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Antibacterial Properties of the Microalgae *Chaetoceros calcitrans*

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Abstract

The antibacterial properties of the microalgae *Chaetoceros calcitrans* were assessed. Samples of *C. calcitrans* were first extracted in methanol, and then in different organic solvents of increasing polarity, n-hexane (n-Hex), dichloromethane (DCM) and ethyl acetate (EA) by liquid-liquid extraction. Solvent extracts were screened for antibacterial activity against four species of bacteria: Gram positive, *Staphylococcus aureus* and *Bacillus subtilis;* and Gram negative, *Escherichia coli* and *Vibrio harveyi*, with Amoxicillin as positive control, N-Hex extract, with significantly lower antibacterial activity than Amoxicillin, showed significantly higher activity than DCM and EA extracts, and least in methanolic extract. High antibacterial activity of n-Hex extract against all the microorganisms indicates that the bioactive components could be non-polar since the activity decreased as the solvent became more polar like methanol, and finally lost in aqueous extract. Results also showed that the extracts have a broad spectrum activity. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of all solvent extracts on all microorganisms tested ranged from 125 to 500 $\mu g \cdot mL^{-1}$. Partial purification and characterisation of the extracts confirmed the antibacterial activity in the non-polar fraction, which could be terpenes. The results suggest a good prospect in using *C. calcitrans* against *Vibrio* and other bacterial species.

Introduction

Microalgae are phytoplankton that comprise the major primary producers of the aquatic ecosystem. They provide the base upon which the aquatic food chains depend. Microalgae are required for larval nutrition during a brief period, either eaten directly by molluscs or shrimps or indirectly as food for live prey fed to small fish larvae (Spolaore et al. 2006).

While microalgae provide food for zooplankton, they also help improve the water quality of the culture medium. The introduction of phytoplankton to rearing ponds (green-water technology) indeed improved the growth and survival of the organisms compared to clear-water technology (Chuntapa et al. 2003; Lio-Po et al. 2005). The reasons are not entirely known but may include the

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products or substances excreted by the microalgae. Microalgae have been recognised as a potential source of a number of products of commercial interest (Cohen 1986; (Borowitzka 1994; Richmond 1990). Studies conducted on selected culture of microalgae (Otero et al. 1997) have reported the occurrence of some significantly important compound classes such as phenolics, alkaloids, flavonoids, glycosides, terpenes, vitamins and fatty acids. These compounds were found to have important biological activities such as antibacterial property (Ohta et al. 1995; Seraspe et al. 2005) against *Vibrio harveyi*.

Microalgae, indeed, have the potential to produce a vast array of products as foodstuff, as nutrient enhancer/supplement or from which novel compounds may be derived for use in the aquaculture industry. Thus, this study was conducted to provide a scientific basis for the utilisation of the microalga, *Chaetoceros calcitrans* in aquaculture where it can be used as an alternative biocontrol agent to antibiotics in the treatment of fish/shrimp diseases.

This study therefore aimed to assess the antibacterial properties of *C. calcitrans*, and to extract and partially characterise the bioactive fractions responsible for the antibacterial properties of this microalgae.

Materials and Methods

Culture and extraction

Pure cultures of *C. calcitrans* starting with an initial algal density of 50,000 cells·mL^{-1} were scaled up from 1 L to 200 L in a fibre glass container supplied with proper aeration and illumination. vitamins, trace metals, Na_2EDTA, Na_2HPO_4, F medium, and $Na_2SiO_2.5H_2O$ were used as fertilisers. The culture was done at the Phycology Laboratory, Southeast Asian Fisheries Development Center Aquaculture Department (SEAFDECAQD), Tigbauan, Iloilo, Philippines.

During the logarithmic phase, pure cultures of the species were harvested by electrolytic flocculation (Poelman et al. 1997). The harvested microalgae were stored in plastic containers and refrigerated to maintain freshness. Over a phytoplankton mesh, they were washed with distilled water until they were chloride-free and then dried using an improvised mechanically-heated cabinet dryer at 35 ^{o}C.

