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Assessment of antigenotoxic effect brown seaweed *Stoechospermum marginatum*

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Abstract
The demand for compounds of natural origin with health promoting effect is increasing throughout the world. Marine macroalgae remain to be a great source of bioactive compounds with potential therapeutic and nutraceutical indications. The present study aimed at evaluating the antimutagenic, anticlastogenic and DNA protective effect of methanolic extract of brown seaweed *Stoechospermum marginatum* using *in vitro* bacterial reverse mutation (Ames) test and *in vivo* rodent micronucleus and alkaline comet assay. In Ames test, the test extract demonstrated a moderate antimutagenic activity of about 35.20% at the highest dose of 5000 µg/plate when tested against direct and indirect-acting mutagens. Additionally, the test extract exhibited a significant anticlastogenic effect of about 27.60% and DNA protective effect of about 32.22% in a dose-dependent manner when tested at the highest dose of 2000 mg/kg body weight against cyclophosphamide-induced micronucleus in mice bone marrow erythrocytes and DNA damage in peripherally lymphocytes. The result obtained from this study indicated that methanolic extract of *Stoechospermum marginatum* possessed an antigenotoxic activity and can confer protective effects to living cell genome against the deleterious effects of various chemical mutagens and clastogens following exposure.

Keywords: Stoechospermum marginatum, comet assay, clastogens, mutagens

1. Introduction
Seaweeds are renewable marine macroalgae consumed in appreciable amounts as part of a dietary supplement by some natives of the Far Eastern region of Asian countries and considered as one of the most nutritionally and therapeutically valuable algal materials (Namvar *et al.*, 2013) [16].

Stoechospermum marginatum is one of the benthic specie of brown marine macroalgae growing between high and low tide along the coastal region of Indian Ocean (Arumugama *et al.*, 2017) [2]. The algal material is a rich source of bioactive phytochemical compounds including polyphenols, terpenoids, polysaccharides, pigments, and numerous known vitamins, and minerals supplements which are used as animal feed/fodder, human food and as raw materials for nutraceuticals, pharmaceuticals, and chemical industries (Anbu *et al.*, 2017) [1]. It was reported to have antimicrobial, antidiabetic, antinflammatory, and antiproliferative activity (Anbu *et al.*, 2017) [1].

Identification of bioactive compounds present in plants with potential activity of preventing or minimizing genomic changes or damage caused by therapeutic drugs, cosmetic ingredients, food additives, insecticides, and other agents used in consumer and industrial applications is of crucial importance in reducing the risk of occurrences of mutation and development cancer in the exposed popultion (Nagarathna *et al.*, 2013) [15]. Antimutagen is described as an agent that reduces the apparent yield of spontaneous and or induced mutations in organism genome (Bhattacharya, 2011) [3]. Seaweeds were found to contained naturally occurring antimutagens and antioxidants compounds which demonstrated great protective effect against oxidative damage caused by free radicals (Arumugama *et al.*, 2017) [2].

The purpose of this study was to investigate the antimutagenic, anticlastogenic and DNA protective effect of methanolic extract of *S. marginatum* using batteries of *in vitro* and *in vivo* assay.

2. Materials and Methods
2.1 Collection and processing of extract
Fresh samples of algae plant (*Stoechospermum marginatum)* were collected in clean polythene bags containing sea water from the coastal line of Mandapam, Ramanathapuram district,

Tamil Nadu, India (latitude 9º18'N and longitude79º6'E) and immediately transported to our Departmental Laboratory for processing. A small portion of the algal plant was sent to Botanical Survey of India (BSI), Coimbatore for identification, it was confirmed as true species and a voucher specimen was deposited there. The algal materials were washed thoroughly under running tap water for removing the attached epiphytes and other marine debris and allowed to dry under shade for 5–7 days, until the moisture was completely removed. The dried material was ground to powdered form and stored in air tight containers at room temperature. Crude extract of *S. marginatum* was obtained using absolute methanol according to the methods described by Vinayak *et al.* (2011) [25] with some modifications.

