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IN-VITRO ANTI-INFLAMMATORY ACTIVITY, ACUTE TOXICITY TO ZEBRAFISH EMBRYOS AND NUTRITIONAL ANALYSIS OF *BOHADSCHIA VITIENSIS* WATER EXTRACT

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Keywords:

Bohadschia vitiensis, *in-vitro* anti-inflammatory activity, Zebra fish acute toxicity, Zoo chemical analysis

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ABSTRACT: Anti-inflammatory activity and the toxicity of *Bohadschia vitiensis* water extract were investigated by *in-vitro* models to evaluate the consumption of the extract by the local community to reduce arthritic-related pain. Specimens of *B. vitiensis* were collected from Mannar, Sri Lanka, and the water extract (WE) was prepared by removing visceral organs and incubating diced samples in distilled water, followed by freeze-drying. The WE was used to test acute toxicity, based on *Danio rerio* embryotoxicity assay and to evaluate anti-inflammatory activity based on *in-vitro* models; egg albumin denaturation, erythrocyte membrane stability, Nitric Oxide (NO) scavenging and Hydrogen Peroxide (HP) scavenging activities. The IC_{50} was calculated for each assay and compared with a standard reference drug. The qualitative zoo-chemical analysis was carried out while the presence of major nutrients was evaluated. According to the results, WE had a LC_{50} of 151.59 µg/ml for *Danio rerio* embryotoxicity assay. The anti-inflammatory activity against egg albumin denaturation (IC_{50} of 277.51 µg/ml) was reported while a maximum percentage inhibition of 32.09% was reported at 250 µg/ml in erythrocyte membrane stability. However, the WE was less potent against NO and HP scavenging activities (IC_{50} = 2577.06 µg/ml, 1908.11 µg/ml respectively). The WE contained terpenoids, saponins, and sterol as zoo-chemicals and proteins and lipids as major nutrients. In conclusion, WE exhibited a considerable anti-inflammatory activity with moderate toxicity and the presence of some important zoo-chemicals, scientifically validating the use of *Bohadschia vitiensis* water extract for anti-inflammatory activity.

INTRODUCTION: The clinical concept of inflammation accounts for the visual changes characterized by five cardinal signs, namely redness (rubor), swelling (tumor), heat, pain (dolor), and loss of function (functiolaesa) [1].

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Currently, inflammation is recognized as a second-line defense mechanism, which is far more complex and a major responsibility of the immune system to tissue damage and infection. The short-term inflammatory response is recognized as an immediate acute reaction, which is launched by the body to assist with its repair [2].

Acute inflammation, the early inflammatory response, is achieved by the increased movement of plasma and leukocytes from blood into the injured tissues [3] and is characterized by increased vascular permeability and cellular infiltration which subsequently leads to oedema formation followed

by extravasation and accumulation of leukocytes in the inflammatory site [4]. Chronic inflammation, on the other hand, is prolonged and persistent inflammation, which is marked chiefly by new connective tissue formation.

Dysregulated inflammation plays a major role in chronic illnesses, including diabetes, cardiovascular disease, arthritis, psoriasis, and cancer [5]. The mechanisms of inflammation are associated with the oxidative stress caused by Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), which play a key role in the development of the inflammatory process [6]. The imbalance between the antioxidants and free radicals' imbalance leads to the damage of important biomolecules and cells, which may result in considerable damage to the whole organism [7].

Despite the existence of numerous Non-Steroidal Anti-inflammatory Drugs (NSAID), anti-inflammatory therapy with natural compounds is renowned as one of the most promising approaches in managing inflammatory diseases since ancient times. They are low-priced, safe, and more effective with a minimum number of side effects in healing the acute inflammatory process, thus preventing the continuing and progressing process which leads to chronic inflammation. Marine invertebrates serve as a virtual cornucopia of novel anti-inflammatory products, varying widely in both chemical structure and biological activity [8, 9].

Bohadschia vitiensis, also known as "brown sandfish," is a sea cucumber which belongs to the Family Holothuriidae and well renowned for its bioactivities against tumors, fungal infection, high blood pressure, arthritis, and muscular disorders [10]while considered as a delicacy and protein component in Asian countries [11]. *B. vitiensis* is abundant in North West and eastern coastal areas of Sri Lanka [12] and traditionally claimed as a therapeutic agent for arthritic related pains. However, scientific validation of its anti-inflammatory activity, toxicity, zoo-chemical, and nutritional analysis has not yet been reported elsewhere.