One kilogram of cabinet-dried sample was soaked in A.R. grade methanol in a 1:3 (weight:volume) ratio for 48h, then filtered and the filtrate was collected and stored. The whole process was repeated until the colour of the filtrate became colourless. The combined filtrate was concentrated using a rotary evaporator under reduced pressure of 60 mm Hg (1.16 psi) and at a temperature of 45 ^{o}C. The methanolic extract was acetone-dried by centrifugation at a speed of 2000 rpm for 5 min.

The crude methanolic extract was further subjected to sequential liquid-liquid extraction using solvents of increasing polarity, n-hexane (n-Hex), dicloromethane (DCM) and ethyl acetate (EA) following the method described by Sylianco (1988). This was done to provide the molecules a range of solvent system where they are best soluble.

Fifty millilitres of n-Hex were added to 100 mL of the crude methanolic extract in a separatory funnel. The content of the funnel was shaken gently, and allowed to stand until equilibrium was attained. The aqueous layer was transferred to another separatory funnel while the n-Hex layer was transferred to a container. Another 50 mL of n-Hex was added to the separatory funnel containing the aqueous layer, and the extraction was carried out until the n-Hex layer became clear. The n-Hex extracts were combined and concentrated using the rotatory evaporator. The same procedure was followed as in n-Hex for DCM and EA extraction. The extraction and fractionation scheme employed is shown in Fig. 1. The three solvent extracts together with the crude methanolic extract were then bioassayed to determine their antibacterial activities.

Antibacterial assay

To determine their antibacterial activities, the different solvent extracts of *C. calcitrans* were bioassayed using the paper disc diffusion method described in a compilation edited by Guevara (2005).

One hundred milligrammes of the different solvent extracts dissolved in 1 mL of their solvent were tested against four species of bacteria: Gram positive, *Staphylococcus aureus* (ATCC No. 25923), *Bacillus subtilis* (ATCC 27853), and Gram negative, *Escherichia coli* (ATCC 25922), and *Vibrio harveyi* (PN 9801) (de la Peña et al. 2001). All these test organisms were recultured every week and maintained for 6 months after which the cultures were tested for their purities. The positive control used was Amoxicillin (100 $mg \cdot mL^{-1}$). Each sample was also assayed against a blank paper disc containing the solvent.

Bacterial suspensions were prepared with nutrient broth media incubated for 18h at 37 ^{o} C standardised with 0.1 McFarland's barium sulfate standard solution containing approximately 300 million colony forming units (cfu) per mL concentration of inoculum. The inoculum of each suspension in 0.1 mL amounts was transferred on prepared nutrient agar (NA) plates. Five millilitres of molten NA, cooled to 45 ^{o}C, was then poured onto the NA plate and was gently swirled to disperse the inoculum. Each blank disc was dipped three times in the extract, dried and placed on top of the seeded agar. The same method was applied to the positive and negative controls. Plates were incubated at 37 ^{o}C for 24 h.

The zones of inhibition of the discs were measured and corresponding antibacterial index for each inhibition was calculated using the following formula (Grove and Randall 1955):

$$\text{Antibacterial index} = \frac{\text{zone of inhibition} - \text{diameter of disc}}{\text{diameter of disc}}$$

