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Full Length Research Paper

Seasonal influence on bioactivity of seaweeds against plant pathogenic bacteria *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin et al.

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In the present study, thin-layer chromatography (TLC) method was adopted for the fractionation of antibacterial substances from the crude extracts obtained in 1:1(v/v) chloroform : methanol of red seaweeds comprising *Portieria hornemannii* (Lyngbye) P. Silva, *Gracilaria crassa* (Harvey) J. Ag., brown *Dictyota bartyrensiana* Lamouroux, *Hydroclathrus clathratus* (C.Agardh) M.A.Howe, *Sargassum wightii Greville*, green *Ulva (Enteromorpha) compressa* (Linnaeus) Nees, and *Halimeda macroloba* Decaisne collected along the Coast of Pamban (Rameswaram, Gulf of Mannar, India) at four different seasons namely post-monsoon (February), summer (May), pre-monsoon (August) and monsoon (November) seasons for the year 2009. Fractionated substances detected under UV (Mechanical + disinfection) and iodine were tested for antibacterial activity against the plant pathogenic bacterium *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin et al. which cause canker in citrus. Number of active fractions with high antibacterial activity was recorded from the crude extracts obtained in seaweeds collected during monsoon and post-monsoon whereas seaweeds collected during summer and pre-monsoon showed low antibacterial activity. UV active substances fractionated at R_f 0.23 from red seaweed *Porteiria hornemannii* showed maximum antibacterial activity among the fractionated substances obtained on TLC from the crude extracts of all the seaweeds investigated.

Key words: Seaweeds, antibacterial activity, *Xanthomonas axonopodis* pv. *Citri*, thin-layer chromatography (TLC) fractions.

INTRODUCTION

Seaweeds are benthic marine macroalgae mainly used for the production of agar, alginate, carrageenan, liquid fertilizers and manures (Kaliaperumal et al., 1998). There are numerous reports from seaweeds derived compounds showing broad range of biological activities such as antiviral, antibiotic, anti-neoplastic, antifouling, antiinflammatory, cytotoxic and antimitotic (Naqvi et al., 1980; Caccamese et al., 1981; Fenical and Paul, 1984; Hodgson, 1984; Ballesteros et al., 1992; Bhosale et al., 2002). Harder (1917) was the first to observe antimicrobial substances in seaweeds. Then, until 1970s no large-scale screening of antimicrobial activity was carried out (Welch, 1962; Hornsey and Hide, 1974; Henríquez et al., 1979). Marine environment is abode of many groups of microorganisms (Nair and Simidu, 1987). The seaweeds living in the sea are constantly exposed to potentially dangerous co-exiting microbes and they have apparently evolved with chemical defense strategies by synthesizing array of secondary metabolites in order to defend against the microbial thread (Kubanek et al, 2003). Thus seaweeds are bestowed with varied source of bioactive natural products that exhibiting biomedical and antimicrobial properties (Padmini et al., 1988, Kulik, 1995; Kubanek et al., 2003; Arunkumar et al., 2005; Arunkumar and Rengasamy, 2000a, b; Ara et al., 2002a, b).

Intensive application of synthetic pesticides in agriculture caused damage to the ecological state of the agricultural system (Abetz and Young, 1983). The pesticides of biological origin are less toxic which generally

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affects only the target pest and closely related organisms. It is effective in very small quantities and are quickly decomposable, thereby it largely avoid the pollution problems caused by conventional pesticides. Though lot of literature is available on bioactivity of seaweeds, anti-bacterial efficacy of seaweeds against plant pathogens are comparatively a new concept (Kulik, 1995) and very few attempts have been made earlier in this line (Ara et al.,1998; Kulik, 1995). Arunkumar et al. (2005) evaluated the bioactive potential of seaweeds against plant pathogenic bacterium *Xanthomonas oryzae* pv. oryzae which cause blight in rice. Kumar et al. (2008) tested crude seaweeds extracts against the phytopathogenic bacterium *Pseudomonas syringae* causing leaf spot disease of the medicinal plant Gymnema *sylvestris*. Hence, the present work was undertaken to evaluate the bioactive potential of seaweeds against another plant pathogenic bacterium *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin et al. causing canker in citrus as well as to fractionate the major active substances from the crude extracts of each seaweeds in order to find the seasonal influence on the bioactivity of seaweeds.

