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In vitro anti-inflammatory, anti-oxidant and cytogenotoxicity of *Axinella* sp., a marine sponge extract

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Abstract

Exploring the oceans for bioactive compounds is an incessant human desire. The present study investigates selected bioactivities; anti-inflammatory, anti-oxidant, cytotoxic, and genotoxic potential of *Axinella* sp. marine sponge crude extract (SCE). Sponge identification was based on morphology and skeletal analysis. The SCE was prepared by methanol/dichloromethane extraction and tested for zoo-chemicals, anti-inflammatory properties by protein denaturation, heat and hypertonicity-induced bovine erythrocyte membrane stability assays. Radical scavenging activity was tested against 2,2-diphenyl-1-picryl-hydrazil (DPPH), 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), nitric oxide (NO), and peroxide radicals. The IC_{50} was calculated for each assay. Cytotoxicity and genotoxicity were tested on *Artemia salina* and *Allium cepa* models, respectively. The LC_{50} and mitotic index (MI) were calculated where appropriate, while chromosomal aberrations were recorded in the *A. cepa* assay. The results indicated the presence of alkaloids, terpenoids, unsaturated sterols, flavonoids, and saponins in SCE. Potent inhibitory activities on egg albumin denaturation were reported by SCE (IC_{50}=39.55±3.21 µg/ml). Heat and hypertonicity induced bovine erythrocyte membrane stability was reported as IC_{50}=44.64±0.56 µg/ml and IC_{50}=35.7±.0.26 µg/ml, respectively. In comparison to reference drugs, the resulting scavenging activities were strong against NO (IC_{50}=50.63±2.85 µg/ml) and more or less similar against DPPH (IC_{50}=42.49±0.85 µg/ml). However, the potency of ABTS and peroxide radical scavenging activities was low in SCE (IC_{50}=42.49±0.74 µg/ml and IC_{50}=323.52±3.71 µg/ml, respectively). The SCE was toxic to *A. salina* nauplii (LC_{50}=106.81 µg/ml) and *A. cepa* root cells (LC_{50}=114.63 µg/ml) with a 7.2% chromosomal aberrations reported in the *A. cepa* genotoxicity assay. The potent anti-inflammatory, anti-oxidant, cytotoxic, and genotoxic effects of SCE proposes its feasibility as a potential drug lead, followed by further comprehensive research.

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1. Introduction

In recent decades, marine pharmacology has received great attention, resulting in a plethora of bioactive compounds with numerous therapeutic properties (Kuruppuarachchi & Gunathilake, 2022). Among all marine invertebrates, sponges are recognised as one of the richest sources of bioactive compounds, accounting for approximately 30% of all marine natural products (Mehbub *et al.*, 2014). Manolide from *Luffariella variabilis*, dysidotronic acid from *Dysidea* sp., Ircinin-1 and -2 from *Ircinia*

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oros, petrosaspongiolides M-R from *Petrosaspongia nigra,* and spongidines A-D from *Spongia* sp. act as phospholipase A inhibitor while jaspaquinol from *Jaspis splendens* and subersic acid from *Suberea* sp. act as lipoxygenase inhibitors (Sipkema *et al.*, 2005). Sponge derived manoalide is by far the best characterised PLA_2 inhibitor from natural sources (Kijjoa & Sawangwong, 2004).

Despite the availability of a vast array of drugs, inflammation continues to be a global health issue, posing a social and economic burden on many societies (Wongrakpanich *et al.*, 2018). The inflammatory response is a defence mechanism that protects the body against infections, damaged cells, toxic substances, irradiation, and other harmful conditions, while allowing injured cells to repair (Medzhitov, 2010). A large number of humoral and cellular mediators known as anti-inflammatory agents lead to inflammatory diseases if disrupted (Dinarello, 2010). Non-steroidal anti-inflammatory drugs (NSAIDs) suppress the symptoms associated with the inflammation, while glucocorticoids inhibit leukocyte function (Punchard *et al.*, 2004). However, most of these drugs are associated with adverse side effects, including gastrointestinal (GI) tract complications, renal disturbances, and cardiovascular events (Wongrakpanich *et al.*, 2018). Thus, novel therapeutic entities that are more effective with fewer side effects and can successfully ameliorate inflammation are important.

In an inflammatory reaction, leukocytes and mast cells undergo a respiratory burst as a result of increased oxygen absorption. This increases the generation and release of reactive oxygen and nitrogen species at the injured region (Arulselvan *et al.*, 2016). These radicals will kill pathogens directly by causing oxidative damage or indirectly by activating various cellular signaling pathways (Delves *et al.*, 2017). However, the excessive release of reactive oxygen and nitrogen species will result in extensive oxidative damage that results in alteration of the structure and biological functions of proteins, which in turn leads to malignant diseases (Ekins *et al.*, 2017). Therefore, it is vital to maintain the balance between the synthesis and removal of these radicals in a healthy system. Thus, screening for radical scavenging activity in crude extracts, along with anti-inflammatory properties, is a common approach. Over the years, treating inflammatory diseases has become an even bigger challenge for scientists as there are no safe drugs commercially available. Thus, the toxicity evaluation of sponge crude extracts prior developing into therapeutic products with fewer side effects is crucial. The use of low-cost, reproducible, short-term biological models that are alternatives to higher animal models are highly recommended in the preliminary drug discovery process (Kuruppuarachchi & Gunathilake, 2022).