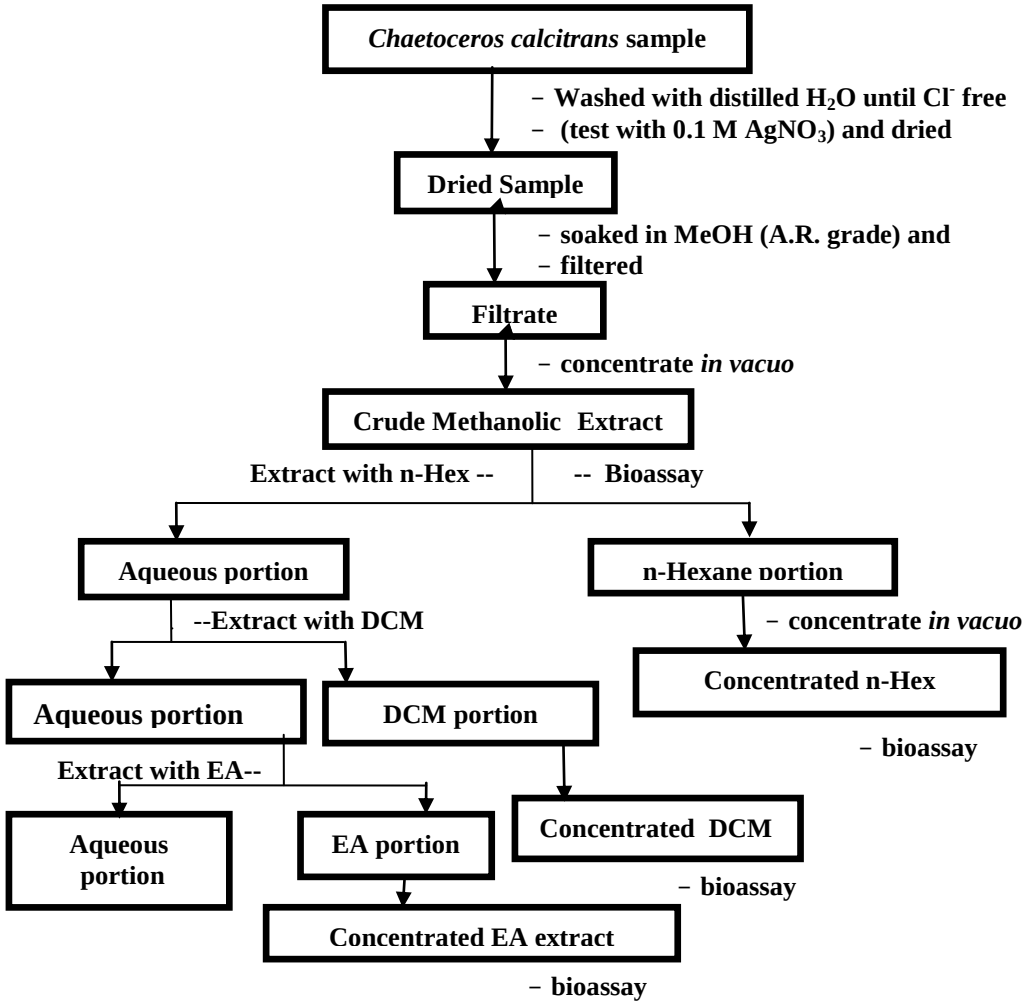

Fig. 1. Extraction and fractionation scheme of *C. calcitrans* samples.

Legend: n-Hex = n-hexane; DCM = dichloromethane ; EA = ethyl acetate; MeOH = methanol.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The tube dilution method was used to determine MIC and MBC. MIC and MBC assays were carried out in duplicates.

Weighed samples of *C. calcitrans* extracts were dissolved in their solvents and diluted to obtain working solutions of 4000 to 7.8 $\mu g \cdot mL^{-1}$. Decreasing amounts of the extract solution was added to a series of sterile 13 x 100 mm screw–capped tubes containing increasing amounts of the diluent, nutrient broth media. One millilitre of diluted bacterial culture standardised by matching to the 0.1 McFarland's barium sulfate standard solution containing approximately 300 million $cfu \cdot mL^{-1}$ was added to each of the tubes, except the last tube which served as the control. The tubes were

incubated for 18-24 h after which they were examined visually for turbidity, i.e., bacterial growth. The lowest concentration of the extract in the series that showed no growth was considered the MIC.

To determine the MBC, 0.1 mL from each tube that showed no turbidity were spread on NA plates in two replicates. Plates were incubated for 18-24 h after which they were examined for the presence of bacterial colonies. Colonies were enumerated by direct counting, and the lowest concentration of the *C. calcitrans* extract that resulted in no growth was considered as the MBC.