Henceforth, the present study was aimed to investigate the anti-inflammatory activity of *B. vitiensis* water extract by some selected *in-vitro* assays such as egg albumin denaturation, mammalian erythrocyte membrane stability, Nitric Oxide (NO) and Hydrogen Peroxide (HP) radical scavenging activity.

Attempts were made to evaluate the toxicity of the water extract of *B. vitiensis* by *Danio rerio* embryotoxicity assay. The zoo-chemical analysis was carried out to investigate secondary metabolites, while the presence of major nutrients such as reducing sugar, proteins, lipids, and carbohydrates were also investigated.

MATERIALS AND METHODS:

***B. vitiensis*; Collection and Identification:** Specimen of *B. vitiensis* (560g) collected from commercial catches of fishermen, from coastal areas of Pallimunei, Mannar, Sri Lanka (8° 03′, 8° 35′N:77° 15′, 77° 36′E) in the month of January 2018. Precise identification of species was carried out by morphology and ossicle analysis based on Purcell (2012) [13]. Samples were packed in plastic bags with ice during transportation. After removing the internal organs, samples were kept at -20 ºC until extractions.

Preparation of the Water Extract (WE) of *B. vitiensis:* The WE were prepared according to the Ridzwan (2003) [14] with slight modifications. Briefly, thawed samples of *B. vitiensis* were thoroughly washed with distilled water. The visceral organs were removed, and the body wall was diced, followed by homogenization with mortar and pestle. The homogenized sample was incubated in distilled water (1:2 w/v) and occasionally shaken for 4 h. The extract was then centrifuged at 3000rpm for 20 min (HERMLE Labortechnik GmbH, D-78564, Wehingen, Germany). The supernatant was carefully collected, freeze-dried, and used for assays.

Chemicals and Reagents: All the chemicals used were of analytical grade unless stated otherwise. Potassium dihydrogen orthophosphate, Disodium hydrogen orthophosphate, Sodium chloride, Ferric chloride, Sodium hydroxide were products of Research lab fine chem industries, Mumbai, India. Sulfuric acid, absolute ethanol, Hydrochloric acid was purchased from Breckland scientific suppliers, Norfolk, UK. Dichloromethane and Griess reagent were products of Sigma Chemical Company Limited Aldrich, USA.

Qualitative Zoo-Chemical Analysis: Qualitative zoo chemical analysis to screen alkaloid, flavonoid, saponin, terpenoid, quinone, anthraquinone, tannins, sterols, unsaturated sterols in WE was carried out using Farnsworth (1966) [15] phyto-chemical screening procedure.

Qualitative Analysis for Major Nutrients: Presence of major nutritional compounds; starch, reducing sugars, proteins, and lipids in WE was investigated according to Mathew *et al.* [16]

Analysis of Starch: A few drops of Iodine solution were added into WE and observed after a few seconds for the appearance of a blue-violet color.

Test for Reducing Sugars: Approximately 2-3 drops of Fehling's reagent were added to WE and boiled for 2 min. A brick-red color can be observed for the presence of reducing sugars.

Test for Proteins: Approximately 0.5 ml of WE was treated with an equal volume of 1% sodium hydroxide, to which a few drops of copper sulphate solution was gently added. Purple color can be observed in the presence of proteins.

Test for Lipids and Oils: Few drops of Sudan III solution were added into WE and shaken well. Pink-colored layers or globules can be observed for the presence of oils and lipids.

Assessment of *Danio rerio* Acute Embryo Toxicity: Acute toxicity assay was carried out by using *Danio rerio* fish embryos, as mentioned in the updated version of OECD guidelines [17]. Healthy, fertilized eggs were randomly collected, washed in tap water, and its existing stage was determined under light microscopy. The test concentration series (50, 62.5, 125, 250, and 300 µg/ml) was determined based on the percentage of mortality by using absolute methanol as positive control. Eggs were distributed to the wells in the following numbers; 10 eggs per each concentration, 24 eggs as solvent control/negative control, 20 eggs per positive control, 4 eggs in distilled water as internal plate control on each of the above plates.

The development of the embryo was monitored at intervals of 24, 48, 72, 96 h. Mortality of embryo was recorded in any observation where coagulation of embryo, lack of somite formation, non-detachment of the tail, lack of heartbeat is reported. The LC_{50} value was determined using calculations by probit analysis.

Assessment of *in-vitro* Anti-inflammatory Activity: The anti-inflammatory activity of WE was experimented by egg albumin denaturation, erythrocyte membrane stability, Nitric oxide (NO) and hydrogen peroxide (HP) radical scavenging assays.