The *Artemia salina* cytotoxicity assay is one of the simplest and most convenient *in vivo* lethality assays (Waghulde *et al.*, 2019). It is widely used to assess the toxicity in many natural compounds, including sponge crude extracts. Similarly, the *Allium cepa in vivo* genotoxicity model is a rapid, low-cost, and convenient model used to evaluate different genetic endpoints (Rosculete *et al.*, 2018). The sensitivity of its genetic makeup, a high proportion of mitotic cells, clearly visible mitotic phases, a low number of large chromosomes (2n=16), a stable chromosome number and karyotype, diverse chromosome morphology, a clear and fast response to genotoxic substances, and rare occurrence of spontaneous chromosomal damages have made this model one of the best *in vivo* models to test genotoxic compounds (Nefic *et al.*, 2013; Firbas & Amon, 2014). Further, the *A. cepa* assay is highly comparable with other mammalian test systems, including the chromosomal aberration assay in rat bone marrow cells, human lymphocytes, and V79 cell line of Chinese hamster (Bonciu *et al.*, 2018).

With the beginning of the United Nations Decade of Ocean Science for Sustainable Development (2021–2030), many scientists are well-lined to explore the ocean in search of potent therapeutic agents (UNESCO/IOC, 2020). Despite Sri Lanka's rich marine biodiversity, a large number of sponge species are understudied, making this field a gold mine for scientists (De Silva *et al.*, 2018). The current work will serve as a paradigm for the discovery of novel anti-inflammatory drug leads from *Axinella* sp., which could ultimately lead to the potential growth of the pharmaceutical industry in Sri Lanka.

2. Materials and Methods

2.1. Materials

Diclofenac sodium (Voltaren® 50, Switzerland) was used as the standard anti-inflammatory drug and purchased from a pharmaceutical shop (Union Chemists Private Limited, Colombo, Sri Lanka). All other chemicals used during the study were of analytical grade.

2.2. Collection, identification, and preparation of the sponge crude extract (SCE)

The sponge sample was collected from Polhena, Matara, Sri Lanka (5.9363 ^{0}N, 80.5263 ^{0}E) via commercial scuba diving at a depth of 10-20 m. The specimen was carefully observed for external morphology, ectosome and choanosme structure,

internal fibre arrangement and identified as *Axinella* sp. (Hooper & Van Soest, 2002). The identification was further confirmed by Dr. Marco Bertolino of the Department of Earth, Environment, and Life (DISTAV), University of Genova, Italy.

After weighing and dicing the sponge material into small pieces and incubating it for 72 h in a methanol/dichloromethane (1:1 v/v) mixture, the SCE was obtained by filtering through Whatmann No: 1 filter paper (LINCO, India), followed by rotary evaporation (BUCHI Rota vapor R-124, Germany) at 40 °C (Zivanovic *et al.*, 2011). After calculating the percentage yield, the SCE was dissolved in 5% (v/v) ethanol to prepare the required doses for anti-inflammatory, radical scavenging, and toxicity assays.

2.3. Qualitative zoo-chemical analysis

The qualitative zoo-chemical analysis was carried out for SCE, where alkaloids, flavonoids, proanthocyanidins, anthraquinones, quinones, saponins, sterols, tannins, terpenoids, and unsaturated sterols were tested using standard protocols with minor modifications (Parekh & Chanda, 2007, Kuruppuarachchi & Gunathilake, 2022).

2.3.1. Test for alkaloids

Approximately 1 ml of ethanolic SCE was combined with a few drops of 50% HCl and a few milliliters of Mayer's reagent and examined for the formation of a yellow-coloured precipitate.

2.3.2. Test for flavonoids

Approximately 2 ml of ethanolic SCE was mixed with 2 ml of Conc. HCl and a few small, cleaned magnesium stripes were added. The solution was examined for an orange-to-red colour appearance.

2.3.3. Test for Proanthocyanidins

The addition of a few drops of Conc. HCl to 1 ml of ethanolic SCE, followed by heating and the subsequent colour change from yellow to red upon the addition of amyl chloride was examined.

2.3.4. Test for anthraquinones

Approximately 1 ml of ethanolic SCE was mixed with a few drops of 10% KOH (v/v). Following agitation, the solution was examined for the presence of a red colour.

2.3.5. Test for quinones

For approximately 1 ml of ethanolic SCE, 10% (v/v) NaOH was added and observed for a yellow, red, or purple colour appearance.

2.3.6. Test for saponins

Approximately 1 ml of ethanolic extract SCE was placed in a glass vial and shaken vigorously. The solution was observed for the formation and persistence of the froth for more than 10 min.

