Research Article

Use of the *Allium cepa* Model to Assess the Cytogenotoxicity of *Luffariella herdmani* Marine Sponge Extract

Alternatives to Laboratory Animals
2023, Vol. 0(0) 1–13

DOI: 10.1177/02611929231171943
journals.sagepub.com/home/atl
SAGE

Sashini U. Kuruppuarachchi[1], Uthpala A. Jayawardena[2] and Varuni K. Gunathilake[1]

Abstract

Marine sponge extracts are known to contain potentially toxic compounds that have biological activities of possible pharmacological interest. Thus, it is vital that biological models are used for the preliminary toxicity screening of such extracts. The present study reports the use of *Allium cepa*, a low-cost plant-based *in vivo* model, to assess the cytotoxicity and genotoxicity of *Luffariella herdmani* marine sponge crude extract (SCE). Pre-germinated onion bulbs, exposed for 96 hours to different concentrations of SCE (ranging from 0.3125 to 20 μg/ml), were used to determine general cytotoxicity. Root length as well as morphological abnormalities were recorded. Genotoxicity was assessed by exposing the root tips to SCE (0.3125–20 μg/ml) and the appropriate controls for 48 hours, and then staining with acetocarmine. The Mitotic Index (MI), Mitotic Phase Indices (MPIs) and chromosomal aberrations were evaluated and recorded. SCE inhibited *A. cepa* root growth (EC_{50} = 10.34 μg/ml) and elicited a mitodepressive effect (LC_{50} = 1.95 μg/ml) in a dose-dependent and significant manner. In addition, macroscopic alterations as well as chromosomal aberrations were detected. Overall, our findings indicate that *L. herdmani* crude extract exhibits cytotoxic and genotoxic activity, suggesting that it might contain substances with anti-proliferative/anticancer potential that could be subject to further characterisation.

Keywords

Allium cepa model, chromosomal aberrations, cytogenotoxicity, *Luffariella herdmani*, marine sponge extract, non-animal alternatives

Introduction

Marine sponges, the most primitive multicellular organisms, are considered a prodigious reservoir of compounds, and many of these possess various bioactivities. A considerable number of these compounds play a pivotal role in marine-based pharmacology, which involves the use of marine sponge-sourced compounds as leads in drug development.[1] This results in an inspiring and potentially productive relationship between marine biology and drug biotechnology. Marine sponge extracts are a rich source of compounds with antibacterial, antiviral, antifungal, antimalarial, antitumour, immunosuppressive and cardiovascular activities.[2] As a result, several drug discovery and development programmes are now focused on marine sponges to help expedite the discovery of novel therapeutic entities against various human diseases.[3] However, despite their potential as a source of various drug leads, marine sponge-derived compounds can be associated with toxicity,[4] most likely as a consequence of lengthy evolutionary adaptations to resist predators, microbial pathogens, parasites and anti-biofouling measures, as well as to restrict overgrowth by other sessile species.[5]

Although the toxicity of such compounds is usually reported at very high concentrations, at low concentrations ($IC_{50} \leq 10$ μM, or 4–5 μg/ml), they exhibit numerous biological activities of pharmacological interest,[4] thus warranting their assessment as potential antitumour agents. Over the years, numerous marine sponge-derived compounds with anti-proliferative/anticancer properties have been discovered — for example, isoaaptamine (from *Aaptos aaptos*) and

[1]Department of Zoology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka
[2]Department of Zoology, Faculty of Natural Sciences, The Open University of Sri Lanka, Nawala, Sri Lanka

Corresponding author:
Varuni K. Gunathilake, Department of Zoology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda 10100, Sri Lanka.
Email: varunig@sjp.ac.lk

debromohymenialdisine (from *Hymeniacidonaldis* sp.) are inhibitors of protein kinase C, which is involved in tumour growth.[6] Other potential candidates for regulating tumour growth include fucosyltransferase inhibitors, such as octa and nonaprenylhydroquinone sulphates isolated from *Sarcotragus* sp.[6] In addition, several other marine sponge-derived non-specific cell growth inhibitors — for example, compounds interfering with microtubule formation (halichondrin B, spongistatin 1, discodermolide, laulimalide), as well as compounds interfering with actin polymerisation (latrunculin A, swinholide A), could potentially be developed into anticancer drugs.[7]

Despite ethical and other concerns, the use of animal models to test the toxicity of natural and synthetic compounds is still routinely practised on a global level. This use of animal models has potentially resulted in long delays in the drug discovery pipeline. For instance, even though many marine sponge-derived compounds with anticancer properties have been discovered, only one sponge-derived anticancer drug, eribulin mesylate (Halaven), has been developed thus far into a commercially viable product.[8] The continued reliance on *in vivo* models has faced criticism, especially directed toward the use of higher animals, such as rodents.[9] It should also be noted that *in vivo* experimental procedures, particularly those that inflict pain and distress on the animals, have the potential to alter the accuracy and translatability of the experimental results.[10] Furthermore, appropriate anaesthesia and surgery skills are required for many of these procedures, which are exceedingly time consuming and might be difficult to repeat for a variety of reasons; cost may also be a factor.[11] Thus, the development of adequate alternatives to replace and reduce the use of animal models in this area has been highlighted previously and is urgently required.

The mechanisms associated with the toxicity of marine sponge-derived compounds vary widely. Many sponge-derived anti-proliferative agents induce DNA damage, arrest the cell cycle, and interact with many different targets involved in cancer development and apoptosis (e.g. the mitochondrial membrane, cytochrome C).[4,8] In preliminary toxicological tests, DNA damage and cell apoptosis are usually assessed.[12] Cell cytotoxicity refers to the tendency of certain agents to harm or kill living cells through a variety of pathways, including cell membrane disruption, prevention of DNA elongation or the inhibition of enzymatic reactions,[13] while genotoxicity refers to the ability to cause mutations or changes in the structure and composition of the genetic material.[14] Genotoxicity is assessed according to various endpoints, including the induction of point mutations, variations in chromosome number (polyploidy or aneuploidy) or changes in chromosome structure.[15] As a result, genotoxicity is often the most difficult adverse effect to identify. In this context, the *Allium cepa* (i.e. onion) model is a promising alternative, as it can be used to detect both cytotoxicity and genotoxicity with the presence of chromosomal alterations.[16] It is a simple, economical and easy to perform test, which is considered to be an extremely efficient indicator of environmental pollution.[17] It has also been effectively used for the testing of cyanobacteria crude extracts, and to assess the *in vivo* genotoxicity of medicinal plants.[17]

Both the International Programme on Chemical Safety and the United Nations Environment Programme (UNEP) have validated the *A. cepa* model as an effective test for analysing the genotoxic effects of various substances.[18,19] The following characteristics of *A. cepa* lead it to be considered as a good non-animal model for use in toxicity testing:

- sensitive genetic makeup;
- high number of mitotic cells;
- clearly visible mitotic phases;
- low number of large chromosomes (2n = 16);
- stable chromosome number and karyotype;
- diverse chromosome morphology;
- clear and rapid response to toxic substances;
- rare occurrence of chromosomal damage;[20,21] and
- high comparability with mammalian test systems.[22]

However, the use of the *A. cepa* model to evaluate marine sponge crude extracts has not been widely adopted. The current study aims to investigate the potential cytotoxic and genotoxic effects of *Luffariella herdmani* sponge extracts by using the *A. cepa* bioassay, in order to validate the extract as a potential therapeutic anti-proliferative/anticancer agent that warrants further, more comprehensive, research.

