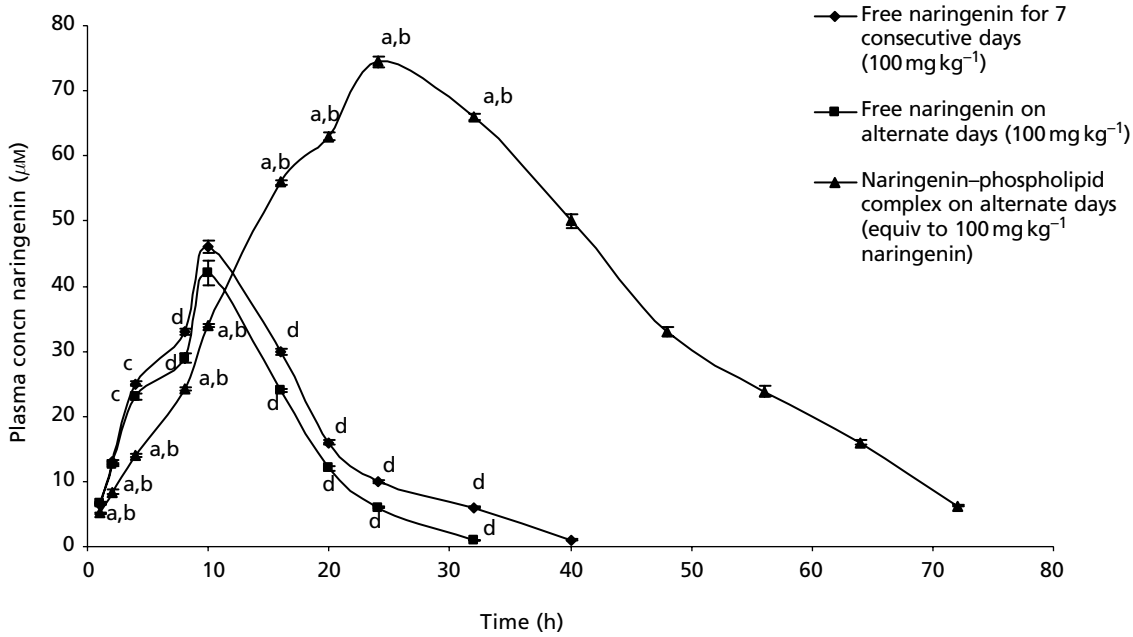

Figure 2 Effect of complexation on plasma concentration of naringenin in rats. Values are mean ± s.e.m. of five animals. [a]$P < 0.001$, values of complex-treated rats statistically significant with respect to the pure naringenin (100 mg kg^{-1})-treated group on alternate days for seven days. [b]$P < 0.001$, values of complex-treated rats statistically significant with respect to the pure naringenin (100 mg kg^{-1})-treated group for seven consecutive days. [c]$P < 0.01$, pure naringenin (100 mg kg^{-1})-treated group on alternate days for seven days and pure naringenin (100 mg kg^{-1})-treated group for seven consecutive days were statistically significant with respect to each other. [d]$P < 0.001$, pure naringenin (100 mg kg^{-1})-treated group on alternate days for seven days and pure naringenin (100 mg kg^{-1})-treated group for seven consecutive days were statistically significant with respect to each other.

the need of some novel compound with the same dose of the conventional dosage form, which could maintain the concentration of naringenin in the blood for a longer time.

This study has dealt with the preparation and evaluation of a phospholipid complex of naringenin, which increased the therapeutic efficacy of this flavanone. Liver function tests and the antioxidant activity studies (Tables 1 and 2) showed that pretreatment with pure naringenin (100 mg kg^{-1}) on alternate days neither reduced the elevated levels of SGOT, SGPT, SALP, total bilirubin and TBARS, nor increased the depleted levels of GPX, SOD and catalase. The results were statistically insignificant as compared with the CCl_4-treated group. Similar results were obtained from the animals treated with 100 mg kg^{-1} pure naringenin daily for seven consecutive days before CCl_4 administration. For both these studies, the complex produced statistically significant results ($P < 0.01$ and $P < 0.05$, Tables 1 and 2) as compared with the CCl_4-treated group. The results of the liver function tests and the antioxidant activity study were further supported by the naringenin–plasma concentration study (Figure 2). This study showed that pretreatment with pure naringenin on consecutive days as well as alternate days failed to produce a sustaining effect and the concentration of naringenin in plasma was detectable up to 40 h (day 9), which was not sufficient to produce a statistically significant effect. However, the complex produced a longer duration of action, as evident from the result, which showed that the concentration of naringenin in plasma was maintained for an extended time and was detected up to 72 h (day 10). Thus, the plasma study supported the hypothesis that the phospholipid complex of naringenin maintained the plasma concentration of the molecule for a longer period, to overcome the problem associated with the fast elimination of the molecule from the body, thus producing better therapeutic efficacy.

Conclusion

Naringenin–phospholipid complex enhanced the free radical scavenging property of the parent molecule. This formulation produced better efficacy in rats as compared with the molecule itself. Further investigation is necessary to elucidate the exact mechanism behind the improved therapeutic efficacy in the light of pharmacokinetic parameters to substantiate the claim of sustained release followed by a prolonged duration of action.

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