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In vitro Toxicity Evaluation of Sterol Isolate from Echinoderm *Stellaster equestris* against Human Peripheral Blood

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ABSTRACT

Background: Ocean acts as a wealthy resource of biological diversity producing prospective novel metabolites from the organisms present in the environment. Novel bioactive compounds from Echinoderms mainly from sea stars have been extensively studied procuring rich diverse compounds which exhibits *in vitro* cytotoxicity comparable to or better than those of the potent anticancer drug. These sea stars which reside in the benthic region serves as their habitat and they are therefore predators and persistently being attacked by various organisms subsequently they acclimatize the survival approach to defend themselves from the external pathogens. Since these metabolites are obscured as a metabolic product during their endurance an initial evaluation of these compounds is necessary to assess human risk. **Objectives:** The present study focuses *in vitro* toxicity evaluation of isolated sterol like isolate from echinoderm sea star *Stellaster equestris* by incorporating hemolytic, chromosomal aberration assay and the cell viability tryphan blue exclusion assay against human peripheral blood. **Methods:** The *in vitro* toxicity evaluation was studied against the human peripheral blood collected from the healthy donors with the defined concentration of the sterol like compound from the sea star *Stellaster equestris*. The hemolytic, cell viability trypan blue exclusion method and chromosomal aberration assay were performed to check the hemolytic, mutagenic and genotoxic effect against the lymphocytes and the red blood cells. **Results and Discussion:** The result suggested that the hemolytic assay and the cell viability assay even in a dose dependent manner were non-hemolytic and percentage of the viability was not affected due to the exposure of the compound. The absence of genotoxicity was evident for the chromosomal aberration assay indicated that the isolated compound from the sea star *Stellaster equestris* might be considered as effective novel compound.

Key words: *In vitro* toxicity, *Stellaster equestris*, Hemolytic assay, Chromosomal aberration assay and cell viability assay.

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INTRODUCTION

Natural products from the marine echinoderms, either primary or secondary metabolites have been exploited because of its possible pharmaceutical importance. The marine natural products lack a traditional history in the flied of medicine mainly due to the difficulty in the collection of samples from the sea.[1] The most renowned echinoderms are members of class Asteroidea or sea stars which are ecologically significant and diverse members in the oceans bed.[2-4] Sea stars produces huge amount of secondary metabolites which is used as a predatory mechanism to prevent other organisms attacking them. These metabolites which are secreted as an innate mechanism for the protection of sea stars serve as a sole source of compounds having medicinal value. Since these metabolites are secreted during their defences these natural product screening systems is essential. *In vitro* testing methods are employed primarily to identify whether the compound from the natural sources is potentially hazardous in the early stages in order to become a new substances such as therapeutic drugs, agricultural chemicals and food additives. *In vitro* toxicity assessment using human peripheral blood has been exposed to show a critical biological response towards any drug tested against them.

The scope of *in vitro* tests is to rapidly assess the formulation's impending to cause acute reactions *in vivo*. Any compound or drug which is tested using the blood should be evaluated for its effects on erythrocytes, white blood cells and the complement system, to identify severe acute toxicities, such as haemolysis, chromosomal aberration and genotoxicity respectively.[5] The marine compounds isolated from marine sources which are nothing but the secondary metabolites which has a notable bioactive component secreted during their survival, hence these metabolites may possess toxicity which might alter the physiology or cell viability this can be measured and quantified. The present study has employed hemolytic, chromosomal aberration assay and cell viability assay with tryphan blue exclusion method for evaluating the sterol isolates from echinoderm *Stellaster equestris* against human peripheral blood.

MATERIALS AND METHODS

Collection and isolation of sterol isolate from sea star *Stellaster equestris*

Sea star samples were procured from the fishing harbour of north Chennai (Latitude 13º 06'N, Longitude 80º 18'E) by using the fishing net. These samples were sent to zoological survey for species identification and to check for the extinct list (red list). Part of the samples was processed and the whole body extraction of active solvents by cold percolation method was done. The extracted active crude chloroform extract of the sea star *Stellaster equestris* was then purified by column chromatography. The purified samples were subjected for characterization to analyze the functional groups present in sample using Fourier transform infrared

spectroscopy (FTIR) and Nuclear magnetic resonance spectroscopic (NMR) analysis.[6] The purified samples were further quantified by the High performance liquid chromatography (HPLC).