Materials and methods

Collection, identification and preparation of the marine sponge crude extract

Approximately 500 g of sponge material was collected in Unawatuna, Sri Lanka (6° 00′ 13.3″ N, 80° 14′ 46.9″ E) at a depth of 9–20 m by a commercial scuba diver (Department of Wildlife, Sri Lanka, permit number: WL/3/2/64/17). After a thorough examination of the specimen's external morphology, fibre arrangement, ectosomal as well as choanosomal structure, it was identified as *L. herdmani*.[23,24] The sponge crude extract (SCE) was prepared by incubating approximately 200 g of sponge material diced into small pieces (~1×1×1 cm) in a 1:1 v/v methanol/dichloromethane mixture (Sigma-Aldrich, St Louis, MI, USA) for 72 hours. It was then filtered through grade 1 filter paper, and rotary-evaporated (Buchi type) at 40°C.[25] By using the serial half-dilution method, a dilution series was obtained for the SCE in 5% v/v ethanol.

The A. cepa bioassay for the evaluation of marine sponge crude extract cytotoxicity

Equal sized (5–6 g) *A. cepa* bulbs were visually inspected and confirmed to be in good condition. The bottom plates and dead scales were removed and, in order to prevent the bulbs from rotting, they were suspended in dechlorinated water with only their basal plates (~0.1–0.3 cm) touching the water. The bulbs were incubated at 27 ± 2°C for 48 hours, without exposing them to direct sunlight, and the water was changed every 24 hours.

After the roots reached approximately 1 cm in length, the bulbs were suspended in different concentrations of ethanolic SCE (20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 µg/ml) for 96 hours with the roots in direct contact with the SCE. Each test concentration was set up as three replicates, in three separate containers. The positive control (PC) and negative (vehicle) control (VC) consisted of 5% v/v dimethyl sulphoxide (DMSO) and 5% v/v ethanol, respectively; the negative (water) control (WC) consisted of the dechlorinated water used in the initial root-growth step. The test solutions were renewed every 24 hours.

At the end of the 96-hour exposure period, appropriately 30 roots for each concentration were randomly selected and their lengths were measured. In addition, visible morphological abnormalities, i.e. gelling, necrosis, presence of hooks or twists, swelling and pigmentation, were recorded. The percentage root growth relative to the negative (vehicle) control (i.e. VC) was calculated for each sample, as well as the effective concentration 50 (EC_{50}) value for the SCE.[26]

$$\text{Percentage root growth} = \frac{\text{Mean root length of sample}}{\text{Mean root length of VC}} \times 100$$

The A. cepa bioassay for the evaluation of marine sponge crude extract genotoxicity

After the roots had reached approximately 1 cm in length (see previous method section), the bulbs were suspended in different concentrations (5, 2.5, 1.25, 0.625 and 0.3125 µg/ml) of ethanolic SCE for 48 hours at 27 ± 2°C. Each test concentration was set up as three replicates, in three separate containers. The test solutions were renewed every 24 hours. The PC was 5% v/v dimethyl sulphoxide (DMSO) and the VC was 5% v/v ethanol; the WC consisted of the dechlorinated water used in the initial root-growth step.

At the end of the 48-hour exposure period, 5–6 root tips (~0.5–1 mm) were obtained from each bulb, immediately placed in a 1:3 solution of 1N HCl: glacial acetic acid (Breckland Scientific Suppliers, Norfolk, UK), and heated in a water bath at 60 ± 2°C for 10 minutes. The root tips were then transferred to another container and exposed to a 1% acetocarmine solution (Sigma-Aldrich) for 15–20 minutes, or until the root tips were properly stained (as determined by the presence of deeply stained ends).

One stained root tip was placed on a glass slide with a drop of distilled water, and a coverslip was placed on top while applying slight pressure to crush the root tip and release the cells. The onion root cells were observed with a compound light microscope under a 400× magnification (OPTIKA®; 40 × 10). In total, 30 root tips were analysed in this manner.

The total number of cells and the number of dividing cells in each mitotic stage were counted. The total number of cells from each root tip that were evaluated for the purposes of the calculations, was 1000. The MI, percentage mitotic inhibition (PMI), mitotic phase indices (MPI) for prophase, metaphase, anaphase and telophase, and the percentage of chromosomal aberrations (PCA) were calculated by using the formulae listed below. The lethal concentration 50 (LC_{50}) value for the SCE was also calculated.[27]

$$\text{MI} = \frac{\text{Total number of cells in division}}{\text{Total number of analysed cells}} \times 100$$

$$\text{PMI} = \frac{(\text{MI of the negative (vehicle) control} - \text{MI of the test sample})}{\text{MI of the negative (vehicle) control}} \times 100$$

$$\text{MPI} = \frac{\text{Total number of cells in each phase}}{\text{Total number of analysed cells}} \times 100$$

$$\text{PCA} = \frac{\text{Total number of aberrant cells}}{\text{Total number of analysed cells}} \times 100$$

Statistical analysis

Minitab 17 statistical software was used to analyse the results. The means with 95% confidence limit and standard error of mean (SEM) for each set of data were calculated. Pearson's correlation, one sample *t*-test and one-way ANOVA (Tukey's test) were applied with 95 CI (significance at $p < 0.05$) for results prediction.

Results and discussion

The identification of new anticancer compounds with appropriate pharmaco-toxicological profiles, to be used alone or in association with conventional chemotherapy strategies, is essential. In this context, natural compounds — such as marine sponge-derived bioactive compounds — could play a pivotal role, since they often show useful activities and are inexpensive and readily available.[8] Toxicological assays for preliminary screening purposes represent a bottleneck in the evaluation of any potential bioactive compounds, before they

can proceed through to preclinical and clinical trials. In addition, the evaluation of a compound's effects on malignant cell lines can also prove to be a time-consuming and resource-intensive process.[4]

A number of biological models, such as marine-derived *Artemia* nauplii and echinoderm eggs/embryos, have been successfully used for the early assessment of potential *in vivo* toxicity of various marine natural products.[4] In the present study, a crude extract of the marine sponge, *Luffariella herdmani*, was investigated for its *in vivo* cytotoxicity and genotoxicity, by using two assays based on the use of *A. cepa*. This plant-based model was chosen for its ethical and environmental considerations, ease of maintenance, reliability in assessing toxic effects, and widespread availability to allow reproducible results to be obtained. *L. herdmani* crude extract was previously shown by the authors to exert toxic effects against the brine shrimp *Artemia salina*.[28]

Cytotoxicity of the marine sponge crude extract, as determined by the A. cepa *bioassay*

The *A. cepa* model was used to assess general cytotoxicity of the SCE by measuring the length, form, colour and turgescence of the roots, after exposure to different concentrations of SCE. The roots were assessed for various macroscopic abnormalities, i.e. gelling, necrosis, presence of hooks or twists, swelling and yellowish brown pigmentation (Figure 1), and the treated samples were compared with the controls. Table 1 summarises the macroscopic findings.

