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Bioassays Application for Mutagenicity and Cytotoxicity Evaluation of Medicinal Plant having Considerable Antioxidant Potential

ABID ALI[1,*], KHALIL-UR-RAHMAN[1], MUHAMMAD SHAHID[1] and MUHAMMAD ARSHAD[2]

[1]Department of Biochemistry, University of Agriculture, Faisalabad-38040, Pakistan
[2]Institute of Microbiology, University of Agriculture, Faisalabad-38040, Pakistan

*Corresponding author: E-mail: bosalvee@yahoo.com

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The main focus of the present study was to explore antioxidant and toxicological profile of *Silybum marianum.* The antioxidant activity of methanolic extract of *S. marianum* was assayed using the diphenyl-picrylhydrazine (DPPH) radical scavenging and H_2O_2 induced DNA protection assay. The cytotoxicity of the plant extract was evaluated by hemolytic, brine shrimp lethality and mutagenic activity by Ames test. The percentage inhibition for DPPH ranged from 51 to 76 at concentration 10 and 100 mg/mL. The methanolic extract exhibited DNA protection on concentration dependent manner and complete protective effect was noted at concentration 10 µL/mL. Toxicological results showed that *S. marianum* was potent against hemolytic, brine shrimps and non-mutagenic against standard mutagens that indicated the presence of some bioactive components in the plant and possessed antioxidant activity that protects DNA against H_2O_2 induced oxidative damage. Thus, the results showed that *S. marianum* exhibited significant bioactive components with least side effects. The present investigation will provide better scientific basis for future pharmacological evaluation due to its novel nature. *In vivo* studies and identification of novel source of pure bioactive compounds will be performed in future studies.

Keywords: *Silybum marianum,* Cytotoxicity, Mutagenicity, Antioxidant, Oxidative damage.

INTRODUCTION

Medicinal plants are rich in secondary metabolites and important potential source of drugs to treat variety of diseases[1,2]. Plant secondary metabolites exert a variety of components having pharmacological actions and therapeutic applications. The phenolic compounds have antioxidant potential that inhibits free radical mediated processes and provide protection against reactive oxygen species[3,4]. Natural and plant based drugs report rare side effects and least data is accordingly available on electronic system[5]. *Silybum marianum* (Milk thistle) is a member of the Asteraceae family with a long phytotherapic history as hepatoprotective[6]. Silymarin is a polyphenolic flavonoid, extracted from fruits and seeds of *S. marianum* consist of a mixture of three structural isomers: silycristin, silydianin and the most active component silibinin[7]. Oxidative damage produced by free radicals may be linked to aging and disorders, such as diabetes, atherosclerosis, cancer and cirrhosis[8]. Almost all organisms have antioxidant defense systems and DNA repair mechanisms. These systems protect against oxidative damage, while this protection is insufficient to prevent the damage entirely[9]. At present, undesirable side effects or loss of efficacy in the long run are the major limitations of existing medication[10]. The bioassays have been used successfully for the toxic potential of medicinal plant as well for the evaluation of environmental quality[11-16]. The need of the time is to establish the safety of *S. marianum* as it is in use to treat various diseases, therefore our work aimed to figure out the toxic potential of this plant using various *in vitro* and *in vivo* standard bioassys.

EXPERIMENTAL

Plant extract: *Silybum marianum* was grown at Postgraduate Agriculture Research Station, University of Agriculture Faisalabad, Pakistan and seeds were cultivated. The plant material was authenticated by a plant taxonomist and voucher specimen (630-14-1) was submitted to the herbarium/ collection at Department of Botany, University of Agriculture Faisalabad, Pakistan. The plant material was cleaned, powdered and macerated in methanol for one week in an orbital shaker. The extract was filtered and concentrates under reduced pressure on rotary evaporator.

DPPH free radical scavenging assay: For the determination of % inhibition DPPH free radical scavenging assay method described by Ho *et al.*[17] was used with some modifications.

The % inhibition was calculated from the plot of the regression equation against percentage scavenging and concentrations of samples used. Three replicates were recorded for each sample.

Antioxidant activity by DNA protection assay: Antioxidant activity of the methanol extract by DNA protection assay was determined[12]. The pUC19 DNA (0.5 µg/µL) was diluted up to two-fold (0.5 µg/3µL) in 50 mM sodium phosphate buffer (pH 7.4) and 3 µL of diluted DNA was treated with 5 µL of the test sample. After this 30 % H_2O_2 (4 µL) was added in the presence and absence of different volumes (1000, 100 and 10 µL) of *S. marianum* extract; final volume was made up to 15 µL with sodium phosphate buffer (pH 7.4). The relative difference in migration between the native and oxidized DNA was then examined on 1 % agarose using horizontal DNA gel electrophoresis (BioRad wide mini system). The gel was documented using gel documentation and analysis system (Syngene, UK).

