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Anti-*Vibrio* and Antioxidant Properties of Two Weeds: *Euphorbia serpens* and *Amaranthus viridis*

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ABSTRACT

Euphorbia serpens and *Amaranthus viridis* are two weed plants, extracts of which were found to be bioactive against antibiotic resistant *Vibrio cholerae*. Amongst 15 *V. cholerae* strains studied, 10 were resistant against 6 different antibiotics, such as trimethoprim, polymyxin B sulphate, vancomycin hydrochloride, amoxicillin and ampicillin. Extracts of both the plants were prepared with five different solvents; hexane, dichloromethane, ethyl acetate, ethanol and water. Aqueous extract of *E. serpens* and ethanolic extract of *A. viridis* showed maximum anti-*Vibrio* activity against all the strains of *V. cholerae*. The anti-*Vibrio* compounds from both the plants were purified by column chromatography and bioactive fractions were found to be stable at extreme temperature and pH. Minimum Inhibitory Concentration (MIC) of *E. serpens* and *A. viridis* were found to be 3.92 and 12.32 mg mL^{-1}, respectively against the most resistant *V. cholerae* strain in our study. The bioactive fractions were further analyzed by HPLC. Column purified fraction with anti-*vibrio* activity caused cell wall indentation and cell ruffling on *V. cholerae* as seen by scanning electron microscopy. Antioxidant content of bioactive fraction of *E. serpens* and *A. viridis* was 28.33 and 13.17 mg g^{-1} of dry weight of extract, respectively as evaluated by TRP assay, 68.93 and 8.65 mg g^{-1} of dry weight of extract, respectively by FRAP assay and 158 mg g^{-1} of extract and 55.32 mg g^{-1} of dry weight of extract, respectively by ABTS assay. The bioactive ingredients in both the plants are essential oil.

Key words: *Euphorbia serpens*, *Amaranthus viridis*, *Vibrio cholerae*, antibacterial, antioxidant

INTRODUCTION

Cholera is a worldwide disease caused by the gram negative bacteria, *Vibrio cholerae*. According to World Health Organization 100,000-120,000 people die globally per year due to cholera. Usually, along with oral rehydration salt, patients are treated with antibiotics, like nalidixic acid, furazolidone, ciprofloxacin, cotrimoxazole, doxycycline, norfloxacin, tetracycline, ampicillin, kanamycin, streptomycin, trimethoprim-sulfamethoxazole etc. (Mukhopadhyay *et al*., 1998). However with time, *V. cholerae* strains develop resistance against antibiotics (Kitaoka *et al*., 2011; Glass *et al*., 1980; King *et al*., 2008). Use of plant based formulation is an alternative for slowing down the development of resistance. The present research study aims towards identifying

anti-*Vibrio* plants, especially to be effective against clinical strains obtained from north-eastern India. Emphasis is also given to screen the plants that are abundantly available in the local area. Here we report anti-*Vibrio* activity of extracts of two weeds, *Euphorbia serpens* and *Amaranthus viridis* against the clinical isolates of *V. cholerae* obtained from several parts of north-eastern India along with standard MTCC strains.

MATERIALS AND METHODS

Preparation of bacterial suspension: Two *Vibrio cholerae* strains were collected from Assam medical college (NE1, NE2), three strains were obtained from IMTECH (MTCC-3904, MTCC-3905, MTCC-3906) and ten strains were provided by NICED, Kolkata (J-6705, J-6951, J-19132, J-20148, C0102, C042, C045, C0111, C0127, C079). All cultures were maintained on Luria-Bertani agar (LB agar) at 37°C. Isolated colonies were inoculated into LB broth and kept overnight into the shaker at 37°C. Next day turbidity of the grown culture was adjusted to McFarland standard 0.5 ($5x\times10^8$ CFU mL^{-1}) and 100 µL of bacterial suspension was used for all assays.

Antibiotic susceptibility of *Vibrio cholerae* strains: Antibiotic sensitivity of all *V. cholerae* strains were checked against five antibiotics: trimethoprim, polymyxin B sulphate, vancomycin hydrochloride, amoxicillin and ampicillin by disc diffusion assay (Mehrotra *et al*., 2010).

Collection and identification of plant: *Euphorbia serpens* and *Amaranthus viridis* plants were collected from Assam, India and were identified by Botanical Survey of India, Eastern Regional Centre, Shillong. The accession numbers were 82903 for *E. serpens* and 82904 for *A. viridis*.

