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EXPLORING THE ANTIPEROXIDATIVE POTENTIAL OF MORIN ON CYCLOPHOSPHAMIDE AND FLUTAMIDE-INDUCED LIPID PEROXIDATION AND CHANGES IN CHOLESTEROL PROFILE IN RABBIT MODEL

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Abstract: Considering drug-induced lipid peroxidation as a possible mediator of drug-induced toxicity and exploiting the free radical scavenging action of antioxidants, the present study was designed to evaluate the antiperoxidative potential of morin on cyclophosphamide/flutamide-induced lipid peroxidation and also to evaluate the effect of morin on cyclophosphamide/flutamide-induced changes in cholesterol content in rabbit blood sample. This evaluation was done by measuring the malondialdehyde (MDA), reduced glutathione (GSH), 4-hydroxy-2-nonenal (4-HNE) and nitric oxide (NO) content of blood samples as markers of lipid peroxidation. In the cholesterol profile total cholesterol and high density lipoprotein cholesterol content of rabbit blood was determined. The study reveals the lipid peroxidation induction capacity of cyclophosphamide/flutamide and the antiperoxidative potential of morin on cyclophosphamide/flutamide-induced lipid peroxidation. It was also observed that morin has protective effect on cyclophosphamide/flutamide-induced changes in cholesterol content.

Keywords: morin, cyclophosphamide, flutamide, lipid peroxidation, malondialdehyde, reduced glutathione, 4-hydroxy-2-nonenal, nitric oxide, cholesterol

Lipid peroxidation is a well known example of oxidative damage of cell membranes, lipoproteins, and other lipid-containing structures (1). This complex process is believed to contribute to human aging and many diseases by disrupting the structural conformation and the packing of lipid components, ultimately the function of biological membranes. The polyunsaturated fatty acids of membrane phospholipids are particularly susceptible to peroxidation and undergo significant modifications, including the rearrangement or loss of double bonds and, in some cases, the reductive degradation of lipid acyl side chains (2, 3). Lipid peroxidation leads to generation of peroxides and hydroperoxide that can decompose to yield a wide range of cytotoxic end products most of which are aldehydes, as exemplified by molondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) etc. (4). Free radicals are highly reactive molecules with odd number of electrons. They are constantly being generated in the body through various mechanisms and also being removed by endogenous antioxidant defense mechanism that acts by scavenging free radicals, decomposing peroxides and/or binding with pro-oxidant metal ion. Free radical mediated oxidative stress results usually from deficient natural antioxidant defense. In case of reduced or impaired defense mechanism and excess generation of free radicals that are not counterbalanced by endogenous antioxidant defense, exogenously administered antioxidants have been proven useful to overcome oxidative damage (5).

Cyclophosphamide, an alkylating agent widely used in cancer chemotherapy, is an inactive cytostatic, which is metabolized into active metabolites mainly in the liver. During bioactivation, reactive oxygen species are also formed, which can modify the components of both healthy and neoplastic cell leading to decreased antioxidative capacity (6). It has been reported that cyclophosphamide produces

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genotoxicity and oxidative stress in mice (7) and early lung injury in rats (8). It also causes fatal cardiotoxicity (9). Hemorrhagic cystitis is a major dose limiting side effect of cyclophosphamide (10). It was also found that cyclophosphamide is toxic for male germ cells (11).

Flutamide, a phototoxic anticancer drug acts as androgen receptor antagonist and is used mainly as an anticancer drug in certain type of prostate cancer. It shows a photo hemolytic effect on human erythrocytes and photo induces lipid peroxidation (12).

Plant flavonoids are emerging as potent therapeutic drugs effective against a wide range of free radical mediated diseases. Morin (3,5,7,2',4'-pentahydroxyflavone), a member of flavonols, exerts antioxidant potential and offers protection against the oxidative stress induced by hydrogen peroxide (13). It was found that there was an enhanced systemic availability of methotrexate in the presence of morin in rats (14). Recently, study shows that morin could exert a significant chemopreventive effect on colon carcinogenesis induced by 1,2-dimethylhydrazine (15). Morin increases the bioavailability of tamoxifen and its main metabolite, 4-hydroxytamoxifen in rats (16). Morin has protective effect on dimethylnitrosamine-induced hepatic fibrosis in rats (17).