Extraction was carried out by soaking 10g of the powdered sample in 100 ml of the solvent (1:10 – w/v) in a conical flask and kept on a shaker (120 rpm) at room temperature for 48hrs. The mixture was filtered using Whatman™ No. I filter paper and the process was repeated until the solvent becomes colorless. The filtrates obtained were combined and concentrated by evaporating the solvent under reduced pressure at it boiling point using rotary evaporator. The solvent was totally removed and the dried extract obtained was weighed and stored at 4ºC until further use. The dried extract yield was approximately 4.5%.

2.2 Chemicals

Ampicillin powder (Sigma-Aldrich), Tetracycline powder (Sigma-Aldrich), Fetal calf serum (Himedia), Dimethyl sulfoxide (DMSO) (Loba chemie), Cyclophosphamide (Sigma-Aldrich), Ethidium bromide (Sigma-Aldrich), Sodium azide (Sigma-Aldrich), Mitomycin C (Sigma-Aldrich), 2-Nitrofluorine (2-NF) (Sigma-Aldrich), 9-Aminoacridine (9-AA) (Sigma-Aldrich), Benzo[a]pyrene (B[a]P) (Sigma-Aldrich), β-nicotinamide adenine dinucleotide Phosphate (NADP) (SRL), D-Glucose-phosphate (SRL), L-Histidine HCL (SRL), D-Biotin (SRL), Low melting point (LMP) agarose (SRL), Normal melting point (NMP) agarose (SRL), Crystal Violet (Himedia), Dextrose (Himedia), Bactor agar (SRL), Oxoide Nutrient broth No. 2 (Himedia), Potassium phosphate Di-basic anhydrous (Loba Chemie), Potassium chloride (Himedia), Magnesium chloride (Himedia) were all utilized in the studies. All other reagents and chemical used were of analytical grade.

2.3 Bacterial tester strains

Five histidine dependent (*His*$^-$) tester strains of *Salmonella typhimurium* viz., TA98, TA100, TA102, and TA1535 and TA1575 were procured from GRL Laboratories privet limited, Chennai, Tamil Nadu and used in this study. The identity of the tester strains was confirmed for the following genotypic characteristics viz., histidine and biotin requirement, crystal violet sensitivity, ultra violet sensitivity, ampicillin and tetracycline resistance as described by Mortelmans and Zeiger, (2000) [14] before conducting the assay.

2.3.1 Preparation of S9 mixture

S9 mixture was prepared according to the method recommended by Mortelmans and Zeiger, (2000) [14]. The components of S9 mixture were, 8 mM magnesium chloride, 33 mM potassium chloride, 5 mM G-6P, 4 mM NADP, 100 mM sodium phosphate (pH 7.4) and 10% S9 fraction (v/v). A positive mutagen, B[a]P was used to check the quality of S9 mixture.

2.3.2 Limit dose / cytotoxicity study

A preliminary limit dose and cytotoxicity study of methanolic extract of *S. marginatum* (500, 1500, and 5000 µg/plate) dissolved in DMSO was done with *Salmonella typhimurium* strain TA98. Overnight grown culture of TA98 was mixed with 0.1 ml of each concentration and plated and grown on nutrient agar plates, as described by Maron and Ames, (1983) [13]. A test concentration with >50% viable cells was considered non-toxic compared with the viability of the negative (solvent) control. All the concentrations tested showed no sign of cytotoxicity and growth inhibition in the tester strain used. The test extract did not produce precipitation or discoloration of the plated medium.