MATERIALS AND METHODS

Preparation of crude seaweeds extracts

Red seaweeds comprising *Portieria hornemannii* (Lyngbye) P. Silva *Gracilaria crassa* (Harvey) J.Ag.; brown *Dictyota bartyrensiana* Lamouroux, *Hydroclathrus clathratus* (C. Agardh) M.A.Howe and *Sargassum wightii* Greville and green *Ulva (Enteromorpha) compressa* (Linnaeus) Nees, and *Halimeda macroloba* Decaisne collected at four seasons namely post-monsoon (February), summer (May), pre-monsoon (August) and monsoon (November) seasons for the one year during 2009 were chosen for this investigation. Each 1 Kg of live, healthy and matured samples of each seaweed collected along the Coast of Pamban (Rameswaram (9°14`N; 79°14`E), Gulf of Mannar, Tamil Nadu, India) were washed thoroughly in seawater followed by tap water to remove extraneous particles and epiphytes. Then they were air dried under shade in laboratory for 3 days. The shade-dried samples were chopped and pulverized. Each 50 g powdered sample was separately extracted for 7 days for thrice in 500 ml of 1:1(v/v) chloroform: methanol using 1 litre Erlenmeyer conical flask under dark condition. The extractants were pooled and concentrated by using flask evaporator under reduced pressure at 45°C and weighed stored at 0°C.

Thin layer chromatography (TLC) study

The silica gel (E. Merck, India) and glass distilled H_2O 1:2 (w/v) were mixed and the slurry was applied on glass plates(20 × 20 cm) with 1.0 mm thickness. The coated plates were activated at 80°C for 3 h and they were cooled at room temperature before use.

Fractionation of crude substances (Arunkumar and Rengasamy, 2000b)

One g of crude extracts re-constituted in 10 ml of methylene chloride was loaded on TLC plates (20 × 10 cm) as line just above 2 cm from the bottom of the plates in using capillary pipette. The plates were left for 30 min at room temperature to evaporate solvent. Then the plates were developed in a glass jar containing hexane: diethyl ether: 1% acetic acid (5:4:1v/v/v). After the solvent front having reached 15 cm height approximately, the plates were removed and allowed the solvent to evaporate at room temperature for 30 min. Then the plates were observed under UV light (240 and 300 nm).After recording the R_f values, the fluorescent bands were scrapped out. Subsequently, the plates were kept in iodine chamber, the coloured bands marked and the R_f values were recorded. Then the plates were kept at room temperature for 30 min for the evaporation of iodine. Different bands which were scrapped out under UV and iodine were dissolved in methylene chloride (1:10 v/v) to extract the substances present in each band. They were filtered through Whatman #1 filter paper to remove the silica gel and this was repeated for three times. Solvent was evaporated and the substances were stored at 0°C and they were used for bioassay.

Antibacterial assay

The plant pathogenic bacterium *X. axonopodis* pv. *citri* (Hasse) Vauterin et al. (syn. *X. citri* pv. *citri* Gabriel et al., 1989) cause canker in citrus obtained from the Center for Advanced Studies in Botany, University of Madras, Chennai-600 025, India was used for conducting bioassay. Sterile paper discs (5.0 mm) loaded with 50 µl (100 µg of the substances) of different fractionated substances using micropipettes were allowed to dry thoroughly under aseptic condition. Each Petri plate of 100 mm diameter was initially poured with 1.5% nutrient agar medium (Peptone - 10 g, Beef extract - 10 g, NaCl- 5 g, Distilled H_2O - 1000 ml and pH 7.0) followed by the 2nd layer of 1.0% nutrient agar medium. Then the substances loaded discs were impregnated on to the plates smeared with 0.05 ml of bacterial culture with exponential phase culture of 1.0 OD at 590 nm and incubated at 28°C for 48 h. The diameters of the agar clear zones of bacterial inhibition around the disc as a result of diffusion of active substances were measured including disc as mm diameter for antibacterial activity. For the antibacterial assay, three replicates were maintained for each experiment and mean values were recorded. The solvents used for reconstituting the substances loaded on paper discs were treated as control which did not show any inhibition zone while testing.

RESULTS

TLC is a less expensive, simple, easy to reproduce and reliable technique still widely followed in phytochemical investigation adopted for the fractionation of major active principles present in the crude extracts of seaweeds in this present study. The fractionated substances obtained through TLC at different R_f level from the crude extracts of seaweeds collected at four seasons were tested for antibacterial activity. Number of active fractions with maximum antibacterial activity was recorded from the fractionated substances obtained from the crude extracts of seaweeds collected during post-monsoon and monsoon.