In view of the above findings and the ongoing search for antioxidant that may reduce drug induced lipid peroxidation (18-21) the present work has been carried out *in vivo* to evaluate the antiperoxidative potential of morin on cyclophosphamide/flutamide-induced lipid peroxidation and also to evaluate the effect of morin on cyclophosphamide/flutamide-induced changes in cholesterol content in rabbit blood sample.

EXPERIMENTAL

Materials

Thiobarbituric acid (TBA), 2,4-dinitrophenylhydrazine (DNPH), sodium nitrite and trichloroacetic acid (TCA) were purchased from Ranbaxy Fine Chemicals Ltd., New Delhi; 5,5'-dithiobis-2-nitrobenzoic acid was from SRL Pvt. Ltd., Mumbai; sulfanilamide was from SD Fine Chem. Ltd., Mumbai; N-naphthylethylenediamine dihydrochloride was from Loba Chemie Pvt. Ltd., Mumbai; 1,1,3,3-tetraethoxypropane and reduced glutathione were from Sigma Chemicals Co. St. Louis, MO, USA. The standard sample of 4-HNE was purchased from ICN Biomedicals Inc., Ohio. Cyclophosphamide injection (Oncomide-200) was from Khandelwal Laboratories Pvt. Ltd., Mumbai, India. Flutamide tablet (Cytomid-250) was purchased from Cipla Ltd. Mumbai, India. Cholesterol test kit was from Span Diagnostic Ltd., Surat, India. All other reagents were of analytical grade.

Animal experiments

The *in vivo* experiments were carried out using male white New Zeeland rabbit (*Oryctolagus caniculus*) as experimental model. The animal experiment was carried out in accordance with the protocol of institutional animal ethics committee of Himalayan Pharmacy Institute, Majhitar, East Sikkim, India (sanctioned by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Govt. of India, Chennai-600041; Registration no. of the institute 1028/C/07/CPCSEA, and dated 24. 01. 2007). Normal healthy rabbits weighing 1.5 – 2.0 kg were taken for the study. All the animals were housed at normal ambient temperatures (25 – 29^{o}C) and acclimatized in the laboratory for at least 72 h. They were maintained on a standard laboratory diet and water *ad libitum*.

Group division of rabbits for *in vivo* lipid peroxidation and lipid profile studies

Rabbits were divided into four groups. There were three animals in each group. The first (C) was a control group (not treated with drug and/or antioxidant), while the second group (D) was treated with drug (cyclophosphamide intramuscularly at a dose of 15 mg/kg b. w. or flutamide orally at a dose of 12.5 mg/kg b. w.). The third group (DA) was treated both with drug and antioxidant (cyclophosphamide intramuscularly at a dose of 15 mg/kg b. w. and morin intramuscularly at a dose of 50 mg/kg b. w. or flutamide orally at a dose of 12.5 mg/kg b. w. and morin orally at a dose of 50 mg/kg b. w. The final group (A) was received only antioxidant (morin intramuscularly or orally at a dose of 50 mg/kg b. w.). After administration of drug and/or antioxidant, the animals were kept for specific time of incubation (2 h for cyclophosphamide or 5 h for flutamide).

Lipid peroxidation study

Determination of malondialdehyde (MDA) level in rabbit blood

The extent of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using thiobarbuturic acid (TBA) method (22). The determinations were performed with rabbit blood taken after 2 or 5 h of incubation time and repeated for five times in accordance with the protocol of Ray et al. (20). The concentrations of MDA for various groups were determined from standard curve. The best-fit

Table 1. Effects of morin on cyclophosphamide/flutamide-induced lipid peroxidation: changes in MDA profile