2.3.3 Mutagenicity assay

The bacterial reverse mutation assay was conducted using plate incorporation method both with and without metabolic activation as described by Mortelmans and Zeiger, (2000) [14] and OECD guideline TG471, (1997). 2-NF, NaN3, MMC, and 9-AA were used as direct acting positive mutagens while B[a]P was used as indirect acting mutagen. The test extract and the corresponding positive control mutagens were dissolved in DMSO as a vehicle. Five different concentrations of the test extract viz., 312.5, 625, 1250, 2500 and 5000µg/ plate were chosen based on a preliminary dose range finding and cytotoxicity study mentioned earlier. Briefly, for each treatment, 0.1 ml of the test extract (for all five doses) or control preparation was introduced into a sterilized test tube, to which 0.1 ml of positive mutagen and 0.1 ml of overnight grown bacterial culture was added. For preparations with S9 mix, 0.5 ml was also added; for preparations without S9 mix, 0.5 ml of 0.1 M sodium phosphate buffer solution was added. After a gentle mixing, 2ml of molten top agar (45ºC) containing 0.5 mm histidine/biotin was added. The mixture was then vortexed and poured on to minimal glucose agar plates. After the top agar solidified, the plates were inverted and incubated for 48hrs at 37ºC. Following incubation, plates were examined for precipitation and toxicity and all the experiment were performed in triplicate. The choice of the positive mutagens used was presented in (Table 1).

2.4 *In vivo* rodent bone marrow micronucleus assay

In vivo rodent bone marrow micronucleus assay was performed using Balb/c mice's bone marrow erythrocytes following guide lines and protocols described by OECD guideline TG474, (2016) and Hayashi et al., 2000 [9].

2.4.1 Animals

Inbred Balb/c mice (*Mus musculus*) of age 6–8 week-old weighing around 22–26 g were obtained from Laboratory Animal Medicine, TANUVAS, Madhavaram Milk Colony Chennai-51, India. The animals were housed in polypropylene cages containing certified paddy husk as bedding and were allowed to acclimatize for one week at our college Centralized Laboratory Animal House. Animals were maintained under good environmental condition with temperature and humidity of the room between 22 ± 3ºC and 50–60% respectively and under a 12 hours light/dark cycles. Commercial standard pelleted diet and wholesome water were provided at *ad libitum.* All protocols used in this experiment were done based on the approval obtained from our Institutional Animal Ethics Committee [1467/DFBS/IAEC/2018/07].

2.4.2 Animals treatment and sacrifice
Experiment was performed using ten animals per group. Mice were dosed orally for seven consecutive days at three different dose levels of the test extract viz., 500 mg/kg, 1000 mg/kg, and 2000 mg/kg BW based on preliminary toxicity test we performed. 1% DMSO in distilled water was used as vehicle control. All the treatment groups and positive control groups received single intra peritoneal (i/p) administration of CP (50 mg/kg) two hours after last dose on day seven. Experiment was terminated by sacrificing five animals from each group at 24 hrs and 48 hrs interval. All mice were anesthetized by isoflurane for blood sample collection by cardiac puncture and were sacrificed by cervical decapitation. All efforts were been made to minimize suffering during the processes.

2.4.3 Bone processing and slides examination
After the animals sacrifice, both side femoral bones were removed after dissection and cutting through the pelvis and tibia. The proximal end of the femur was carefully shortened until a small opening to the marrow canal became visible. Immediately femur was submerged in a 0.5 ml of chilled FCS contained in microcentrifuge tube and bone marrow was aspirated after several flushing were performed. The cells were centrifuged at 1000 rpm for 5–10 minutes and two drops of cell suspension from each animal were placed onto a clean, grease free, dry slide and smeared, fixed in methanol and stained with May-grunwald and Giemsa stain. The slides were examined using light microscope under oil immersion objective lens for the presence of micronucleus. For each animal, a minimum of 2000 polychromatic erythrocytes (PCE) were screened for determining the presence of micronuclei and the percentage of cells containing micronucleus on the total number of cells. Simultaneously, the normochromatic erythrocytes (NCE) were scanned from each animal to determine the ratio of PCE and NCE.

2.5 *In vivo* alkaline comet assay
In vivo alkaline comet assay performed using the whole blood collected from the treated mice following the method described by Tice *at al.*, 2000 [24]. The whole blood was collected in K_2EDTA blood collection tube and equal volume of freshly prepared cold RPMI 1640 medium containing 10% DMSO was added. The mixture was transferred to 2 ml cryovials, capped and immediately frozen at -80ºC. The whole was thawed in a water bath at 37ºC for 1–2 min just before commencement of comet assay.

