Genotoxic and antigenotoxic activity of Silymarin by an in vivo Bone marrow micronucleus assay and Comet assay

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ABSTRACT

Introduction: Silymarin is obtained from Silybum marianum (milk thistle), an edible plant that has long been used medicinally for the treatment of liver-related disorders. Silymarin is a powerful hepatoprotective and antioxidant but the anticlastogenic activity, which is an important aspect of its cancer chemoprevention is not known; hence the present investigation was carried out to study its anticlastogenic and DNA protective activity in Balb/c Mouse.

Material & Methods: After silymarin injections, the mice were administered either cyclophosphamide (CP) or mitomycin C (MMC) intraperitoneally and were sacrificed at 24 hrs, 48 hrs and 72 hrs after the last dose. In vivo bone marrow micronucleus assay and Comet assay in whole blood were performed.

Results: Silymarin produced an increased number of micronucleated polychromatic erythrocytes in in vivo bone marrow micronucleus assay. Similarly it showed anticlastogenic activity against mutagens cyclophosphamide (CP) and mitomycin C (MMC) and caused significant reduction in the number of micronucleated polychromatic erythrocytes. In comet assay, it produced DNA damage, however silymarin showed DNA protective action against mutagens CP and MMC.

Conclusion: Silymarin has chemopreventive potentials against CP and MMC induced clastogenicity in Balb/c mice. Ironically, it has also been found to induce clastogenicity and DNA damaging effects. Hence, large-scale investigations are essential to confirm the clastogenic effect before continuing the use of silymarin for therapeutic purposes.

Key words: Silymarin, Clastogens, Micronucleus Tests , Bone Marrow, Comet Assay

INTRODUCTION

Modern life exposes humans constantly to a large number of chemical, physical and biological agents either through pollution in the environment or therapeutic agent. These agents can therefore interact in many ways with the human organism and as a consequence act in a beneficial, neutral or harmful manner. Health problems and their solutions as a result of these agents interaction have stirred interest among the scientific community (1).

Natural products have been traditionally accepted as remedies due to popular belief that they present minor adverse effects. Therefore, understanding their beneficial potentials or adverse influences is important in implementing safety measures for public health. Now-a-days beneficial aspect of dietary constituents of plant origin is being extensively studied (2).

Silymarin is a plant flavonoid extracted from the fruits and seeds of the milk thistle Silybum marianum. It consists of a mixture of mainly three flavonoids

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namely silibinin, silydianin and silychristin. The main chemical difference between silymarin and other flavonoids is that its isomers are substituted by a coniferyl alcohol group. Of the three isomers that constitute silymarin, silibinin is the most active. From a medical point of view, silymarin and silibinin have been found to provide cytoprotection and hepatoprotection (3)

Silymarin is used for the treatment of numerous liver disorders characterised by degenerative necrosis and functional impairment. In various in vivo studies in rats, silymarin has shown to have hepatoprotective and antioxidant activities (4), modulate the cellular immunoresponse (5), anti inflammatory and antifibrotic actions (6), hypocholesteremic effect (7), cardioprotective effect (8), antidiabetic effect (9), neuro protective effect on foetal rat's brain and liver (10) and anti nephrotoxic effect (11). Silymarin also produces in vitro antiangiogenic effect in colon cancer Lovo cell line (12) and DNA protective effect (13). Recently we found that silymarin has antimutagenic activity in Ames bacterial reverse mutation assay (14); however, the anticlastogenic activity, which is an important aspect of cancer chemoprevention, of silymarin, is not known.

Therefore, the present investigation was carried out with an aim to study the anticlastogenic activity using in vivo bone marrow micronucleus assay and comet assay in whole blood.

MATERIALS AND METHODS
Chemicals

Silymarin (M/s. Natural Remedies Pvt. Ltd., Bangalore, India); Cyclophosphamide (CAS No.: 50-18-0), Mitomycin-C (CAS No.: 50-07-7), Low melting point agarose, Disodium EDTA, TRIS, Ethidium bromide, TritonX100 (Sigma, USA); Giemsa (Fisher, India); May Grunwald stain and fetal bovine serum (FBS) (Himedia, India) and Dulbecco's PBS (Ca2+ and Mg2+ free) (Gibco BRL,

USA). Other common chemicals used in this study were of analytical grade and were purchased locally.
In vivo rodent bone marrow micronucleus assay