Preparation of plant extract: Extracts were prepared as described earlier (Mehrotra *et al*., 2011). In brief, plant materials were dried in hot air oven at 60°C and 10 g of dried plant materials were crushed by using mortar and pestle and soaked overnight in shaker with hexane. Next day, it was filtered through whatman filter paper no.1 and filtrates were concentrated under reduced temperature and pressure in rota-evaporator and stored at 4°C for future use. The residue was allowed to air dry and soaked in the next solvent and the remaining extraction steps were repeated. The plant extracts were prepared in five different solvents-hexane, dichloromethane, ethyl acetate, ethanol and water.

Anti-*Vibrio* activity of plants: Disc diffusion assay was performed to check the anti-*Vibrio* activity of both the plant extracts prepared in different solvent systems. Ethanolic extract of both the plants were checked against fifteen antibiotic resistant *V. cholerae* strains (Mehrotra *et al*., 2011; Kirar *et al*., 2015; Mamta *et al*., 2015).

Aqueous and ethanolic extract of *Euphorbia serpens*: Ethanolic and aqueous extract of *E. serpens* was loaded on precoated thin layer chromatography plate and separated in formic acid: Methanol (1:1) solvent. Contact bioautography was done for whole TLC strips against MTCC-3904 *Vibrio cholerae* strain. The Rf value of bioactive spots were noted (Mehrotra *et al*., 2010).

Partial separation of bioactive compounds by column chromatography: Ethanolic extract of both the plants were loaded separately in silica column of 60-120 mesh size. The column was run with ten different combinations of three solvents: hexane, ethyl acetate and methanol. Ten fractions

for each solvents were collected. Each fraction was loaded on precoated TLC plate and separated in 5% methanol in chloroform. Based on TLC separation profile, fractions were pooled up and bioactivity against *V. cholerae* was checked.

The bioactive column fractions of both plants were kept overnight at 4°C followed by centrifugation. Bioactivity of pellet and supernatant were checked.

Minimum inhibitory concentration: The LB broth was inoculated with *V. cholerae* strain and incubated for 4 h in the shaker at 37°C. The grown culture was treated with different concentration of column purified bioactive fraction and incubated overnight in shaker. 10^{-2}, 10^{-4} and 10^{-6} serial dilutions were made for each treated culture and each dilution was streaked on LB agar plate. Next day colonies were counted.

Analysis of bioactive fraction by HPLC: Bioactive column fractions of *E. serpens* and *A. viridis* were analysed by HPLC (Infinity 1220, Agilent, USA) using 5 micron C-18 reverse phase column. The dried samples were dissolved in the 200 µL of HPLC mobile phase (0.2 M sodium acetate in 10% methanol) and 100 µL was injected in the column by autosampler. The HPLC was run at 30°C with a flow rate of 0.8 mL min^{-1}. The peaks were recorded at 310 nm wavelength and were collected by the system included fraction collector. Bioactivity of each fraction was checked against *V. cholerae* strain.

Effect of column purified bioactive fraction on morphology of *Vibrio cholerae*: *Vibrio cholerae* strain was allowed to grow until turbidity of McFarland standard 0.5 (5×10^{8} CFU mL^{-1}) was achieved followed by treatment of culture with the bioactive fractions of both the plants. *Vibrio cholerae* cells were harvested and fixed in 2.5% glutaraldehyde and stored at 4°C. Samples were dehydrated with ethanol before scanning electron microscopic study.

Temperature and pH tolerance of column purified bioactive fraction: The targeted column fractions of both the plants were placed in a thermal cycler at 4, 25, 60 and 100°C for 1 h and antimicrobial activity was checked by disc diffusion assay against *V. cholerae* strain (Mehrotra *et al.*, 2010). The TLC strips containing bioactive column fractions from both the plants were treated separately with 100 mM citrate buffer of pH 2, pH 7 and pH 8. pH 2 and 8 were neutralized to pH 7 and contact bioautography was performed to check the growth inhibition of *V. cholerae* strain (Mehrotra *et al.*, 2010).

Antioxidant assay of column purified bioactive fraction: Total Reducing Power (TRP) assay, Ferric Reducing Ability of Plasma (FRAP) assay and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay were done to check the antioxidant property of the bioactive fractions of both the plants using ascorbic acid and trolox as standard (Mehrotra *et al.*, 2011).