2.6 Slide processing and examination
Comet slides were prepared by dipping a grease free frosted glass slide to a coplin jar containing 1% normal melting point (NMP) agarose at 45ºC up to two-third of the frosted end. The slides were removed and the undersides were wiped with clean gauze for removing the agar and were allowed to dry. 10 µl of whole blood was mixed with 75 µl of 0.5% low melting point (LMP) agarose at 37ºC in an eppendorf tube and mixed gently. 80 µl of the mixture was added to the surface of pre-coated slides and a cover slip was placed. The slides were allowed to solidify at 4ºC for 5minutes. Later the cover slips were carefully removed and a third layer of 80 µl of 0.5 per cent LMP agarose was added and cover slips were placed again. The slides were allowed to solidify at 4ºC for 5 minutes. The slides (without cover slips) were immersed in freshly prepared, cold lyzing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCl (pH 10), 1% Triton X–100 and 10% DMSO) and refrigerated for one hour or overnight for the cell soluble component to lyzed. The slides were then removed from the lyzing solution and placed on a horizontal gel electrophoresis unit. The unit was filled with freshly prepared electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH) to a level 0.25 cm above the slides. The slides were allowed to set in this high pH buffer for 20–40 minutes to allow unwinding of DNA before electrophoresis. Electrophoresis was carried out in the same alkaline buffer for 25 min at 25 V (0.66 V/cm) adjusted to 300 mA by raising or lowering the buffer level in the tank. Slides were then drained, placed on a tray and rinsed slowly in neutral buffer (0.4 M Tris HCl, pH 7.5) for 5 minutes and later rinsed with PBS. The slides were stained with 80 µl of ethidium bromide (final concentration 2 µg/ml) for 5 minutes. The slides were rinsed in ice cold PBS and covered with a cover slips. The prepared slides were observed using fluorescent microscope under green fluorescence light for scoring 100 nuclei. The extent of DNA damage was assessed using a visual classification method. Cell nuclei were analyzed and given a score from 0 (undamaged nucleus) to 4 (severely damaged nucleus). The slides were prepared in duplicate for each sample and the whole procedure was carried out in dim light to minimized artefactual DNA damage.

2.7 Data analysis
2.7.1 Ames test
Atimutagenicity and anticlastogenicity results were evaluated for per cent inhibition (P.I: i.e. the ability of the test extract to reduce the action of the positive mutagen used) following the formula described by Resende *et al.* (2012) [22]. Per cent Inhibition $(\mathrm{P.I}) = \left(1 - \frac{\mathrm{T}}{\mathrm{M}}\right) \mathrm{X100}$ Where T is the number of revertant bacterial colonies in the plate containing mutagen and test extract or number of micronucleus polychromatic erythrocytes formed in presence of mutagen and test extract**.** M is the number of revertant bacterial colonies in the plate containing only the mutagen or number of micronucleus polychromatic erythrocytes induced by positive mutagen only. The result obtained were interpreted as follows: No antimutagenic effect was detected when the inhibition was lower than 25%, a moderate effect for a value between 25% and 40%, and strong Antimutagenicity for values greater than 40%.

2.8 Statistical analysis
The data generated from this study were expressed as mean plus or minus standard error (Mean ±SE). The data were further analyzed for statistical significance using one way analysis of variance (ANOVA) and the differences between the mean values were compared using Tukey Kramer multiple comparison test at $P< 0.05$.

3. Results and Discussion
3.1 Ames test
Methanolic extract of *S. marginatum* was evaluated for its potential antimutagenic activity against mutation induced by positive mutagens in five *His*$^-$ mutant strains of *S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 using Ames test both with and without metabolic activation (Table 2). From the result obtained, there exists a dose dependent increase in antimutagenic activity of the test extract in almost all the tester strains both with and without metabolic activation. The test extract has demonstrated moderate

inhibitory activity in preventing the induction of mutation by positive mutagens in the bacterial tester strains where highest inhibitory activity of about 35.20% in TA1537 was observed in the absence of metabolic activation. No significant antimutagenic activity of the test extract was observed against the action of B[a]P in TA102 strain with the highest per cent inhibition observed was 13.11%. The tester strain TA102 is normally used to detect oxidative damage and cross linking mutagens that causes transition and reversion (Mortelmans and Zeiger, 2000) [14].