2.3.7. Test for sterols

Approximately 1 ml of acetic anhydride and 1 ml of Conc. H_2SO_4 were added to 1 ml of ethanolic SCE successively, and the colour change from red brown to purplish-brown was observed.

2.3.8. Test for tannins

Approximately 1 ml of ethanolic SCE was mixed with a few drops of 1% (v/v) $FeCl_3$ and examined for a blue-black colour appearance, which identifies the presence of gallic tannins, and a greenish-brown colour appearance, which identifies the presence of tannins.

2.3.9. Test for terpenoids

Approximately 1 ml of ethanolic SCE was combined with 2 ml of Conc. H_2SO_4 and heated in a water bath at 40 ^{0}C for 2 min. The solution was observed for a brown to red colour appearance.

2.3.10. Test for unsaturated sterols

A few drops of Conc. H_2SO_4 were added drop wise along the wall into 1 ml of ethanolic SCE, and the formation of a red colour ring at the interface was observed.

2.4. Evaluation of the in vitro anti-inflammatory activity of SCE

2.4.1. Protein denaturation assay

A reaction mixture containing 2 ml of SCE (100, 50, 25, 12.5, and 6.25 µg/ml) or standard drug diclofenac sodium (2500, 1250, 625, 312.5, and 156.25 µg/ml), 2.8 ml of phosphate buffered saline (PBS) (pH 6.4), and 0.2 ml of fresh egg albumin was incubated at 37 ^{0}C for 15 min. It was further incubated at 70 ^{0}C in a water bath for 5 min. The absorbance was measured by UV-visible spectrophotometer (UVD-2960) at 660 nm using PBS as a blank and 5% ethanol as the negative control. The test was triplicated, and the percentage inhibition of protein denaturation was calculated according to Eq. 1 (Chandra *et al.*, 2012).

Percentage inhibition of protein denaturation =

$$[(Abc - Abs) / Abc] \times 100 \quad \text{Eq. 1}$$

Where; Abc = Absorbance of the control and Abs = Absorbance of the sample

2.4.2. Erythrocyte membrane stabilisation assay

Fresh bovine blood mixed with 3.8% trisodium citrate was centrifuged at 3000 rpm for 10 min and the supernatant was carefully removed. The volume

of blood was measured, and a 2% v/v erythrocyte suspension was prepared by diluting in normal saline (Ranasinghe *et al.*, 2012).

2.4.3. Heat induced hemolysis assay

The reaction mixture containing 1 ml 2% RBC suspension and 1 ml SCE (100, 50, 25, 12.5, and 6.25 µg/ml) or standard drug aspirin (2500, 1250, 625, 312.5, and 156.25 µg/ml) and the control containing normal saline were incubated at 56 ^{0}C water bath for 30 min, followed by 2500 rpm centrifugation for 5 min. The absorbance was measured at 560 nm. The test was performed in triplicate, and the percentage inhibition of heat induced hemolysis was calculated according to Eq. 2 (Ranasinghe *et al.*, 2012).

Percentage inhibition of hemolysis = [(Abc – Abs) / Abc] × 100 Eq. 2

Where; Abc = Absorbance of the control and Abs = Absorbance of the sample

2.4.4. Hypotonicity induced hemolysis assay

Accurately, 2 ml hyposaline, 1 ml phosphate buffer and 0.5 ml RBCs suspension were mixed with SCE (100, 50, 25, 12.5, and 6.25 µg/ml) or diclofenac sodium (standard drug) (2500, 1250, 625, 312.5, and 156.25 µg/ml) followed by an incubation at 37 ^{0}C for 30 min. The mixture was centrifuged at 3000 rpm for 10 min and the absorbance was measured at 560 nm. The test was triplicated, and the percentage inhibition was calculated according to Eq. 2 (Ranasinghe *et al.*, 2012).

2.5. Free radical scavenging activity of the SCE

2.5.1. 2, 2-diphenyl-1-picryl-hydrazil (DPPH) scavenging assay

Accurately, 100 µl of each dose of SCE (100, 50, 25, 12.5, and 6.25 µg/ml) or standard drug ascorbic acid (2500, 1250, 625, 312.5, and 156.25 µg/ml) was incubated with 100 µl of DPPH solution (125 µM in ethanol) for 30 min in the dark at room temperature. The colour change was measured at 517 nm using a microplate reader (s/n MR05405, USA). 5% ethanol was used as the negative control. The test was triplicated, and the percentage inhibition was calculated according to Eq. 3 (Prastya *et al.*, 2019).

Radical scavenging activity (%) = [(Abc – Abs) / Abc] × 100 Eq. 3

Where; Abc = Absorbance of the control and Abs = Absorbance of test sample

2.5.2. 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) scavenging assay

Accurately, 180 µl of ABTS mixed with 20 µl of different concentrations of SCE (100, 50, 25, 12.5, and 6.25 µg/ml) or standard ascorbic acid (2500, 1250, 625, 312.5, and 156.25 µg/ml) were incubated for 30 min at room temperature. The absorbance was measured at 734 nm. The test was triplicated, and the percentage inhibition was calculated according to Eq. 3 (Prastya *et al.*, 2019).