Egg Albumin Denaturation Assay: Egg albumin denaturation assay was carried out according to Akinwunmi and Oyedapo (2014) [18] for WE and for the reference drug, Diclofenac sodium, with slight modifications. Briefly, 2 ml of different concentrations of WE (1000 µg/ml, 500 µg/ml, 250 µg/ml, 200 µg/ml, 125 µg/ml, 100 µg/ml, 50 µg/ml) or Diclofenac sodium (2000 µg/ml, 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml) and 2.8 mL of phosphate-buffered saline (pH 6.4) was mixed with 2 mL of egg albumin collected from fresh hen's egg. The reaction mixture was incubated at room temperature (27±1 °C) for 15 min. After incubation, the reaction mixture was kept at 70 °C in a water bath for 10 min. After cooling, absorbance was measured by a spectrophotometer (Genesys 10S UV Vis, USA) at 660 nm where PBS was used as the blank.

The percentage inhibition was calculated using the following formula;

Percentage inhibition = Ab. of control – Ab. of test × 100 / Ab. of control

Ab - Absorbance

The IC_{50} was calculated for WE and for reference drug, Diclofenac sodium.

Assessment of Erythrocyte Membrane Stability Activity: Fresh, uncoagulated goat red blood cells were used to test the effect of WE on erythrocyte membrane stability following Akinwunmi and Oyedapo (2014) [18]. Briefly, fresh, uncoagulated goat blood was collected from a slaughterhouse. Erythrocytes were isolated by centrifugation at 4000 rpm for 10 min in normal saline at room temperature. This process was repeated until the supernatant becomes clear. Two percent of (w/v) red blood cells were prepared by appropriate dilution of RBCs in normal saline.

To assess the erythrocyte membrane stability, a reaction mixture was prepared with 0.5ml of hyposaline,0.25 ml of 0.5M phosphate buffer (pH 7.4), 0.5ml of WE (500 µg/ml, 250µg/ml, 125 µg/ml, 62.5 µg/ml, and 31.25 µg/ml, 15.625 µg/ml) or reference drug Ibuprofen (1500 µg/ml, 750 µg/ml, 375 µg/ml, 187.50 µg/ml, 93.75 µg/ml, 46.875 µg/ml, 23.4375 µg/ml) and 0.25 ml of 2% erythrocyte suspension. The volume was adjusted to a total volume of 1.5 ml by adding normal saline. The blood control was prepared as above with distilled water instead of standard drug or WE. The drug control was prepared by using all other reagents except the erythrocyte suspension.

The reaction mixture was incubated at 56 ºC for 30 min. Followed by cooling to room temperature, they were centrifuged at 3500 rpm for 10 min. the supernatant was collected and absorbance was taken at 560 nm using a spectrophotometer (Genesys 10S UV Vis, USA) against the blank (PBS). The percentage membrane stability was calculated by using the formula;

Percentage membrane stability = 100-(Ab. of test drug – Ab. of drug control) ×100 / Ab. of blood control

Where Ab-Absorbance

IC_{50} values were calculated for WE and Ibuprofen.

Assessment Nitric Oxide (NO) Scavenging Activity: The method described by Kumar *et al*. [19] was followed with slight modifications. Accurately, 60 µl of a serial diluted sample (5000 µg/ml, 2500 µg/ml, 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml) or L-Ascorbic acid (2000 µg/ml, 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.625 µg/ml, 7.8125 µg/ml) and 60 µl of 10mM sodium nitroprusside in PBS were added into a 96 well plate. The plate was incubated at room temperature for 150 min. After incubation, an equal volume of Griess reagent (Sigma, USA) was added to each well in order to measure nitrite content. After chromophore was formed at room temperature in 10 minutes, the absorbance at 595 nm was measured in a microplate reader (Thermo Scientific, Multiskan EX). The NO scavenging activity was calculated by the following formula;

Scavenging activity = (Ab. of control- Ab. of the sample) × 100 / Ab. of control

Where Ab is the absorbance.

IC_{50} value was determined for WE and reference compounds.