Root gelling was the most abundant macroscopic abnormality, which was observed in all test samples (except in the VC and the WC). The presence of hooks, swelling and necrosis was observed in varying numbers of roots, at all tested concentrations of SCE. Overall, the occurrence of macroscopic abnormalities was random in nature (i.e. spontaneous) and lacked any discernible pattern, and multiple abnormalities were observed in some of the treated roots at all concentrations of SCE and in the controls (Table 1). All of these spontaneous alterations are common (i.e. expected to occur normally), and are consistent with previous studies.[26,29]

As the SCE was insoluble in water, 5% ethanol was used as the solvent for the extract — thus 5% ethanol was used as the negative (vehicle) control during the experiments. It was ascertained that the average root length of *A. cepa* exposed to 5% ethanol (VC) was not significantly different (one-sample *t*-test, $p = 0.213$) to that exposed to water only (WC) (2.99 ± 0.04 cm *versus* 3.05 ± 0.05 cm, respectively). Dieleman et al.[30] stated that the use of ethanol as a solvent for cytokinins strongly inhibited the growth and development of the rose plants used in their study. However, a recent study by Miller et al.[31] demonstrated that, although root zone ethanol concentrations of 1% to 5% (v/v) reduced the height of *Narcissus tazetta* (paper white narcissus), there was no visible phytotoxicity to the roots. Both *Allium* sp. and *Narcissus* sp. belong to the family Amaryllidaceae, and numerous studies have used *A. cepa* to investigate the toxicity of ethanolic extracts.[32,33] As all sample endpoints in these experiments were compared to those of the ethanol (i.e. negative (vehicle)) control (VC), the results obtained take into account any potential effects of ethanol on the roots.

Increasing concentrations of SCE reduced root growth in a dose-dependent manner. The dose–response curve obtained by comparing the effects of exposure to different concentrations of SCE on the percentage root growth (relative to the VC) is shown in Figure 2. This relative percentage root growth had a strong negative linear relationship with increasing concentration of SCE (Pearson's $r = -0.883$, $p = 0.008$).

The mean root lengths of *A. cepa* treated with SCE showed statistically significant differences at all concentrations tested, as compared to the VC ($p < 0.05$), and also compared to the PC, except at 20 µg/ml ($p = 0.656$) (Table 1). Thus, SCE appeared to exert a significant inhibitory effect on *A. cepa* root growth. This could be due to alterations in various biological processes involved in cellular expansion, such as water uptake, nitrogen mobilisation, increased sugar synthesis, and plasma and tonoplast membrane flexibility.[34]

Determination of the EC_{50} value: The effective concentration at which the SCE caused a 50% decline in root growth compared to the VC (i.e. the EC_{50} value) was found to be 10.34 µg/ml. The crude extract of *L. herdmani* that was previously determined as being toxic to *A. salina* larvae had an LC_{50} value of 14.34 µg/ml.[28] Overall, the current study further confirms the extract's potent toxic nature. The EC_{50} value can also be used to select the range of test concentrations for genotoxicity analysis.[35] Consequently, in the current study, all further test concentrations were maintained below the EC_{50}, since concentrations above 5 µg/ml had direct cytotoxic effects, causing cellular apoptosis or necrosis. This, in turn, lowered the number of mitotic divisions and the number of detectable chromosomal aberrations (data not shown).

Genotoxicity of the marine sponge crude extract, as determined by the A. cepa *bioassay*

The *A. cepa* assay also enables the assessment of different genetic endpoints, which include the mitotic index (MI), mitotic phase indices (MPI), and the number of chromosomal aberrations.[27]

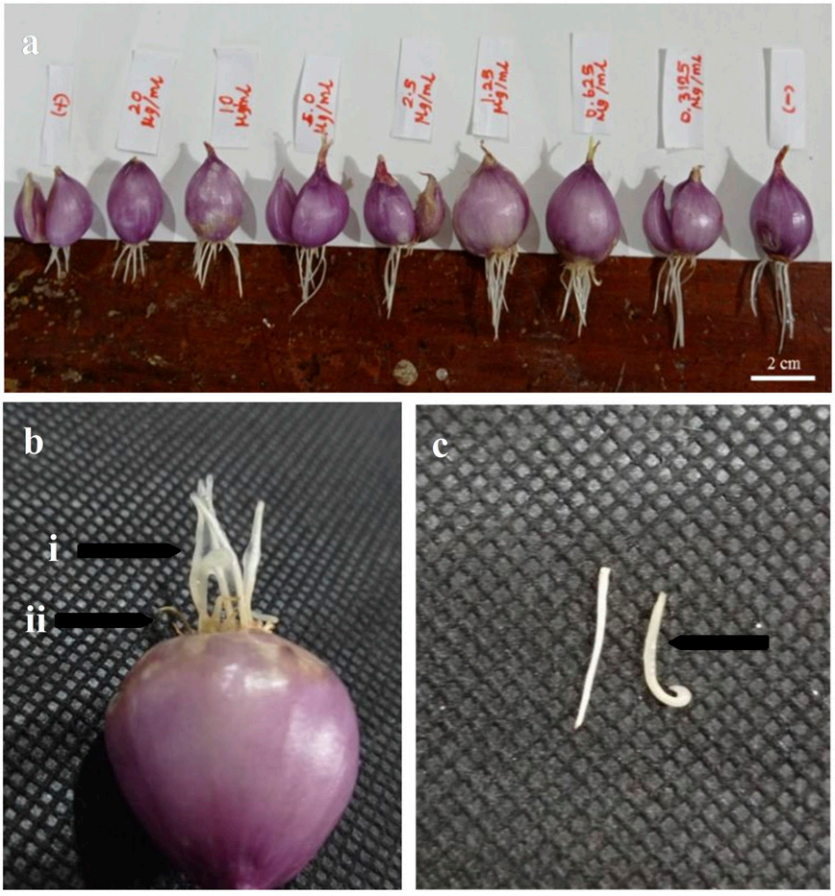

Figure 1. General toxicity indicators assessed in the *A. cepa* test system. a) Variation in root lengths after a 96-hour exposure to different concentrations of SCE and controls (scale bar = 2 cm). (+) = Positive control; (–) = negative (vehicle) control (ethanol). b) Examples of root gelling (i) and necrosis (ii) are shown in (b); a comparison of a normal root and a hooked root (indicated by the arrowhead) is shown in (c).

Mitotic index (MI): In genotoxicity studies, the MI estimates the proportion of cells in the M-phase of the cell cycle.[17] A drop in MI value might be viewed as being indicative of cellular death or a delay in cell proliferation kinetics.[36] As the mitotic division in *A. cepa* root cells resembles the cell division in normal human and cancer cells, the MI is a good indicator of a compound's antimitotic potential.[37]

The MI values decreased with increasing concentrations of SCE and showed a clear negative relationship (Table 2). The highest MI (22.0 ± 0.86) was obtained after exposure to 0.3125 µg/ml SCE, while the lowest (4.17 ± 0.15) was obtained with 5 µg/ml SCE (Table 2). The MI obtained for *A. cepa* exposed to 5% ethanol (VC) was not significantly different to that of the water only control (WC) (24.40 ± 0.27 *versus* 25.47 ± 0.30, respectively). A similar study carried out with stem bark extracts of the medicinal plant, *Ficus benghalensis*, recorded its lowest MI of 28 ± 0.577 at the highest extract concentration (4 mg/ml) — a concentration which is significantly higher than that of the *L. herdmani* extract used in the current study.[37]

The percentage inhibition of the MI was plotted against the different concentrations of SCE, as shown in Figure 3. There was a significant positive linear correlation between the concentration of SCE and the percentage of MI inhibition, as indicated by the Pearson's r value of 0.879 (p = 0.050).