Hemolytic assay

The hemolytic assay is a cell based assay where the red blood cells is employed as a primary culture since RBC lacks nucleus the hemoglobin in the cytoplasm is released as and when they are exposed to toxic substances causing lyses of the red blood cells that alters the osmotic equilibrium which can be quantified.[7] 5ml of peripheral blood was collected in heparin vacationer. The collected blood was transferred to a 15 ml centrifuge tube which was then centrifuged at 1000 rpm for 10 min. The supernatant was discarded and equal volume of 1XPBS was added. The contents were centrifuged at 1000 rpm for 10 min. The step was repeated until white supernatant was obtained. The supernatant was discarded. The whole blood was taken from which 100 µl of blood was added to 900 µl of PBS (1% RBC suspension). 1 ml of 1% RBC and varied concentration of purified sample (50 µl, 100 µl, 200 µl and 400 µl) was added. 100 µl (0.1% Triton X) was added to 1ml 1% RBC was used as positive control and 1ml of 1% RBC as the negative control. After 2 hr of incubation the contents were centrifuged at 1000 rpm for 10 mins. 100µl of supernatant was transferred into a 96 well flat bottom plate and OD was measured at 545 nm.

Percentage of lyses was calculated using the formula:

$$\text{Percentage of haemolysis} = \frac{\text{OD of the test sample - OD of the negative control}}{\text{OD of the positive control - OD of the negative control}} \text{ X } 100$$

Cell viability assay by trypan blue exclusion method

The cell viability assay is a cytotoxicity assay which is used to determine the toxicity of the compound exposed to the cells *in vitro* which determines the number of viable in a cell suspension which has been exposed to compounds. The blood was collected from a healthy individual in heparinized tubes. The separation of the lymphocytes from the whole blood was done by Ficoll-density gradient centrifugation method.[8] The toxicity evaluation of purified compound with defined concentration (50, 100, 200 and 400 µg/ml) with Positive control of 100 µl (0.1% Triton X) and negative control of live cells was left for incubation for 2 hr. These exposed lymphocytes were then treated with 0.4% Trypan blue solution an aliquot of 10µl of treated cell pellet were mixed with 10µl of trypan blue this set up was left for 3 min.[9] The stained cell pellets were then loaded on to the neubar chamber which was then counted to check the cell viability percentage.

$$\text{Cell viability percentage \%} = \frac{\text{Number of viable cell}}{\text{Total number of cells counted}} \text{ X } 100$$

Chromosomal aberration assay

The chromosomal aberration assay also represents the chromosome or chromatid breaks and gaps which are also scored as an endpoint; this indicates the toxicity of the compound. Culture initiation was done with 80% of RPMI 1640 media, 20% of FBS, 400µL of Phytohemagglutinin (PHA) and 1ml of peripheral blood was added to the culture flask along with varied concentration of purified sample (50, 100, 200 and 400 µg/ml) and Positive control ethyl methanesulphonate (EMS) and a media, serum and blood with the PHA was maintained as negative control.[10] The culture flasks were incubated at 37°C for 48 hr with 5% CO_2. Colchicine (0.2 ml) was added to block the cells in metaphase. At the end of 48th hr, they were processed for harvesting. The content was centrifuged at 1000rpm for 10 min to which 8ml of freshly prepared pre-warmed hypotonic solution was added and left for incubation (20 min) in room temperature. The cell pellet was fixed with prechilled Carnoy's Fixative and washed 2-3 times to obtain a clear white pellet. The pellet was dropped on to a clean pre-chilled microscopic glass slide. The slides were left at 40°C overnight for it to be aged before staining. The slides were stained in 2% Giemsa solution and were subjected for analysis. Chromosomal aberration (CA) was calculated using the formula mentioned below

$$\text{Chromosomal Aberration (CA) frequency} = \frac{\text{Total number of aberration}}{\text{Total number of cell metaphase scored}}$$