Antioxidant	Drug	Time of incubation (h)	Animal sets	% changes in MDA content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Morin	Cyclophosphamide	2	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	45.31[a] 43.48[a] 42.61[a] 16.77[a] 18.73[a] **33.38** (± 6.40)	13.17[a] 13.42[a] 12.12[a] -5.16[b] -5.33[a] **5.64** (± 4.45)	-3.09[d] -3.55[b] -4.65[b] -5.35[c] -6.76[a] **-4.68** (± 0.65)	F1 = 43.59 [df = (2, 8)] F2 = 4.89 [df = (4, 8)] Pooled variance: (S^2)* = 44.44 Critical difference: (p = 0.05)[#] LSD = 12.55 Ranked means[**] (D) (DA) (A)
Morin	Flutamide	5	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	20.15[a] 20.03[a] 19.63[a] 17.78[a] 20.39[a] **19.59** (± 0.47)	5.23[d] 5.24[b] 3.42[d] 10.82[b] 11.64[a] **7.27** (± 1.65)	-35.11[a] -35.65[a] -34.21[a] -25.46[a] -24.28[a] **-30.94** (± 2.49)	F1 = 350.35 [df = (2, 8)] F2 = 2.64 [df = (4, 8)] Pooled variance: (S^2)* = 9.909 Critical difference: (p = 0.05)[#] LSD = 5.93 Ranked means[**] (D) (DA) (A)

Percent changes with respect to controls of corresponding hours are shown. C, D, DA and A indicate control (not treated with cyclophosphamide/flutamide or morin), only cyclophosphamide/flutamide-treated, cyclophosphamide/flutamide and morin -treated and only morin-treated samples, respectively; Av. = averages of five animal sets. SEM = Standard error of estimate (df = 4). Significance of '*t*' values of the changes of MDA content (df = 2) are shown as: a > 99%; b = 97.5-99%; c = 95-97.5%; d = 90-95%; e = 80-90%; f = 70-80%; g = 60-70%; h < 60%. Theoretical values of F: p = 0.05 level, F1= 4.46 [df = (2,8)], F2 = 3.84 [df = (4, 8)]; p = 0.01 level, F1 = 8.65 [df = (2, 8)], F2 = 7.01 [df = (4, 8)], F1 and F2 corresponding to variance ratio between groups and within groups, respectively.
* Error mean square, # Critical difference according to least significant difference procedure (27, 28), **Two means not included within the same parenthesis are statistically significantly different at p = 0.05 level

equation was A = 0.005631M, where M = nanomoles of MDA, A = absorbance, r = 0.991, SEM = 0.0289 and F = 490.83 (df = 1, 9).

Determinations of 4-hydroxy-2-nonenal (4-HNE) level in rabbit blood

4-HNE content was determined by reaction with 2,4-dinitrophenyl hydrazine (DNPH) (23). The determinations were performed with rabbit blood taken after 2 or 5 h of incubation time and repeated for five times in accordance with the protocol of Ray et al. (20). The concentrations of 4-HNE for various groups were estimated from the standard curve. The best-fit equation was: nanomoles of 4-HNE = (A_{350} – 0.005603185) / 0.003262215, where A_{350} = absorbance at 350 nm, r = 0.999, SEM = 0.007.

Determinations of reduced glutathione (GSH) level in rabbit blood

Reduced glutathione (GSH) was measured in accordance with Ellman's method (24). The determinations were performed with rabbit blood taking after 2 or 5 h of incubation time and repeated for five times in accordance with the protocol of Ray et al. (20). The concentrations of reduced glutathione were determined from standard curve. The best-fit equation was A = 0.000531M, where M = nanomoles of reduced glutathione, A = absorbance, r = 0.991, SEM = 0.0059 and F = 574.07 (df = 1, 10).

Determinations of nitric oxide (NO) level in rabbit blood

NO content was determined by reaction with Griess reagent. Griess reagent was prepared by mixing equal volumes of sulfanilamide (1% w/v in 3 M HCl) and (0.1% w/v N-naphthylethylenediamine dihydrochloride) (25). The determinations were performed with rabbit blood taking 2 or 5 h of incubation time and repeated for five times in accordance with the protocol of Ray et al. (20). The values were calculated from standard curve. The best-fit equation was A = 0.014061M, where M = nanomoles of NO, A = absorbance, r = 0.9994, SEM = 0.0029 and F = 8122.28 (df = 1, 9).

