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SHORT COMMUNICATION

The Cytotoxic Effect and Antioxidant Properties of Actiniarian Sea Anemones

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Abstract

The Actiniarian sea anemones possess great potential as a source for developing useful compounds for the pharmaceutical industry. The present study was conducted to investigate the biological activity of crude extracts from the sea anemones *Heteractis magnifica* (Quoy and Gaimard, 1833) and *Heteractis crispa* (Hemprich and Ehrenberg in Ehrenberg, 1834) collected from Andaman Islands, India. The crude proteins of *H. magnifica* and *H. crispa* were obtained by using methanol and aqueous extracts. The cytotoxic effect of the crude extracts was evaluated on brine shrimp. The methanol extracts of *H. crispa* displayed high cytotoxicity of $LC_{50} = 416.9 \mu\text{g.mL}^{-1}$, while for *H. magnifica* it was $LC_{50} = 575.4 \mu\text{g.mL}^{-1}$. The antioxidant evaluations were carried out on the crude extracts of *H. magnifica* and *H. crispa*, with use of free radical, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and total phenolic content methods. The extracts of *H. magnifica* showed high total phenolic content which is an indication of significant free radical scavenging activity.

Keywords: sea anemones, cytotoxicity, antioxidant, India, Andaman & Nicobar Islands

Introduction

There are many creatures with venoms that have fascinating and diverse bioactive metabolites which could be used for novel pharmaceutical compounds. The sea anemone, a member of the phylum Cnidaria and the class Anthozoa invertebrates has venom produced from tentacles containing specialized stinging cells or cnidocytes. Beress (1982) noted that toxins produced by sea anemones in molecular weight range from 3,000 to 300,000 Da. Sea anemones contain several toxins such as neurotoxins, phospholipases, cytolsins (Anderluh and Macek 2002; Beress 2004; Norton et al. 2004).

The investigation of bioactive compounds from sea anemones from the Indian waters has been scantily studied especially for their bioactive properties. Based on the above peculiar pharmacological feature of sea anemones, the present study was undertaken to investigate the screening of the pharmacological potentials for bioactive study of two actiniarian sea anemone species *Heteractis magnifica* (Quoy and

Gaimard, 1833) and *Heteractis crispa* (Hemprich and Ehrenberg, 1834) from Andaman Islands, India.

Materials and Methods

Chemicals

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, methanol (MeOH), 0.2 M di-potassium hydrogen phosphate (K_2HPO_4), 0.2M potassium dihydrogen phosphate (KH_2PO_4), potassium ferricyanide [$K_3[Fe(CN)_6]$], ferric chloride ($FeCl_3$), sodium carbonate, gallic acid, Mueller Hinton agar (M173), bovine serum albumin, Coomassie Blue G-250, ethanol and phosphoric acid (Himedia, India); TCA and Folin-Ciocalteu reagent (Merck, India) were used for the analysis.

Apparatus

Spectrophotometer (UV/VIS, Perkin Elmer Lambda 25, India), filter paper A1 (Sigma-Aldrich, India), rotary evaporator (VC 100A, Lark Innovative, India).

Sample collection

Five specimens of *H. magnifica* and three of *H. crispa* were collected with the help of scalpel at the depth of 15 m from Rutland Island site I (Lat: 11°26.506'N; 092°36.86'E), Rutland Island site II (Lat: 11°27.307'N; Long: 092°36.098'E), South Andaman. Soon after collection the specimens were kept in aerated plastic containers filled with seawater of ambient salinity and transported to the laboratory. On arrival in the lab, the specimens were immediately washed and cleaned thoroughly to get rid of other attached organisms. The sea anemone specimens were identified to the species level following Dunn (1981).

Preparation of crude extracts

Aqueous extract

Aqueous extraction was prepared according to method of Kem et al. (1989). The tentacles were excised manually from live specimens after which the whole body was immediately macerated and submerged in distilled water for 5 h at 4 °C. The ratio of organic tissue to distilled water was approximately 1:2. After a complete detachment of the epidermis the tissue was removed from the suspension containing both epidermis and undischarged nematocysts derived from the osmotic rupture of nematocysts. The nematocysts, attached to the epidermal tissue, were separated by stirring. The nematocysts suspension filtered through Whatman® No. 1 filter paper (0.4 µM) to remove most of the tissue debris, and then centrifuged at 4 °C (5000 rpm for 10 min). The supernatant was collected and freeze dried. The extracted crude toxin was stored at - 20 °C for further use.