RESULTS AND DISCUSSION

Antibiotic resistance profile of *Vibrio cholerae* strains: Amongst fifteen *V. cholerae* strains, ten strains (NE2, MTCC-3904, MTCC-3906, J-6705, J-20148, C0102, C042, C045, C0111 and C0127) were resistant to all the antibiotics tested (Table 1). All the strains were resistant to trimethoprim. The NE-1, MTCC-3095, J-6951, J-19132 and C079 strains showed variable level of resistance against the antibiotics tested (Table 1). The results suggested that most of the clinical isolates that were used in the study are multi-drug resistant.

Anti-*Vibrio* activity of plant extracts: The principle research interest of our laboratory has been screening medicinal plants to be effective against human pathogens including *V. cholerae* for several years (Mehrotra *et al*., 2010; Mandal *et al*., 2014). There was no report available for anti-*Vibrio* effect of *E. serpens* and *A. viridis* in literature. Thus we explored the efficacy of extracts of these two plants against multi-drug resistant *V. cholerae* in this study. Dry leaves of *E. serpens* and *A. viridis* were extracted with the solvents of increasing polarity, such as hexane, dichloromethane, ethyl acetate, ethanol and water. It was observed that ethanolic extract of both the plants showed good anti-*Vibrio* activity against all the 15 *V. cholerae* strains tested (Fig. 1). Aqueous extracts of *E. serpens* also showed zone of inhibition almost to a similar extent that was observed with ethanolic extract. To determine, whether the bioactive compounds present in water and ethanolic extracts were same, we analyzed the extracts by TLC and found by contact bioautography bioactive spots of both the extracts possess the same Rf value. Thus, in most likelihood, the same compound was responsible for anti-*Vibrio* activity in aqueous and ethanolic extracts. Hexane extract of *E. serpens* also showed anti-*Vibrio* activity but much less than ethanolic extract. There were no anti-*Vibrio* activity for dichloromethane and ethyl acetate extracts of *E. serpens*. In case of

Table 1: Antibiotic resistance profile of *Vibrio cholerae* strains

Antibiotics	Trimethoprim	Polymyxin B sulfate	Vancomycin hydrochloride	Amoxicillin	Ampicillin
NE-1	-	+++	-	++	+++
NE-2	-	-	-	-	-
MTCC-3904	-	-	-	-	-
MTCC-3905	-	+	++	-	-
MTCC-3906	-	-	-	-	-
J-6705	-	-	-	-	-
J-6951	-	+	-	++	-
J-19132	-	++	-	-	-
J-20148	-	-	-	-	-
C-0102	-	-	-	-	-
C-042	-	-	-	-	-
C-045	-	-	-	-	-
C-0111	-	-	-	-	-
C-0127	-	-	-	-	-
C-079	-	-	++	++	+++

Concentration of antibiotic is 1 μg disc^{-1}. -: Absence of zone, +: Hair line zone, ++: Zone of inhibition (9-15 mm) , +++: Zone of inhibition (25-30 mm)

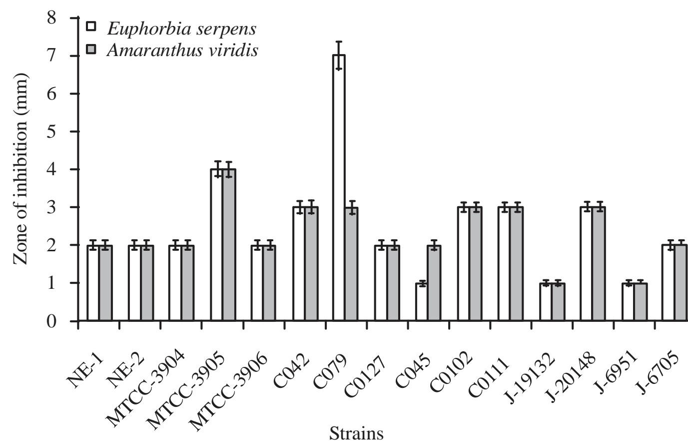

Fig. 1: Growth inhibition of *Vibrio cholerae* strains with ethanolic extract of *Euphorbia serpens* and *Amaranthus viridis* after subtracting the disc diameter on which extracts were loaded