Table 2. Effects of morin on cyclophosphamide/flutamide-induced lipid peroxidation: changes in 4-HNE profile

Antioxidant	Drug	Time of incu-bation (h)	Animal sets	% changes in 4-HNE content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Morin	Cyclophos-phamide	2	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	7.82[a] 13.14[a] 8.33[a] 7.46[a] 10.51[a] **9.45** (± 1.06)	-2.79[b] -5.42[b] -5.86[a] -3.46[b] -5.95[c] **-4.69** (± 0.65)	-7.45[a] -9.69[a] -9.91[b] -7.10[c] -11.67[a] **-9.16** (± 0.84)	F1 = 104.46 [df = (2, 8)] F2 = 9.45 [df = (4, 8)] Pooled variance: (S^2)* = 4.52 Critical difference: (p = 0.05)[#] LSD = 4.003 Ranked means[**] (D) (DA) (A)
Morin	Flutamide	5	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	8.64[a] 9.35[a] 4.65[a] 6.57[a] 6.42[a] **7.13** (± 0.84)	-3.26[b] -4.43[a] -2.89[b] -2.44[b] -4.13[c] **-3.43** (± 0.37)	-7.79[a] -8.18[a] -7.07[a] -4.49[a] -6.93[b] **-6.93** (± 0.64)	F1 = 107.07 [df = (2, 8)] F2 = 0.527 [df = (4, 8)] Pooled variance: (S^2)* = 2.50 Critical difference: (p = 0.05)[#] LSD = 2.98 Ranked means[**] (D) (DA) (A)

Percent changes with respect to controls of corresponding hours are shown. C, D, DA and A as in Table 1. Av. = averages of five animal sets. SEM = standard error of estimate (df = 4). Significance of 't' values of the changes of 4-HNE content (df = 2) are shown as in Table 1. Theoretical values of F: p = 0.05 level, F1 = 4.46 [df = (2, 8)], F2 = 3.84 [df = (4,8)]; p = 0.01 level, F1 = 8.65 [df = (2, 8)], F2 = 7.01 [df = (4, 8)], F1 and F2 correspond-ing to variance ratio between groups and within groups, respectively. *, #, ** as in Table 1

Lipid profile analysis

Determination of total cholesterol and HDL cholesterol in rabbit blood

Determination of cholesterol concentration was performed in one step method (26) with the help of cholesterol test kit. The determinations were done after 2 or 5 h of incubation and it was repeated for five times. In each case there were three samples. After the specified hours of incubation, 2 mL of blood was withdrawn from the ear vein of rabbits. The blood samples were centrifuged at 2000 rpm for 15 min and the supernatant (plasma) was separated out. After that, total cholesterol and high density lipoprotein (HDL) cholesterol of the rabbit blood were determined.

The total cholesterol (TC) was calculated using the following formula:

TC (mg/dL) = (O.D. of test / O.D. of standard) × 200

HDL cholesterol was determined in two steps:

Step I – HDL cholesterol separation: 0.2 mL of the supernatant was transferred into a centrifuge tube and to it 0.2 mL of reagent 3 from the test kit was added. After shaking, the tubes were kept at room temperature for 10 min and then centrifuged at 2000 rpm for 15 min to obtain a clear supernatant.

Step II – HDL cholesterol determination: The test sample was prepared by mixing 3 mL of reagent 1 from test kit with 0.12 mL of the supernatant obtained from the step I. The centrifuge tubes were shaken well and kept in the boiling water bath exactly for 90 s. The tubes were cooled immediately to room temperature under running tap water. The O.D. of standard (S) and test (T) were measured at 560 nm against reagent 1 as a blank. The content of HDL cholesterol was calculated using the formula:

HDL cholesterol (mg / dL) = (O.D. of test / O.D. of standard) × 50

Statistical analysis

Interpretation of the results was supported by Student "*t*" test. Analysis of variance (ANOVA) and multiple comparison analysis using least significant difference procedure (27, 28) was also performed on the percent changes data of various groups such as flutamide/cyclophosphamide-treated (D), flutamide/cyclophosphamide and morin-treated (DA) and only morin-treated (A) animals with respect to control group at the corresponding time.

