

**EFFECT OF HIBISCUS ROSA SINENSIS EXTRACT ON MODIFYING
CYCLOPHOSPHAMIDE INDUCED GENOTOXICITY AND SCAVENGING
FREE RADICALS IN SWISS ALBINO MICE.**

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Summary

The objective of the present study was to evaluate the anti-oxidant and antigenotoxic effects of ethanol extract of *hibiscus rosa sinensis* (HRS) flower. The potential of the extract to scavenge the free radicals and inhibit lipid peroxidation invitro was assessed to study its anti oxidant property. For the genotoxic study the ethanol extract in the dose of 250mg/kg was administered orally. Single dose for acute study and multiple doses (repeated every 24 hr for 7 days) in additional group of mice (n=5) for sub acute study was administered after inducing genotoxicity with cyclophosphamide. At the end of the treatment 0.5ml of blood was collected from retro-orbital sinus for comet assay and later the mice were sacrificed to aspirate the femoral bone marrow for micronucleus test. The ethanol extract not only showed a dose dependent increase in radical scavenging ability against various free radicals but also exhibited a significant inhibition of lipid peroxidation invitro. The extract rendered significant ($p < 0.001$) protection against cyclophosphamide induced genotoxicity in both micronucleus and comet assay indicating significant anti-genotoxic effects.

Key words: Anti oxidant, comet assay, genotoxocity, hibiscus rosa sinensis, micronucleus assay.

Introduction

Life and propagation of life are determined by the information stored in the genes.^[1] However, often these genes are exposed to various environmental and pharmaceutical pollutants that posses the ability to produce unexpected and unidirectional changes in the genome. These changes if not repaired can lead to deleterious gene mutations resulting in carcinogenesis which could be due to various mechanisms. One such mechanism is generation of reactive oxygen species (ROS), viz. super oxide (O_2^-), nitric oxide (NO), hydrogen peroxide (H_2O_2) and peroxy radicals.^[2]

Mammalian cells posses a defense mechanism of scavenging these ROS by virtue of various enzymes like super oxide dismutase (SOD), catalyse (CAT) and glutathione (GPX).^[3] However, due to aging these protective anti-oxidant processes may not cop up with excess amounts of ROS produced in the body leading to various chronic diseases like rheumatoid arthritis, heart diseases, stroke, arteriosclerosis, diabetes, neurodegenerative disorders, carcinoma etc.^[4] Use of anti-oxidants therefore would help to decrease the oxidative reactions caused by ROS thereby preventing /delaying the cell damage. Therefore, the commercial development of plants as source of antioxidants to enhance health is of current interest.^{[5], [6]}

Many medicinal plants such as curcuma domestica, daucus carota, Foeniculum vulgare, Magnifera indica known to posses natural antioxidant polyphenols like anthraquinones, flavonoids, aromatic acids and tannins have been reported to posses ROS scavenging and lipid peroxidation preventing effects.^[6]

Hibiscus rosa sinensis Linn (Family: Malvaceae) is an ornamental evergreen plant with flowers widely distributed throughout India. All the parts of the plant have been used in traditional medicine viz flowers for gonorrhoea, roots for menorrhagia, petals for fever.^[7]

All the parts of the plant have also been reported to possess various pharmacological actions like flowers are reported to have anti-fertility^{[8],[9],[10],[11]} anti-ovulatory,^[12] anti-spermatogenic, androgenic,^{[13],[14]} analgesic,^[15] anti-inflammatory,^[15] wound healing^[16] and antidiabetic^[17] properties.

Hibiscus rosa sinensis contains numerous compounds like anthocyanins, flavonoids, cyclopeptide alkaloids, quercetin, vitamins which are known to possess antioxidant property.^[18] By virtue of its anti-oxidant property, on co-administration, it could be expected to minimize the genotoxic effects of anticancer drugs but, it might itself induce genotoxicity due to its chemo preventive activity.^[19] However, there is scanty information regarding its genotoxic effect and interaction with anticancer drugs. In view of scarcity of information the present study was planned to explore the anti-oxidant activity and anti-genotoxic activity of *hibiscus rosa sinensis* flower extract on cyclophosphamide induced genotoxicity in Swiss Albino mice.