RESULTS

Collection and isolation of sterol isolate from sea star *Stellaster equestris*

The sea star *Stellaster equestris* were collected by fishing nets at the fish harbour of north Chennai coast (Latitude 13º 06'N, Longitude 80º 18'E). The collected sample was identified as the above mentioned species *Stellaster equestris* (*Retzius,* 1805) by Marine Biology Regional Centre, Zoological Survey of India, and Chennai. The crude active metabolite was extracted by cold percolation method using solvents (hexane, dichloromethane, chloroform, ethlyacetate and methanol). The activities of the crude extracts were established by viability assay using PA1 cell line. The active crude chloroform extract was further purified by column chromatography and subjected for characterization and quantification by Fourier transform infrared spectroscopy (FTIR) and Nuclear magnetic resonance spectroscopic (NMR) and High performance liquid chromatography (HPLC) analysis. The Fourier transform infrared spectroscopy (FTIR) and Nuclear magnetic resonance spectroscopic analysis indicated that the observed absorption bands the absorption frequency resembles the absorption frequency of steroidal compounds. The quantification by High performance liquid chromatography (HPLC) method indicated that the sharp peak was observed for the presence of sterol with the retention time of 7.999 and a purity of 98.5% of sterol content present in the sample compared with the standards which was subjected respectively.

Hemolytic assay

The hemolytic assay is a significant screening assay for establishing the biocompatibility of purified sample of sea star *Stellastar equestris*. The hemolytic assay was performed with defined concentrations of compound (50, 100, 200 and 400µg/ml) of purified sample.

The percentage of haemolysis indicated in (Table 1) shows concentration of the samples were 0 for 50µg/ml, 0.38% for 100 µg/ml, 0.45% for 200 µg/ml and 0.65% for 400µg/ml, respectively with the positive control

Table 1: Hemolytic Assay.

		Concentration (µg/ml)					
Percentage of Hemolysis	Sample	50	100	200	400	Positive Control (0.1% Triton X)	Negative control
	Purified compound from *Stellaster equestris*	0	0.38	0.45	0.65	100	0

showing 100% of lysis as shown in (Figure 1). Based on above mentioned values the compound isolated from the sea star are considered to be non-hemolytic.

Cell viability assay by trypan blue exclusion method

The cell viability assay by trypan blue exclusion method was performed to assess the toxicity of the defined concentrations (50, 100, 200 and 400 µg/ml) of purified sample exposed against the lymphocytes from the peripheral blood. The percentage of cell viability as indicated in (Table 2) shows that for concentration of the samples for the cell viability assay were 99% for 50µg/ml, 98.8% for 100 µg/ml, 97.4 % for 200 µg/ml, and 95% for 400µg/ml, respectively with the positive control showing 25% of cell viability. No significant toxicity was observed hence the percentage of cell viability was good on comparison with the positive control. Hence the compound isolated from the sea star is not considered to be cytotoxic as shown in (Figure 2). All experiments were carried out in duplicates. The data are the mean % viable cells ± SD of duplicates performed.

Chromosomal aberration

Human peripheral blood lymphocytes were subjected for the toxicity assessment using chromosomal aberration assay with defined concentrations (50, 100, 200 and 400 µg/ml) of purified sample. 100 metaphases were scored and evaluated under each concentration.

As mentioned in (Table 3) the microscopic analyses revealed no structural or numerical abnormalities in all the four defined concentration. In 50 µg/ml and 100 µg/ml with 100 number of metaphases were scored in which no numerical or structural aberration was noted as shown in (Figure 3 and 4). The defined concentration of 200 µg/ml and 400 µg/ml as shown in (Figure 5 and 6) had shown no structural aberration but numerical deviation in 3 and 5 spreads out of 100 metaphases scored and analysed which might be due to the counting error. Hence all the four concentration were found to be non genotoxic on comparison with the positive control as shown in (Figure 7).

Table 2: Cell Viability assay by trypan blue exclusion method against lymphocytes (Average mean % viable cells ± SD).

S. No.	Concentration of sample (µg/ml)	Cell viability %	Average mean ± standard deviation
1	50	99	1 ± 0.50
2	100	98.8	0.36 ± 0.08
3	200	97.4	0.46 ± 0.06
5	400	95	0.16 ± 0.50
6	Positive control	25	0.30 ± 0.100
7	Negative control	100	0.00

Table 3: Chromosomal Aberration Assay.