The discovery and extrapolation of the chemical compounds with antioxidant, antimutagenic and anticarcinogenic potency at present time is of great importance because of the undesirable consequence of an increased rate of mutations and the possibility of increased risks of developing cancer and other degenerative diseases (Nagarathna *et al.,* 2013) [15]. In previous decade, there is an increase demand by the growing population for consuming herbal products with potential antioxidant properties due to the toxic side effects of synthetic antioxidants chemicals (Mansour *et al.,* 2017) [12]. Seaweeds are rich in bioactive compounds such as carotenoids, pigments, polyphenols, enzymes and diverse functional polysaccharides which demonstrated great antioxidant activity and can prevent the deleterious consequences of oxidative stress and improve antioxidant status (Vinayak *et al.,* 2011) [25].

Several studies have reported the antimutagenic activity of various classes of seaweeds and our finding in this study was similar to what reported by Kim *et al.* (2005) [10] where he screened five Korean seaweed viz., seaweed fusiforme, sea tangle, Laver, green laver and sea mustard for their antimutagenic activity against 2-NF and NaN_3 induced mutation in *His⁻ S. typhimurium* tester strains. The extract test exhibited a strong antimutagenic activity in preventing induction of mutation by the positive mutagens in the genome of the bacterial tester strains. In a similar studies, two brown seaweeds *Undaria pinnatifida* and *laminaria japonica* were also assessed for their antimutagenic activity and were found to have a strong antimutagenic against several mutagens when assayed in *S. typhimurium* tester strains (Okai *et al.,* (1993) [19]. In another study, he reported that a red algae *Porphyra tenure* have a strong antimutagenic activity of against mutagen induced *umu*-C gene expression in *His⁻ S. typhimurium* tester strain (Okai *et al.,* 1996) [20]. This antimutagenic effect observed with seaweeds extract may be likely due to the presence of various bioactive constituents such as pigments phenols and functional polysaccharides which were reported to have a great antioxidant effects (Deepak *et al.,* 2017) [6]. The probable antimutagenic mechanism of action of the test extract may be one of several ways such as inhibiting the interaction between genes and biochemically reactive mutagens, inhibiting metabolic activation of indirectly acting mutagens by inactivation of metabolizing enzymes, or interacting with the pro-mutagens to make them unavailable for the enzymatic process (Resende *et al.,* 2012) [22].

3.2 Anticlastogenic activity

The anticlastogenic and cytoprotective effects of *S. marginatum* extract were evaluated against CP-induced micronucleus and cytotoxicity in mice bone marrow erythrocytes using micronucleus assay both at 24 hrs and 48 hrs time interval after cyclophosphamide administration. The data obtained were calculated and presented as mean ±SEM (Table 3).

There exists a statistically significant difference at $P < 0.05$ in the ratio of PCE/NCE and per cent frequency of MNPCE between the positive control group and treatment Group-3 but no significant difference was observed between positive control group and treatment Group-1, Group-2. The inhibitory activity of the test extract against micronucleus induction and cytotoxicity were found to lie between 8.66% and 27.64% for both the two sampling period. The extract exhibited a significant dose dependent inhibition of formation of micronuclei and cytotoxicity against CP-induced cytotoxicity and clastogenicity in the bone marrow erythrocytes.

Cyclophosphamide (CP) is an alkylating agent and indirectly acting mutagen and genotoxin that was commonly used as an antineoplastic and immunosuppressive agent. The mutagenic potential of CP has been clearly demonstrated in mice, rats and hamsters. It has already been reported in the literature that the cytotoxicity of CP is mediated through the mechanism of free radical production and free radicals are well known for producing DNA damage and mutagenesis in human and animal cells (Chandrasekran *et al.,* 2009) [5].