Inbred female Balb/c mice, 7- 9 weeks old weighing around 25-35 g obtained from Laboratory Animal Medicine, TANUVAS, Madhavaram Milk Colony, Chennai – 51, India were used for the study. The animals were housed in polypropylene cages at a population density of five per cage. Animals were acclimatized for two weeks at Centralized Laboratory Animal House, Madras Veterinary College, under controlled environmental conditions of temperature (22 3 C) with normal light and dark cycle. Commercial standard pellet diet and water were provided ad libitum.
Clastogenicity and Anticlastogenicity assay

Clastogenicity study was conducted for assessing the cytotoxicity and clastogenicity of silymarin with different doses and formulation was administered intraperitoneally for 7 days. In case of anticlastogenicity assay, one hour after the last dose of silymarin mice were administered either cyclophosphamide (CP) or mitomycin C (MMC) intraperitoneally at the dose of 50 and 4 mg/kg, respectively. However either CP or MMC alone were administered separately for the positive control. Animals were sacrificed by cervical decapitation at 24 hrs, 48 hrs and 72 hrs after the last dose and both femurs from each animal were removed, cleaned of adherent tissue and the ends removed from the shanks. Bone marrow was flushed from the marrow cavity with approximately 3 ml of foetal bovine serum (FBS) into labelled centrifuge tubes using a syringe and needle.

The bone marrow cell suspension was centrifuged at 1500 rpm for 5 minutes; the supernatant was aspirated to leave one or two drops and the cell pellet. The pellet was mixed in a small volume of serum and from each tube one drop of suspension was placed at the end of each of slides. Smear was made from a drop by drawing the end of a clean slide along the labelled slide. All

the slides were allowed to air dry and then fixed for 3 minutes in absolute methanol before being stained. Air-dried slides were stained with May- Grunwald stain for 4 minutes and rinsed in distilled water for 2 minutes. Immediately after the slides were stained with 10% Giemsa stain for 9 minutes and rinsed in distilled water for 2 minutes. After staining, slides were air-dried and mounted with DPX (Distrene-80 Plasticizer and Xylene) before microscopic examination.

Evaluation of the slides was performed using microscope with 100x oil immersion objectives. Slides were coded and evaluated for the ratio of polychromatic erythrocytes (PCE) to Total erythrocytes (TE) and minimum 2000 polychromatic erythrocytes were counted for micronucleated PCE from each animal.

Comet assay (alkaline single cell gel electrophoresis)

In comet assay the same rodent groups utilized for the micronucleus test were used. All the study procedures were the same for control and treated animals to avoid difference between the groups. The groups were sacrificed on the same day. Before conducting DNA protective effect with the test substance, it is customary to do the DNA damaging activity of the compound. The test substance silymarin was used at three different doses (125, 250, 500 mg/kg BW) for assessing the DNA damage and its protective activity. Blood sample was collected from the orbital sinus of each animal at 24, 48 and 72 and comet assay was performed (15). Cell viability determined by the "Trypan Blue Exclusion Technique" (16) ranged from 92 to 96%.

Slides were prepared in duplicate per sample. Fully frosted microscopic slides were covered with 140 µL of 0.75 % regular-melting agarose (40 – 42℃). After application of a coverslip, the slides were allowed to gel at 4℃ for 10 min. Meanwhile, 20 µL of whole blood was then added to 0.5 % of 110 µL of LMA (37℃). After carefully removing the coverslips, a second layer of 110 µL of sample mixture was pipetted out on the precoated slides and allowed to

solidify at 4℃ for 10 min. The coverslips were removed and a third layer of 110 µL of LMA was pipetted out on the slides and allowed to solidify at 4℃ for 10 min. The slides (without coverslips) were immersed in freshly prepared, cold lysing solution [2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–HCl (pH 10), 1 % Triton X-100 and 10 % dimethyl sulfoxide (DMSO), DMSO added just before use] and refrigerated overnight.

Slides were then placed in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min to allow unwinding of the DNA to occur. Electrophoresis was conducted for 25 min at 25 V adjusted to 300 mA by raising or lowering the buffer level in the tank. Slides were then drained, placed on a tray and washed slowly with three changes of 5 min each of neutralization buffer (0.4 M Tris–HCl, pH 7.5). DNA was precipitated and slides were dehydrated in absolute methanol for 10 min and were left at room temperature to dry. The whole procedure was carried out in dim light to minimize artifactual DNA damage.