Table 3. Effects of morin on cyclophosphamide/flutamide-induced lipid peroxidation: changes in GSH profile

Antioxidant	Drug	Time of incubation (h)	Animal sets	% changes in GSH content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Morin	Cyclophosphamide	2	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	-7.76[a] -7.67[a] -5.59[a] -5.61[a] -6.80[a] **6.69** (± 0.47)	-0.74[c] -1.79[b] -3.85[a] -5.61[d] -0.92[c] **-2.58** (± 0.94)	6.84[a] 6.00[a] 5.68[a] 7.29[a] 4.41[a] **6.04** (± 0.49)	F1 = 65.08 [df = (2, 8)] F2 = 0.084 [df = (4, 8)] Pooled variance: (S^2)* = 3.24 Critical difference: (p = 0.05)[#] LSD = 3.389 Ranked means[**] (D) (DA) (A)
Morin	Flutamide	5	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	-20.30[a] -20.92[a] -20.84[a] -20.80[a] -20.63[a] **-20.69** (± 0.11)	7.40[a] 7.13[a] 6.53[a] 6.30[a] 7.58[a] **6.98** (± 0.24)	10.57[a] 11.92[a] 23.37[a] 19.75[a] 24.84[a] **18.09** (± 2.92)	F1 = 134.81 [df = (2, 8)] F2 = 0.91 [df = (4, 8)] Pooled variance: (S^2)* = 14.799 Critical difference: (p = 0.05)[#] LSD = 7.24 Ranked means[**] (D) (DA) (A)

Percent changes with respect to controls of corresponding hours are shown. C, D, DA and A as in Table 1. Av. = averages of five animal sets. SEM = standard error of estimate (df = 4). Significance of 't' values of the changes of GSH content (df = 2) are shown as in Table 1. Theoretical values of F: p = 0.05 level, F1 = 4.46 [df = (2,8)], F2 = 3.84 [df=(4,8)]; p = 0.01 level, F1 = 8.65 [df = (2,8)], F2 = 7.01 [df = (4,8)], F1 and F2 corresponding to variance ratio between groups and within groups, respectively. *, #, ** as in Table 1

RESULTS

The percent changes in MDA, 4-HNE, GSH, NO, total cholesterol and HDL cholesterol content of different samples at different time of incubation were calculated with respect to the control of the corresponding time of incubation and was considered as indicator of the extent of lipid and cholesterol peroxidation. The results of the studies on cyclophosphamide/flutamide-induced lipid peroxidation and its inhibition with morin were shown in Tables 1-4. The results of the studies on cyclophosphamide/flutamide-induced changes in cholesterol content (i.e. changes in total cholesterol and HDL cholesterol) and the effects of morin on these changes were also listed in Tables 5-6. From Table 1 and 2, it was evident that in rabbits treatment with cyclophosphamide/flutamide caused an increase in MDA and 4-HNE content in blood samples with respect to control to a significant extent after incubation for varying period of time. The observations suggest that cyclophosphamide/flutamide could significantly induce the lipid peroxidation process. But the MDA and 4-HNE contents were significantly reduced with respect to cyclophosphamide/flutamide-treated group when the rabbits were treated with cyclophosphamide/flutamide in combination with morin. When the rabbits were treated only with morin, the MDA and 4-HNE levels were reduced in comparison to the control and the drug treated group. This decrease may be due to the free radical scavenging property of morin.

It was evident from Tables 3 and 4 that treatment of rabbits with cyclophosphamide/flutamide caused a decrease in GSH and NO content with respect to control to a significant extent. The decrease in GSH and NO content was associated with an increase in lipid peroxidation. When the rabbits were treated with cyclophosphamide/flutamide and morin, the GSH and NO levels increased in comparison to drug treated group at corresponding time. When rabbits were treated only with morin, an increase in the GSH and NO contents in comparison to the control samples was observed, what suggests the antiperoxidative potential of morin.

The experimental data in Tables 5 and 6 indicate that incubation of rabbits with cyclophosphamide/flutamide caused an increase in total cholesterol content with respect to corresponding control, but the HDL cholesterol level was reduced in com-

Table 4. Effects of morin on cyclophosphamide /flutamide-induced lipid peroxidation: changes in NO profile