Cytotoxicity studies: The cytotoxicity was tested through standard procedures such as brine shrimps lethality, hemolytic activity and mutagenic activity of the plant extract[18-20].

Statistical analysis: Statistical analysis was done using statistical software and expressed as means ± SEM. Values of $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

The extract of *S. marianum* seeds was able to reduce the stable radical DPPH to the yellow coloured DPPH. In this study, the extract exhibited a concentration dependent antiradical activity (percentage inhibition). Highest DPPH antioxidant activity of *S. marianum* extract in term of percentage inhibition was 76.23 at 100 mg/mL concentration that designates strong radical-scavenging capacity (Fig. 1).

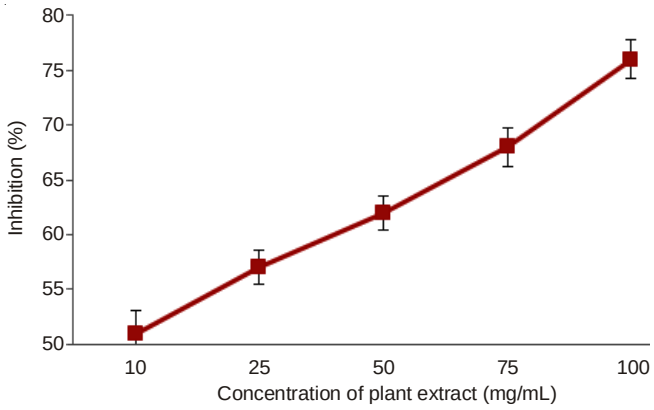

Fig. 1. Percentage inhibition of *S. marianum* (seeds) at different concentration by DPPH

Protective effect of H_2O_2 induced oxidative damage on pUC19 DNA: The antioxidant potential of *Silybum marianum* extract was evaluated by DNA protection assay. Three different concentrations (10 µL/mL, 100 µL/mL and 1000 µL/mL) were made and tested in DNA protection assay. Hydrogen peroxide was used as source of free radical. Its effect was studied on supercoiled pUC19 (Fig. 2) is the representation of DNA in the presence and absence of different concentrations of *S. marianum.* DNA remained intact in its supercoiled form without any kind of treatment (lane 1). The hydroxyl radical of H_2O_2 damaged the pUC19 DNA and resulted in streaking band due to strand cleavage (lane 2). *S. marianum* at 10 µL/mL concentration showed complete protective effect (lane 5) because in the presence of H_2O_2 there were no sign of degradation in DNA. As the concentration of plants extract increased its protective effect gradually decreased and moderate protective effect was observed at 100 µL/mL and poor at 1000 µL/mL concentrations (lane 6-7) because disruption in DNA was observed on both 100 and 1000 µL/mL concentrations.