Slides were stained with 50 µL of ethidium bromide (20 mg/mL) and viewed under a fluorescence microscope. Analysis was performed using a 400 X objective with a Leica optiphase microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. A total of 100 individual cells were screened per sample (duplicate, each with 50 cells). Undamaged cells resemble an intact nucleus without a tail and damaged cell has the appearance of a comet. The length of the DNA migrated in the comet tail, which is an estimate of DNA damage was measured using an ocular micrometer and ranking was assigned based on the length of damage as 0, 1, 2 and 3.

Per cent Inhibition

The calculation of per cent inhibition for all experiments was done as the formula given below (17).

Percent Inhibition (P.I.) = $(1-T / M) \times 100$.

Where, T is the number of MNPCE/DNA damage

in the presence of clastogen and the test sample and M is the number of MNPCE/DNA damage in the clastogen treatment.

Statistical analysis

The results are presented as the mean and standard deviation. The data were further analysed for statistical significance using one way analysis of variance and the difference among means were compared by Dunnet's multiple comparison test to determine means of the treatments differed significantly from the positive mutagenic control using statistical package, SPSS Version 10.

RESULTS

In vivo rodent bone marrow micronucleus assay

The results obtained with three doses of silymarin in clastogenicity assay and for anticlastogenic effect against CP and MMC in Balb/c mice are furnished in Table 1 and 2.

A significant difference between treated vs vehicle control animals was observed for silymarin at different dose levels. It was observed that silymarin produced significant clastogenic effect in a dose dependent manner in all the three doses. However no cytotoxicity was observed at any dose of silymarin. The two

Table 1: Clastogenic effect of silymarin using in vivo rodent bone marrow micronucleus assay

Group	Dose (mg/kg)	24 hrs			48 hrs			72 hrs		
		MNPCE (Mean ± SD)		P/E ratio	MNPCE (Mean ± SD)		P/E ratio	MNPCE (Mean SD)		P/E Ratio
		No	%		No	%		No	%	
VC	–	4.50±0.65	0.45±0.06	0.39±0.02	6.00±0.91	0.60±0.09	0.40±0.18	4.75±0.85	0.48±0.09	0.40±0.20
S	125	22.50±2.53	2.25±0.25**	0.42±0.01	24.00±1.87	2.40±0.19**	0.45±0.24	21.00±2.72	2.05±0.27**	0.42±0.21
S	250	28.50±1.44	2.85±0.14**	0.42±0.01	27.50±1.71	2.75±0.17**	0.39±0.20	24.00±2.48	2.40±0.25**	0.37±0.18
S	500	30.50±0.96	3.05±0.10**	0.38±0.01	30.75±1.75	3.08±0.18**	0.43±0.21	30.25±1.11	3.02±0.11**	0.42±0.16

VC - Vehicle control, S – Silymarin, ** - P<0.01

Table 2: Anticlastogenic effect of silymarin using in vivo rodent bone marrow micro nucleus assay

Group	Dose (mg/kg)	24 hrs				48 hrs				72 hrs			
		MNPCE		P/E ratio	PI	MNPCE		P/E ratio	PI	MNPCE		P/E Ratio	PI
		No	%			No	%			No	%		
VC	–	5.5±1.19	0.55±0.12	0.41±0.24	–	5.25±0.63	0.53±0.06	0.42±0.2	–	6.25±0.75	0.63±0.08	0.38±0.2	–
S+CP	125+50	46.75±2.50	4.65±0.25	0.45±0.20	27.69**	44.50±2.64	4.50±0.26	0.44±0.24	28.57**	48.50±1.71	4.85±0.17	0.42±0.21	24.62**
S+CP	250+50	45.50±1.32	4.55±0.13	0.43±0.22	29.23**	43.75±1.65	4.37±0.17	0.47±0.22	30.16**	47.50±1.04	4.75±0.10	0.40±0.20	26.15**
S+CP	500+50	43.50±1.71	4.35±0.17	0.44±0.21	32.31**	42.00±2.16	4.20±0.22	0.45±0.24	33.33**	43.50±1.55	4.35±0.16	0.40±0.23	32.31**
S+MMC	125+4	36.25±2.17	3.63±0.22	0.40±0.16	28.00**	44.50±3.30	4.45±0.33	0.44±0.22	16.67 NS	40.00±4.06	4.00±0.41	0.38±0.19	37.5**
S+MMC	250+4	33.75±1.65	3.38±0.17	0.43±0.21	32.00**	35.75±3.71	3.58±0.37	0.44±0.21	33.33**	36.25±1.75	3.63**±0.18	0.35±0.16	43.75**
S+MMC	500+4	30.75±1.85	3.08±0.18	0.37±0.20	38.00**	33.75±2.32	3.33±0.23	0.41±0.19	37.04**	37.50±1.70	3.68±0.17	0.41±0.21	42.19**
CP	50	65.00±3.30	6.50±0.33	0.41±0.20	–	63.00±2.60	6.30±0.26	0.42±0.19	–	65.00±3.40	6.50±0.34	0.45±0.21	–
MMC	4	50.00±1.90	5.00±0.19	0.42±0.20	–	53.75±2.53	5.38±0.25	0.41±0.23	–	63.50±3.12	6.35±0.31	0.41±0.2	–