Antioxidant	Drug	Time of incubation (h)	Animal sets	% changes in NO content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Morin	Cyclophosphamide	2	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	-19.81[b] -22.22[a] -16.53[b] -19.94[b] -23.85[a] **20.47** (± 1.24)	-6.83[c] -8.89[d] -10.47[c] -7.53[c] -5.69[d] **-7.88** (± 0.83)	12.98[c] 13.05[a] 11.29[d] 16.18[c] 15.45[b] **13.79** (± 0.89)	F1 = 231.16 [df = (2, 8)] F2 = 0.324 [df = (4, 8)] Pooled variance: (S^2)* = 6.496 Critical difference: (p = 0.05)[#] LSD = 4.798 Ranked means[**] (D) (DA) (A)
Morin	Flutamide	5	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	-10.57[a] -8.31[b] -8.98[a] -8.92[b] -5.36[a] **-8.43** (± 0.85)	-4.54[d] -2.34[c] -3.06[b] -2.94[c] -2.36[c] **-3.05** (± 0.40)	5.28[c] 6.82[b] 6.77[a] 5.98[b] 6.87[a] **6.34** (± 0.31)	F1 = 406.77 [df = (2, 8)] F2 = 5.17 [df = (4, 8)] Pooled variance: (S^2)* = 6.87 Critical difference: (p = 0.05)[#] LSD = 1.56 Ranked means[**] (D) (DA) (A)

Percent changes with respect to controls of corresponding hours are shown. C, D, DA and A as in Table 1. Av. = averages of five animal sets. SEM = standard error of estimate (df = 4). Significance of 't' values of the changes of NO content (df = 2) are shown as in Table 1. Theoretical values of F: p = 0.05 level, F1 = 4.46 [df = (2, 8)], F2 = 3.84 [df = (4, 8)]; p = 0.01 level, F1 = 8.65 [df = (2, 8)], F2 = 7.01 [df = (4, 8)], F1 and F2 corresponding to variance ratio between groups and within groups, respectively; *, #, ** as in Table 1

parison to control group. These observations suggest that cyclophosphamide can change the cholesterol profile. It was further found that incubation of blood sample with cyclophosphamide/flutamide and morin produce a decrease/increase in total cholesterol/HDL-cholesterol content, respectively, with respect to cyclophosphamide/flutamide-treated group. Incubation of blood samples only with morin also shows a tendency of a decrease/increase in total cholesterol/HDL cholesterol content, respectively, with respect to control and cyclophosphamide/flutamide-treated group. These results suggest that morin could inhibit cyclophosphamide/flutamide-induced changes in cholesterol profile.

From Tables 1-6, it is seen that there are significant differences among various groups (F1), but within a particular group, differences (F2) are insignificant. The Tables also indicate that the content of MDA / 4-HNE / GSH / NO / total cholesterol / HDL cholesterol in cyclophosphamide/flutamide-treated, cyclophosphamide/flutamide and morin-treated and only morin-treated groups are statistically significantly different from each other with the exception of MDA content and total cholesterol content in cyclophosphamide-treated group, which is only statistically significantly different from the cyclophosphamide and morin-treated group and only morin-treated group. But there is no statistically significant difference among the cyclophosphamide and morin-treated group and only morin-treated group.

DISCUSSION

It has been understood that lipid peroxidation induction capacity of drugs may be related to their toxic potential. This is an analogy to cardiotoxicity of doxorubicin (29) and indomethacin-induced gastric mucosal injury (30). Malondialdehyde is a naturally occurring product of lipid peroxidation and prostaglandin biosynthesis that is mutagenic and carcinogenic. It reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine (31). An increase in the accumulation of MDA in cells can result in cellular degradation, some biochemical changes and even cell death (32). 4-Hydroxy-2-nonenal (4-HNE), a lipid aldehyde that form due to lipid peroxidation occurring during episodes of oxidative stress, readily forms adducts with cellular proteins; these adducts can be assessed as a marker of oxidative stress in the form of lipid peroxidation (33). It is an oxidation product of the ω–6 unsaturat-

Table 5. Effects of morin on cyclophosphamide/flutamide-induced changes in cholesterol content: changes in total cholesterol profile

Antioxidant	Drug	Time of incubation (h)	Animal sets	% changes in total cholesterol content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Morin	Cyclophosphamide	2	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	55.80[a] 23.15[a] 46.38[a] 32.71[a] 26.86[a] **36.98** (± 6.14)	25.58[a] 16.85[a] 23.51[a] 21.66[a] 12.96[a] **20.11** (± 2.29)	8.54[a] 7.35[a] 11.22[a] 10.38[a] 5.30[a] **8.56** (± 1.06)	F1 = 23.07 [df = (2, 8)] F2 = 2.98 [df = (4, 8)] Pooled variance: (S^2)* = 44.27 Critical difference: (p = 0.05)[#] LSD = 12.54 Ranked means[**] (D) (DA) (A)
Morin	Flutamide	5	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	22.73[a] 23.63[a] 21.59[a] 33.58[a] 29.66[a] **26.24** (± 2.30)	18.18[a] 13.17[a] 5.46[b] 18.46[a] 19.28[a] **14.91** (± 2.59)	9.10[a] 2.84[b] 1.71[b] 2.55[a] 14.19[a] **6.08** (± 2.42)	F1 = 33.94 [df = (2, 8)] F2 = 3.95 [df = (4, 8)] Pooled variance: (S^2)* = 15.02 Critical difference: (p = 0.05)[#] LSD = 7.297 Ranked means[**] (D) (DA) (A)