In the present investigation, six active fractions were commonly identified from the crude extracts of red seaweed *P. hornemannii* collected in all the four seasons. In addition to 6 fractions, 2 additional fractions (R_f 0.37 and 0.57 UV active) were detected from *P. hornemannii* collected during post-monsoon and monsoon seasons.

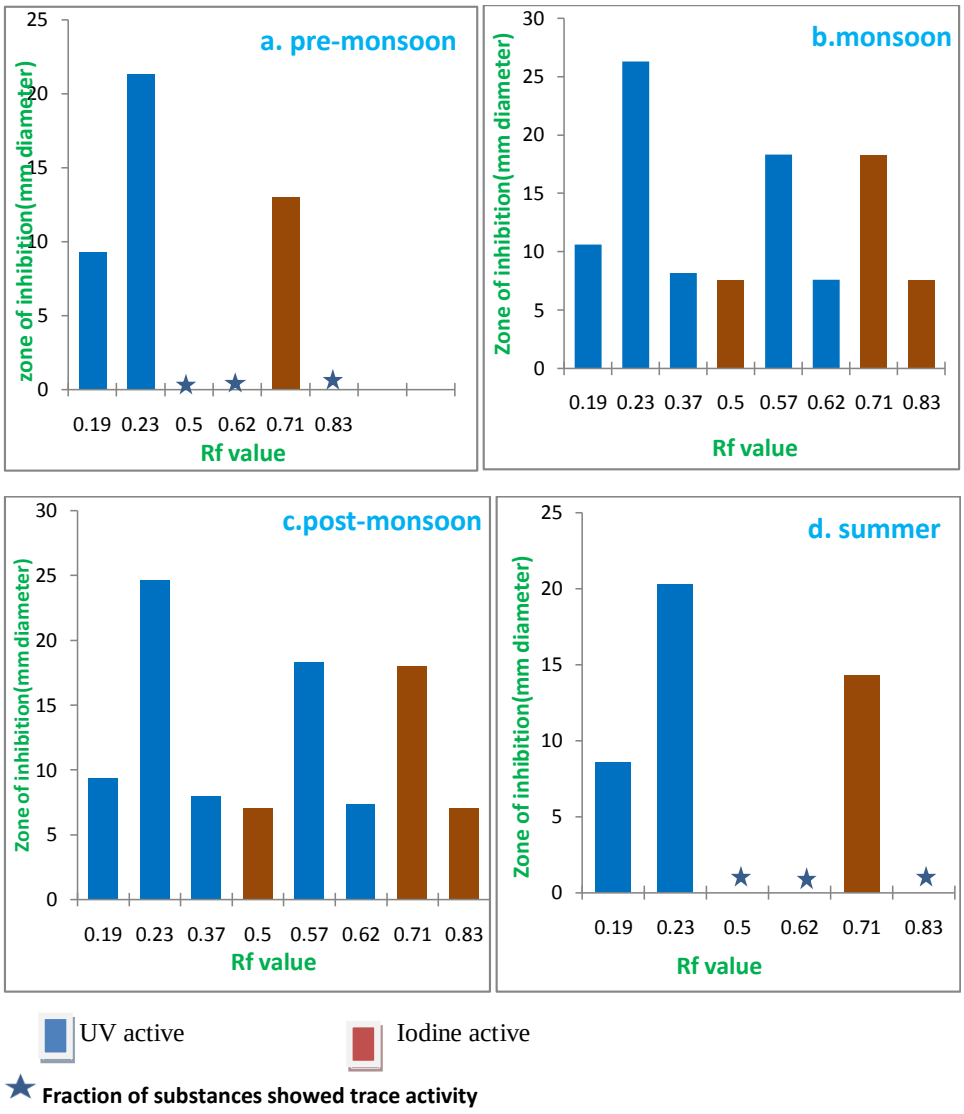

Figure 1. Antibacterial activity of TLC fractionated substances obtained from the chloroform: methanol (1:1 v/v) extract of red alga *P. hornemanii*.

The highest antibacterial activity was recorded from the UV active substances fractionated at R_f 0.23 from *P. hornemannii* collected during monsoon season among the fractionated substances obtained from all seaweeds investigated (Figure 1). Of the 4 to 5 bands recorded from the red alga *G. crassa,* maximum antibacterial activity was recorded from the Iodine active substances fractionated at R_f 0.23 (Figure 2).