CP – Cyclophosphamide, MMC – Mitomycin C, VC – Vehicle control, S – Silymarin, ** - P<0.01

anticancerous drugs induced statistically significant increases in micronuclei (MN). There was no significant variation of inhibition by silymarin at different dose levels against CP and MMC. The PI of silymarin against the MMC was slightly higher in animals

sacrificed at 72 hrs after dose administration.

Comet assay (alkaline single cell gel electrophoresis)

The results of DNA damaging and DNA protective effect of silymarin are presented in Table 3 and 4. It was observed that significant DNA damaging

Table 3: DNA damaging activity of silymarin in whole blood of mice using comet assay

Group	Dose (mg/kg)	24 hrs					48 hrs					72 hrs				
		Comet class				Scores (mean±SD)	Comet Class				Scores (mean±SD)	Comet class				Scores (mean±SD)
		0	1	2	3		0	1	2	3		0	1	2	3	
VC	-	22.25	1.50	1.25	-	4.00 1.08	23.25	0.50	1.00	0.25	3.25 0.48	23.25	0.50	1.00	0.25	3.25 0.75
S	125	3.25	0.50	18.50	2.75	45.75** 1.97	1.50	1.50	16.00	6.00	51.50** 3.86	1.75	0.75	17.25	5.25	51.00** 0.71
S	250	2.00	-	19.00	4.00	50.00** 1.63	2.25	-	19.25	3.50	49.00** 1.78	0.25	1.00	19.75	4.00	52.50** 1.32
S	500	1.75	0.75	18.50	4.00	49.75** 0.75	1.75	-	19.00	4.25	50.75** 2.10	0.75	0.75	19.50	4.00	51.75** 1.80

VC - Vehicle control, S – Silymarin, ** - P<0.01

Table 4: DNA damaging activity of silymarin in whole blood of mice using comet assay

Group	Dose (mg/kg)	24 hrs						48 hrs						72 hrs					
		Comet class				Scores (mean SD)	PI	Comet Class				Scores (mean SD)	PI	Comet class				Scores (mean SD)	PI
		0	1	2	3			0	1	2	3			0	1	2	3		
VC	-	23.50	-	1.50	-	3.00±1.29	-	23.25	0.25	1.25	0.25	3.5.00±1.32	-	22.50	1.00	1.25	0.25	4.25±1.11	-
S+CP	125+50	0.50	1.00	21.25	2.25	50.25**±1.80	31.51	-	1.00	20.75	3.25	52.25**±0.85	28.77	0.25	1.25	19.75	3.75	52.00**±1.08	28.77
S+CP	250+50	-	1.50	20.25	3.25	51.75**±0.95	28.77	-	2.50	20.25	2.25	49.75**±0.48	31.51	0.75	-	20.25	4.00	52.50**±1.44	27.40
S+CP	500+50	-	1.50	18.25	5.25	53.75**±1.11	26.03	0.75	0.75	18.00	5.50	53.25**±1.25	27.40	0.75	3.00	17.75	3.50	51.50**±1.73	32.88
S+MMC	125+4	4.75	2.25	12.00	6.00	44.25**±2.66	27.78	-	0.50	22.00	2.50	52.00**±0.41	38.89	-	2.25	14.75	8.00	55.75**±1.03	18.84
S+MMC	250+4	1.25	2.00	19.00	2.75	48.25**±1.93	22.22	-	1.00	17.00	7.00	56.00**±0.71	33.33	0.75	11.00	8.50	4.75	42.25**±2.29	39.13
S+MMC	500+4	-	-	18.75	6.25	56.25**±1.18	23.61	-	1.00	17.75	6.25	55.25**±1.6	22.22	1.00	17.50	2.50	4.00	34.50**±1.26	49.28
CP	50	0.25	-	1.25	23.50	73.00±0.71	-	-	0.50	1.25	23.25	72.75±0.48	-	-	0.50	1.00	23.50	5.25±1.25	-
MMC	4	-	-	3.50	21.50	71.50±0.65	-	-	0.75	1.25	23.00	72.25±0.48	-	-	-	6.00	19.00	69.00±1.08	-