2.5.3. Nitric oxide (NO) radicals scavenging assay

A 60 µl reaction mixture comprising of 10 mM sodium nitroprusside in PBS (pH 7.4) and SCE (100, 50, 25, 12.5, and 6.25 µg/ml) or standard ascorbic acid (2500, 1250, 625, 312.5, and 156.25 µg/ml) was incubated at 25 ^{0}C for 150 min. A 10 µl of Griess reagent (1% sulphanilamide, 2% orthophosphoric acid, and 0.1% Napthylethyline diamine hydrochloride) was added, and the absorbance was measured at 546 nm. The test was triplicated, and the percentage inhibition was calculated according to Eq. 3 (Kumar *et al.*, 2013).

2.5.4. Peroxide radicals scavenging assay

Accurately, 150 µl of 4 mM hydrogen peroxide solution in phosphate buffer (pH 7.4) mixed with 850 µl of the SCE (100, 50, 25, 12.5, and 6.25 µg/ml) or standard butylated hydroxyl toluene (BHT) (2500, 1250, 625, 312.5, and 156.25 µg/ml) was incubated for 10 min at room temperature and the absorbance was measured at 230 nm. The test was triplicated, and the percentage inhibition was calculated according to Eq. 3 (Prastya *et al.*, 2019).

2.6. Cytotoxicity assessment by Artemia salina bio assay

Cytotoxicity test was carried out on newly-hatched *A. salina* nauplii in sterile artificial seawater (24 h incubation period at 27±2 ^{0}C). Ten nauplii were delivered to 5 ml of each concentration of SCE (100, 50, 25, 12.5, and 6.25 µg/ml), 10% DMSO (positive control), and 5% ethanol (negative control) for another 24 h. The number of dead and live nauplii were counted, and the percentage mortality was calculated according to Eq. 4 (Olowa & Nuñeza, 2013).

Percentage mortality of *A. salina* nauplii = $[(N_0 - N_1) / N_0] \times 100$ Eq. 4

Where; N_0 = Total number of nauplii and N_1 = Number of live nauplii

2.7. Genotoxicity assessment by A. cepa bio assay

Equal sized (5-6 g) onion bulbs rooted in dechlorinated water and kept in dark for 48 h were treated with different concentrations of SCE (100, 50, 25, 12.5, and 6.25 µg/ml), 5% DMSO (positive control), and 5% ethanol (negative control) for another 48 h. At the end of the incubation period, onion root tips measuring 0.5 cm in length (5-6 tips from each bulb) were treated with a solution of aceto alcohol (1:3 ratio of 1N HCl: glacial acetic acid). The treated root tips were then incubated at 60°C for 10 min, followed by incubation in 1N HCl at 60°C for another 10 min. The root tips were transferred to an acetocarmine solution until they were deeply stained. Each root tip was placed on a glass slide and was evenly spread to prepare a root squash. The total number of cells and the number of dividing cells were counted under a compound light microscope (OPTIKA®, Italy), and the images were taken using Magnus Live USB 2.0 viewer software of the Microscopic Image Projection System (Magnus MIPS, India). The test was done in triplicate (Ghosh *et al.,* 2010).

The mitotic index (MI) was calculated as the number of dividing cells per 1000 observed cells according to Eq. 5.

MI = [no. of dividing cells/ Total no. of cells] × 100% Eq. 5

The percentage of mitotic inhibition was calculated using Eq. 6.

Percentage mitotic inhibition = [(MI control – MI sample) / MI control] × 100 Eq. 6

The 1000 cells were further observed for chromosomal aberrations and the percentage chromosomal aberrations was calculated according to Eq. 7.

Chromosomal aberrations (%) = [No. of aberrant cells/ Total no. of cells] × 100 Eq. 7

2.8. Statistical analysis

Minitab 17 statistical software was used to analyse the results, and the data were expressed as Mean ± Standard Error Mean (SEM). The nature and magnitude of the relationship between given variables were assessed by Pearson's product – moment correlation. One sample t test was used to compare the mean value of the sample population with the control values. The applied parametric tests had a 95% confidence interval, and statistical differences were considered significant for a p value < 0.05. In all assays, the IC_{50} and LC_{50} values were calculated appropriately and compared with those of the reference drugs.