Materials and methods

Plant materials

The flowers of *Hibiscus rosa sinensis* were collected from local area of Belgaum district and authenticated by Dr. Harsha Hegde, Research Officer, Regional Medical Research Centre (RMRC) Belgaum, where a herbarium of the plant is deposited.

Preparation of extract

The flowers of *Hibiscus rosa sinensis* were shade dried and powdered coarsely through mechanical grinding. The powdered material was passed through a No. 40 sieve to get uniform powder. The powder was then defatted using petroleum ether at 60-80 °C and filtered. The residue was extracted with absolute ethanol at 50 °C in a Soxhlet apparatus for 72 hours. The extract was then cooled and concentrated under reduced pressure using rotary evaporator at 20 °C. The yield of the extract was 5.0% w/w which was stored in refrigerator at -4°C till the experiment was conducted.

In vitro Antioxidant activity

The chemicals, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; i.e. ABTS), Ethylenediamine tetra acetic acid (EDTA), were obtained from Qualigens Fine Chemicals, India while, Butylated hydroxytoluene (BHT) was obtained from Merck (Germany).

Radical generation and scavenging studies:

A stock solution of 1mg/ml of ethanolic extract of *Hibiscus rosa sinensis* was prepared in deionized water and diluted to get various concentrations (100–1000 µg/ml) in a final volume of the reaction mixture. The free-radical scavenging activity of the extract was analyzed by following the various standard *in vitro* radical generating model systems viz., DPPH, ABTS, and NO. In all the experiments, deionized water served as blank and reaction mixtures without ethanolic extract served as control samples. The changes in the absorbance of the reaction mixtures were measured using a spectrophotometer (VIS-260, Shimadzu Corp., Japan) and the percentage scavenging or inhibition was calculated according to the following formula.

Percent scavenging or inhibition = $\frac{(\text{Absorbance of control} - \text{Absorbance of test}) \times 100}{\text{Absorbance of control}}$

All the experiments were performed three times and the data was expressed as Mean \pm SEM.

DPPH scavenging activity of the ethanolic extract of *Hibiscus rosasinensis*

The effect of ethanolic extract of *Hibiscus rosasinensis* on the DPPH radical was estimated *in vitro* according to the method of Hou et al.^[20]. The principle this assay is that antioxidants react with the stable DPPH radical and convert it into 1, 1-diphenyl-2-picryl hydrazine. The ability to scavenge the stable DPPH radical is measured by a decrease in the absorbance. Aliquots containing various concentrations (100–1000 $\mu\text{g/ml}$) of ethanolic extract of *Hibiscus rosa sinensis* in the final volume of 2 ml were mixed with 2 ml of 0.05 mM DPPH (in methanol). DPPH (2 ml) with equal amount of PBS served as control. Reaction mixtures were incubated at 37 °C for 20 min and the absorbance of reaction mixtures was recorded spectrophotometrically at 517 nm.

ABTS radical decolourization assay

ABTS diammonium salt radical cation decolourization test was performed *in vitro* using spectrophotometric method of Pellegrini et al.^[21] The principle of this assay is that the antioxidants react with ABTS resulting in the decolourization of the ABTS radical in aqueous phase. The ABTS stock reagent mixture was prepared by mixing 88ml of 140 mM potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) with 5ml of 7mM of ABTS stock solution (pH 7.4). The working ABTS reagent was prepared by diluting the stock solution with ethanol to give an absorbance of 0.7 ± 0.5 at 734 nm. Various concentrations of ethanolic extract of *Hibiscus rosa sinensis* (100–1000 $\mu\text{g/ml}$) in a final volume of 1ml of PBS were mixed with 1ml of the ABTS cation working solution. The ABTS working solution with equal volume of PBS served as control. The reaction mixtures were incubated at room temperature (28 °C) for 30 min and the absorbance was measured at 734 nm spectrophotometrically.

Scavenging of nitric oxide

The nitric oxide scavenging potential of HRS was determined *in vitro* according to the method of Sreejayan and Rao.^[22] with minor modifications. Briefly, 1 ml of sodium nitroprusside (5mM) in PBS containing various concentrations of ethanolic extract of *Hibiscus rosa sinensis* (100–1000 $\mu\text{g/ml}$) along with the control (sodium nitroprusside without HRS) were incubated at room temperature (28 °C) for 2.5 h, followed by the addition of 1 ml of Greiss reagent (prepared by mixing an equal volume of 1% sulfanilamide in 2% phosphoric acid with 0.1 % N- (naphthyl) ethylene diamine hydrochloride in water). The absorbance of the chromophores formed during diazotization of nitrate with sulfanilamide and the subsequent coupling with N- (1-naphthyl) ethyenediamide was read at 546 nm spectrophotometrically.