S. No.	Concentration of sample (µg/ml)	Total number of metaphase scored	Total number of aberration scored	Aberration per cell ± standard error
1	50	100	0	0.00
2	100	100	0	0.00
3	200	100	3	0.03±0.025
4	400	100	5	0.08±0.025
5	Positive control	100	300	3.00±0.100
6	Negative control	100	0	0.00

DISCUSSION

The marine echinoderms sea stars are rich resources of natural bioactive compounds that have prospective biomedical applications. The sea star research has contributed to isolate diverse compounds with complex structure hence the researcher focuses on isolation, elucidation and pharmacological screening of the isolated bioactive compounds and also evaluating the toxicity of the compounds. *In vitro* toxicity assessment plays a major role in assessing the compound and provides information about the toxic effect of compound. Since these compounds possessing biomedical properties can serve as a potent drug and likely to interact with cells therefore it is important to rule out the toxicity and its adverse effect before they are to be applied in the flied of medicine. *In vitro* studies are mainly conducted with cell lines or cells or even animal tissues in a laboratory. These results provide us information about the interaction of the exposed compound towards the biological material. But the *in vivo* studies are performed with whole organism providing information on the organ toxicity as well as genotoxicity.

The present study has employed hemolytic chromosomal aberration assay and cell viability assay by trypan blue exclusion method for testing the compound from sea star *Stellaster equestris* against human peripheral blood. The extracted, isolated, purified and characterised compound at a defined concentration (four concentration 50, 100, 200, and 400µg /ml from the stock of 1mg/ml) was evaluated for its toxicity. The percentage

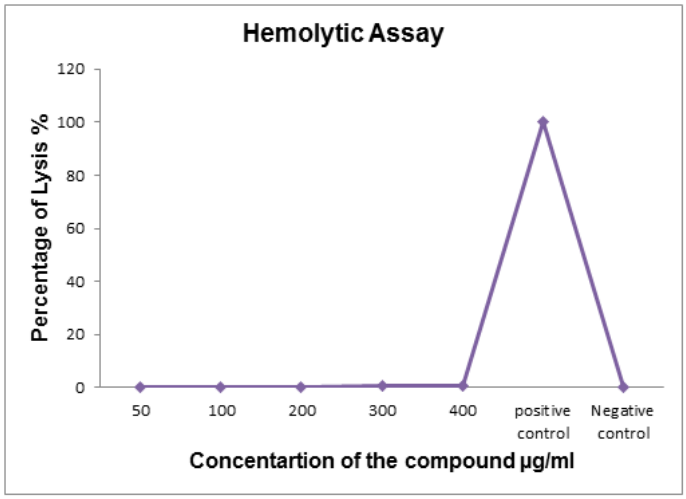

Figure 1: Hemolytic assay of purified sample of sea star *Stellaster equestris*.

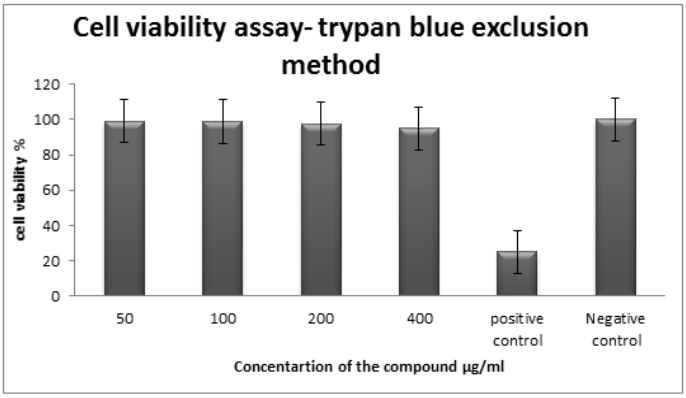

Figure 2: Cell Viability assay by trypan blue exclusion method against lymphocytes (Average mean % viable cells ± SD).

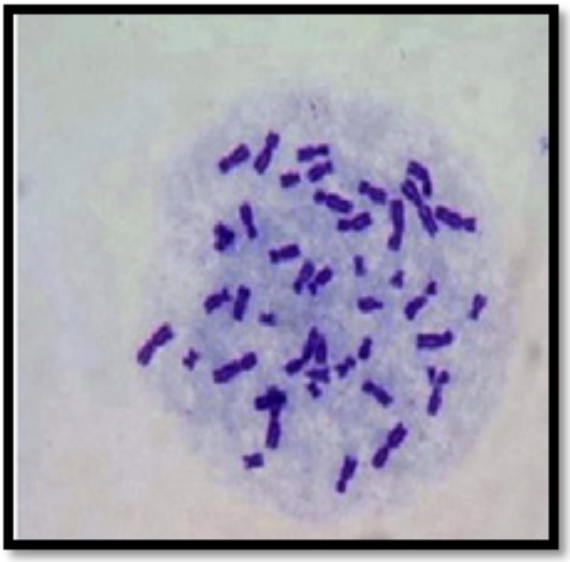

Figure 3: Metaphase 50µg/ ml of purified sample.