Mitotic phase indices (MPI): The fact that the MI was reduced in a dose-dependent manner indicates that the compounds present in the extract were able to progressively inhibit the ability of the cells to progress through the cell cycle phases. Cells might be unable to progress through mitosis as efficiently as normal for a variety of reasons — for example, hindrance of the onset of prophase, arrest of one or more mitotic phases, or retardation of the rate of cell progression through mitosis.[27] Overall, the observed mitodepressive nature of the SCE is a strong indicator that it could potentially exert effects on the cell cycle by blocking the G1 phase, subsequently inhibiting DNA synthesis and blocking the G2 phase, preventing the cells from entering mitosis, or blocking the synthesis of nucleoproteins.[27]

Table 1. The effects of SCE exposure on *A. cepa* root growth and form.

					Test sample concentration (μg/ml)						
		PC	VC	WC	0.3125	0.625	1.25	2.5	5	10	20
Mean root length ± SEM (cm)		1.06 ± 0.04	2.99 ± 0.04	3.05 ± 0.05	**2.53 ± 0.04**	2.03 ± 0.06	1.97 ± 0.04	1.81 ± 0.04	1.51 ± 0.04	1.31 ± 0.04	**1.08 ± 0.03**
Macroscopic abnormalities	Hook	4	6	2	9	7	11	4	8	3	7
	Gelling	27	—	—	18	20	19	21	23	28	25
	Necrosis	7	1	2	4	4	3	2	2	2	4
	Swelling	5	—	1	—	1	—	3	2	5	3

The highest and the lowest values of mean root length, following a 96-hour exposure to SCE, are highlighted in bold. — denotes absence of macroscopic abnormalities. Root tips analysed: $n = 30$. PC = positive control; VC = negative (vehicle) control; WC = water control.

The mitotic phase indices (MPI) for prophase, metaphase, anaphase and telophase, indicating the proportion of cells observed in each phase of the cell division process, are shown in Figure 4. Healthy cell division phases (prophase, metaphase, anaphase and telophase) of *A. cepa* meristematic cells were clearly visible. In *A. cepa* cells treated with SCE, prophase represented the highest proportion of observed cells, followed by telophase, while metaphase and anaphase cells were the least observed. Similar results, where prophase was predominant and other phases were less common, were obtained in other studies.[37]

In the current study, the prophase index values showed statistically significant differences (ANOVA, Tukey's test, $p < 0.05$) compared to the negative control, at all SCE concentrations except for 0.3125 µg/ml. The prophase index values showed significant differences compared to the positive control, only at the lower SCE concentrations (0.3125 and 0.625 µg/ml). The index values for metaphase

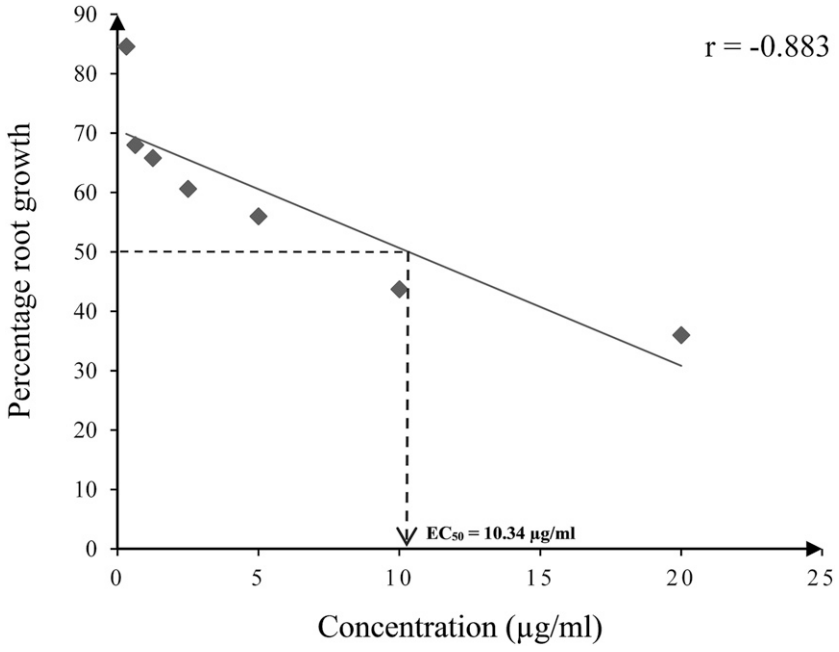

Figure 2. The effect of SCE exposure on *A. cepa* root growth.
The roots were exposed to SCE at the indicated concentrations for 96 hours. The values shown refer to root growth after exposure to SCE, relative to that of the negative (vehicle) control (i.e. ethanol).

Table 2. Genotoxic effects of SCE exposure on *A. cepa* root meristem cells.

		Mean chromosome aberration ± SEM					
Sample concentration	MI ± SEM (%)	c-Mitosis	Stickiness	Numerical aberrations	Laggard/ vagrant	Anaphase bridge	PCA ± SEM (%)
0.3125 µg/ml	**22.00 ± 0.86**	**8.67 ± 2.33^{b}**	**6.00 ± 0.58^{d}**	**5.67 ± 0.88^{b}**	0.33 ± 0.33^{a}	—	**2.07 ± 0.27**
0.625 µg/ml	17.4 ± 1.21	22.33 ± 1.67^{ab}	7.33 ± 0.88^{d}	16.00 ± 3.06^{ab}	—	—	4.57 ± 0.38
1.25 µg/ml	11.33 ± 0.87	32.00 ± 3.00^{ab}	24.00 ± 7.55^{cd}	34.67 ± 2.85^{ab}	—	—	9.07 ± 0.38
2.5 µg/ml	6.27 ± 0.83	**39.00 ± 16.20^{ab}**	84.00 ± 24.20^{b}	**36.70 ± 16.10^{ab}**	**1.00 ± 1.00^{a}**	**1.33 ± 0.88^{a}**	16.20 ± 0.67
5 µg/ml	**4.17 ± 0.15**	26.33 ± 3.53^{ab}	**172.00 ± 4.04^{a}**	25.33 ± 3.28^{ab}	0.67 ± 0.67^{a}	0.33 ± 0.33^{a}	**22.47 ± 0.32**
PC	7.97 ± 0.43	51.00 ± 3.79^{a}	68.30 ± 18.80^{bc}	57.30 ± 16.30^{a}	0.33 ± 0.33^{a}	0.33 ± 0.33^{a}	17.73 ± 0.47
VC	24.40 ± 0.27	16.33 ± 1.86^{b}	0.33 ± 0.33^{d}	10.00 ± 3.06^{b}	—	—	2.67 ± 0.15
WC	25.47 ± 0.30	16.67 ± 2.60^{b}	—	1.33 ± 0.33^{b}	—	—	1.80 ± 0.27

A. cepa roots were exposed to SCE and the root meristem cells were assessed for chromosomal abnormalities. The values shown are the mean ± SEM (n = 3); values in the same column with the same superscript letters indicates that they are not significantly different from each other (p 0.05) and vice versa. These letters were generated and assigned by using one-way ANOVA (Tukey's test) in Minitab 17. The highest and the lowest values for each type of chromosomal aberration are shown in bold. — denotes absence of microscopic abnormalities; MI = mitotic index; PCA = percentage chromosomal aberrations; PC = positive control; VC = negative (vehicle) control; WC = water control.