Several studies have revealed the efficacy of different classes of seaweeds in conferring protective effect against cytotoxic and genotoxic effects of CP. For instance, *Dunaliella saline* was reported to have a significant antioxidant activity because of its high carotenoids pigment it contained. It demonstrated a strong antigenotoxic effect against y-ray induced micronucleus and chromosomal aberration formation in human peripheral lymphocytes (Ma *et al.,* 1998) [11]. In a similar study, Dolpady and Vijayaxmi, (2018) [7] have reported that ethanolic extract of a green seaweed, *Ulva fasciata* possessed a strong anticlastogenic effect against the chromosomal aberration (CA) and micronucleus (MN) induction by anticancer drugs like CP. they suggested that the activity observed was due to the synergistic and or additive effects of biologically active ingredients present in the seaweed extract. It was reported that pre-treatment with different doses of *S. fusiformis* significantly reduced the formation of chromosomal damage and lipid peroxidation with concomitant changes in the antioxidative and detoxification status systems (Premkumar *et al.,* 2001) [21]. The functional sulfated polysaccharides such as laminarans and fucoidan present in brown seaweeds were reported to found to have a significant antimutagenic and chemoprotective activity against various genotoxins in living system (Celikler *et al.,* 2008) [4]. From our observations made, it revealed that pretreatment with methanolic extract of *S. marginatum* has a beneficial effect against CP-induced micronuclei formation in bone marrow erythrocytes. This may likely be due to the activities of antioxidant compounds present in the test extract by preventing it from causing genotoxic effect.

These findings were in agreement with what we observed in the present study where the test extract exhibited a significant anticlastogenic and cytoprotective effects of in preventing CP-induced damage to bone marrow erythrocytes.

3.3 DNA protective effect

DNA protective effect of methanolic extract of *S. marginatum* was assessed against CP-induced DNA damage in peripheral lymphocytes after 24 hrs and 48 hrs of last dose administration of the test extract. A visual scoring system of 0 (normal) to 4 (severely damaged) was used to classify the level of DNA damage. The data obtained were tabulated and

presented as mean ±SEM (Table 4).

There exists a statistically significant difference between positive control group and treatment Groups-2 and 3 for both 24 hrs and 48 hrs sampling interval but no significance difference was observed between positive control group and treatment Group-1. The test extract exhibited significant dose dependent inhibition of DNA damage induced by CP in all the treatment groups with highest per cent inhibition of 32.22% observed at the highest dose tested. Both the negative control groups and treatment control groups exhibited types 0, 1, 2, and 2 comet forms. Seaweeds are used worldwide in the management and treatment of many diseases including the prevention and management of cancer condition. They are rich in bioactive compounds that are capable of reducing or reversing and inhibiting various kind of genomic damage caused by certain chemical genotoxins (Gamal-Eldeen *et al.*, 2013) [8]. Chlorophyll pigment extracted from seaweed was evaluated for its DNA damaging effect in peripheral lymphocytes of mice and was found to be devoid of any DNA damaging effect, the pigment rather exhibited a strong protective effect against positive mutagens (Serpeloni *et al.*, (2011) [23]. The activity of the pigment observed may likely be attributed to the antioxidant property of the compound. In a similar study, hinokinin a compound isolated from pepper having good antitrypanosomal activity was evaluated for its potential DNA damaging and protecting effect against DNA damaging effect of doxorubicin in V76 cell line using alkaline comet assay and was found to have insignificant DNA damaging effect rather it demonstrated a significant DNA protective effect in protecting the cells from DNA damage (Resende *et al.*, 2012) [22]. This activity observed may be likely due to the presence of various phytochemical constituents in the test extract which prevent the DNA alkylating effect of CP or reverse it effect after DNA adduct.