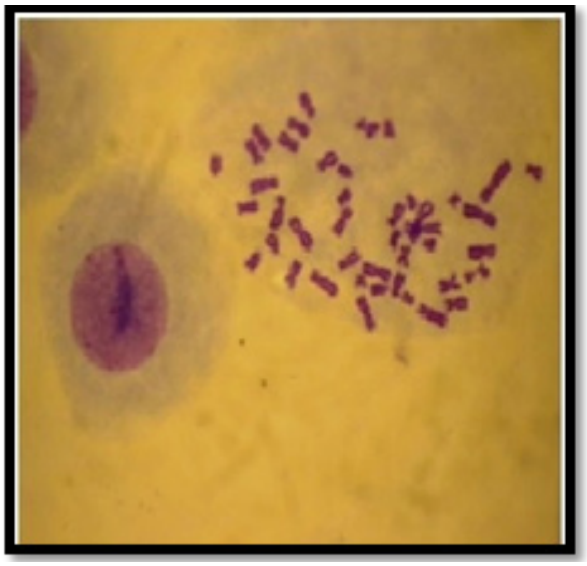

Figure 4: Metaphase 100 µg/ ml of purified sample.

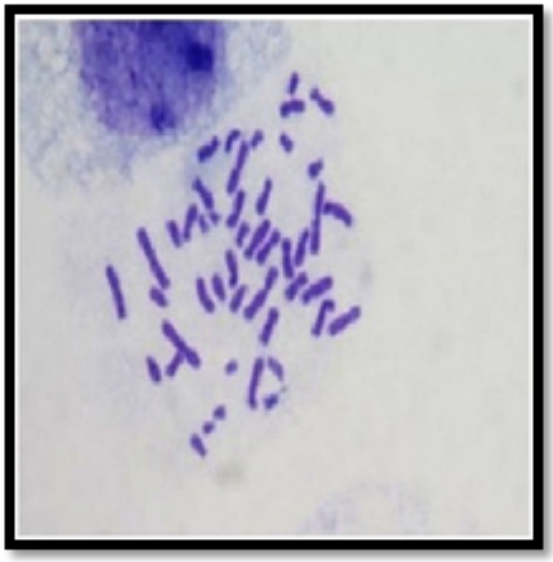

Figure 5: Metaphase 200 µg/ ml of purified sample.

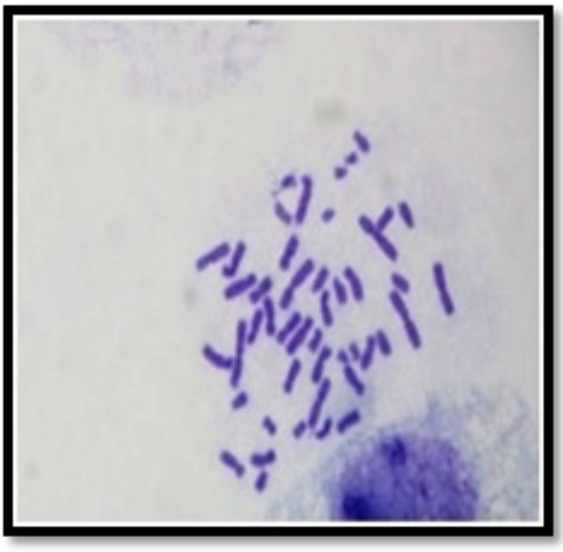

Figure 6: Metaphase 400 µg/ ml of purified sample.

of hemolysis for the defined concentration of the compound isolated from the sea star are considered to be non-hemolytic.

Previous studies reported on the another sea star *Acanthaster planci* which was involved in lethal and hemolytic activities was evident due to the presence of venom which was secreted as the secondary metabolite during the attack of predators. This was hence the first study reported on the hemolytic assay using red blood cells of the sheep and rabbit erythrocytes on micro titre plates and blood agar plates. The capacity of crude extract namely the venom secreted by the sea star *Acanthaster planci* lysed the red blood cells and hence was found to be hemolytic.[11] One another study reported on crude extracts of brittle sea star *Ophiocnemis marmorata* was assayed on chicken, sheep and human erythrocytes which possessed hemolytic activity against the goat blood showing a 100% of lysis than the other. The results thus indicated that the crude ethanolic extract of brittle sea star *Ophiocnemis marmorata* exhibited haemolytic properties.[12]