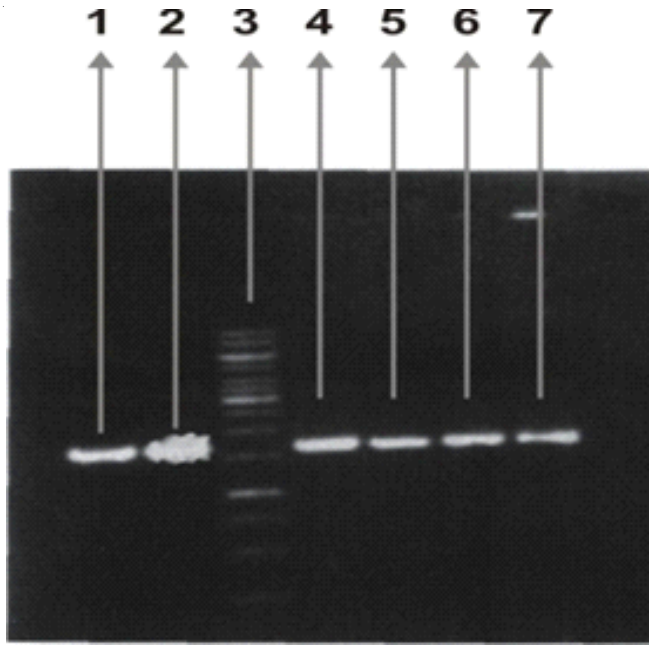

Fig. 2. DNA protection pattern of pUC19 plasmid DNA treated with 30 mM H_2O_2 in the presence and absence of different concentrations of plants extracts on agarose gel electrophoresis; Lane 1: Normal pUC19 DNA, Lane 2: pUC19 DNA + 30 mM H_2O_2, Lane 3: 1 kb Ladder, Lane 4: pUC19 DNA + P_1 (*S. marianum*) [100 µL/mL], Lane 5: pUC19 DNA+ 30 mM H_2O_2+ P_1 (*S. marianum*) [10 µL/mL], Lane 6: pUC19 DNA + 30 mM H_2O_2+ P_1 (*S. marianum*) [100 µL/mL], Lane 7: pUC19 DNA + 30 mM H_2O_2 + P_1 (*S. marianum*) [1000 µL/mL]

Hemolytic activity: Hemolytic activity of the methanolic extract of *Silybum marianum* was screened against normal human erythrocytes using triton X-100 as positive control. Hemolytic activity of the plant is expressed in percentage hemolysis and reported as mean ± standard deviation. The percentage hemolysis with *Silybum marianum* at different concentrations was 1 ± 0.84, 3 ± 0.27, 4 ± 0.36 and 6 ± 0.18 at 10, 25, 50 and 100 mg/mL respectively. The hemolytic activity showed that *Silybum marianum* is more potent and safe for use as medicine.

Brine shrimp lethality: The toxicity of *S. marianum* extract was evaluated using brine shrimps lethality test. Each tube contained 15 larvae of brine shrimp and volume was made up to 5 mL with artificial sea water. Live larvae were counted after 24 h and the percentage mortality was calculated (Table-1). The percentage mortality was concentration dependent, lowest percentage mortality was 10 and highest percentage mortality was 17 at concentration 10 mg and 100 mg/mL respectively.

TABLE-1
BRINE SHRIMP LETHALITY OUTCOMES SHOWING SURVIVED SHRIMPS AND % MORTALITY TREATED WITH EXTRACT OF *S. marianum*

Fraction (mg/mL)	Number of survived shrimps in following intervals (h)									Mortality (%)
	Initial	3	6	9	12	15	18	21	24	
Control	20	20±0	20±0	20±0	20±0	20±0	20±0	20±0	20±0	No activity
10	20	20±0	19.5±0.5	19.5±0.5	19±0	19±0	18.5±0.5	18.5±0.5	18±0	10
25	20	20±0	19±0	19±0	18.5±0.5	18.5±0.5	18±0	17.5±0.5	17.5±0.5	12
50	20	20±0	19.5±0.5	19.5±0.5	19±0	19±0	18±0	17±0	17±0	15
100	20	20±0	19±0	19±0	18±0	18±0	17±0	17±0	16.5±0.5	17

Mutagenic activity: Mutagenic activity was performed by using test strains *S. typhimurium* TA98 and *S. typhimurium* TA98. In standard of both *S. typhimurium* TA98 and TA 100 showed significant increase in number of positive wells. The numbers of positive wells in background of *S. typhimurium* TA98 were 21/96 and 13/96 in *S. typhimurium* TA100. The plant extract was non mutagenic and toxic to test strains (Table-2).

In DPPH assay the antioxidants interact with the stable free radical, *i.e.*, 1,1-diphenyl-2-picrylhydrazyl. The DPPH values in aqueous extract were found slightly different in another study whereas almost similar radical scavenging activity was observed in methanol extract of *S. marianum*[21]. Present study clearly indicates that the extract of *S. marianum* is capable of inhibiting lipid peroxidation thus shield humans against infection and degenerative diseases. Potential of the plant to protect DNA can be manifested in anticancerous drug development. Plants and vegetables phenolic compounds are potential antioxidants proved in many studies[22]. Previous studies have shown that medicinal plants exhibit concentration dependent antioxidant ability. Free radicals can damage cellular and genetic material by disrupting DNA another bio macromolecule. Molecular studies have revealed that reactive oxygen species damaged sugar moiety of nucleotides in DNA, hence either it alters nucleotides or break the strand. If these changes remain unrepaired, they not only disturb the cell structure and alter the functionality. Medicinal plants have the ability to protect DNA by scavenging ROS and this attribute is due to their antioxidant potential[23].