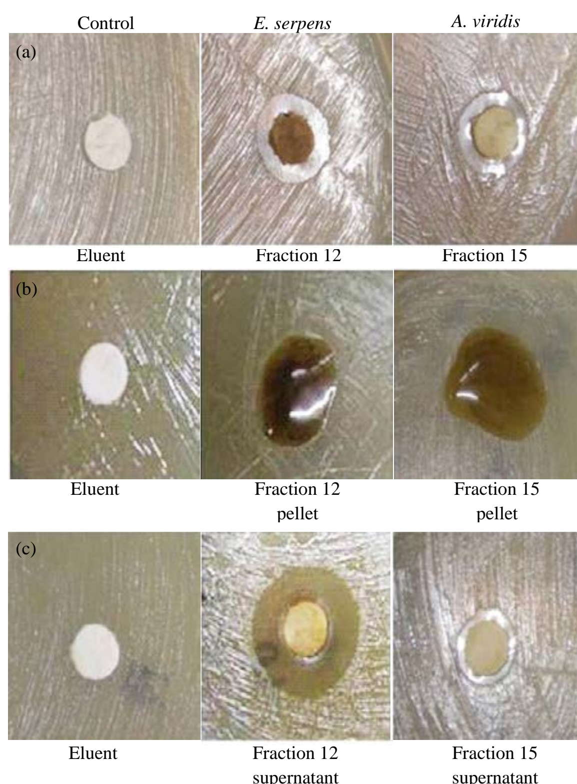

Fig. 2(a-c): Bioassay with column purified fraction. Control (running solvent treated, respectively as sample), (a) Top panel-fractions showing bioactivity, (b) Middle panel-pellet after centrifugation of the column fraction as in top panel and (c) Lowest panel-supernatant after centrifugation of column fractions as in top panel, Diameter of discs is 5.5 mm

A. viridis only ethanolic extract showed anti-*Vibrio* activity and extracts prepared with hexane, dichloromethane, ethyl acetate and water did not show anti-*Vibrio* activity.

Purification of bioactive fractions by column chromatography: The extracts were fractionated in a silica column with the solvents as mentioned in materials and methods. Using bioassay guided fractionation, bioactive fraction with highest growth inhibition of *V. cholerae* were identified. Maximum bioactivity was observed in the 12th fraction of *E. serpens* when eluted with ethyl acetate : methanol (1:1) and in 15th fraction of *A. viridis* with methanol. When, such bioactive column fractions were stored overnight at 4°C, a precipitation developed in the tubes. After centrifugation, the supernatant showed anti-*Vibrio* activity but not the pellets (Fig. 2). The supernatants were used for further experiments as column purified fractions of respective plants. Bioactive fractions of *E. serpens* and *A. viridis* plants were stable over range of temperature, 0, 25, 60 and 100°C and pH 2.0, 7.0 and 8.0. Spraying with phosphomolibidic acid on bioactive TLC (Kosalec *et al*., 2005) spot suggested that active ingredient in both the plants was essential oil.

Minimum inhibitory concentration: The MIC of the bioactive components were determined as minimum concentration that inhibited growth of *Vibrio* strains in liquid LB medium. Bioactive fraction of *E. serpens* (Fig. 3a) and *A. viridis* (Fig. 3b) showed MIC values of 3.92 and

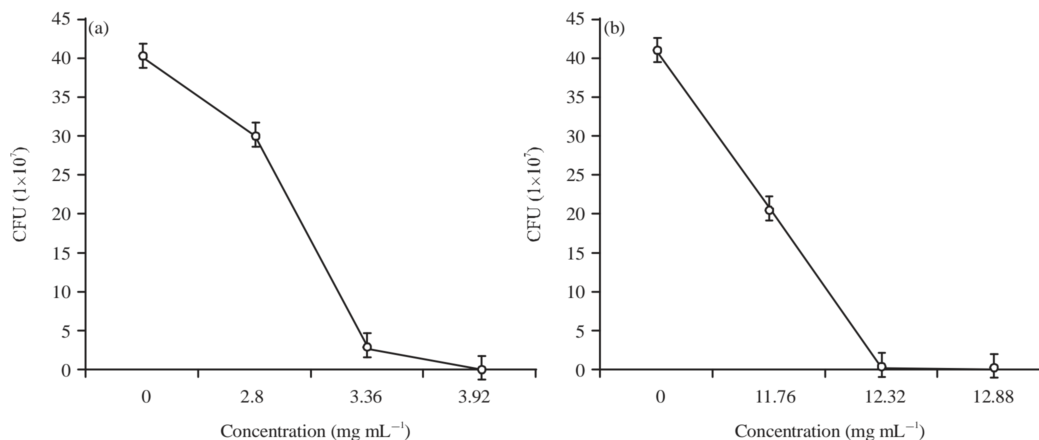

Fig. 3(a-b): Growth inhibition curve of *Vibrio cholerae* with column purified fraction of (a) *Euphorbia serpens* and (b) *Amaranthus viridis*