Assessment Hydrogen Peroxide (HP) Scavenging Activity: Hydrogen peroxide scavenging activity of WE was assessed by the method of Kumar *et al*. [19] with slight modifications. Approximately, 1.0 ml of 0.1 mM H_2O_2 and 1.0 ml of various concentrations of WE extract (4000 µg/ml, 2000 µg/ml, 1000 µg/ml, 500 µg/ml, 250 µg/ml) or ascorbic acid (1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.50 µg/ml, 31.25 µg/ml) were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2M H_2SO_4 and 0.7 ml of 1.8M KI. The reaction mixture was titrated with 5.09 mM $Na_2S_2O_3$ until the yellow color was disappeared. For control, all the reagents were added except the extract or ascorbic acid. The scavenging activity was calculated by the following formula, while the IC_{50} value was calculated for WE and Ascorbic acid separately.

Scavenging activity = Volume of $Na_2S_2O_3$ for control – Volume of $Na_2S_2O_3$ for test × 100 / Volume of $Na_2S_2O_3$ for control

Statistical Analysis: The inhibitory concentration 50% (IC_{50}), the scavenging activity for extract, and drug control were calculated from the dose-response curve using EXCEL 2013.

RESULTS:

Qualitative Zoo-Chemical Analysis: According to the qualitative zoo-chemical analysis, the WE contained sterols, saponins, and terpenoids while alkaloids, tannins, anthraquinones, quinone, unsaturated sterols, flavonoids were absent.

Qualitative Analysis for Major Nutrients: The results of the analysis for major nutrients revealed the presence of proteins and lipids in the WE while reducing sugar and carbohydrates were absent.

Toxicity to *Danio rerio* Acute Embryo: Results of acute *Danio rerio* embryotoxicity revealed that the WE was toxic to the embryos at LC_{50} of 151.59 µg/ml. The percentage mortalities of different concentrations of WE, negative control (distilled water) and positive control (absolute methanol) at the end of 24 h (Day 1), 48 h (Day 2), 72 h (Day 3), and 96 h (Day 4) are displayed in **Fig. 1**.

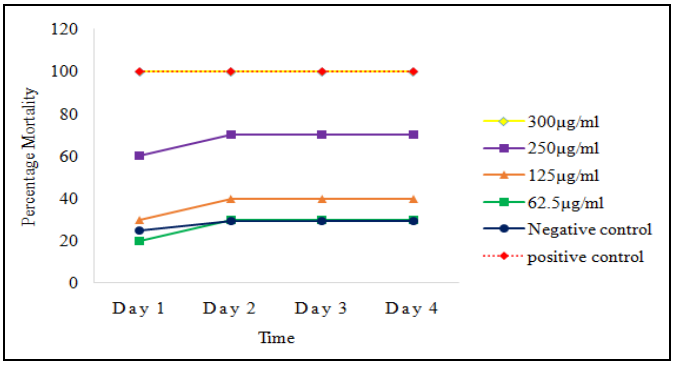

FIG. 1: PERCENTAGE MORTALITIES OF ZEBRA FISH EMBRYOS OVER 96 h EXPOSURE TO THE CONTROLS, 300µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml OF WE

The percentage mortality was reported for the negative control at 96 h as 29.1% while it was 100% for the positive control. Mortality of embryo was recorded in any observation where coagulation of embryo, lack of somite formation, non-detachment of the tail, lack of heartbeat was reported as in **Fig. 2**.

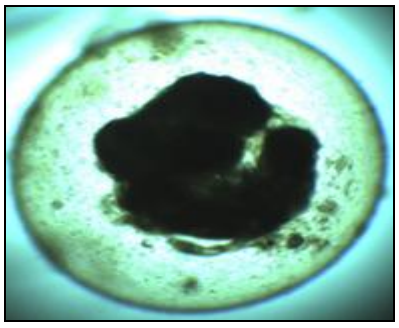

FIG. 2: COAGULATED ZEBRA FISH EMBRYOS WHILE EXPOSURE OF 250 µg/mL OF WE AT DAY 4 (X4)

***In-vitro* Anti-inflammatory Activity:**

Egg Albumin Denaturation Assay: The WE inhibited egg albumin denaturation in a dose-dependent manner with an IC_{50} value of 277.51 µg/ml **Fig. 3**. The potency of WE was higher than the reference drug, diclofenac sodium, which reported IC_{50} of 665.49 µg/ml.