Apart from 5 to 8 common fractions, 3 additional UV active fractions ($R_{f\ 0.19}$, 0.51 and 0.67) were recorded from the crude extracts of brown alga *Dictyota bartyresiana* sampled during post-monsoon and monsoon. High antibacterial activity was recorded from the UV active substances fractionated at R_f 0.51of monsoon sample among the fractions of *D. bartyresiana* (Figure 3). In *H. clathratus,* 5 to 7 fractions were detected and high antibacterial activity was recorded from the UV active substances of monsoon collection fractionated at R_f 0.36 (Figure 4). Among the 4 to 6 common bands detected from all crude extracts, high antibacterial activity was recorded from the R_f 0.23 Iodine active fraction obtained from the crude extract of *S. wightii* sampled during monsoon season (Figure 5). In the crude extract of green alga *Ulva compressa*, of the 6 to 9 common fractions separated, iodine active substances fractionated at R_f 0.60 showed the highest antibacterial activity among the obtained iodine active substances from all the seaweeds investigated (Figure 6). Out of 4 to 7 fractionated substances detected from the crude extracts of *H. macroloba,* high antibacterial activity was recorded from the UV active substances fractionated at R_f 0.23 of monsoon collection (Figure 7).

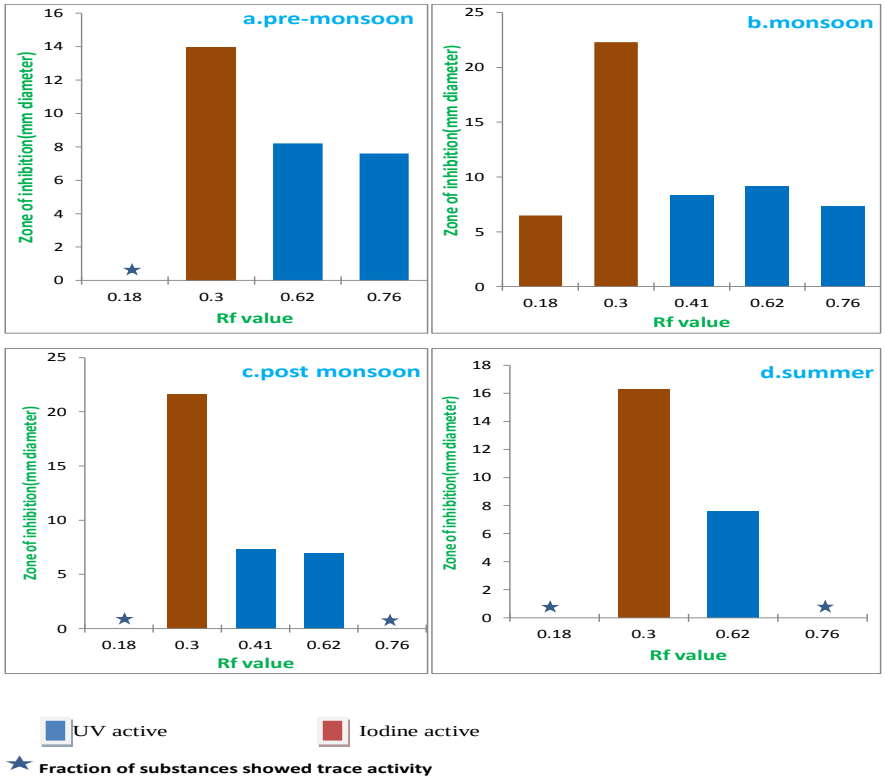

Figure 2. Antibacterial activity of TLC fractionated substances obtained from the chloroform: methanol (1:1 v/v) extract of red alga *Gracilaria crassa* .