Partial characterisation of the bioactive compounds

The n-Hex extract which exhibited the highest antibacterial activity among the other solvent extracts of *C. calcitrans* was fractionated using vacuum liquid chromatography (VLC). The glass column with a diameter of 15 mm and a height of 22 cm was packed with 10 g silica gel (70-230 mesh). About 1.0 g sample was homogenised in 1.5 g of silica gel (Silica gel $60F_{254}$) and was added to the column containing 10 g of silica gel. Solvent systems (50 mL) of increasing polarity composed of different ratios of n-Hex, DCM and EA were added to the column. The percent composition by volume of the solvent systems used is shown in Table 1. Different fractions were eluted under partial vacuum and each of the fractions collected were concentrated *in vacuo* using a rotatory evaporator.

Table 1. Solvent systems used in vacuum liquid chromatography.

Solvent System	% Composition		
	n-Hex	DCM	EA
1	100	0	0
2	98	2	0
3	96	4	0
4	94	6	0
5	92	8	0
6	90	10	0
7	85	15	0
8	80	20	0
9	75	25	0
10	70	30	0
11	65	35	0
12	60	40	0
13	50	50	0
14	30	70	0
15	10	90	0
16	0	100	0
17	0	80	20
18	0	60	40
19	0	30	70
20	0	10	90
21	0	0	100

Legend: n-Hex = n-hexane; DCM = dichloromethane; EA = ethyl acetate

The fractions obtained from the VLC were subjected to thin-layer chromatography (TLC). Fractions with similar TLC profiles were pooled and subjected to antibacterial assay.

The TLC pure fraction that showed high antibacterial activity was characterised using Fourier-transform infrared spectroscopy (AVATAR 330 FT-IR Thermo Nicolet) to determine the functional groups present in the compound.

The sample was homogenised with potassium bromide (KBr) powder in the agate mortar and pestle. It was pelletised into a transparent film and placed into the attentuated total reflectance (ATR) plate. The ATR plate with the transparent film was loaded into the FT-IR machine. The first run done on the KBr film was the control in order to obtain the background spectra. The next run was for the sample homogenised into the KBr film. The spectra obtained were interpreted by determining the functional groups of the signals detected.

Analysis of data

The data were analysed by analysis of variance (ANOVA) followed with Duncan's Multiple Range Test (DMRT) using SPSS software version 16. Differences between treatment means were considered statistically significant at $p<0.05$. The data were transformed to arc sine for percentage values and log transformed for non-percentage values to normalise the data, and subjected to test for normality before ANOVA was done.

Results

Recovery of cultured *C. calcitrans*

The fresh *C. calcitrans* concentrate is deep brown in colour and slimy when in the culture medium. The dried sample has a very fine texture and a greenish brown colour. Pure culture with an initial density of 50,000 cells·mL^{-1} and scaled up from 1 L to 200 L yields approximately 363,000 cells·mL^{-1}. After drying, a dry weight of 101.3 g was obtained and by computation each cell weighs about 1.39 pg·cell^{-1}. Therefore, 1 g of dried *C. calcitrans* contains 1.39 x 10^9 cells. Moisture content is about 84.19%.

Antibacterial activities

The crude methanolic extract as well as the four fractions (n-Hex, DCM, EA and aqueous fractions) obtained from the sequential liquid-liquid extraction were bioassayed for antibacterial activities. All the solvent extracts of *C. calcitrans* except the aqueous fraction exhibited antibacterial activities towards the test organisms, *S. aureus, B. subtilis, E. coli and V. harveyi* (Table 2). The n-Hex fraction showed significantly the highest antibacterial activity compared to the other extracts. DCM and EA had almost the same level of activity. The crude methanolic extract had the lowest activity. DCM and n-Hex extracts were effective against Gram positive bacteria, *S. aureus* and *B.*

subtilis. Only the n-Hex extract was effective against *E. coli*. Of the four solvent extracts, the DCM extract had the lowest activity against *V. harveyi*. However, the activities of the solvent extracts of *C. calcitrans* were lower than that of the positive control, Amoxicillin. Only the n-Hex extract had an activity more or less comparable to Amoxicillin. In general, the antibacterial properties of *C. calcitrans* are high in non-polar solvent (n-Hex) and activity decreased as solvent becomes polar like methanol. Water extract of *C. calcitrans* did not show any antibacterial activity.