and anaphase were significantly different at all SCE concentrations, as compared with the negative control, but were not significantly different when compared with each other. The telophase index values for all SCE concentrations were not significantly different, except at the 5 µg/ml concentration, when compared to the negative control. Index values for metaphase, anaphase and telophase were not significantly different, as compared to the positive control (all ANOVA, Tukey's test, $p > 0.05$). Thus, overall, all the phase indices of the *A. cepa* root cells treated with

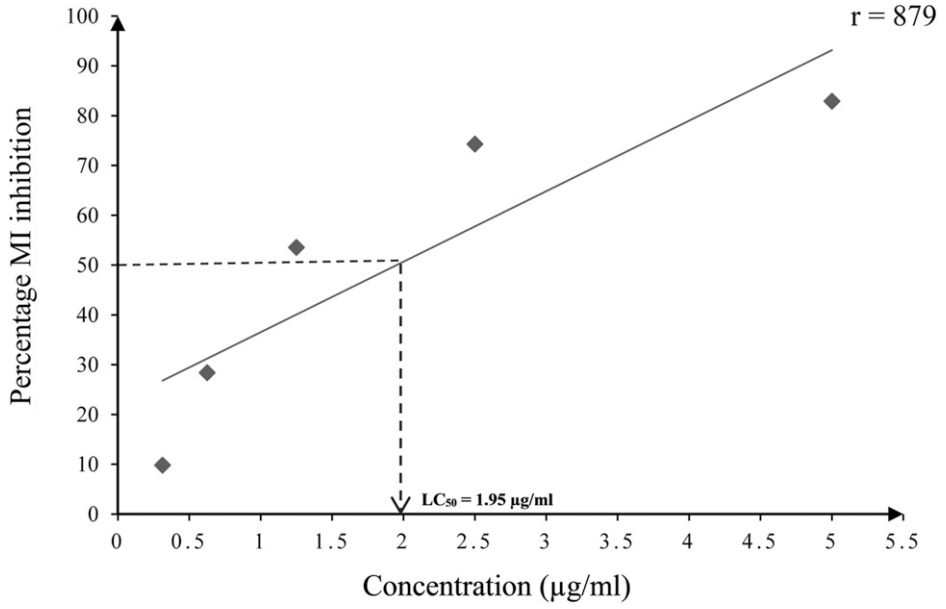

Figure 3. The effect of SCE exposure on the mitotic index (MI) of *A. cepa* root meristem cells.
The roots were exposed to SCE at the indicated concentrations for 48 hours and the mitotic index (MI) of the root meristem cells was calculated. The values shown refer to the percentage inhibition, relative to the negative (vehicle) control (i.e. ethanol).

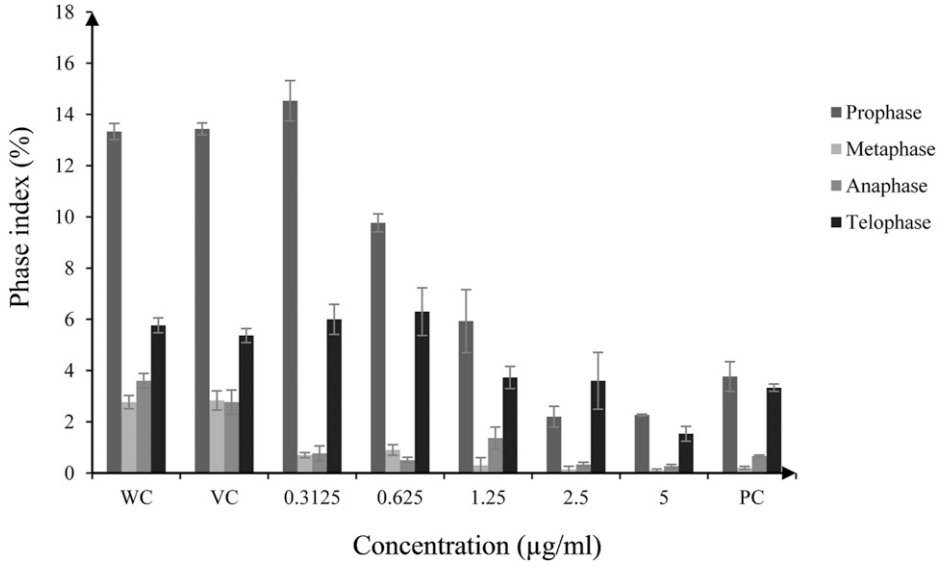

Figure 4. Phase indices of major cell division stages of *A. cepa* root meristem cells treated with different concentrations of SCE.
A. cepa roots were exposed to different concentrations of SCE (5, 2.5, 1.25, 0.625 and 0.3125 µg/ml), as well as the relevant controls, for 48 hours. WC = water only control; VC = negative (vehicle) control (i.e. ethanol); PC = positive control (i.e. DMSO). Following treatment, the phase indices, which indicate the proportion of root cells observed in each phase of the cell division process, were determined. Data are presented as the mean ± SEM.

SCE showed a decreasing trend with concentration (see Figure 4).

Determination of the LC_{50} value: The LC_{50} value is often used to evaluate the toxicity of a natural extract, for comparison in either Meyer's toxicity index or Clarkson's toxicity index.[24] Extracts with LC_{50} values of < 1000 µg/ml are considered toxic according to Meyer's toxicity index, whereas Clarkson's toxicity index classifies extracts with LC_{50} values of 0–100 µg/ml as very toxic.[24] According to both toxicity indices, the *L. herdmani* crude extract exhibits potent toxicity. The LC_{50} value corresponding to a 50% mitotic inhibition of *A. cepa* meristem cells by SCE was 1.95 µg/ml. This mitodepressive effect was positively and significantly correlated with the root length (see Figure 5; Pearson, r = 0.938, p = 0.019).

A recent study that evaluated the MI inhibitory activities of extracts derived from two Sri Lankan marine sponge species (*Stylissa carteri* and *Axinella* sp.) determined their LC_{50} values to be 94.06 µg/ml and 114.63 µg/ml, respectively. Compared to these values, the crude extract of *L. herdmani* evaluated in our study elicited a more potent mitodepressive effect.[38]

Chromosomal aberrations: Changes in the structure of chromosomes due to the break or exchange of chromosomal material are known as chromosomal aberrations.[27] The assessment of various forms of chromosomal aberrations in all mitotic phases of SCE-treated samples provides an effective evaluation of the SCE's clastogenic, aneugenic and tubergenic effects.[39] The chromosomal abnormalities observed in *A. cepa* root meristem cells exposed to SCE are summarised in Table 2 and illustrated in Figure 6. The most abundant chromosomal aberrations were stickiness, c-mitosis and numerical aberrations.

Chromosome stickiness may be caused by sub-chromatid linkage between chromosomes, depolymerisation of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fibre units of chromatids or removal of the protein covering of the DNA in the chromosomes.[27] Observation of this effect indicates that the SCE can cause irreversible toxic effects.[40]

C-mitosis is a potentially toxic, reversible effect that occurs as a result of a disrupted spindle apparatus. If not reversed, it can cause aneuploidy or polyploidy.[27] C-mitotic compounds are also classified as spindle poisons, mitotic poisons or antimitotic compounds.[40] Since microtubules and microfilaments are crucially active in cell division and are a primary target in cancer therapy, *L. herdmani* crude extract could be further investigated as a potential anti-mitotic compound.