Methanolic extract

Crude protein was prepared following the method of Sunahara et al. (1987). The sea anemones were chopped into pieces and macerated fully in absolute methanol and maintained for 10 days, then the material was removed by squeezing the macerated anemone pieces, and the solvent was filtered through Whatman No. 1 filter paper (0.4 µm); it was then evaporated at low pressure using a rotary evaporator at 30 °C. The resultant compound was stored at 4 °C for further screening.

Brine shrimp lethality assay

Culture of *Artemia salina* (Linnaeus, 1758)

A total of 15 mg of dried *Artemia* cysts were washed with chlorine water for decapsulation. Then, all the soft cysts were immersed in fresh water for one hour with aeration and finally cultured in conical

flask containing 500 mL filtered seawater and supplied with oxygen for 24 h at 27 °C.

Larvae collection

After 24 h hatching, phototropic nauplii stages attracted with the light source (at the top of the beaker) were collected using a sterile pipette from the lighted side and transferred into sterile beaker containing 5 mL fresh filtered seawater. Once more, the nauplii were transferred aseptically from beaker to another sterile beaker containing 5 mL fresh filtered seawater.

Cytotoxicity assay

Brine shrimp lethality test for larvae nauplii was used to determine the toxicity of methanol and aqueous extracts of *H. magnifica* and *H. crispa* (Manilal et al. 2009). A concentration of 250, 500, 750, 1000, 1250, 1500 and 2000 µg.mL⁻¹ of sea anemone extracts were prepared in methanol and aqueous, and 2 mL from each extract were transferred in glass vial and left open for 2 h to evaporate the organic solvent before adding the nauplii. Controls were kept without adding methanol and aqueous extracts. Filtered seawater (2 mL) was added in each vial of methanol and aqueous extracts before adding the nauplii. Twenty brine shrimp nauplii were transferred in each vial. Observations were recorded after 24 h and survivors were counted and percentage of death at each dose level and control were determined. Larvae were considered dead if no movement of the appendage was observed within the vial. The lethality concentration (LC₅₀) were calculated using probit analysis (Finney 1971). As mentioned by Meyer et al. (1982), LC₅₀ value of less than 1000 µg. mL⁻¹ is toxic while LC₅₀ value of greater than 1000 µg.mL⁻¹ is non-toxic. The percentage of mortality (% M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the extracts. The experiment was carried out in triplicate.

Antioxidant activity

Determination of total phenolic content

The total phenolic content of sea anemone extracts was measured using Folin-Ciocalteu method (Singleton and Rossi 1965). A 200 µL of diluted sample prepared from methanol and aqueous extracts of *H. magnifica* and *H. crispa* with concentration of (1000 µg.mL⁻¹), were added to 1 mL of 1:10 diluted Folin-Ciocalteu reagent. After 4 min, 800 µL of saturated sodium carbonate (75 g.L⁻¹) was added. Blank was concomitantly prepared,

containing 200 µL methanol, 1 mL 10 % Folin-Ciocalteu's reagent and 800 µL of saturated sodium carbonate. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured using the spectrophotometer. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg. mL⁻¹) from the calibration line; then the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GAE.g⁻¹ dry weight of extract) and calculated as mean value ± SD (n = 3).

Ferric reducing antioxidant power (FRAP)

The reducing power of methanol and aqueous extracts of *H. magnifica* and *H. crispa* and ascorbic acid was evaluated according to the method of Oyaizu (1986) and Manivannan et al. (2012). Briefly, 1.0 mL of extract containing different concentration of samples (500, 750, 1000, 1250, 1500 and 2000 µg. mL⁻¹) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1 %). Reaction mixture was incubated at 50 °C for 20 min. After incubation, 2.5 mL of trichloro acetic acid (10 %) was added and centrifuged (650 g) for 10 min. From the upper layer, 2.5 mL solution was collected and mixed with 2.5 mL distilled water and 0.5 mL FeCl₃ (0.1 %). Absorbance of all the sample solutions was measured at 700 nm in spectrophotometer. Increased absorbance indicated increased reducing power.