Table 2. Average antibacterial indices of the different solvent extracts of *C. calcitrans* bioassayed on the four bacterial species. Values expressed in mean±SEM and those with the same superscript are not statistically significant.

Solvent Extract/ Control	Test Organisms				Mean Total
	S. aureus	*B. subtilis*	*E. coli*	*V. harveyi*	
Methanolic	0.39 ± 0.03	0.25 ± 0.05	0.20 ± 0.03	0.53 ± 0.03	0.34 ± 0.04[c]
n-Hex	0.67 ± 0.00	0.70 ± 0.03	0.70 ± 0.03	0.55 ± 0.03	0.65 ± 0.02[a]
DCM	0.70 ± 0.03	0.70 ± 0.03	0.22 ± 0.03	0.22 ± 0.03	0.46 ± 0.07[b]
EA	0.42 ± 0.05	0.28 ± 0.03	0.30 ± 0.03	0.70 ± 0.03	0.42 ± 0.05[b]
Aqueous fraction	0	0	0	0	0.00
(+) Control Amoxicillin	0.84 ± 0.13	1.02 ± 0.08	0.82 ± 0.13	0.93 ± 0.16	0.90 ± 0.05[d]
Solvent (- Control)	0	0	0	0	0.00

Legend: n-Hex = n-hexane extract ; DCM = dichloromethane extract; EA = ethyl acetate extract

The MIC of the *C. calcitrans* solvent extracts was about 250 $\mu g \cdot mL^{-1}$, except in DCM extract against *S. aureus* (500 $\mu g \cdot mL^{-1}$) and n-Hex extract against Gram negative bacteria, *E. coli* and *V. harveyi* (125 $\mu g \cdot mL^{-1}$). The methanolic, n-Hex, and DCM extracts had an MBC of 500 $\mu g \cdot mL^{-1}$ while EA was about 250 $\mu g \cdot mL^{-1}$ for Gram positive bacteria, *S. aureus* and *B. subtilis*. The growth of *E. coli* was inhibited at an MBC of 250 $\mu g \cdot mL^{-1}$ for all the solvent extracts. The n-Hex extract inhibited the growth of *V. harveyi* with the lowest MBC of 125 $\mu g \cdot mL^{-1}$. However, the growth of *V.*

harveyi was inhibited at an MBC of 250 µg·mL^{-1} of methanolic and EA extracts, and 500 µg·mL^{-1} of DCM extract. It is only the EA extract that was consistent in its MIC and MBC of 250 µg·mL^{-1} for all the test organisms (Table 3).

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the different solvent extracts of *C. calcitrans* against four species of bacteria. Amoxicillin served as the positive control.

	Concentration (ug·mL^{-1})									
Test	**Methanol**		**n-Hex**		**DCM**		**EA**		**Amoxicillin**	
Organism	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
S. aureus	250	500	250	500	500	500	250	250	100	100
B. subtilis	250	500	250	500	250	500	250	250	100	100
E. coli	250	250	125	250	250	250	250	250	100	100
V. harveyi	250	250	125	125	250	500	250	250	100	100

Partial purification of the bioactive compounds

The antibacterial properties of *C. calcitrans* was used to guide the isolation and partial purification of the bioactive compounds. There were 21 VLC fractions produced and the TLC profile of these fractions is shown in Table 4. Fractions 1 to 6 had the highest Rf values of 0.94 and had brown coloration that goes with the blue violet colour after spraying with vanillin-sulfate. All these fractions were pooled together and labeled as C1.

Fractions 7 to 10 with violet and bluish violet colours had the same Rf values of 0.84. These were all combined and labelled as C2. For fractions 11 to 13, the spots were also violet but had Rf values equal to 0.74. Thus, these fractions were separated and pooled together as C3. Two coloured spots, green and pink violet were observed for each fractions from 14 to 16. The green spots had an Rf value of 0.96. The Rf value of pink violet spot was 0.81. Fractions 14 to 16 were pooled together and labelled as C4. The rest of the VLC fractions from 17 to 21 were not pooled together. Fraction 17 showed three different Rf values. The remaining fractions did not have any spot.