At higher concentrations of SCE, a few anaphase bridges, laggard and vagrant chromosomes were detected. In the anaphase and telophase stages, chromosomal bridges can form as a result of chromosome breakage and fusion, as well as during the translocation of uneven chromatid exchange.[41] Vagrant chromosomes can be induced by a spindle irregularity, resulting in the formation of irregular-shaped nuclei during interphase and unequal-sized daughter cells.[42] Vagrant chromosomes have a weak c-mitotic effect, suggesting that there might be a chance of aneuploidy.[27] Laggard chromosomes are those that do not entirely pinch out from their opposite daughter cell during the process of cell

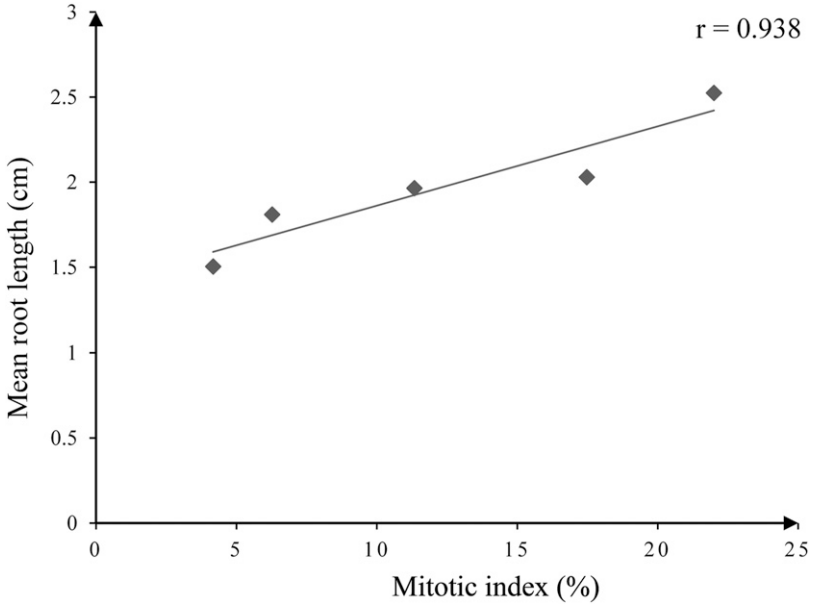

Figure 5. Correlation between the mitotic index (MI) and mean root length following exposure to SCE. The roots were exposed to different SCE concentrations for 48 hours and the root tips stained with acetocarmine.

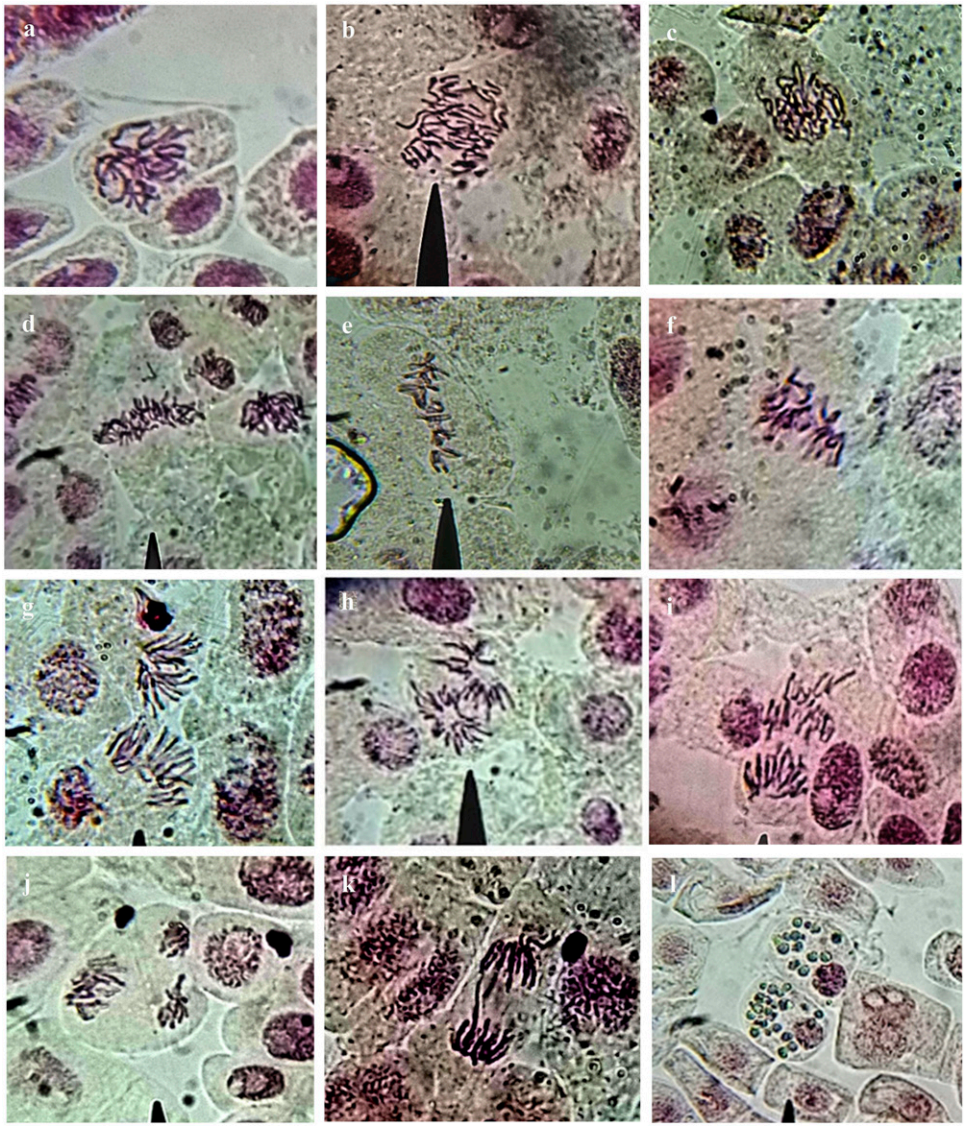

Figure 6. Visualisation of chromosome aberrations in *A. cepa* root meristem cells, after exposure to SCE.
A. cepa meristematic cells were exposed to different concentrations of SCE and stained with acetocarmine, prior to visualisation under a light microscope. The images shown are representative, and illustrate: a) and b) c-mitosis; c) and d) numerical aberrations; e) and f) stickiness; g), h) and i) laggard and vagrant chromosomes; j) multi polar anaphase; k) anaphase bridge; and l) necrotic cells.

division.[40] Chromosomal breaks were rarely observed in the current study.

Concentrations of SCE above 5 µg/ml caused abundant cellular apoptosis or necrosis, which resulted in a reduction in the number of mitotic divisions and thus in the number of detectable chromosomal aberrations. The percentage of chromosomal aberrations increased with increasing SCE concentration (Pearson's r = 0.971, p = 0.006; and see Table 2).