3. Results and Discussion

In recent years, a significant number of marine natural products with potent pharmacological properties have been discovered, where marine sponges account for 30% of all of them (Mehbub *et al.*, 2014). Despite the global scenario, to date, marine spongology remains in its infancy in Sri Lanka (De Silva *et al.*, 2018). The reason for this may be merely due to the non-exploration of Sri Lankan waters or due to the lack of proper taxonomic authentication of the species. During the present study, the collected sponge specimen was identified as *Axinella* sp., which belongs to Family - Axinellidae Carter, 1875; Order - Halicondrida Gray, 1867; and Class - Demospongiae Sollas, 1885. The sponge was a brightly red, branched, flabelliform species that was attached to the substrate with a narrow stalk (Figure 1 A). Both choanosomal and ectosomal skeletons were present, and in both, spicules (mainly large megasclerers; oxeas and styles) (Figure 1 B) produced clusters and arranged around an axis in a plumose pattern (Figure 1 C and D). Species of Genus *Axinella* have been previously recorded in many regions of the world, including in Sri Lanka (e.g. *Axinella labyrinthica*, *Axinella ceylonensis*, and *Axinella lamellate*) (Hooper & Van Soest, 2002). However, due to the limited morphological features, the identification of the collected sponge sample during the current study was limited to its genus level. Further studies are required to identify this particular species up to the species level.

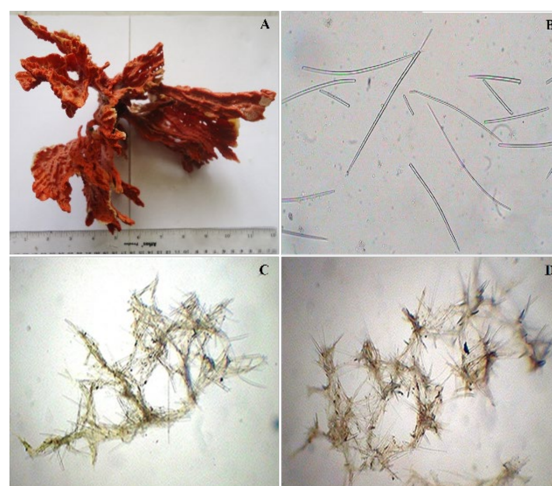

Figure 1. The external morphology and skeletal structure of the sponge. (A) Live specimen; (B) common view of different spicules (C) Structure of the choanosomal skeleton; and (D) Structure of the ectosomal skeleton.

It is well accepted that marine sponges harbour an array of bioactive compounds that may be responsible for their anti-inflammatory properties (Mehbub *et al.*, 2014). Flavonoids have been shown to inhibit enzymes involved in inflammation, including cyclooxygenase and lipoxygenase, while alkaloids have been shown to reduce the synthesis or effect of some pro-inflammatory cytokines, as well as limit histamine release and nitric oxide generation (Mayefis *et al.*, 2021). The genus *Axinella* is particularly known for having a variety of metabolites, including bromo compounds, cyclopeptides, polyethers, sterols, and terpenes (Yalçin, 2007).

During the current study, the *Axinella* sp. crude extract with a yield of 4.00% resulted in a considerable amount of alkaloids and unsaturated sterols, while terpenoids, flavonoids, and saponins were present in moderate amounts (Table 1).

Table 1. Qualitative zoo-chemical analysis of SCE of *Axinella* sp.

Zoo- chemical	Qualitative analysis
Alkaloids	++
Terpenoids	+
Sterols	–
Tannins	–
Antharquinones	–
Quinones	–
Unsaturated Sterols	++
Flavonoids	+
Saponins	+
Proanthocyanidins	–

Tested zoo-chemical present in considerable amount/positive within 5 minutes (+++); present in moderate amount/positive within 5-10 minutes (++); present in trace amount/ positive within 10-15 minutes (+) and completely absent (-).

However, sterols, tannins, antharquinones, quinones, and proanthocyanidins were absent. A recent study on a locally identified *Axinella* sp. has also shown an abundance of alkaloids, terpenoids, and sterols, which may be responsible for its anti-inflammatory properties (De Silva *et al.*, 2018). Another anti-inflammatory study carried out using *Axinella carter* originated from Riau Islands also reported an abundance of alkaloids, flavonoids, terpenoids, and saponins (Mayefis *et al.*, 2021). Thus, the bioactivities observed during the study may be the effect of a single compound, or a combination of many compounds, or both. Further research on compound isolation and bioactivity-guided fractionation will be useful to solve this puzzle.

Hemodynamic changes, increases in vascular permeability, and the accumulation of inflammatory cells are the three basic mechanisms of an inflammation response (Yalçin, 2007). Protein denaturation and plasma leakage may occur as a result of these processes being disrupted by inflammation-induced heat generation, plasma ion imbalances, etc. (Anosike *et al.*, 2012). Most of these adverse conditions can be recreated in simple, low cost *in vitro* systems (Sarveswaran *et al.*, 2017). The current study used these *in vitro* models to evaluate SCE's potential to prevent protein denaturation as well as its inhibitory effects on lysosomal membrane breakdown, which results in the release of lysosomal enzymes (Mounnissamy *et al.*, 2007). The SCE showed an increasing inhibitory effect on thermally induced egg albumin denaturation (Pearson, $r = 0.985$ and $p = 0.002$), heat induced (Pearson, $r = 0.990$ and $p = 0.001$), and hypotonicity induced (Pearson, $r = 0.980$ and $p = 0.003$) hemolysis with respect to its concentration. All the anti-inflammatory assays resulted in lower IC_{50} values than that of the relevant reference drug, as summarised in Table 2. Results of a similar experiment on *Axinell*a sp. from the western coast of Sri Lanka had an IC_{50} value of 3.98 µg/ml for its inhibitory effect on protein denaturation, indicating potent anti-inflammatory properties (De Silva *et al.*, 2018). The deviation of the IC_{50} values may be due to differences in locality (variations in secondary metabolite synthesis) or being a different species of the genus *Axinella*.