Table 1: Positive mutagens used in Ames test

Mutagens	Dose/µg/plate	Strains	S9(±)
B[a]P	5	TA98, TA100, TA102, TA 1535, TA1537	+
2-NF	1	TA98	-
NaN_3	0.5	TA100	-
	0.5	TA1535	-
MMC	0.5	TA102	-
9-AA	50	TA1537	-

Table 2: Evaluation of antimutagenic effect of methanolic extract of *S. marginatum* against mutation induction by positive mutagens in *His⁻ S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537) using Ames test

Strains	Test chemicals	Concentration (µg/plate)	Number of revertant (Mean ±SME)/ plate and P.I (%)			
			-S9 (Mean ±SME)	P.I (%)	+S9 (Mean ±SME)	P.I (%)
TA98	DMSO	-	31.00 ± 1.73	-	29.00 ± 1.53	-
	2-NF[a]/B[a]P[b]	-	265.00 ± 2.00	-	237.00 ± 1.15	-
	SM	312.5	245.00 ± 2.65	7.55	215.67 ± 1.45	9.00
	"	625	231.00 ± 1.15	12.83	201.33 ± 2.03	15.05
	"	1250	218.00 ± 1.15	17.74	190.00 ± 5.13	19.83
	"	2500	194.67 ± 2.33	26.54	170.00 ± 0.58	28.27
	"	5000	188.00 ± 1.53	29.06	161.00 ± 1.53	32.07
TA100	DMSO	-	127.00 ± 3.61	-	128.00 ± 2.65	-
	NaN_3[a]/B[a]P[b]	-	994.00 ± 2.08		1005.00± 1.73	-
	SM	312.5	938.00 ± 0.88	5.57	914.67 ± 1.45	8.99
	"	625	927.00 ± 2.31	6.74	895.00 ± 1.15	10.95
	"	1250	918.00 ± 1.15	7.65	876.00 ± 1.73	12.84
	"	2500	864.33 ± 2.33	13.04	844.00 ± 1.53	16.02
	"	5000	738.33 ± 1.20	25.72	723.33 ± 1.45	28.03
TA102	DMSO	-	238.00 ± 1.53	-	240.00 ± 1.15	-
	MMC[a]/B[a]P[b]	-	1110.00 ± 5.03	-	1269.00 ± 0.58	-
	SM	312.5	1085.00 ± 1.15	2.25	1242.67 ± 1.20	2.08
	"	625	1052.00 ± 1.15	5.23	1222.00 ± 1.53	3.70
	"	1250	1032.00 ± 0.58	7.03	1205.33 ± 0.88	5.02
	"	2500	1016.00 ± 1.15	8.47	1172.00 ± 2.08	7.64
	"	5000	987.00 ± 2.08	11.08	1102.67± 2.19	13.11
TA1535	DMSO	-	22.00 ±2.08	-	21.00 ± 1.15	-
	NaN_3[a]/B[a]P[b]	-	320.00 ± 2.65	-	294.00 ± 2.08	-
	SM	312.5	300.00 ± 1.73	6.25	270.33 ± 0.88	8.05
	"	625	281.00 ± 1.15	12.19	255.00 ± 1.15	13.27
	"	1250	253.00 ± 1.53	20.94	233.00 ± 1.53	20.75
	"	2500	227.00 ± 2.31	29.06	220.00 ± 0.58	25.17
	"	5000	211.33 ± 2.40	33.96	204.00 ± 1.15	30.61
TA1537	DMSO	-	12.00 ± 0.58	-	11.33 ± 0.88	-
	9-AA[a]/B[a]P[b]	-	143.00 ± 1.73	-	113.00 ± 1.15	-
	SM	312.5	132.00 ± 1.15	7.69	104.67 ± 2.33	7.37

	"	625	127.00 ± 1.15	11.19	98.00 ± 1.15	13.27
	"	1250	115.00 ± 1.15	19.58	89.00 ± 0.58	21.24
	"	2500	105.33 ± 0.88	26.34	80.33 ± 1.20	28.91
	"	5000	92.67 ± 1.45	35.20	73.33 ± 0.67	35.10

P.I: per cent inhibition, DMSO: vehicle, S9: soluble liver microsomal enzymes fraction, NaN_3 sodium azide, 2-NF: 2-nitrofluorine, MMC: mitomycin C, 9-AA: 9-aminoacridine, Benzo[a]pyrene B[a]P, a: -S9, b: +S9, n = 3

Table 3: Evaluation of anticlastogenic effect of methanolic extract of *S. marginatum* against cyclophosphamide induced micronucleus in mice bone marrow erythrocytes using *in vivo* micronucleus assay.