An analysis of crude extracts from *Stylissa carteri* and *Axinella* sp. showed that, respectively, they induced a level of 11.9% and 7.2% chromosomal aberration at the maximum concentration tested (100 µg/ml). These aberrations were mainly characterised as stickiness, c-mitosis, vagrants, fragments and chromosomal bridges.[38] Higher percentages of aberrant cells were found at both the 5 and 2.5 µg/ml SCE concentrations in the present study. Furthermore, SCE induced more aneugenic aberrations (stickiness, c-mitosis) than clastogenic aberrations (breaks and bridges), and also had the potential for inducing polyploidy. The effects of *L. herdmani* SCE on the MI and chromosomal aberrations of *A. cepa* root cells are summarised in Table 2.

Similar studies have demonstrated that various medicinal plant species, including *Citrus sinensis*,[43] *Artemisia verlotorum*,[44] *Pterocaulon polystachyum*[45] and *Mikania glomerata*,[46] exert cytogenotoxic effects on the *A. cepa* root

system. In contrast to the present study, chromosomal abnormalities found to be caused by other plant extracts were wider ranging, and included anaphase bridges, breakages and laggards, fragment formations,[44–46] as well as binucleated cells and micronuclei.[44] These variations in the types of chromosomal abnormality may have implications for the genotoxicity of different extracts and their potential effects on DNA.

Overall, the *L. herdmani* marine sponge crude extract was found in the current study to be both cytotoxic and genotoxic to *A. cepa* root tips, suggesting potential anti-proliferative properties. In agreement with these results, another sea sponge (*L. variabilis*) has displayed anti-proliferative effects on a human breast cancer cell line (MCF7WT) [47] and on the HTLV-1-related leukaemia cell line (S1T).[48] Furthermore, *L. geometrica* has also shown moderate anti-proliferative effects on the tumour cell lines HMO2 and HepG2, with a GI_{50} (i.e. growth inhibitory power) of 1.7 and 1.8 µg/ml, respectively.[49]

Summary and conclusions

Inevitably, a single assay will not be sufficient to evaluate the potential genotoxicity of a compound, due to the existence of a wide range of genetic endpoints.[40] Plant extracts of *Maytenus ilicifolia* and *Bauhinia candicans*, which caused a decrease in the MI of *A. cepa* root cells, produced similar results in Wistar rat bone-marrow cells.[50] Thus, the current results should be viewed as preliminary, and further experiments involving different endpoints — such as the Comet assay, DNA fragmentation, and the testing of anti-proliferative effects on selective *in vitro* cell lines (e.g. OVCAR-3, HCT-116, HCT-115, SK-OV-03) — are recommended prior to consideration of this extract as a potential drug candidate.

The present study highlighted the successful use of two *A. cepa*-based assays, in the preliminary testing of *L. herdmani* SCE for cytotoxicity and genotoxicity. The extract was found to: significantly inhibit *A. cepa* root growth (EC_{50} = 10.34 µg/ml); induce mitodepressive effects on all stages of cell division (LC_{50} = 1.95 µg/ml); and induce both macroscopic and chromosomal aberrations in *A. cepa* root cells. The types of chromosomal aberrations induced by the SCE suggest that it could be aneugenic, and also that it might have the potential to induce polyploidy. Thus, the results obtained suggest that the *L. herdmani* SCE has potential anti-proliferative properties that are undoubtedly worthy of further study.

Acknowledgements

The authors would like to express their gratitude to Dr Marco Bertollino of the Department of Earth Environment and Life Sciences (DISTAV), at the Università degli Studi di Genova in Genova, Italy, for his assistance in identification of sponge species.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Ethical approval

Ethics approval was not required for this research paper.

Informed consent

Informed consent was not required for this research paper.

References

1. Mannelli LDC, Esposito FP, Sangiovanni E, et al. Pharmacological activities of extracts and compounds isolated from Mediterranean sponge sources. *Pharmaceuticals* 2021; 14: 1329.
2. Mehbub MF, Lei J, Franco C, et al. Marine sponge derived natural products between 2001 and 2010: Trends and opportunities for discovery of bioactives. *Mar Drugs* 2014; 12: 4539–4577.
3. Gunathilake V, Bertolino M, Bavestrello G, et al. Immunomodulatory activity of the marine sponge, *Haliclona (Soestella*) sp. (Haplosclerida: Chalinidae), from Sri Lanka in Wistar albino rats: Immunosuppression and Th1-skewed cytokine response. *J Immunol Res* 2020; 2020: 1–11.
4. Mioso R, Marante FJ, Bezerra RD, et al. Cytotoxic compounds derived from marine sponges. A review (2010–2012). *Molecules* 2017; 22: 208.
5. Van Soest RW, Boury-Esnault N, Vacelet J, et al. Global diversity of sponges (Porifera). *PLoS One* 2012; 7: e35105.
6. Sipkema D, Franssen MC, Osinga R, et al. Marine sponges as pharmacy. *Mar Biotechnol* 2005; 7: 142–162.
7. Anjum K, Abbas SQ, Shah SA, et al. Marine sponges as a drug treasure. *Biomol Ther* 2016; 24: 347–362.
8. Calcabrini C, Catanzaro E, Bishayee A, et al. Marine sponge natural products with anticancer potential: An updated review. *Mar Drugs* 2017; 15: 310.
9. Sarveswaran R, Jayasuriya WJ and Suresh TS. *In vitro* assays to investigate the anti-inflammatory activity of herbal extracts a review. *World J Pharm Res* 2017; 6: 131–141.
10. Doke SK and Dhawale SC. Alternatives to animal testing: A review. *Saudi Pharm J* 2015; 23: 223–229.
11. Patil KR, Mahajan UB, Unger BS, et al. Animal models of inflammation for screening of anti-inflammatory drugs: Implications for the discovery and development of phytopharmaceuticals. *Int J Mol Sci* 2019; 20: 4367.