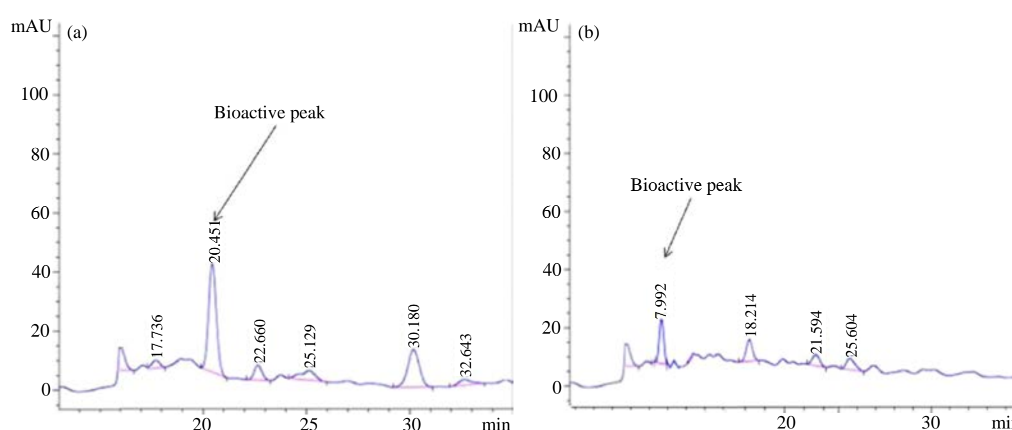

Fig. 4(a-b): Analysis of bioactive fraction by HPLC (a) Bioactive HPLC peak of *Euphorbia serpens* at retention time 20.451 min and (b) Bioactive HPLC peak of *Amaranthus viridis* at retention time 7.992 min

12.32 mg mL^{-1}, respectively. According to an earlier study, MIC values of anti-*Vibrio* plant extracts ranges between 2.5-20 mg mL^{-1} (Sharma *et al*., 2009). Our results with *E. serpens* and *A. viridis* is within this range. Thus, *E. serpens* may be a better source for generation of anti-*Vibrio* formulation.

Identification of bioactive peak by HPLC: Multiple peaks were obtained in HPLC chromatogram for column purified fractions of both the plant (Fig. 4). Each peak was collected by fraction collector and assayed for anti-*Vibrio* activity. Peak with retention time 20.451 min for *E. serpens* (Fig. 4a) and 7.992 min (Fig. 4b) for *A. viridis* showed anti-*Vibrio* activity (Fig. 4).

Effect of column purified bioactive fraction of plants on morphology of *Vibrio cholerae*: Morphology of *V. cholerae* got affected by bioactive fractions of both the above mentioned plants (Fig. 5). Indentation on surface of the bacterial cell wall and production of thread like projection