Inhibition of lipid per oxidation

Lipid per oxidation was carried out *in vitro* according to the method of Shoji et al.^[23] with minor modifications. Briefly, 10% (w/v) of mouse brain homogenate was

prepared in 150mM potassium chloride. The reaction mixture contained 300ml of brain homogenate, 100ml of 150mM potassium chloride and various concentrations of ethanolic extract of *Hibiscus rosa sinensis* (100–1000 µg/ml) in a final volume of 1ml. The reaction mixtures without ethanolic extract of *Hibiscus rosa sinensis* served as control. Peroxidation was initiated by addition of 10ml of 20mM ferrous sulfate (FeSO₄). After incubating the mixture for 20 min at 37 °C, the reaction was stopped by the addition of 1 ml of ice-cold solution of 0.25N hydrochloric acid (HCl) containing 15 % trichloroacetic acid (TCA), 0.38% thiobarbituric acid (TBA) and 0.05% BHT and heated in a water bath at 65 °C for 30min. The reaction mixtures were cooled and centrifuged at 1000 r.p.m. for 10min and absorbance of the thiobarbituric acid reactive substance (TBARS) in the supernatant was recorded spectrophotometrically at 532 nm.

In vivo Antigenotoxic studies:

Drugs and chemicals: Cyclophosphamide (Endoxan) was purchased from local market. Low melting agarose (LMA), normal melting agarose (NMA) and fetal bovine serum were obtained from HI-MEDIA, Mumbai. Histopaque 1077 was obtained from Sigma (St. Louis, MO, USA). All other chemical reagents used were of analytical grade. The dose and the route of administered of each treatment is shown in table 1.

Table 1. Various treatment groups with doses and routes of administration

Treatment groups	Doses (per Kg body weight)	Route of drug administration	Number of mice used	
			Acute Study	Sub acute study
Normal saline (negative control)	8ml	p.o.	5	5
Cyclophosphamide (positive Control)	40mg	i.p.	5	5
Cyclophosphamide + Standard anti-oxidant (Vit.E)	40mg+50IU	both i.p.	5	5
HRS extract	250mg	p.o.	5	5
Cyclophosphamide + HRS Extract	40mg+250mg	i.p.+p.o.	5	5
Standard anti-oxidant(Vitamin E)	50IU	i.p.	5	5

Animals: Sixty Swiss Albino mice of either sex, weighing 20-25g procured from central animal house of the J.N. Medical College, Belgaum were used in the experiments. Animals were on standard rodent feed (Amrut feeds, Sangli) with drinking water *ad libitum* and maintained on a 12-h light/dark cycle. In addition, mice were acclimatized for 1 wk to laboratory environment prior to the study. The study protocol was approved by the Institutional Animal Ethical Committee. The animals were divided in groups (n=5 in each) to receive one of the treatments, both in acute as well as sub acute studies. Control animals received equal volume of normal saline (NS).

Study design: Of the 12 groups, 6 were in acute study where single dose of drugs was administered and remaining 6 groups were included in sub acute study to receive treatment for 7 days (one dose every 24 h). Blood and bone marrow samples were collected 24 h after the last dose of drug.