Table 2. Comparison of the IC50 values of the anti-inflammatory assays (Each value represents the mean of three readings ± SEM).

Anti-inflammatory assay	$[IC_{50}]$ µg/ml	
	SCE	Standard Drug
Egg albumin denaturation	39.55±3.21	40.00±1.02 (Diclofenac sodium)
Heat induced hemolysis	44.64±0.56	220.87±2.87 (Aspirin)
Hypotonicity-induced hemolysis	35.7±.0.26	304.78±4.02 (Diclofenac Sodium)

Table 3. Comparison of the IC50 values of radical scavenging assays (Each value represents the mean of three readings ± SEM).

Radical scavenging assay	$[IC_{50}]$ µg/ml	
	SCE	Standard Drug
DPPH scavenging	42.49±0.85	42.17±2.98 (Ascorbic acid)
ABTS radical scavenging	42.49±0.74	28.77±0.93 (Ascorbic acid)
Nitric Oxide scavenging	50.63±2.85	150.7±3.11 (Ascorbic acid)
Peroxide radicals scavenging	323.52±3.71	35.67±0.48 (BHT)

Increased quantities of reactive oxygen and reactive nitrogen species produced during the inflammatory process are highly toxic to cells that can alter the structure and function of proteins (Reddy *et al.*, 2008). Therefore, the anti-radical potential of extracts must be tested. Radicals such as DPPH and ABTS are non-physiological stable radicals where the colour change can be easily visualised during the experiment (Prastya *et al.*, 2019), whereas NO is a potent inhibitor of physiological processes including smooth muscle relaxation, neuronal signalling, and inhibition of platelet aggregation (Kumar *et al.*, 2013), and hydrogen peroxide interacts with Fe^{2+} and Cu^{2+} ions to form hydroxyl and other free radicals (Gülçin *et al.*, 2010).

The SCE showed a strong positive correlation between its concentration and the radical scavenging activity against DPPH (Pearson, $r = 0.957$ and $p = 0.011$), ABTS (Pearson, $r = 0.957$ and $p = 0.011$), NO (Pearson, $r = 0.996$ and $p = 0.000$), and peroxide radicals (Pearson, $r = 0.933$ and $p = 0.020$).

However, only the IC_{50} value of the NO scavenging assay was lower than that of its reference drug. Though all other radical scavenging assays resulted in comparatively higher IC_{50} values (Table 3), the DPPH scavenging assay had an IC_{50} value which was more or less similar to that of the standard drug. Similar to the current study, two *Axinella* sp., *Axinella damicornis* and *Axinella cannabina,* found from Mediterranean coasts of Turkey expressed moderate NO scavenging activity with IC_{50} values of 1150 µg/ml and 1668.9 µg/ml, respectively (Aktas *et al.*, 2013). Another study showed that the hexane, $CHCl_3$, and MeOH extracts of the marine sponge *Axinella*, which originated from Turkey, possessed efficient scavenging potential on DPPH (Yalçin, 2007). The abundance of phenolic chemicals, such as flavonoids, may be the reason for the resulted high scavenging capacity.

Despite the therapeutic advantages, numerous natural compounds have been demonstrated to be potentially toxic, which might lead to malignancies or other degenerative disorders. Thus, the toxic effects of natural extracts need to be tested in order to comply with international laws that safeguard human and animal health (Tamokou & Kuete, 2014). The SCE showed larvicidal activities on *A. salina* nauplii with a LC_{50} of 106.81 µg/ml.

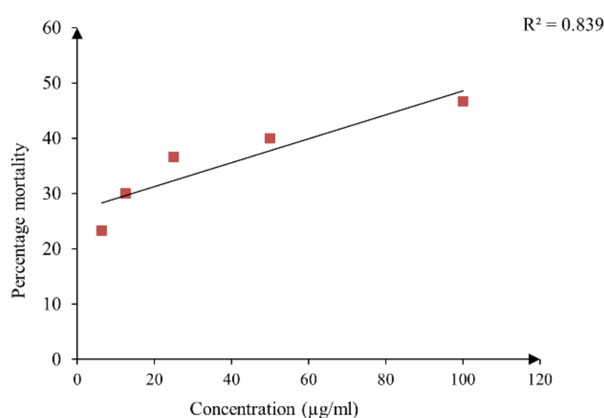

Figure 2. Relationship between different concentrations of SCE and percentage mortality of *A. salina* nauplii.