One other study reported on the saponins like compound isolated from brittle sea star *Ophiocoma erinaceus.* This study was investigated on the

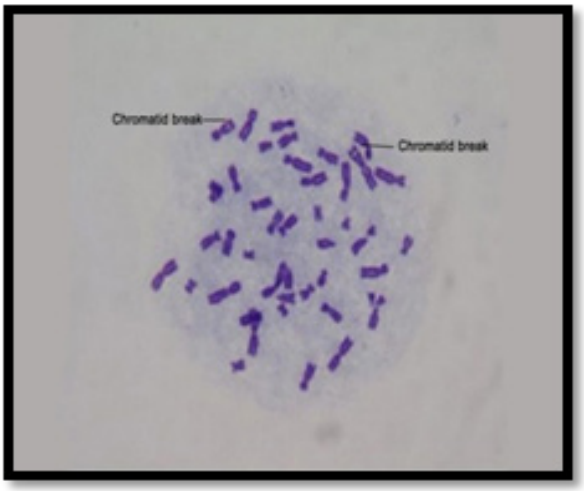

Figure 7: Positive control.

different fractions namely ethanol, butanol and water fraction obtained from the arm of brittle sea star *Ophiocoma erinaceus* against the cardiac blood of the rats. No significant hemolytic activity was shown at 50% and 100% of ethanolic fraction. But the 80% ethanolic fraction showed 50% of haemolysis at a concentration of 500 µg/ml. Hence indicating the presence of saponins might be the cause for haemolysis.[13]

Yet another study investigated on the coelomic fluid of sea star *Astropecten indicus*. The hemolytic assay was performed against the human and the goat red blood cells in dose dependent manner. 5% of haemolysis was observed in human blood on exposure to all doses of the sea star coelomic fluid. As for the goat blood a significant 50% haemolysis was observed at 20µg/ml of the coelomic fluid of sea star *Astropecten indicus*. The results hence indicated that the crude coelomic fluid from the sea star *Astropecten indicus* is a safe entrant for drug discovery.[14]

However the results obtained from the present study for the *in vitro* analysis of hemolytic assay correlates with the finding that the compound isolated sea star *Stellaster equestris* even in a dose dependent manner were non-hemolytic and seems to be safe.

The present study revealed that the purified sample did not show any cytotoxic effect on the exposed lymphocytes even in the dose dependant manner on comparison with the positive control. The viability was comparatively significant on comparison with the negative control which was the untreated cells. Previous study on the cell viability of the novel protein isolated from the coelomic fluid of sea stars *Astropecten indicus* reported to show increased cell viability in the time dependent manner indicating the protein molecule isolated from the coelomic fluid was not toxic.[14] Hence the present study correlates with the cell viability assay by trypan blue exclusion assay reporting to be non-toxic.

In vitro analysis specifically the chromosomal aberration assay was performed in the present study using human peripheral for analysing the chromosomal breaks, increase or decrease in chromosomal number, deletion and growth ability in the culture.[15] Chromosomal aberration assay with defined concentrations (50,100, 200 and 400 µg/ml) of purified sample was exposed to the human peripheral blood. The chromosomes were isolated, casted and stained and 100 metaphases and 100 interphase were scored and analysed under each concentration. The microscopic analyses revealed no structural or numerical abnormalities in all the four defined concentration except 200 µg/ml and 400 µg/ml as shown had shown no structural aberration but numerical deviation in 3 and 5 spreads respectively out of 100 metaphases scored each and analysed which might be due to the counting error. Hence all the four concentration were found to be non-genotoxic on comparison with the positive control as shown in (Figure 7) which illustrated the chromosomal breaks due to the exposure of the chemical mutagenic agent ethyl methanesulphonate (EMS) T the *in vitro* end points consistently verified that the purified compound of sea star *Stellaster equestris* was not toxic (both genotoxic nor mutagenic).

CONCLUSION

The outcome of the present study reported on the *in vitro* toxicity evaluation of sterol isolate from echinoderm *Stellaster equestris* against human peripheral blood has validated that the isolated compound was not toxic and hence require additional experimentation for the compound to be used as a potential drug for biomedical application.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PBS: Phosphate buffer; **EMS:** Ethyl methanesulphonate; **CA:** Chromosomal aberration; **FTIR:** Fourier transform infrared spectroscopy; **NMR:** Nuclear Magnetic Resonance Spectroscopic; **HPLC:** High performance liquid chromatography.

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