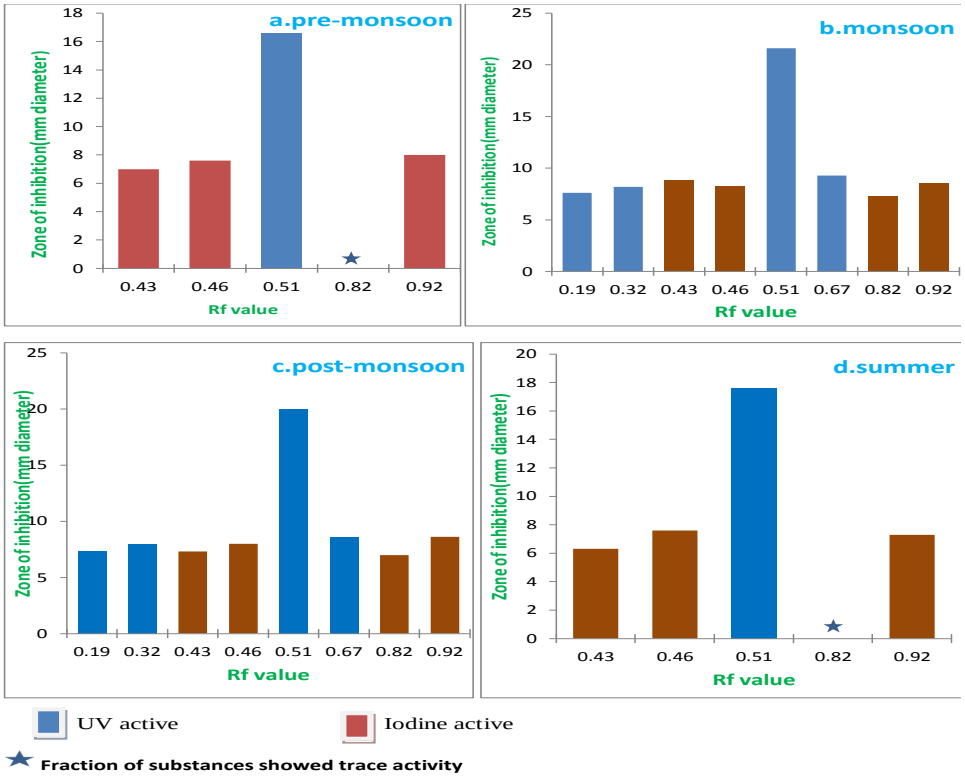

Figure 3. Antibacterial activity of TLC fractionated substances obtained from the chloroform: methanol (1:1 v/v) extract of brown alga *Dictyota bartyresiana*.

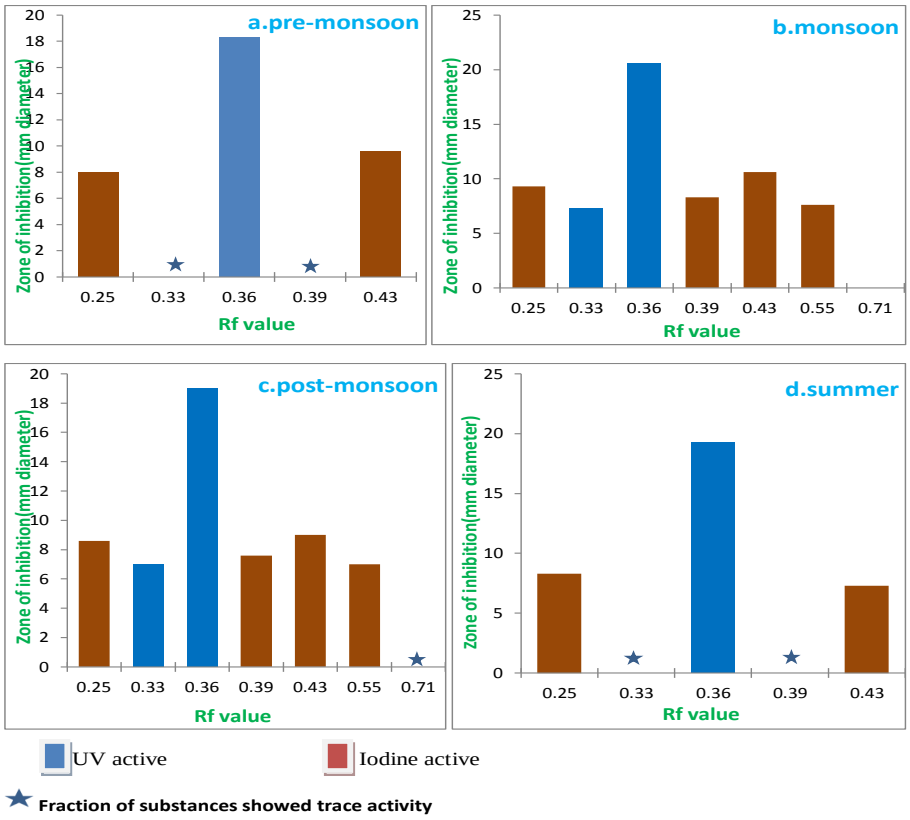

Figure 4. Antibacterial activity of TLC fractionated substances obtained from the chloroform: methanol (1:1 v/v) extract of brown alga *H. clathratus.*