CP – Cyclophosphamide, MMC – Mitomycin C, VC - Vehicle control, S – Silymarin, ** - P<0.01

effect was exhibited by silymarin at all the three doses tested. The protective effect studied at 24 hrs after silymarin had a maximum Percent Inhibition (PI) exhibited at 125 mg/kg dose. However, at 48 hrs after silymarin a maximum PI was observed at 250 mg/kg with CP and at 125 mg/kg with MMC. To the contrary, maximum PI of 32.88 and 49.28 were observed with 500 mg/kg dose in the observation made at 72 hrs after silymarin with CP and MMC respectively.

DISCUSSION

A considerable emphasis is being laid down on the use of dietary substances as chemo-protective measures for controling genetic diseases. Bone marrow cytogenetics and single cell analysis are useful fast techniques, for elucidating mechanism as well as to assess substances for their clastogenic and anticlastogenic activity (18). The majority of the mutagenic/carcinogenic compounds e.g. polycyclic

aromatic hydrocarbons act by generating electrophillic intermediates by microsomal enzymatic reactions causing mutations (19, 20). Antigenotoxic agents , especially those present in natural substances, act through different cellular pathways involving endogenous sequestration of mutagens by various enzymes (21).

In the present study, silymarin produced a significant clastogenic activity in rodent bone marrow micronucleus assay. Similar results have been observed with 5, 3', 4' – trihydroxy – 3, 6, 7, 8 – tetra methoxy flavone which induces significant increment of PCEs carrying MN suggestive of clastogenic activity (23). Silymarin being a flavonoid has also produced clastogenic activity in rodent bone marrow micronucleus assay. Similarly we have also previously reported that, silymarin showed significant mutagenicity in frame shift mutant strains (TA97a and TA98) with metabolic activation (14).

Comet assay is potentially useful as an in vivo genotoxicity test to investigate target organs as well as 'first – site – of – contact' genotoxicity. This assay is useful to assess the in vivo genotoxic potential of in vitro genotoxic compounds at the site of their deleterious action (24). In the present study, silymarin showed significant DNA damaging activity. In a similar study, Flavonoids i.e., quercetin, myricetin and silymarin all increased the frequency of DNA breaks in HeLa cells, quercetin being the most effective at lower concentrations, whereas myricetin and silymarin only began to show genotoxicity at greater concentrations. It has also been suggested that flavonoids may exert their DNA damaging activity in a metal catalyzed oxidation with the subsequent generation of oxygen species such as H_2O_2 and the hydroxyl radical (25). Silymarin showed DNA protective effect against CP and MMC induced DNA damage in the present study. In a similar study, it has been reported that co-administration of silymarin significantly decreased the DNA damage when compared with ethanol induced DNA damage in rats. It has also been suggested by the authors that the protective effect of silymarin can be explained by the scavenging of free radicals before they cause DNA damage (13). Furthermore silymarin has shown the ability to protect the DNA against reactive oxygen species induced damage (26).

The present study showed that silymarin exhibited both DNA damaging and DNA protective activity against CP and MMC at the same doses. In a similar study, rutin displayed both DNA damaging and DNA protective effect against MMC at same concentration in which the drug was being tested (27) and Syzigium cordatum was itself slightly genotoxic at 500 μg/mL, but also protected against MMC genotoxicity (28).

Citral protected mouse bone marrow cells by reducing the micronucleus against CP and MMC in a time related manner. It was also suggested that citral could exhibit an antioxidant activity to attenuate the nuclear damage induced by the clastogen (29). Similar results were obtained in the present study with silymarin. Similarly the antioxidant action of silymarin may be responsible for its anticlastogenic activity observed in the present study.

CONCLUSION

During the in vivo bone marrow micronucleus assay, silymarin showed increased incidence of MNPCEs. But the compound also reduced the number of MNPCEs against CP and MMC induced micronucleus damage in mice. Silymarin showed significant DNA damaging activity when tested using comet assay, while it showed significant DNA protective action against CP and MMC induced DNA damage in blood cells of Balb/c mice. From the observations, it may be concluded that silymarin obtained from Silybum marianum, which is being used as a hepato-protective agent possesses anticlastogentic and DNA protective properties. Ironically it has also been found to induce

clastogenicity and DNA damaging effects. Hence, large-scale investigations are essential to confirm the clastogenic effect before continuing the use of silymarin for therapeutic purposes.

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