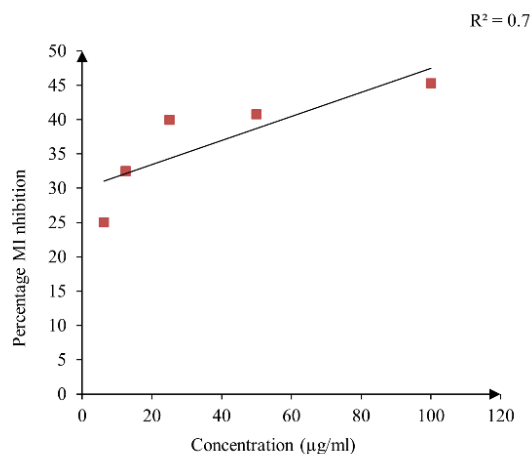

Figure 3. Percentage inhibition of the MI of A. cepa root cells by exposure to different concentration of SCE.

The percentage mortalities of *A. salina* nauplii in SCE increased with increasing concentration and had a strong positive linear relationship (Pearson, $r = 0.916$ and $p = 0.029$) as shown in Figure 2.

For the *A. cepa* assay, the LC_{50} was 114.63 µg/ml and further, the mitotic index (MI) decreased along the concentration series (Pearson, $r= 0.837$ and $p = 0.077$) (Figure 3). MI is the proportion of cells in the M-phase of the cell cycle, where its inhibition could be interpreted as cellular death or a delay in the cell proliferation kinetics (Celik & Aslantürk, 2010). The reduction of MI with the increase of extract concentrations reveals that the ability to get into the phases of the cell cycle was gradually arrested by the metabolites present in the extract. During the study, healthy cell division phases of *A. cepa* meristematic cells (prophase, metaphase, anaphase, telophase, and interphase) were clearly visible and are given in Figure 4.

Changes in chromosome structure in these cells due to a break or an exchange of chromosomal materials are known as chromosomal aberrations (CA) (Ping *et al.*, 2012). Despite the fact that the percentage CA (Table 4) is low at the maximum concentration of SCE (7.2%), several chromosomal abnormalities were observed (Figure 5). Among them, c-mitosis (Figure 5 A) and stickiness (Figure 5 B) were significant. C-mitosis occurs when a cell becomes polyploid and the causative chemical prevents spindle microtubule assembly by dissociation of disulfide bonds, whereas stickiness is a permanent chromosome abnormality that causes cell death (Ping *et al.*, 2012). Thus, the tested crude extract induced more aneugenic aberrations (stickiness, c-mitosis) than clastogenic aberrations (breaks

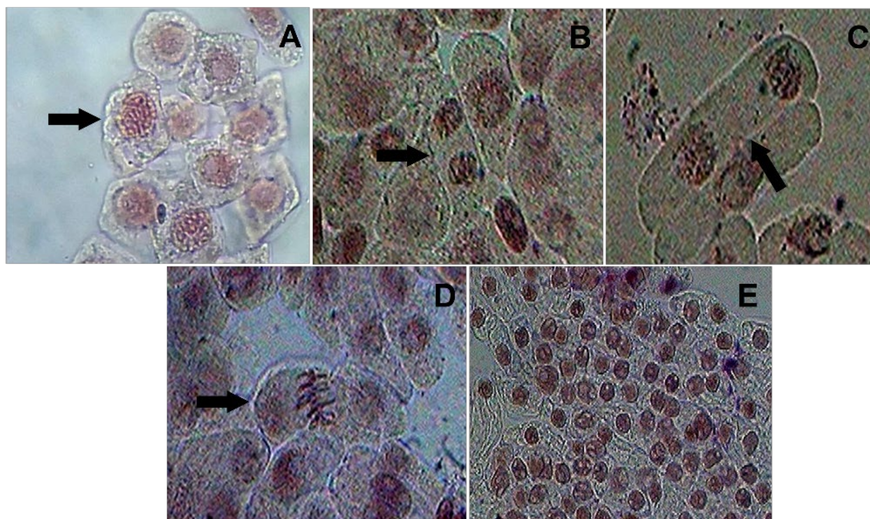

Figure 4. *A. cepa* root meristem cells undergoing different phases of the mitotic cell division. (A) Prophase; (B) Anaphase; (C) Metaphase; (D) Telophase; and (E) Interphase.