Treatment	Sampling time	Dosage (mg/kg/Day)	Total MNPCE Mean± SEM	MNPCE/2000 PCE Mean± SEM (%)	PCE/PCE+NCE Mean± SEM (%)	P.I (%)
CYP	24hrs	50	25.40± 1.75	1.27± 0.09[a]	53.20± 0.54[b]	-
G4	24hrs	500	23.20± 1.80	1.16± 0.09	56.70± 0.94	8.66
G5	24hrs	1000	21.20± 0.86	1.06± 0.04	61.00± 0.80	16.54
G6	24hrs	2000	18.60± 1.03	0.93± 0.05[a]	65.10± 0.64[b]	26.77
CYP	48hrs	50	24.60± 1.63	1.23± 0.08[c]	54.60± 0.68[d]	-
G4	48hrs	500	22.40± 0.87	1.12± 0.04	58.20± 1.14	8.94
G5	48hrs	1000	20.04± 0.87	1.02± 0.04	63.30± 0.92	17.07
G6	48hrs	2000	17.80± 1.16	0.89± 0.06[c]	66.00± 1.13[d]	27.64

CPY: cyclophosphamide, G: group, NCE: normochromatic erythrocytes, PCE: polychromatic erythrocytes, MN: micronucleus, P.I: per cent inhibition, SEM, standard error of mean, n = 5. Mean values with the same superscript differs significantly $P< 0.05$.

Table 4: Evaluation of DNA protective effect of methanolic extract of *S. marginatum* in peripheral blood using *in vivo* alkaline comet assay

Treatments	Dosage (mg/kg)	Time	DNA score mean ±SEM					Total DNA damage Mean± SME	P.I (%)
			0	1	2	3	4		
CYP	50	24hrs	45.00 ±0.32	11.00 ±0.71	11.60 ±0.51	15.00±0.32	17.40±0.75	55.00±0.35[a]	-
G4	500	24hrs	50.00 ±0.45	15.60 ±0.68	12.40 ±0.40	10.60±0.68	11.40±0.60	50.00±0.45[b]	9.09
G5	1000	24hrs	56.00 ±0.71	15.80 ±0.37	11.40 ±0.68	8.80±0.20	8.00±0.55	44.00±0.71[a]	20.00
G6	2000	24hrs	62.40 ±0.68	14.80 ±0.37	10.00 ±0.84	7.60±0.60	5.20±0.37	37.60±0.68[ab]	31.64
CYP	50	48hrs	46.00 ±0.55	11.40 ±0.51	10.80 ±0.37	14.40±0.40	17.40±0.75	54.00±0.55[c]	-
G4	500	48hrs	51.60 ±0.51	15.80 ±0.49	12.60 ±0.24	10.40±0.68	9.60±0.24	48.40±0.51[d]	10.37
G5	1000	48hrs	57.40 ±0.51	14.00 ±0.32	11.00±0.45	10.00±0.32	7.60±0.24	42.60±0.51[c]	21.11
G6	2000	48hrs	63.40 ±0.60	14.40 ±0.40	11.00±0.71	8.00±0.45	4.20±0.37	36.60±0.60[cd]	32.22

CYP: cyclophosphamide, SD: standard deviation, G: group, n = 5
Mean values with the same superscript differs significantly, $P<0.05$

3.4 Conclusion

The result obtained in the present study revealed that methanolic extract of *S. marginatum* has exhibited a moderate antimutagenic, anticlastogenic and DNA protective effects against all the positive mutagens used in this study in both *in vitro* and *in vivo* testing system. The test extract shown to have potential beneficial effects and can serve as an adjuvant in chemotherapeutics for minimizing unwanted toxicity side effect associated with conventional anticancer agents. Further study is required to evaluate the possible bioactive compound responsible for its antimutagenic, anticlastogenic and DNA protective effect and its mechanisms of action.

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Conflict of interest

The authors have read and approved the final draft of this manuscript and have no conflict of interest to declare with regards to publication of this manuscript.

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