In vivo micronucleus assay: The animals were killed by overdose of ether anesthesia and the bone marrow was collected for micronucleus assay.^[24] Both the femora were removed *in to*, by cutting through pelvis and tibia and the bones were then freed from muscle fibers by the use of gauze and fingers. By gentle traction, the distal epiphysis was torn off, together with the rest of the tibia and the surrounding muscle. The proximal end of femur was carefully shortened with scissors until a small opening to the marrow canal became visible. The bone marrow was aspirated with the syringe and needle of appropriate size by inserting it for few mm into the proximal part of the marrow canal. Then the femur was submerged completely in fetal bovine serum (5ml) contained in a centrifuge tube and subsequently, the marrow was aspirated by several gentle aspirations and flushing. This process was also repeated from the distal end of the femur and the tube was shaken, so that bone marrow cells get into the serum as a fine suspension and not settle down in the form of gross particles. The tube was centrifuged at 1000r.p.m. for 5 min to isolate the bone marrow cell as a pellet. This pellet was suspended in 1-2 drops of fetal bovine serum, and drops of this suspension were put on glass slides so as to prepare bone marrow smears. These slides were air dried and stained with undiluted May Gruenwald (MG) stain (Lobo) for 3 min followed by diluted MG stain (1: 1; with distilled water) for 2 min. Later, the slides were stained with diluted Geimsa stain (1:6; with distilled water) for 10 min. The slides were then rinsed in distilled water and backside was cleared by methanol, air dried and mounted permanently with cover slips. These slides were observed under microscope (Olympus BX 41) (oil immersion) for the presence of micronuclei in 2000 polychromatic erythrocytes (Fig.1). Bone marrow toxicity was assessed by the incidence of polychromatic erythrocytes (PCE) per 200 total erythrocytes.

Comet assay (Single cell gel electrophoresis - SCGE):

0.5ml of blood was collected from retro-orbital sinus, and was mixed with double amount of phosphate buffer saline (PBS) and was processed for SCGE (comet assay).^[25] The blood sample was gently layered over the histopaque in a centrifugation tube, centrifuged to obtain a white band containing lymphocytes. This band was aspirated and mixed with 0.5 per cent LMA (to form as second layer on the slides). Frosted glass slides were taken to form a 3 layered agar bed. These slides were then kept in lysis buffer for 2-3 h and then incubated for 20mins in electrophoresis buffer (100mM EDTA, 300mM NaOH, pH>13). Subsequently after electrophoresis, silver staining was carried out by the procedure of Nadin *et al.*^[26] Whole procedure was carried out in dim light to minimize artificial DNA damage. All the slides were coded before evaluation and were observed under microscope (45 X- Fig 2). One hundred comets were scored per animal, (50 in each of two replicate slides). Comet head diameter and total comet length were measured using ocular micrometer which was calibrated with the help of stage micrometer and tail length was calculated by the following formula: Comet tail length (μm) = total comet length - head diameter

Statistical analysis: Results were expressed as mean \pm S.E.M. The statistical significance of any difference in each parameter among the groups was evaluated by one-way ANOVA, followed by Turkey's multiple comparison *post hoc* test.

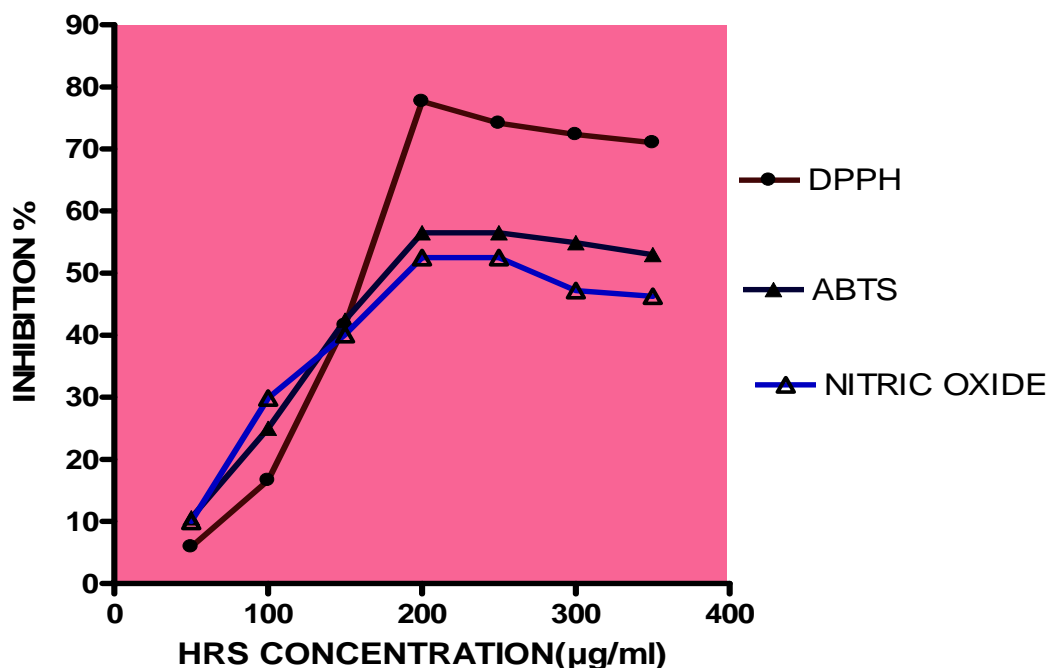
Results

In vitro Antioxidant activity - Radical generation and scavenging studies.