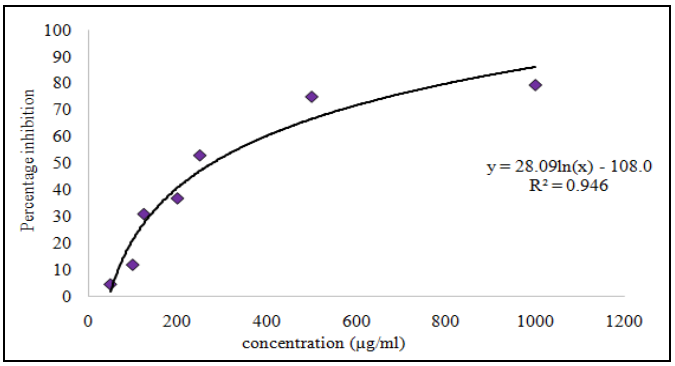

FIG. 3: EFFECT OF WE ON ALBUMIN DENATURATION, DATA PRESENTED AS MEANS ± SEM

Erythrocyte Membrane Stability Assay: According to the results, the WE was able to inhibit the membrane stability of goat erythrocytes in a dose-dependent manner up to the maximum concentration of 250 µg/ml. The maximum percentage inhibition at 250 µg/mL was reported as 32.09%, while the erythrocyte membrane stability showed declining thereafter, **Fig. 4**. The IC_{50} value of standard drug Ibuprofen was reported as 468.44 µg/ml.

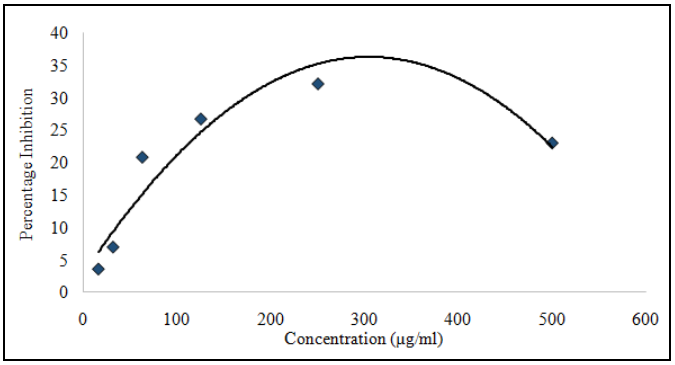

FIG. 4: EFFECT OF WE ON THE ERYTHROCYTE MEMBRANE STABILITY, DATA PRESENTED AS MEANS ± SEM

NO Scavenging Activity: According to the results, WE exhibited nitric oxide scavenging activity in a dose-dependent manner. However, the IC_{50} value of WE (2577.06 µg/ml) was approximately 4.1 folds higher than the standard reference (623.95 µg/ml) **Fig. 5**.

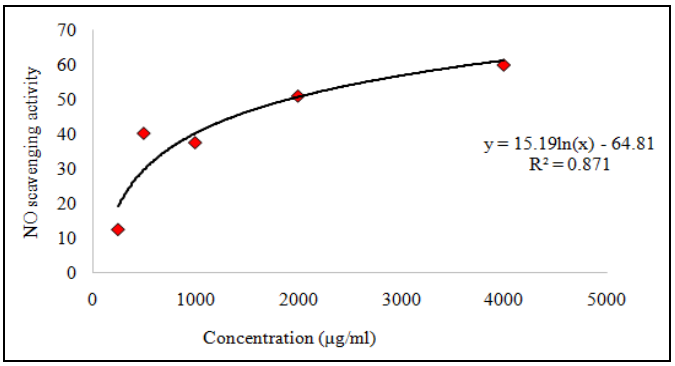

FIG. 5: EFFECT OF WE ON NO RADICAL SCAVENGING ACTIVITY; DATA PRESENTED AS MEANS ± SEM

HP Radical Scavenging Activity: The results of HP radical scavenging activity indicated that WE was less potent in scavenging HP radicals than the standard reference, ascorbic acid **Fig. 6**. The IC_{50} value of 1908.11 µg/ml was reported for WE and while it was 378.26 µg/ml for ascorbic acid. Thus, the potency of the WE was approximately 5 folds lower than the reference drug.

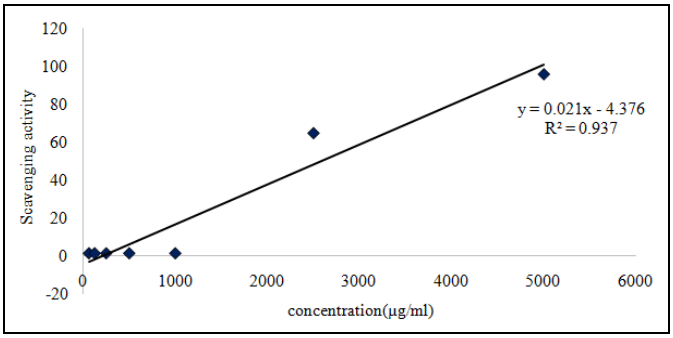

FIG. 6: EFFECT OF WE ON HP RADICAL SCAVENGING ACTIVITY; DATA PRESENTED AS MEANS ± SEM

DISCUSSION: The extract of sea cucumber species, *B. vitiensis* is consumed by the local community as a remedy for arthritic related pain. The present study was aimed to scientifically validate the use of this extract as an anti-inflammatory agent by investigating its anti-inflammatory activity on selected *in-vitro* models.