12. Dorato MA and Buckley LA. Toxicology testing in drug discovery and development. *Curr Proto Toxicol* 2007; 31: 1–19.
13. Aslantürk ÖS. *In vitro* cytotoxicity and cell viability assays: Principles, advantages, and disadvantages. In: Larramendy M and Soloneski S (eds) *Genotoxicity: A predictable risk to our actual world*. 2nd ed. London: Intech Open Limited, 2018, pp. 64–80.
14. Ifeoma O and Oluwakanyinsola S. Screening of herbal medicines for potential toxicities. In: Gowder SJT (ed) *New insights into toxicity and drug testing*. London: Intech Open Limited, 2013, pp. 63–88.
15. Tamokou JDD and Kuete V. Mutagenicity and carcinogenicity of African medicinal plants. In: Kuete V (ed) *Toxicological survey of African medicinal plants*. Amsterdam: Elsevier, 2014, pp. 277–322.
16. Jayathilake NJ and Jayewardena UA. Genotoxic potential of aqueous extract: A sea cucumber species, *Bohadschia vitiensis* using genotoxicity model *Allium cepa*. *Adv Pharm J* 2021; 6: 9–14.
17. Tedesco SB and Laughinghouse IV HD. Bioindicator of genotoxicity: The *Allium cepa* test. In: Srivastava JK (ed) *Environmental contamination*. London: Intech Open Limited, 2012, pp. 137–156.
18. Frescura VD, Laughinghouse IV HD and Tedesco SB. Antiproliferative effect of the tree and medicinal species *Luehea divaricata* on the *Allium cepa* cell cycle. *Caryologia* 2012; 65: 27–33.
19. Pastori T, Flores FC, Boligon AA, et al. Genotoxic effects of *Campomanesia xanthocarpa* extracts on *Allium cepa* vegetal system. *Pharm Biol*. 2013; 51: 1249–1255.
20. Nefic H, Musanovic J, Metovic A, et al. Chromosomal and nuclear alterations in root tip cells of *Allium cepa* L. induced by alprazolam. *Med Arh* 2013; 67: 388–392.
21. Firbas P and Amon T. Chromosome damage studies in the onion plant *Allium cepa* L. *Caryologia* 2014; 67: 25–35.
22. Bonciu E, Firbas P, Fontanetti CS, et al. An evaluation for the standardization of the *Allium cepa* test as cytotoxicity and genotoxicity assay. *Caryologia* 2018; 71: 191–209.
23. Gülçin İ, Huyut Z, Elmastaş M, et al. Radical scavenging and antioxidant activity of tannic acid. *Arab J Chem* 2010; 3: 43–53.
24. Kumar AN, Bevara GB, Laxmikoteswramma K, et al. Antioxidant, cytoprotective and anti-inflammatory activities of stem bark extract of *Semecarpus anacardium*. *Asian J Pharm Clin Res* 2013; 6: 213–219.
25. Prastya ME, Astuti RI, Batubara I, et al. Antioxidant, antiglycation and *in vivo* antiaging effects of metabolite extracts from marine sponge-associated bacteria. *Indian J Pharm Sci* 2019; 81: 344–353.
26. Sabini MC, Cariddi LN, Escobar FM, et al. Evaluation of cytogenotoxic effects of cold aqueous extract from *Achyrocline satureioides* by *Allium cepa* L test. *Nat Prod Commun* 2011; 6: 995–998.
27. Yuet Ping K, Darah I, Yusuf UK, et al. Genotoxicity of *Euphorbia hirta*: An *Allium cepa* assay. *Molecules* 2012; 17: 7782–7791.
28. Kuruppuarachchi SU and Gunathilake VK. Zoo-chemical profiling, *in vivo* toxicity and *in vitro* anti-inflammatory properties of *Luffariella herdmani* marine sponge extract. *J Adv Biotechnol Exp Ther* 2022; 5: 269–282.
29. Bidau CJ, Amat AG, Yajía M, et al. Evaluation of the genotoxicity of aqueous extracts of *Ilex paraguariensis* St. Hil. (*Aquifoliaceae*) using the *Allium* test. *Cytologia* 2004; 69: 109–117.
30. Dieleman JA, Perik RR and Kuiper D. Effect of ethanol on the growth of axillary rose buds *in vitro*. *J Plant Physiol* 1995; 145: 377–378.
31. Miller WB and Finan E. Root-zone alcohol is an effective growth retardant for paperwhite narcissus. *HortTechnology* 2006; 16: 294–296.
32. Cabrera GL and Rodriguez DM. Genotoxicity of soil from farmland irrigated with wastewater using three plant bioassays. *Mutat Res* 1999; 426: 211–214.
33. Mekki L. Genoprotectivity of methanol and ethanol extracted leaf sap of *Trigonella foenum-graecum* in *Allium cepa* root assay. *Acta Biol Hung* 2014; 65: 85–95.
34. Iwalokun BA, Oyenuga AO, Saibu GM, et al. Analyses of cytotoxic and genotoxic potentials of *Loranthus micranthus* using the *Allium cepa* test. *Curr Res J Biol Sci* 2011; 3: 459–467.
35. Rank J and Nielsen MH. *Allium cepa* anaphase–telophase root tip chromosome aberration assay on N-methyl-N-nitrosourea, maleic hydrazide, sodium azide, and ethyl methanesulfonate. *Mutat Res* 1997; 390: 121–127.
36. Aşkin Çelik T and Aslantürk ÖS. Evaluation of cytotoxicity and genotoxicity of *Inula viscosa* leaf extracts with *Allium* test. *J Biotechnol Biomed* 2010; 2010: 1–8.
37. Raheel R, Saddiqe Z, Iram M, et al. *In vitro* antimitotic, antiproliferative and antioxidant activity of stem bark extracts of *Ficus benghalensis* L. *S Afr J Bot* 2017; 111: 248–257.
38. Ranatunga NP and Gunathilake KVK. The genotoxicity assessment of *Stylissa carteria* and *Axinella* sp. marine sponge extracts by *Allium cepa* bio assay. In: *The 8th Annual Scientific Session of Sri Lanka Association for Laboratory Animal Science* (SLALAS), Colombo, Sri Lanka, 23–24 January 2021, Paper no. 02, pp. 26.
39. Khalil AM, Salman WK and Al-Qaoud KM. Preliminary evaluation of acute cytogenotoxicity of a novel phenylboronic acid derivative; 2-(bromoacetamido) phenylboronic acid using the *Allium cepa* chromosome aberrations assay. *Caryologia* 2017; 70: 34–41.

40. Timothy O, Idu M, Olorunfemi DI, et al. Cytotoxic and genotoxic properties of leaf extract of *Icacina trichantha* Oliv. *S Afr J Bot* 2014; 91: 71–74.
41. Chukwujekwu JC and van Staden J. Cytotoxic and genotoxic effects of water extract of *Distephanus angulifolius* on *Allium cepa* Linn. *S Afr J Bot* 2014; 92: 147–150.
42. Özkara A, Akyıl D, Eren Y, et al. Potential cytotoxic effect of *Anilofos* by using *Allium cepa* assay. *Cytotechnology* 2015; 67: 783–791.
43. Tedesco M, Kuhn AW, Boligon AA, et al. Chromatographic analysis, antiproliferative effect and genotoxicity of aqueous extracts of *Citrus sinensis* (L.) Osbeck on the *Allium cepa* L. test system. *Biosci J* 2015; 31: 1213–1221.
44. Bittencourt de Souza LF, Laughinghouse IV HD, et al. Genotoxic potential of aqueous extracts of *Artemisia verlotorum* on the cell cycle of *Allium cepa*. *Int J Environ Sci* 2010; 67: 871–877.
45. Knoll MF, Silva AC, Canto-Dorow TS, et al. Effects of *Pterocaulon polystachyum* DC. (Asteraceae) on onion (*Allium cepa*) root-tip cells. *Genet Mol Biol* 2006; 29: 539–542.
46. Dalla Nora G, Pastori T, Laughinghouse IV HD, et al. Antiproliferative and genotoxic effects of *Mikania glomerata* (Asteraceae). *Biocell* 2010; 34: 95–101.
47. Gauvin A, Smadja J, Aknin M, et al. Isolation of bioactive 5α, 8α-epidioxy sterols from the marine sponge *Luffariella* cf. *variabilis*. *Can J Chem* 2000; 78: 986–992.
48. Hamada T, Harada D, Hirata M, et al. Manoalide-related sesterterpene from the marine sponge *Luffariella variabilis*. *Nat Prod Commun* 2015; 10: 863–864.
49. Kehraus S, König GM and Wright AD. A new cytotoxic calyculinamide derivative, geometricin A, from the Australian sponge *Luffariella geometrica*. *J Nat Prod* 2002; 65: 1056–1058.
50. Camparoto ML, Teixeira RD, Mantovani MS, et al. Effects of *Maytenus ilicifolia* Mart. and *Bauhinia candicans Benth* infusions on onion root-tip and rat bone-marrow cells. *Genet Mol Biol* 2002; 25: 85–89.