Scavenging of stable DPPH, ABTS and NO free radicals by HRS:

HRS extract scavenged DPPH, ABTS and NO radicals in a concentration dependent manner with a maximum scavenging activity of 77.66%, 56.5% and 52.5% respectively at 200 μ g/ml demonstrating saturation with further increase in HRS concentration as shown in graph no.1.

Graph 1

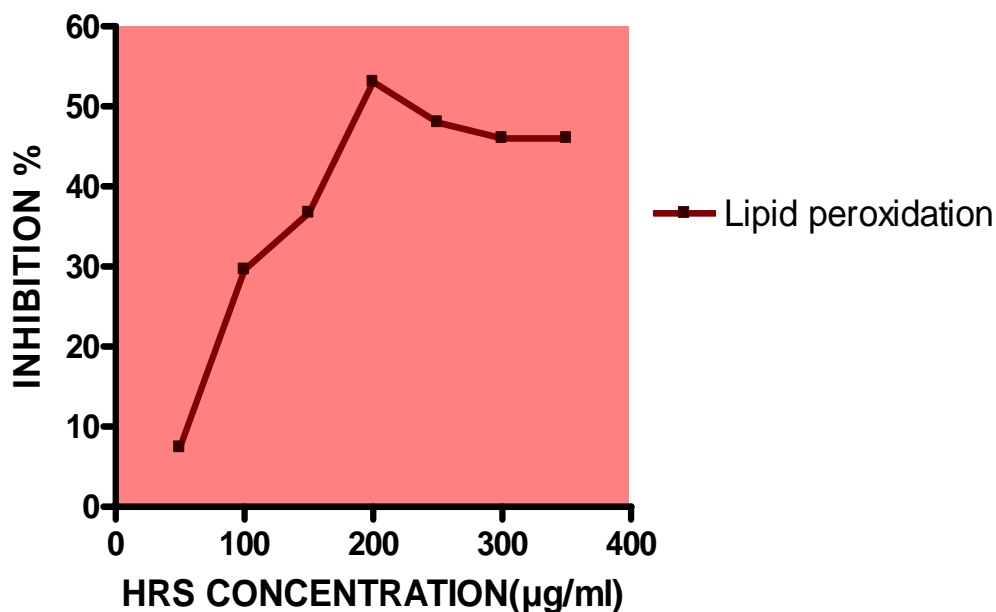


. Interaction of various concentrations of *Hibiscus rosa sinensis* ethanolic extract (HRS) with DPPH (2, 2-diphenyl-2-picryl hydrazyl) (filled circle), ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonicacid) [filled triangle], and nitric oxide [open triangle]

Effect of HRS extract on lipid per oxidation:

The addition of HRS extract to the reaction mixture resulted in a moderate dose – dependent inhibition of lipid per oxidation with a maximum inhibition of 53.07% at 200 μ g/ml and saturation thereafter as shown in graph.2.

Graph 2.



Inhibition of lipid per oxidation by various concentrations of Ethanolic extract of *Hibiscus rosa sinensis* (HRS)

In vivo Antigenotoxic effects

In vivo micronucleus assay: The % of micronucleated polychromatic erythrocytes (MnPCE) per 2000 polychromatic erythrocytes (PCE) was scored in saline and drug treated groups both in acute and sub acute studies. As expected, cyclophosphamide induced statistical significant ($P < 0.001$) increase in % MnPCE as compared to saline treatment whereas, HRS combined treatment with cyclophosphamide significantly ($P < 0.001$) decreased the MnPCE as compared to cyclophosphamide alone treated group both in acute and sub acute studies. While, alone HRS and vitamin E treatment did not represent any significant results. The corresponding data expressed as mean \pm SEM has been represented in table 2.