Preliminary screening tests to detect zoo-chemicals in a natural extract are useful since these compounds are responsible for the resulted bioactivity. These compounds may lead to the discovery and development of drugs once appropriate quantitative estimation, isolation and chemical characterization is accomplished. Zoo-chemical investigations of the WE resulted in the presence of terpenoids, saponins, and sterols. Sterols are well reputed for lowering blood cholesterol, thus reduce cardiovascular diseases and inflammation [20]. The anti-inflammatory and other medicinal effects of saponins from various natural resources such as lower invertebrates, bacteria, and plants are also well documented [21, 22]. Terpenoids are also proven its effectiveness in protecting organisms from environmental stress and heal injuries [23]. The occurrence of sterols, saponins, and terpenoids in the WE may be undoubtedly responsible for the resulted anti-inflammatory activity of the WE.

Denaturation of proteins is responsible for the cause of inflammation in conditions like rheumatoid arthritis, diabetes, cancer, *etc*. [24.] Therefore, the prevention of protein denaturation is vital in reducing inflammation. The WE resulted in a dose-dependent inhibitory effect in protein denaturation, indicating the possibility of it to reduce protein denaturation in inflammation. The potency of the WE was even two-fold higher than the existing NSAID, diclofenac sodium.

Erythrocytes membrane is the model system used for many *in-vitro* investigations of drug and membrane interactions. Maintaining the stability of the erythrocyte membrane is considered as very important in many diseases related to plasma leakage such as dengue hemorrhagic fever [25]. Further, any agent which stabilizes the erythrocyte membrane is considered to be good sources for anti-inflammatory activity [26]. Other than that, the erythrocyte membrane is analog to the lysosomal membrane system [27]. Lysosomes play a key role in the inflammation. Therefore, stabilization of lysosomal membranes inhibits releasing of lysosomal hydrolytic enzymes. They are released into the sites, which cause damage to the surrounding organelles and tissues. Our results highlighted that WE do not demonstrate a linear dose-response relationship. Instead, the observed dose-response relationship forms a hormetic dose-response relationship where the beneficial effects observed at low doses and absent at higher concentrations. Such dose-response relationships have been reported to occur with a wide range of chemotherapeutics including antibiotics, antiviral, and antitumor agents [28]. Although the precise mechanism of membrane stabilization is yet to be elucidated, it is evident that the *B. vitiensis* water extract produces this effect at its lower doses.

It is widely recognized that many modern non-communicable diseases are due to the oxidative stress that results from an imbalance between the formation of ROS/RNS and their neutralization when endogenous antioxidant mechanisms are unable to quench the free radicals. Accumulation of NO and peroxide radicals in the body is also implicated for inflammation, cancer, and other pathological conditions [29]. Natural compounds have the potential to scavenge those free radicals leading to low cellular stress. The scavenging activity may also help to arrest the chain of reactions initiated by excess generation of free radicals. The WE had shown a dose-response increase in NO and HP radical scavenging activity, yet less effective than the reference drug.

CONCLUSION: The present study investigated the anti-inflammatory activity, *Danio reiro* embryotoxicity, qualitative zoo-chemicals and major nutrients in water extract of *Bohadschia vitiensis*, a sea cucumber.

Our results proved that the WE contained potent anti-inflammatory activity with respect to albumin denaturation assay with an IC_{50} value of 277.51 µg/ml. However, the WE was less potent than the standard NSAID with respect to erythrocyte membrane stability, Nitric oxide radical scavenging activity and hydrogen peroxide scavenging activity. The WE was moderately toxic on zebrafish embryos.

The resulted anti-inflammatory activity of WE may probably be due to the presence of the zoo chemicals; saponin, sterols, terpenoids, and high polar compounds present in water extract as resulted by zoo chemical analysis. The WE was safe to consume with respect to its nutritional value as well as it is devoid of reducing sugars and carbohydrates. Thus, these results support the scientific validation of the traditional use of this sea cucumber extracts by the local Sri Lankan community.

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COMPETING INTERESTS: Authors have declared that no competing interests exist.

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