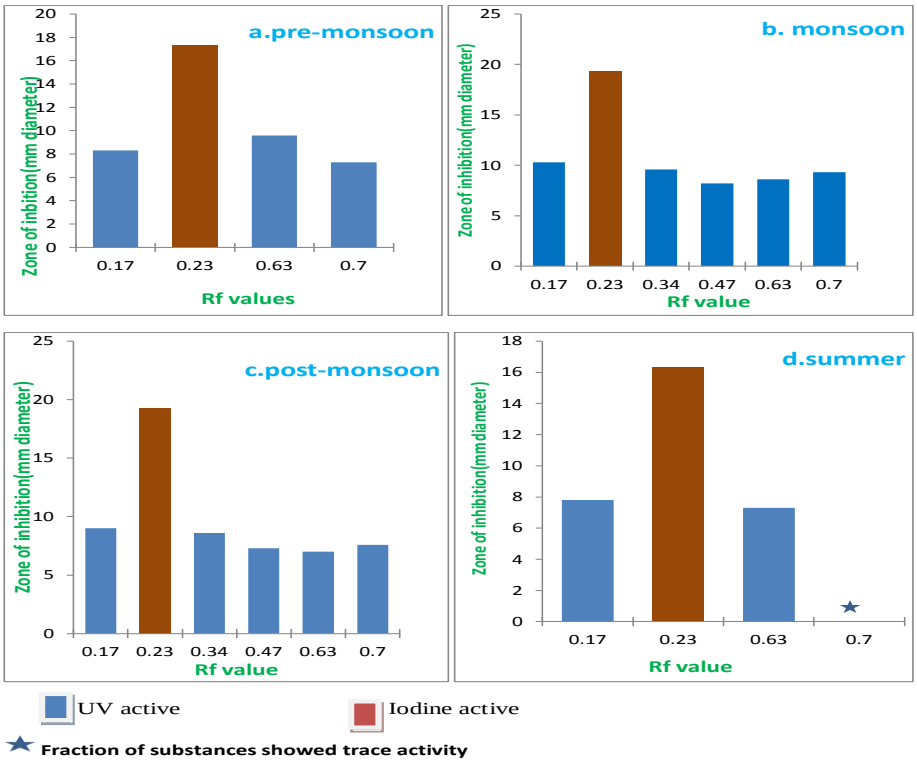

Figure 5. Antibacterial activity of TLC fractionated substances obtained from the chloroform: methanol (1:1 v/v) extract of brown alga *S. wightii.*