Table 2. Percentage of MnPCE and PCE in acute and sub acute studies

Treatment groups	Acute study		Sub acute study	
	%MnPCE	%PCE	%MnPCE	%PCE
Normal saline (negative control)	0.460±0.022	58.60±1.364	0.450±0.005	55.50±1.00
Cyclophosphamide (Positive control) 40mg/Kg	4.31±0.134*	56.20±1.158	8.130±0.232*	53.40±1.269
CYP+HRS (40+250mg/Kg)	1.540±0.093**	55.90±2.033	4.600±0.175**	54.70±1.347
HRS (250mg/Kg)	0.3700±0.030	55.50±1.313	0.470±0.055	55.50±1.492
CYP+Vit.E(40mg/Kg +50IU/Kg)	1.480±0.489**	56.50±1.225	4.450±0.076**	54.70±1.663
Vit E (50IU/Kg)	0.310±0.027	55.70±1.224	0.440±0.045	55.52±1.196

One-way ANOVA, F=330.2(Acute study); 615.9(sub acute study)

*P<0.001 when compared with negative control.

**P<0.001 when compared with positive control

Comet assay: The parameter studied in this assay was comet tail length (μm).Cyclophosphamide significantly ($P<0.001$) increased as compared to saline group while CYP+HRS group exhibited significant ($p<0.001$) decrease as compared to CYP group, and HRS,Vitamin E groups treatment alone did not significantly increase the comet tail length. The corresponding data expressed as mean \pm SEM has been represented in table 3. Microscopic representation of the comet assay is shown in Fig 1.

Table 3. Comet tail length (μm) in acute and sub acute studies

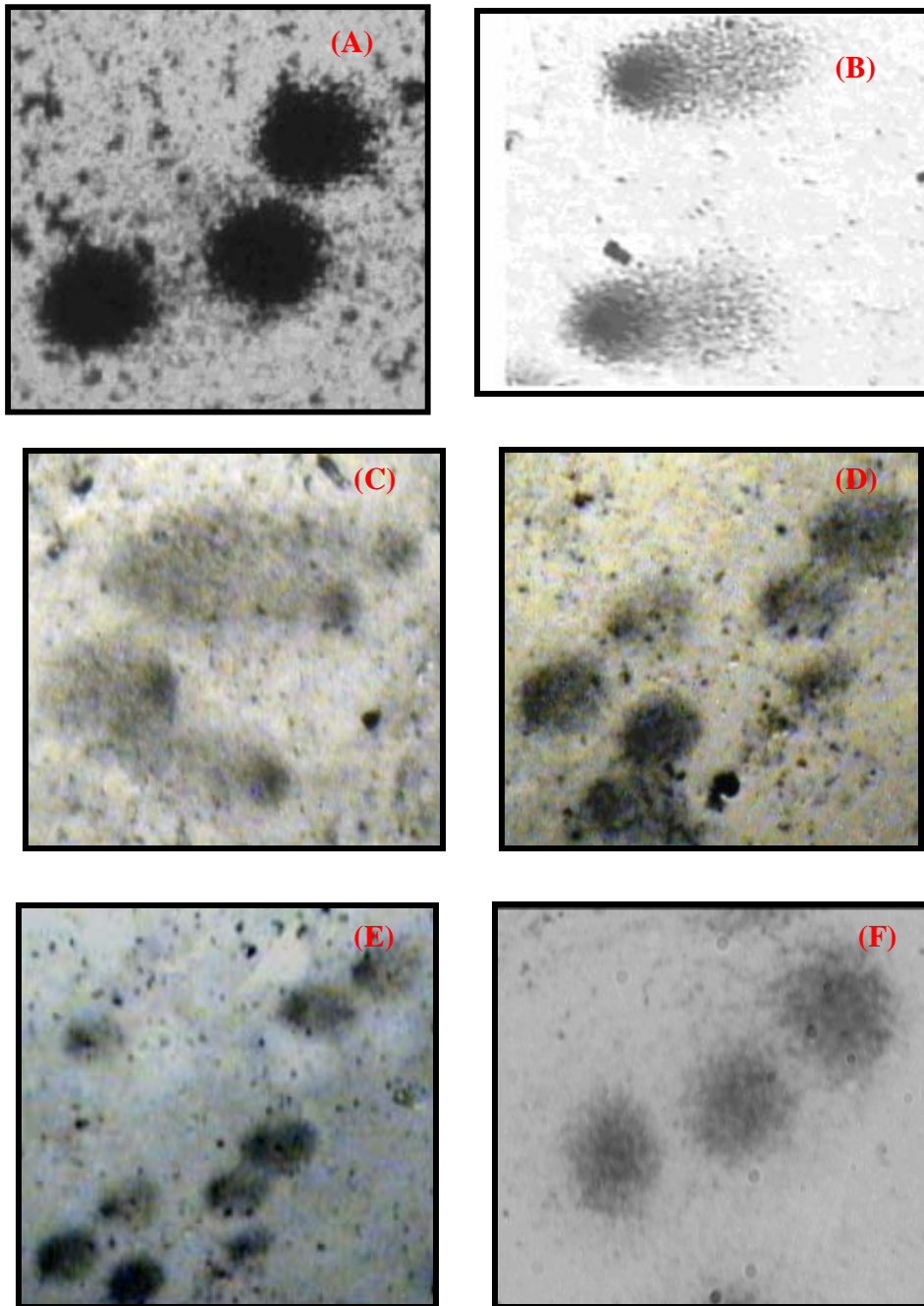
Treatment groups	Acute study	Sub acute study
Normal saline (negative control)	3.920±0.232	4.930±0.229
Cyclophosphamide (Positive control) 40mg/Kg	24.90±0.674*	25.70±0.700*
CYP+HRS (40+250mg/Kg)	10.40±0.763**	11.100±0.546**
HRS (250mg/Kg)	3.300±0.290	4.310±0.287
CYP+Vit.E(40mg/Kg+50IU /Kg)	10.200±0.489**	11.400±0.636**
Vit E (50IU/Kg)	3.520±0.270	3.90±0.221