DPPH radical scavenging activity

The free radical scavenging activity of methanol and aqueous extracts of *H. magnifica*, *H. crispa* and L-ascorbic acid (standard) were assessed using DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical (Blois 1958, Gordon et al. 2001). The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. About 3.9 mL of DPPH (0.06 mM, 25 mg dissolved in 1 L methanol) was added to 0.1 mL of each of the extract with different concentration of samples (250, 500, 1000, 1500 and 2000 µg.mL⁻¹). About 3.9 mL DPPH solution along with 0.1 mL of methanol was used as control, and 3.9 mL of methanol along with 0.1 mL of sample solution was added as blank. All the sample solutions were kept for 30 min incubation. After incubation, the sample solutions were mixed properly. Further, the decrease in absorbance was recorded at 517 nm on a spectrophotometer, against methanol without DPPH as the blank reference. All experiments were carried out at room temperature.

The EC₅₀ value was calculated. Radical scavenging potential was expressed as EC₅₀ value, which represents the sample concentration at which 50 %

of the DPPH radicals scavenged. Similarly, DPPH scavenging activity was assessed for the concentration gradient of the standard ascorbic acid (25, 50, 75 and 100 µg).

Absorbance (A) was converted to the DPPH radical-scavenging effect according to the equation:

$$\text{DPPH radical scavenging effect (\% inhibition)} = [(A_0 - (A_1 - A_2)/A_0)] \times 100.$$

Where, A₀ is the absorbance of the control reaction (DPPH only); A₁ is the absorbance in presence of all of the extract samples; and A₂ is the absorbance of the blank reference without DPPH. The experiment was carried out in triplicate.

Statistical analysis

For all the experiments, data were presented as mean ± Standard deviation (S.D). EC₅₀ and LC₅₀ values respectively for DPPH and cytotoxic assays were found by Linear Regression Probit analysis (Finney 1971).

Results and Discussion

Brine shrimp lethality assay

Methanol extracts of *H. crispa* (HC-MeOH) showed higher toxicity (55.7 % mortality at 500 µg.mL⁻¹ and 70 % mortality at 750 µg. mL⁻¹), compared to methanol extracts of *H. magnifica* (HM-MeOH) which caused 34 % mortality at 500 µg.mL⁻¹ and 52 % mortality at 750 µg.mL⁻¹. The extracts of sea anemone evaluated for their cytotoxicity at different concentrations were classified as non-cytotoxic (NCT < 50 % mortality), mild cytotoxic (MCT > 50 % mortality but < 75 % mortality) and highly cytotoxic (HCT > 75 % mortality) at 1000 µg.mL⁻¹ based on their lethality to brine shrimp. The aqueous extracts of *H. magnifica* (HM-Aq) and *H. crispa* (HC-Aq) at 1000 µg.mL⁻¹ were considered as non-cytotoxic with mortality rate of less than 50 % (NCT), whereas the methanol extracts of *H. crispa* was highly cytotoxic (85.1 ± 2.2 % mortality) at 1000 µg.mL⁻¹ and *H. magnifica* showed mild cytotoxicity (69.9 ± 1.6 % mortality) at 1000 µg.mL⁻¹ (Table 1).

Figure 1 shows the LC₅₀ values of four different extracts, i.e., HC-MeOH, HM-MeOH, HM-Aq and HC-Aq with values of 416.9, 575.4, 1259 and 1514 µg.mL⁻¹ respectively. Out of the four extracts, only two extracts HC-MeOH and HM-MeOH showed LC₅₀ < 1000 µg (Fig. 1). The lower LC₅₀ value of HC-MeOH indicated high cytotoxicity against all the extracts.

Antioxidant activity

Total phenolic content

There was a wide variation in the amount of total phenolics in sea anemones ranging from 3.75 to

25.1 mg GAE.g⁻¹ dry material. Among the two species, the highest total phenolic was found in aqueous extracts of *H. magnifica* (25.09 ± 0.06 mg GAE.g⁻¹) followed by methanol extracts of *H. magnifica* (14.09 ± 0.02 mg GAE.g⁻¹), and methanol extracts of *H. crispa* (11.0 ± 0.05 mg GAE.g⁻¹). The lowest total phenolic was in aqueous extracts of *H. crispa* (3.75 ± 0.06 mg GAE.g⁻¹) (Table 2). The calibration equation for gallic acid was $y = 0.043x + 0.009$ ($R^2 = 0.9968$) (Fig. 2). The amount of total phenolic content of sea anemone was in the following descending order viz. HM-Aq > HM-MeOH > HC-MeOH > HC-Aq.

Table 1. Mortality rate of brine shrimp at different concentration of crude extracts of sea anemone *Heteractis magnifica* and *Heteractis crispa* after 24 h.