Percent changes with respect to controls of corresponding hours are shown. C, D, DA and A as in Table 1. Av. = averages of five animal sets. SEM = standard error of estimate (df = 4). Significance of 't' values of the changes of total cholesterol content (df = 2) are shown as in Table 1. Theoretical values of F: p = 0.05 level, F1 = 4.46 [df = (2, 8)], F2 = 3.84 [df = (4, 8)]; p = 0.01 level, F1 = 8.65 [df = (2, 8)], F2 = 7.01 [df = (4, 8)], F1 and F2 corresponding to variance ratio between groups and within groups, respectively; *, #, ** as in Table 1

ed fatty acids and contains a double bond at the C3 position which reacts readily with thiol groups *via* a Michael addition and a C1 aldehyde group that can form Schiff's bases with His and Lys residues. These reactive groups render 4-HNE capable of forming adducts with proteins, thereby altering their properties and/or denaturing them (34). In addition to the direct effects of 4-HNE in disrupting enzyme catalyzed processes, aldehyde-protein adducts are also believed to act as neo-antigens promoting tissue inflammation and disease severity (35). These capabilities have implicated 4-HNE in a number of pathologies, such as atherosclerosis, cancer, kidney disease and neurological disorders, where increased levels of 4-HNE-protein adduct have been detected (36). So the decrease in MDA and 4-HNE content in rabbit blood samples, when treated with cyclophosphamide/flutamide and morin as well as only with morin implies the free radical scavenging property of morin. Reduced glutathione is an important antioxidant and plays a very important role in the defense mechanism for tissue against the reactive oxygen species (37). GSH is related to the peroxidation of the unsaturated fatty acids in microsomal fractions and mitochondria in several different ways (38). The depletion of GSH is associated with an increase in lipid peroxidation. The decrease in GSH level may be a consequence of enhanced utilization of this compound by the antioxidant enzymes, glutathione peroxidase and glutathione-S-transferase. NO plays a very important role in host defense (39). Nitric oxide has versatile role in biology because it can be a signaling molecule in vasodilatation (40-43), a toxin (43), a pro-oxidant (44) and a potential antioxidant (45-49). So the increase in GSH and NO content of rabbit blood, when treated with drug and antioxidant as well as only with antioxidant, implies the free radical scavenging activity of the antioxidant.

An increase in total cholesterol level and a decrease in HDL cholesterol level in cyclophosphamide/flutamide-treated group indicates that cyclophosphamide/flutamide has the ability to change cholesterol profile may be by inducing oxidation of cholesterol. Yalcin et al. observed an increase in the lipid peroxidation level in hyperlipidemic patients (50). Similarly, increased levels of lipid peroxidation in hypercholesterolemic subjects have also been observed (51) High density lipoproteins (HDL) are susceptible to structural modifica-

Table 6. Effects of morin on cyclophosphamide/flutamide-induced changes in cholesterol content: changes in HDL cholesterol profile