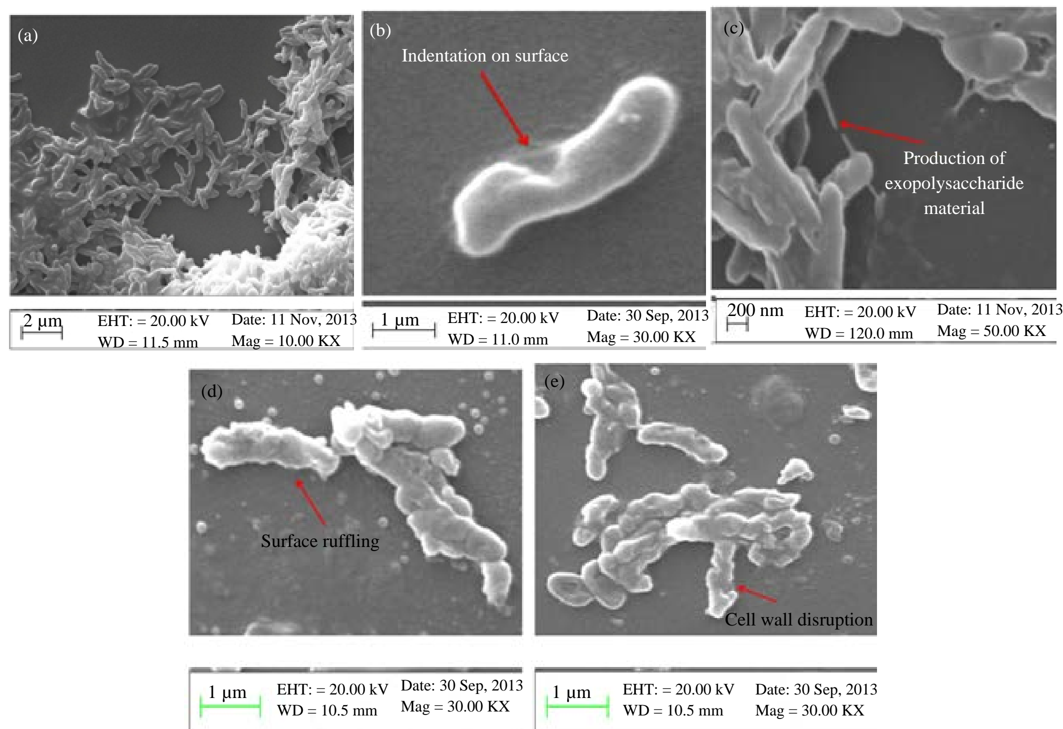

Fig. 5(a-e): Scanning electron micrograph of *Vibrio cholerae* after treatment with column purified plant fractions (a) Control (untreated *Vibrio cholerae*), (b, c) *Vibrio cholerae* treated with *Euphorbia serpens* and (d, e) *Vibrio cholerae* treated with *Overeats*

material from the surface were observed in both the cases. Bacterial cell surface ruffling and cell wall disruption were also observed after treatment with the purified bioactive fractions from *E. serpens* and *A. viridis*. The anti-*Vibrio* compounds from both the plants provided a stressful environment for *V. cholerae* which is visible as change in the morphology of the bacteria. According to previous research work, indentation on the surface of bacterial cell was also observed, when it was treated with cetylpyridinium chloride and nisin (Thongbai *et al*., 2006), *V. cholerae* cells showed projection of thread like extension made of exopolysaccharide material from the surface of the cell when it was allowed to grow in starvation medium (Wai *et al*., 1998), bacterial cell treated with eugenol also showed cell surface deformation (Devi *et al*., 2010) and cell wall disruption was observed when bacterial cells were treated with chlorohexidine gluconate (Shalamanov, 2005).

Antioxidant assay: Three different antioxidant assays have been performed: TRP, FRAP and ABTS assay (Salminen and Heinonen, 2008; Bunkova *et al*., 2005; Higashi-Okai *et al*., 2004). The TRP assay systems measures total reducing power in equivalence with ascorbic acid. The FRAP assay systems measures maximum reductive ability to transform Fe^{3+} to Fe^{2+} and commercially available trolox used as a standard for this assay. The ABTS assay systems measures electron transfer radical scavenging capacity. All the assays showed that extracts of *E. serpens* had much better antioxidant capability than the extracts of *A. viridis* (Fig. 6).

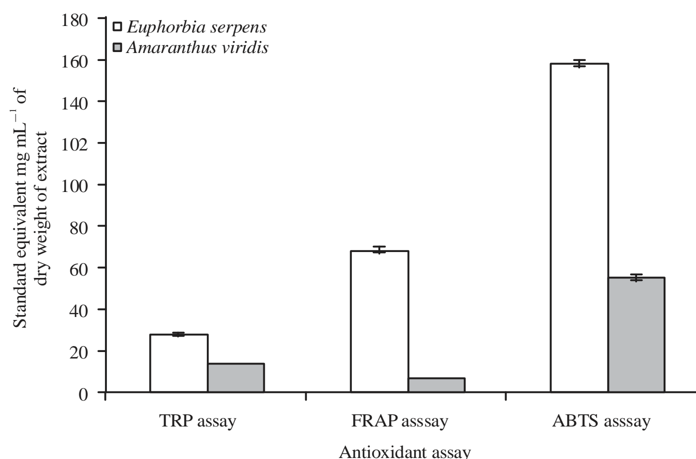

Fig. 6: Comparative study of antioxidant property of *Euphorbia serpens* and *Amaranthus viridis* by TRP, FRAP and ABTS assay

CONCLUSION

Our studies demonstrated the presence of anti-*Vibrio* activity in *E. serpens* and *A. viridis*. To our knowledge this is the first report about anti-*Vibrio* activity from these two plants. These plants are abundantly available in large parts of India especially in north-eastern region. Thus for making plant-based anti-*Vibrio* formulations, these two plants can be used. In addition, high-antioxidant property of *E. serpens* will have additional health benefit. At present we do not know the chemical.

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