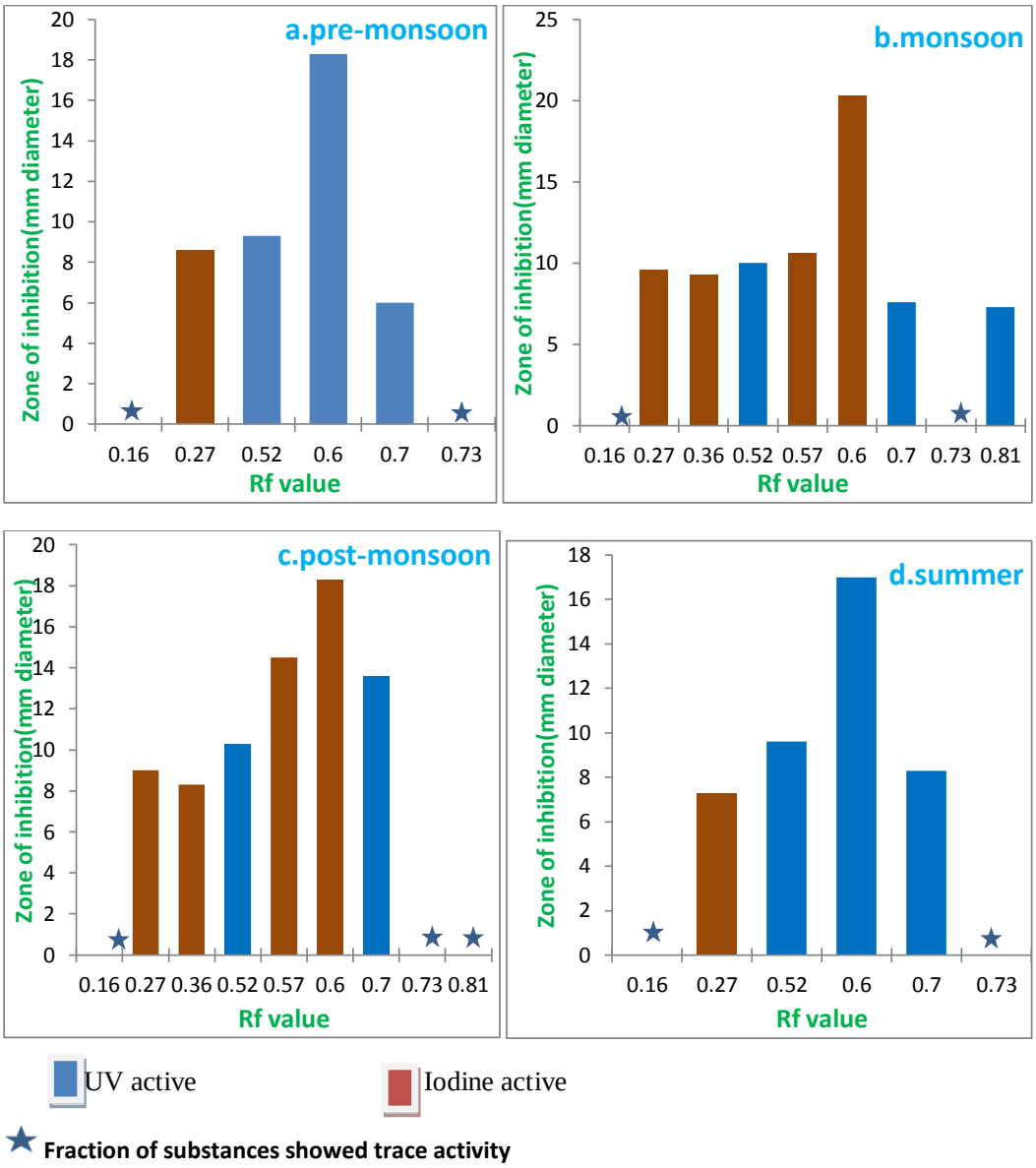

Figure 6. Antibacterial activity of TLC fractionated substances obtained from the chloroform: methanol (1:1 v/v) extract of green alga *U. compressa*.

DISCUSSION

The term 'bioactive substance' refers to the compounds of biological origin at low concentration; it is beneficial or harmful to all living organisms. Generally, the term refers to secondary metabolites of biological origin that attracted the attention of both scientists and industrialists recently. Seaweeds have been identified as a new and rich source of bioactive compounds (Pulz and Gross, 2004). In the present investigation, bioactive compounds with difference on TLC characteristics were separated from the crude extracts of seaweeds collected at different seasons against a plant pathogenic bacterium *X. axonopodis* pv. *citri* cause canker in citrus plant.

In phytochemical analysis, TLC provides a significant clue for chemical identification (Robles-Centeno et al., 1996). The solvent system used in the present study for TLC was selected on the basis of maximum separation, reproducibility, stability and non-destructive properties (Olesen et al., 1963). Arunkumar and Rengasamy (2000a) reported that the developing mixture of hexane: ethyl acetate (4:6 v/v) was found suitable for the separation of antibacterial substances of petroleum ether extract and unsaponified fraction of red and green seaweeds and unsaponified fraction of brown seaweeds, whereas methanol extract and lipophilic fraction of brown seaweeds showed good separation in the developing mixture of hexane: diethyl ether:1% acetic acid