In present study, H_2O_2 generated free radicals which damage DNA whereas in previous studies e-radiation and UV photolysis had been used for damaging DNA *in vitro*. No matter whatever the way was adopted for damaging DNA, it was helpful in studying protective effect of different concentrations of plant extracts. Hesperidin protected mice against DNA damage which was induced in bone marrow cells by exposing to various intensities of e-radiation[24]. Similarly, in this study protective effect of *S. marianum* extract is recorded when DNA damage was caused by ROS generated by H_2O_2. The aqueous and methanolic extracts of *Rheum emodi* were compared for DNA protection assay, aqueous extract displayed significant protective effect against DNA damage caused by UV photolysis as well as H_2O_2 treatment on pBR322. Their study revealed that 50 µg of aqueous extract of *Rheum emodi* partially protected entirely obliterated DNA[25]. Similarly in present study H_2O_2 induced DNA damage was protected by extract of *S. marianum* by inhibiting lipid peroxidation. The possible mechanism which can be explaining this effect of DNA protection could be the prevention of hydroxyl radicals to attack lipids by scavenging the free radicals. Hemolytic activity was studied because even if the plant possesses therapeutic properties its use in medicine will not be possible until its safety is established. Hemolytic activity could be used as a primary tool for studying the toxicity of plant extract as it provides primary information on the interaction between molecules and biological entities at cellular level. The hemolytic activity was higher in another plant species *Aerva lanata* than *S. marianu*[26]. Hemolytic activity of different solvent extracts of *Allium stracheyi* was high towards human red blood cells[27]. Chloroform and aqueous extract of leaves of *Acanthus ilicifolius* were reported to possess significant hemolytic activity towards the chick red blood cells[28]. Antimutagenicity assays of liquid *Ambar orientalis* using TA98 and TA100 strains was performed at 2.5, 0.25 and 0.025 mg/plate concentrations and found strongest antimutagenic activity at 2.5 mg/plate concentration against *S. typhimurium* TA 98 strain, only one concentration (0.025 mg/plate) of the extract did not exhibit any antimutagenic effect against *S. typhimurium* TA 100. The results show that extract had a better antimutagenic effect (37.7–85.67 %) on the TA98 strain. It was observed that antimutagenic activity was dose dependent[29]. Mutagenic activity of medicinal plants was studied against various disorders by Venda people and found that all the plant extracts were non-mutagenic towards *Salmonella typhimurium* strain TA98 except for *Elephantorrhiza burkei* and *Ekebergia capensis* that showed weak mutagenicity. *Ekebergia capensis* bark induced 50 revertant colonies at 500 mg/mL and 50 mg/mL. The mutagenic activity was not dose dependent[30]. The freshly collected *Acokanthera oppositifolia* plant extracts at 5 mg/mL exhibited mutagenic effects against TA1535 strain against three bacterial strains[31]. Whereas researcher found that mutagenic potential observed with

TABLE-2
MUTAGENIC ACTIVITY OF METHANOLIC EXTRACT OF *S. marianum*

Plant	Number of positive wells/Total number of wells		Interpretation
	S. typhimurium TA98	*S. typhimurium* TA100	
Background	21/96	13/96	
Standard ($K_2Cr_2O_7$)	96/96	88/96	Mutagenic$^+$
Silybum marianum (Methanolic extract)	0/96	0/96	Non-mutagenic$^-$

$^+$Significant increase in the number of positive wells compared to the related control ($p < 0.05$); (–), no significant effect observed.

TA1535 strain is associated with the substitution of leucine with proline in the bacterial genome[32]. Mutagenic and anti-mutagenic activities of aqueous and methanol extracts of *Euphorbia hirta* was studied and found that quercetin (25 g/mL) have strong mutagenic activity in *S. typhimurium* TA98. While both the methanol and aqueous extracts at concentration up to 100 g/mL in the absence and presence of S-9 metabolic activation were non-mutagen when tested with *S. typhimurium* TA98 and TA100 strains[33] since bioassays are authentic test for toxicity evaluation[34-39].

Conclusion

Silybum marianum extract showed significant antioxidant properties in terms of scavenging free radicals and thus, shield humans against infection and degenerative diseases. The H_2O_2 induced oxidative damage in plasmid pUC19 DNA was evaluated and it was found that methanolic extract of *S. marianum* protected the DNA againt stress, which may be due to the presence of phyto constituents that confirmed its antioxidant properties. The results of hemolytic, brine shrimp lethality and mutagenic assays showed that the methanolic extract of *Silybum marianum* non-toxic, hence *S. marianum* can be used as antioxidant natural source to cope with oxidative stresses. The toxicity study against different cancerous cell lines will contributes in understanding the potential of *S. marianum* for further *in vivo* studies as a source of biological active compounds.

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