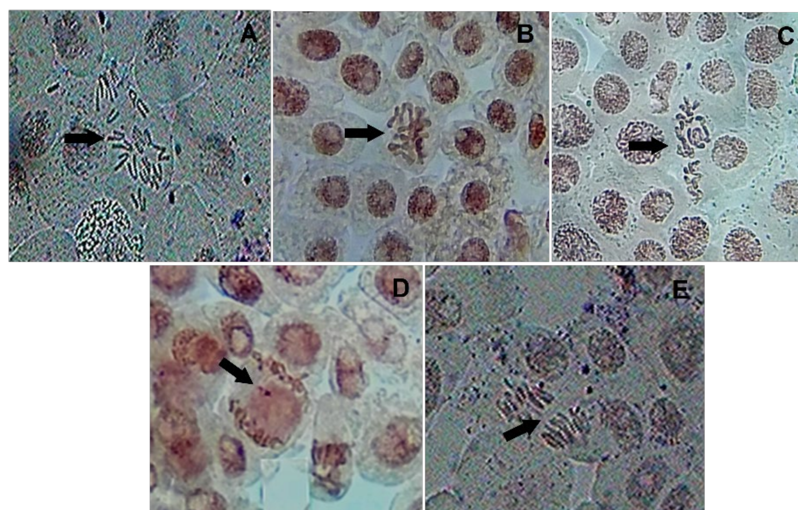

Figure 5. Chromosomal aberrations induced by different concentrations of SCE in A. cepa root meristem cells. (A) C mitosis; (B) Stickiness; (C) Numerical alterations; (D) Vagrant; and (E) Anaphase with chromosome bridge and laggards.

Table 4. Different types of chromosomal aberrations in the root meristem cells of A. cepa exposed to various test samples.

Concentration (µg/ ml)	Mean chromosome aberration ± SEM					Total aberrations	PCA ± SEM (%)
	C mitosis	Stickiness	Vagrants	Chromosomal bridges	Chromosomal breaks/ fragments		
Negative control	8	5	-	-	2	15	1.5
100	15	20	13	5	19	72	7.2
50	7	17	8	2	11	45	4.5
25	12	9	-	-	5	26	2.6
12.5	3	11	-	-	2	16	1.6
6.25	2	-	-	-	1	3	0.3
Positive control	48	65	37	25	34	209	20.9

and bridges). The number of CA increased with the concentration (Pearson, r=0.984 and p= 0.002) and the positive correlation indicates the dose dependency of the occurrence of aberrations.

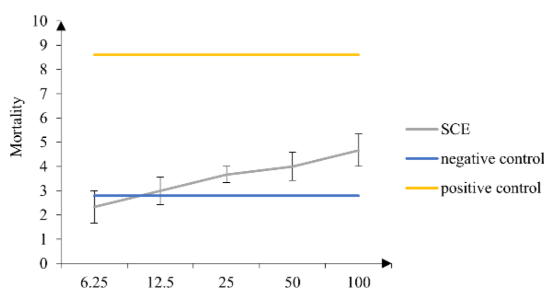

Figure 6. Larvicidal activity of different test samples. Each value represented the mean ± SEM of three (n=3) consecutive readings. $P < 0.05$ considered statistically significant.

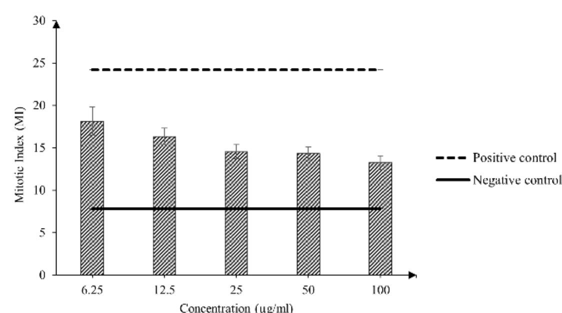

Figure 7. Effect of test samples on MI of *A. cepa* root cells. Each value represents the mean ± SEM. Solid line represents negative control while broken line positive control.

However, compared to the positive control (Figure 6 and Figure 7, respectively), the effect of SCE on the mortality of *A. salina* nauplii and cell division of *A. cepa* root cells was at a moderate level. According to the Clarkson's toxicity index also, extracts with LC_{50} of 100 - 500 µg/ml are moderately toxic (Hamidi *et al.*, 2014). Though the cytotoxic effects of *Axinella* sp. have been well documented by earlier studies (Yalçin, 2007), its genotoxicity has yet to be investigated. Although the SCE has lower cytotoxic and genotoxic effects than the positive control, its effects should be addressed while it is being developed into a potential therapeutic drug.

4. Conclusion

Overall, the *Axinella* sp. crude extract was proven to have bioactivities; anti-inflammatory properties with respect to egg albumin denaturation and erythrocyte membrane stabilisation. It also demonstrated strong radical scavenging activities against NO and more or less similar scavenging activities against DPPH compared to their respective reference drugs. However, it showed comparatively less potency with respect to ABTS and peroxide radical scavenging activities. The SCE showed moderate larvicidal activity on *A. salina* nauplii. Further, the *A. cepa* genotoxicity assay revealed that *Axinella* sp. crude extract induces a moderate mitodepressive effect on all stages of cell division and has the potential to be aneugenic. The presence of alkaloids, unsaturated sterols, terpenoids, flavonoids, and saponins in *Axinella* sp. extract may be responsible for the resulted bioactivities. To support the findings of the current study, further

comprehensive *in vitro/ex vivo* analysis, followed by bioactivity activity guided fractionation and chemical characterisation is proposed.

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6. Conflict of Interest

There is no conflict of interest among the authors and have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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