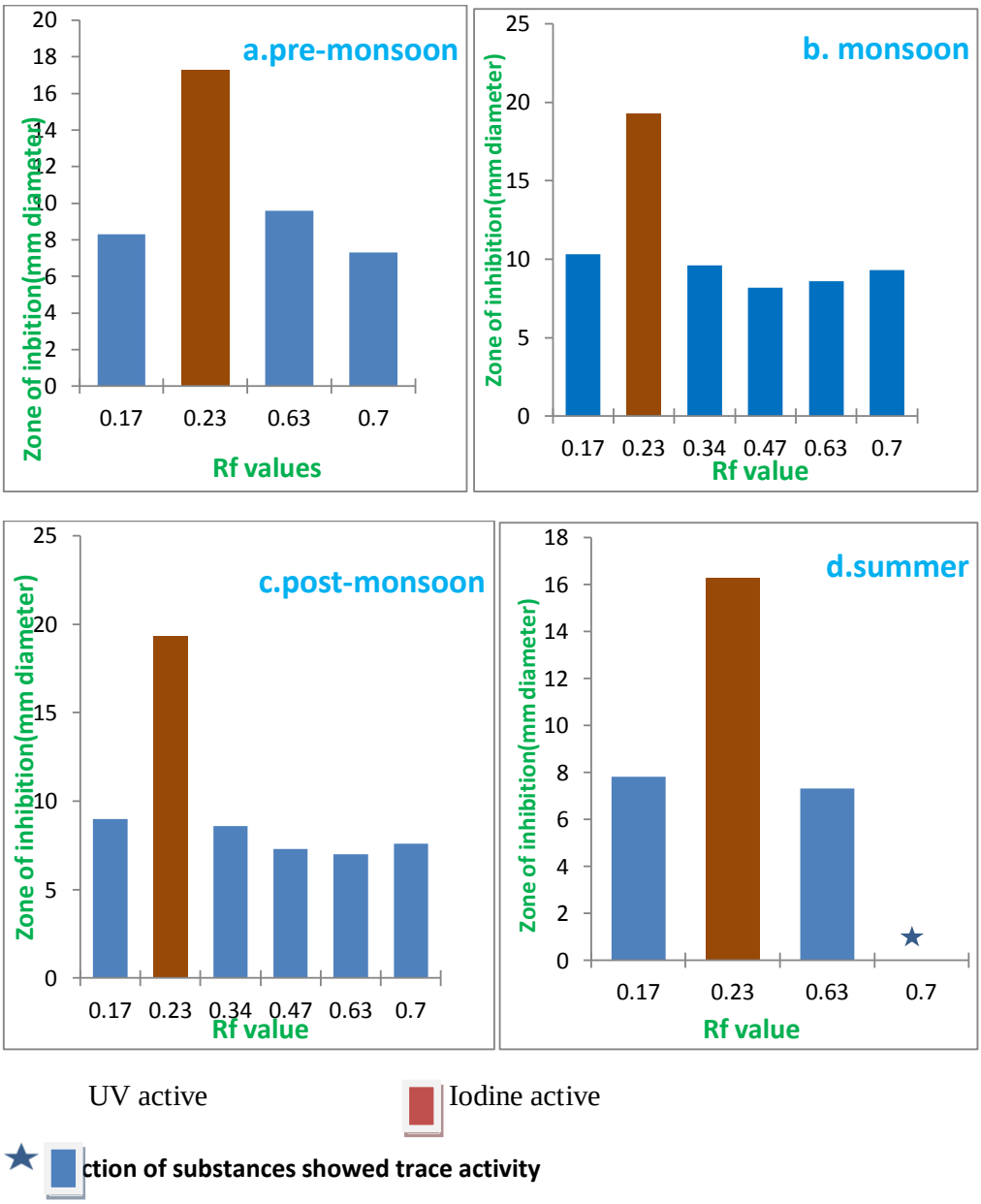

Figure 7. Antibacterial activity of TLC fractionated substances obtained from the chloroform: methanol (1:1 v/v) extract of green alga *H. macroloba.*

(5:4:1 v/v-/v) which was used to separate 1:1 (v/v) chloroform: methanol extracts of all seaweeds in the present investigation.

Ballantine et al. (1987) screened the potential of 102 algal species for antimicrobial activity. They found that a number of species showed temporal variability in bioactivity and concluded that the factors such as reproductive state, sampling locale and seasonality influence the ability of substance for antimicrobial activity. The green seaweed *Halimeda optuntia* collected from different habitations and seasons at Puerto Rico showed the marked seasonal variation in bioactivity against *Escherichia coli* (Almodovar, 1964). Robles-Centeno et al. (1996) reported that the difference in bioactivity in the crude extracts of seaweeds occurring between habitats were presumably due to environmental influence on specific metabolite synthesis. Arunkumar and Rengasamy (2000a) suggested that the obtained seaweed extracts from the backwaters possessed higher antibacterial activity than the same collected from open coasts. Further, the crude extracts of same seaweed collected at different localities of the coasts showed a significant difference in antibacterial activity. In the present study, seaweeds collected during monsoon and

post-monsoon seasons possessed high number of active fractions with high antibacterial activity whereas pre-monsoon and summer collection showed low activity. This indicted that seasonal variation would influence the synthesis of specific metabolites in seaweeds. Sreenivasa and Parekh (1981), Vidyavathi and Sridhar (1991) and Arunkumar and Rengasamy (2000a) reported low bioactivity during summer when the biomass of seaweed population was less. The high antibacterial activity that was observed during monsoon might be due to rapid growth with the fast proliferation, photosynthetic activity, physiological and chemical stresses as well as retention of the bioactive principles (Khaleafa et al., 1975) along with less loading of epiphytic bacteria during active growth when the protective mechanism of seaweeds was strong (Sieburth and Jenson, 1968).

In conclusion, a number TLC active fractions with the highest antibacterial activity recorded in the crude extracts of seaweeds collected during monsoon and post-monsoon seasons suggested that bioactive potential in each seaweed vested with more than one compounds found to be a promising source to control the plant pathogenic bacterium *X. axonopodis* pv. *citri cause* canker in citrus.

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