Conc. (µg)	% Mortality					
	HM-MeOH	HC-MeOH	HM-Aq	HC-Aq	Cntrl-MeOH	Cntrl-Aq
250	15.3 ± 1.9	32.9 ± 3.1	9.4 ± 0.25	4.5 ± 0.11	0	0
500	34.3 ± 4.4	55.7 ± 2.3	13.7 ± 0.6	10.8 ± 0.11	0	0
750	52.3 ± 2.3	70.2 ± 2.3	25.8 ± 2.5	22.3 ± 3.97	0	0
1000	69.9 ± 1.6	85.1 ± 2.2	38.5 ± 2.3	27.6 ± 4.0	0	0
1250	84.3 ± 3.0	98.5 ± 2.6	49.2 ± 2.6	38.8 ± 2.3	0	0
1500	100 ± 0	100 ± 0	64.8 ± 3.7	50.7 ± 2.7	0	0
2000	100 ± 0	100 ± 0	78.4 ± 3.0	64.3 ± 3.0	0	0
LC ₅₀	575.4 µg	416.9 µg	1259 µg	1514 µg		

[HM-MeOH = Methanol extraction of *H. magnifica*; HC-MeOH = Methanol extraction of *H. crispa*; HM-Aq = Aqueous extraction of *H. magnifica*; HC-Aq = Aqueous extraction of *H. crispa*; Cntrl-MeOH = control of methanol extraction and Cntrl-Aq = control of aqueous extraction].

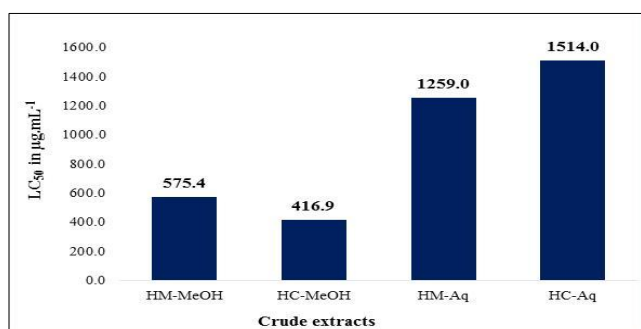


Fig. 1. The lethality concentration (LC₅₀) value of different extracts of sea anemone *Heteractis magnifica* and *Heteractis crispa*.

[HM-MeOH = Methanol extraction of *H. magnifica*; HC-MeOH = Methanol extraction of *H. crispa*; HM-Aq =

Aqueous extraction of *H. magnifica*; HC-Aq = Aqueous extraction of *H. crispa*].

Table 2. Mean of total phenolic content (mg GAE.g⁻¹) of different extracts of sea anemones *Heteractis magnifica* and *Heteractis crispa*.

Sample	mg GAE.g ⁻¹
HM-Aq	25.09 ± 0.06
HM-MeOH	14.09 ± 0.02
HC-MeOH	11.0 ± 0.05
HC-Aq	3.75 ± 0.06

[HM-MeOH = Methanol extract of *H. magnifica*; HC-MeOH = Methanol extract of *H. crispa*; HM-Aq = Aqueous extract of *H. magnifica*; HC-Aq = Aqueous extract of *H. crispa*].

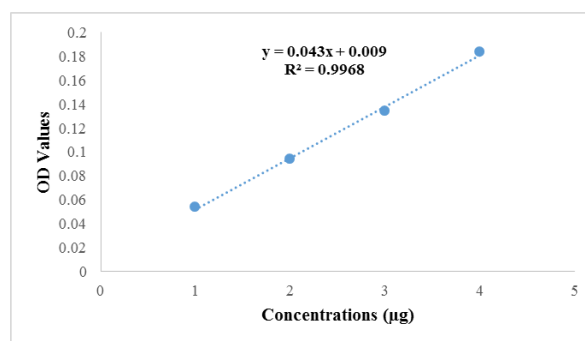


Fig. 2. Standard curve for gallic acid for measuring total phenolic content.

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power assay is shown in the figure 3 that aqueous extracts of *H. magnifica* (HM-Aq) showed high reducing power followed by HM-MeOH, HC-MeOH and HC-Aq. HM-Aq extract exhibited the highest absorbance reading between 0.233 ± 0.011 and 0.928 ± 0.034 in concentration gradient (500-2000 µg.mL⁻¹) whereas, HC-Aq extract showed lowest absorbance reading between 0.025 ± 0.004 and 0.288 ± 0.004. All the extracts exhibited concentration - dependent activity (Fig. 3). Similarly, ascorbic acid exhibited a stronger reducing power compared to the extracts of sea anemones and its absorbance reading was between 0.011 ± 0.003 to 0.041 ± 0.004 in concentration gradient (10-40 µg.mL⁻¹). The regression equation for ascorbic acid was $y = 0.001x + 0.0001$ ($R^2 = 0.9975$) and P -value, 0.001 was smaller than alpha value of 0.05 (Fig. 4).