Antioxidant	Drug	Time of incubation (h)	Animal sets	% changes in HDL cholesterol content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Morin	Cyclophosphamide	2	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	-25.26[a] -22.15[a] -25.97[a] -29.39[a] -20.39[a] **-22.61** (± 1.58)	-6.06[b] -2.42[c] -12.15[a] -13.08[a] -6.88[a] **-8.12** (± 1.98)	13.14[a] 10.18[a] 3.45[a] 11.84[a] 8.94[a] **9.51** (± 1.67)	F1 = 145.06 [df = (2, 8)] F2 = 2.61 [df = (4, 8)] Pooled variance: (S^2)* = 10.03 Critical difference: (p = 0.05)[#] LSD = 5.96 Ranked means[**] (D) (DA) (A)
Morin	Flutamide	5	An 1 An 2 An 3 An 4 An 5 Av. (± SEM)	-17.33[a] -22.18[a] -21.20[a] -22.32[a] -23.30[a] **-21.27** (± 1.04)	-10.26[b] -10.44[a] -17.33[a] -9.37[c] -16.62[a] **-12.80** (± 1.72)	9.21[a] 9.59[a] 8.44[a] 8.15[a] 4.39[a] **7.96** (± 0.93)	F1 = 240.71 [df = (2, 8)] F2 = 3.20 [df = (4, 8)] Pooled variance: (S^2)* = 4.696 Critical difference: (p = 0.05)[#] LSD = 4.08 Ranked means[**] (D) (DA) (A)

Percent changes with respect to controls of corresponding hours are shown. C, D, DA and A as in Table 1. Av. = averages of five animal sets. SEM = standard error of estimate (df = 4); Significance of 't' values of the changes of HDL cholesterol content (df = 2) are shown as in Table 1. Theoretical values of F: p = 0.05 level, F1 = 4.46 [df = (2, 8)], F2 = 3.84 [df = (4, 8)]; p = 0.01 level, F1 = 8.65 [df = (2, 8)], F2 = 7.01 [df = (4,8)], F1 and F2 corresponding to variance ratio between groups and within groups, respectively; *, #, ** as in Table 1

tions mediated by various mechanisms including oxidation, glycation, homocysteinylation or enzymatic degradation. Structural alterations of HDL may affect their functional and atheroprotective properties. Oxidants, such as hypochlorous acid, peroxyl radicals, metal ions, peroxynitrite, lipoxygenases and smoke extracts, can alter both surface and core components of HDL (52). But the increase in HDL-cholesterol level in cyclophosphamide/flutamide along with morin- treated group as well as only morin-treated group implies that morin and HDL cholesterol has protective effect against lipid peroxidation. It was also reported that vitamin C, due to its antioxidant property, prevents oxidation of LDL cholesterol, decreases total and LDL cholesterol and triglyceride and also raises HDL cholesterol level (53-55). The potential of N-acetylcysteine as an extraneous antioxidant toward hyperoxaluric manifestations in liver as well as in maintaining ratio of cholesterol/phospholipid content with in a short period of time (12 h) was also reported (56). Vitamin C treatment improves endothelial dysfunction at all time points and attenuates postprandial lipemia (PPL)-induced oxidative stress as well as lipid profile at 4 h (57). It was reported that copper exerts an oxidant effect on cultured astrocytes as shown by the significant increase in the levels of hydroperoxides in astrocytes oxidized with 10 µM Cu^{2+} for 4 h with respect to control cells. These results also demonstrate that preincubation of astrocytes with HDL for 20 h makes cells more resistant to the Cu^{2+} oxidative effect (58). It has become clear that HDL has the potential to limit oxidative modification of low density lipoprotein (LDL) whether induced by transition metals or by cells in tissue culture. HDL is the major carrier of cholesteryl ester hydroperoxides, but more than this it appears to have the prolonged capacity to decrease the total amount of lipid peroxides generated on LDL during oxidation, while the quantity accumulating on HDL itself reaches an early plateau. Several enzymes are present on HDL, such as paraoxonase, lecithin-cholesterol acyl transferase, platelet activating factor acetylhydrolase, phospholipase D and protease. Apolipoproteins, such as apolipoprotein AI, could also have enzymic activity. An evidence suggests that some of these might act to metabolize lipid peroxidation products, such as oxidized phospholipids

and lyso-phosphatidylcholine and produce beneficial effect (59).

CONCLUSION

The finding from *in vivo* model indicates the lipid peroxidation induction potential of cyclophosphamide/flutamide, which may be related to its toxic potential. The results also suggest the antiperoxidative effects of morin and demonstrate its potential to reduce cyclophosphamide/flutamide-induced lipid peroxidation and thus to increase therapeutic index of the drug by way of reducing toxicity that may be mediated through free radical mechanisms. It is also observed that cyclophosphamide/flutamide also has the ability to change the cholesterol profile and morin has a protective effect on these changes. However, a detailed study of total lipid profile is required in this regard.

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