Table 4. TLC profile of the VLC fractions of n-Hex extract using n-Hex:DCM:EA (1:1.25:1.25) as solvent system and vanillin-sulfuric acid as the visualising agent.

VLC Fraction	n-hex:DCM:EA	Rf values	Color	Pooled Fraction
1	100:0:0	0.94	blue violet with a little tinge of brown	
2	98:2:0	0.94	blue violet with a little tinge of brown	
3	96:4:0	0.94	blue violet with a little tinge of brown	C1
4	94:6:0	0.94	blue violet with a little tinge of brown	
5	92:8:0	0.94	blue violet with a little tinge of brown	
6	90:10:0	0.94	blue violet with a little tinge of brown	
7	85:15:0	0.84	blue violet	
8	80:20:0	0.84	blue violet	C2
9	75:25:0	0.84	violet	
10	70:30:0	0.84	violet	
11	65:35:0	0.74	violet	
12	60:40:0	0.74	violet	C3
13	50:50:0	0.74	violet	
		0.96	green	
14	30:70:0			
		0.81	pink violet	
		0.96	green	
15	10:90:0			C4
		0.81	pink violet	
		0.98	green	
16	0:100:0			
		0.81	pink violet	
		0.9		
17	0: 80:20	0.85		
		0.7		
18	0:60:40			
19	0:30:70			
20	0:10:90			
21	0:0:100			

Legend: TLC = Thin Liquid Chromatography; VLC = vacuum Liquid Chromatography; n-Hex = n-hexane ; DCM = dichloromethane; EA = ethyl acetate

The four pooled fractions (C1, C2, C3, and C4) were tested for their antibacterial activities (Table 5). The pooled C2 fraction was relatively the most active fraction. It showed significant antibacterial activities especially against *S. aureus* and *V. harveyi*.

Table 5. Average antibacterial indices of the pooled vacuum liquid chromatography fractions (C1, C2, C3 and C4) bioassayed on the four species of bacteria. Values expressed as mean ± SEM and those with the same superscript are not statistically significant.

Pooled Extract/ Control	Test Organisms				Mean Total
	S. aureus	*B. subtilis*	*E. coli*	*V. harveyi*	
C1	0.47 ± 0.03	0.20 ± 0.03	0.36 ± 0.03	0.53 ± 0.03	0.39 ± 0.07[b]
C2	1.53 ± 0.03	0.28 ± 0.03	0.47 ± 0.03	0.50 ± 0.08	0.70 ± 0.28[a]
C3	0.17 ± 0.00	0.17 ± 0.00	0.36 ± 0.03	0.53 ± 0.0	0.31 ± 0.09[b]
C4	0.17 ± 0.00	0.11 ± 0.03	0.14 ± 0.03	0.14 ± 0.03	0.14 ± 0.12[c]
(+)Control Amoxicillin	1.08 ± 0.30	1.42 ± 0.30	0.68 ± 0.04	0.66 ± 0.13	0.96 ± 0.18[a]
(-)Control Solvent	0	0	0	0	0

Fig. 2 shows the FT-IR spectra of the C2 fraction. The spectra showed an absorption band at 3424.94·cm^{-1} for the presence of a hydroxyl group (O-H). A C-H stretching at 2860.84·cm^{-1} and banding vibrations at 1462.61·cm^{-1} and 1360.75·cm^{-1} conveyed an impression that the compound is aliphatic in nature. Supporting the unsaturated character of the substance was a signal at 2928.16·cm^{-1} and 2955.18·cm^{-1}. The probable presence of a carbonyl group (C=O) can be inferred from the signal at 1728.56·cm^{-1}. Vinylidene group may be present as detected by signals at 1280.84·cm^{-1} and 1122.58·cm^{-1}. With all these observations, it can be deduced that one of the active components may be terpenes. It can also be ascertained that the bioactive compounds indeed have a non-polar characteristic.