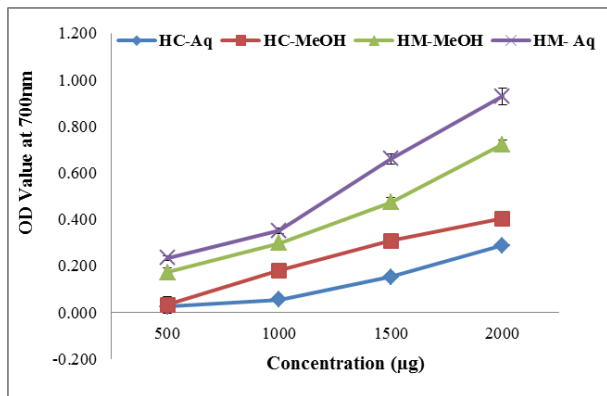


Fig. 3. Total reducing power of crude extracts of sea anemones *Heteractis magnifica* and *Heteractis crispa* with increase in concentrations.

[HM-Aq = Aqueous extract of *H. magnifica*; HM-MeOH = Methanol extract of *H. magnifica*; HC-MeOH = Methanol extract of *H. crispa*; HC-Aq = Aqueous extract of *H. crispa*]

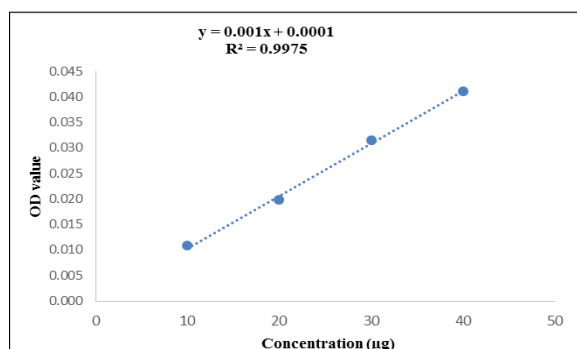


Fig. 4. Total reducing power of ascorbic acid.

DPPH free radical scavenging assay

DPPH radical scavenging property is shown in the Table 3 that aqueous and methanol extracts of *H. magnifica* indicated only scavenging activity (%) of DPPH radical, whereas *H. crispa* showed very little activity at single concentration (1000 µg.mL⁻¹) compared against the standard ascorbic acid at concentration of 100 µg.mL⁻¹. The standard reference ascorbic acid showed highest scavenging activity with the value of 98.3 ± 0.53. Among the four extracts, the aqueous extracts of *H. magnifica* (HM-Aq) showed highest scavenging activity with the value of 52.6 ± 1.07 followed by HM-MeOH (39.2 ± 1.23), HC-MeOH (16.7 ± 1.13) and HC-Aq (8.0 ± 1.19)(Table 3).

Furthermore, the extracts of HM-Aq and HM-MeOH caused an increase in DPPH free radical scavenging activity (% inhibition) in increasing dose. HM-Aq extract showed highest scavenging activity of DPPH radical that ranged from 16.1 ± 1.2 to 98.5 ± 0.6, whereas scavenging activity of HM-MeOH extract ranged from 8.9 ± 0.6 to 86.9 ± 0.9 with increase in concentrations tested (250 - 2000 µg.mL⁻¹)(Table 4). Similarly, ascorbic acid exhibited a dose-dependent pattern in DPPH radical

scavenging activity in the range of concentrations tested (25 - 100 µg.mL⁻¹) (Table 5). The result of ANOVA shows that the *F* value (9.710) was greater than *F*_{crit} value (3.885); *df* = 12; and the *P*-value, 0.003 was smaller than α value 0.05.

Table 3. Scavenging activity (%) of DPPH radicals of different extracts of sea anemones *Heteractis magnifica* and *Heteractis crispa* with standard reference (Ascorbic acid).