One-way ANOVA, F=277.2(Acute study); 344.7(sub acute study)

*P<0.001 when compared with negative control.

**P<0.001 when compared with positive control

Fig 1. Various level of DNA damage seen in various groups. (Comet Assay)



- (A) Negative control group (normal saline) no damage is seen
- (B) & (C) Positive control group (Cyclophosphamide) maximal damage is seen
- (D) Cyclophosphamide + Vit. E (intermediate damage is seen)
- (E) Cyclophosphamide + HRS extract (intermediate damage is seen)
- (F) HRS alone (no damage is seen)

Discussion

The present study was planned to evaluate the influence of *hibiscus rosa sinensis* (HRS) flower extract on oxidative radicals and CYP induced genotoxicity. Results of the present study indicate a significant anti-oxidant and anti-genotoxic effects of HRS flower extract. Results of the in vitro studies clearly exhibit the potential of HRS extract to scavenge the free radicals namely DPPH, ABTS and NO in a dose dependent manner thus pointing at its anti-oxidant property. Such free radical scavengers have been known to play a key role in cytoprotection and cyclophosphamide induced cyto-toxicity is known to be mediated mainly through generation of free radicals and its action on DNA within the biological system.^[27] In the present study pretreatment with oral HRS extract (250mg/Kg) in CYP induced genotoxic mice showed significant reduction in %MnPCE and comet tail length. Similarly, pretreatment with standard anti-oxidant Vitamin E showed significant protection against CYP induced genotoxicity. Also on their own HRS extract or vitamin E proved to possess non-genotoxic effects. Therefore, results of the present study clearly indicate that HRS when co administered with cyclophosphamide and on its own conferred protection against genotoxic effects. Thus, free-radical scavenging action appears to be the likely mechanism of cyto-protection of HRS in case of cyclophosphamide induced genotoxicity. However, previous studies have shown *hibiscus rosa sinensis* to possess chemo preventive action in croton oil induced carcinoma model, and most of the anti-cancer drugs including cyclophosphamide are known to induce DNA damage^[19] by means of generation of free radicals. Standard anti-oxidants therefore could provide protection against DNA damage by scavenging these free radicals. Therefore it could be explained that HRS extract though confers protection against genotoxicity due to its anti-oxidant property may have chemo-preventive effect due to some other mechanism. It is obvious to know which of the active principles of HRS is responsible for cyto-protection, and experimental studies with isolated and purified constituents are therefore needed. Moreover before extrapolating the observations of the present study to human situations it needs to be confirmed clinically.

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