Samples	Scavenging activity(%)
HM-MeOH(1000 µg.mL ⁻¹)	39.2 ± 1.23
HM-Aq(1000 µg.mL ⁻¹)	52.6 ± 1.07
HC-MeOH(1000 µg.mL ⁻¹)	16.7 ± 1.13
HC-Aq(1000 µg.mL ⁻¹)	8.0 ± 1.19
AA(100 µg.mL ⁻¹)	98.3 ± 0.53

[HM-MeOH = Methanol extract of *H. magnifica*; HC-MeOH = Methanol extract of *H. crispa*; HM-H₂O = Aqueous extract of *H. magnifica*; HC-H₂O = Aqueous extract of *H. crispa*; AA = Ascorbic acid]

Table 4. Scavenging activity of different extracts of sea anemone *Heteractis magnifica* with increase doses (250 - 2000 µg).

Concentration (µg)	Scavenging activity(%)	
	HM-MeOH	HM-Aq
250	8.9 ± 0.6	16.1 ± 1.2
500	19.3 ± 0.9	28.4 ± 1.2
1000	39.8 ± 1.7	52.8 ± 0.9
1500	67.7 ± 1.1	83.4 ± 1.1
2000	86.9 ± 0.9	98.5 ± 0.6

[HM-MeOH = Methanol extract of *H. magnifica*; HM-Aq = Aqueous extract of *H. magnifica*]

Table 5. Scavenging activity of the standard ascorbic acid with increase in dose.

Concentration (µg)	Scavenging activity(%) of AA
25	18.81 ± 0.39
50	48.63 ± 0.21
75	74.46 ± 0.09
100	98.79 ± 0.19

AA = Ascorbic acid.

The comparison of the mean concentration for 50 % free radical scavenging activity (EC₅₀) of ascorbic acid and *H. magnifica* extracts are were shown in Table 6. The value of EC₅₀ of HM-Aq and HM-MeOH extracts were 707.9 ± 1.65 and 1032.7 ± 2.5 µg.mL⁻¹ respectively and the value of EC₅₀ of ascorbic acid

was $47.1 \pm 0.6 \mu\text{g.mL}^{-1}$ which is a well-known antioxidant. Low EC_{50} value indicates strong ability of the extract of HM-Aq to act as DPPH scavenger. Methanol extracts showed less activity with high EC_{50} values i.e., $1032.7 \pm 2.52 \mu\text{g.mL}^{-1}$.

The results of the present study indicate that sea anemones especially *H. magnifica* could be used for significant and varied applications in the pharmaceutical industries because of its high antioxidant activities.

Table 6. EC_{50} value of the extracts (HM-MeOH = Methanol extract of *Heteractis magnifica*; HM-Aq = Aqueous extract of *H. magnifica*) compared with ascorbic acid.

Extracts	$\text{EC}_{50}(\mu\text{g.mL}^{-1})$
AA(Standard)	47.1 ± 0.63
HM-Aq	707.9 ± 1.65
HM-MeOH	1032.7 ± 2.52

This is the first report on antioxidant activities shown by the aqueous extract of *H. magnifica* in India. The DPPH assay showed that, the extracts of *H. magnifica* were able to reduce stable radical DPPH to the yellow coloured diphenylpicryl hydrazine, which was visually noticeable by the discolouration of test samples from purple to yellow. The degree of discolouration indicated the potential of the aqueous extract of *H. magnifica* to scavenge free radical due to its ability to donate hydrogen proton. Further, the total phenolic contents were determined and stated as gallic acid equivalent (GAE) in order to make a comparison between different extracts of sea anemones and identify a natural marine source for phenolic compounds. The aqueous extract of *H. magnifica* exhibited higher quantity of phenols compared to other extracts. The high antioxidant activity of aqueous extract of *H. magnifica* was due to the combined effect of potential phenolic contents. The concentration of phenolic contents and radical scavenging properties were lower in methanol extracts. In converse, the aqueous extract of *H. crispa* had the lowest total phenolic levels and scavenging properties as compared to *H. magnifica*. In FRAP method, the consequences showed the aqueous extract of *H. magnifica* had high reducing power than other extracts. There was an evidence of positive correlation between DPPH radical scavenging ability and the reducing power assay, which indicated that the reducing ability of the *H. magnifica* contributed in part to the antioxidant activity.

The results of the present study revealed that the crude extracts of *H. crispa* showed more pronounced cytotoxic effect, whereas *H. magnifica* had potential antioxidant activity. These results

encourage further pharmacological studies, to show the mechanism of action of the extracts, as well as to isolate active compounds present in *H. magnifica* and *H. crispa*.

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