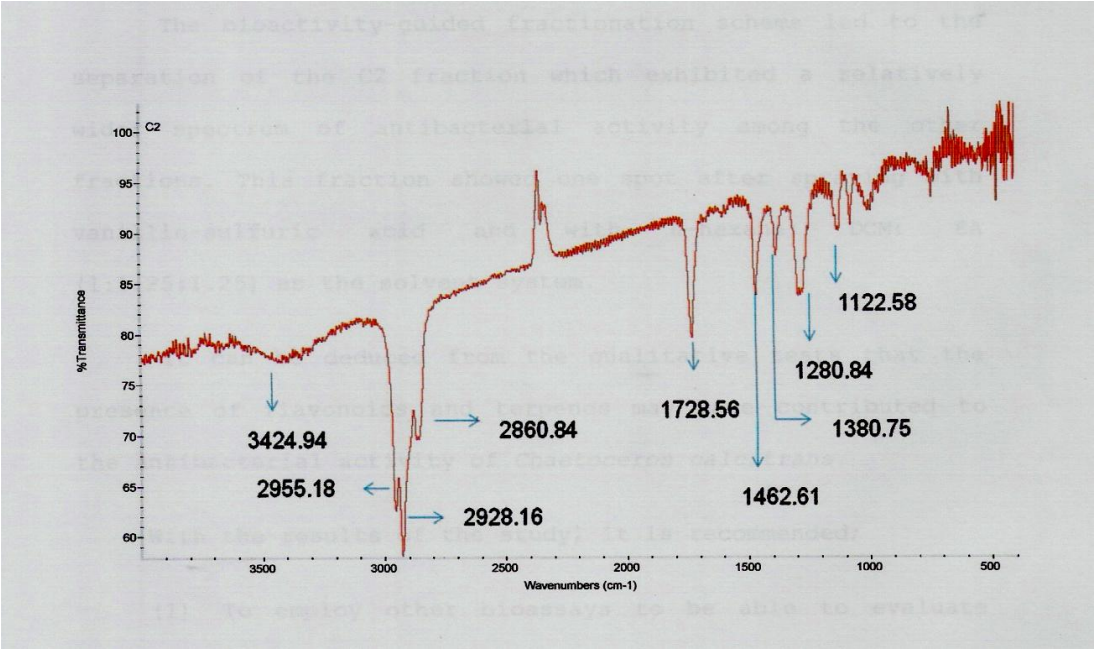

Fig. 2. FT – IR spectra of the C2 fraction of the n-hexane extract of *C. calcitrans* after thin layer chromatography (TLC). With all these absorption bands shown, it can be deduced that one of the active components may be a terpene.

Discussion

Chaetoceros calcitrans is a unicellular floating marine diatom used as larval food in aquaculture. Hatcheries, for over 20 years now, still use this microalga as live feeds for their production (Brown 2002). The metabolites percentage dry weight of *C. calcitrans* cell is composed of 35% protein, 6.6% carbohydrates, and 6.9% fats (Parsons et al. 1961). This proximate analysis in dry matter confirmed the potential of *C. calcitrans* as larval food.

Chaetoceros calcitrans is indeed a potential species for aquaculture use because of its antibacterial properties. Extracts of *C. calcitrans* in different solvents except the aqueous fraction (Table 2) inhibited the growth of *V. harveyi, E. coli, S. aureus and B. subtilis*. Previous studies of Lio-Po et al. (2005) and Seraspe et al. (2007) similarly showed the antibacterial action of *C. calcitrans* against *Vibrio* species and other bacterial pathogens. Results of the present study also showed that the extracts have generally a broad spectrum activity. It inhibited the growth of both the Gram positive (*S. aureus* and *B. subtilis)* and Gram negative *(V. harveyi* and *E. coli)* bacteria. The MIC and MBC of all solvent extracts on the microorganisms tested are in the range of 125 - 500 $\mu g \cdot mL^{-1}$ (Table 3).

The n-hexane extract of *C. calcitrans* had the highest antibacterial activity as compared to the other solvent extracts. This confirms the observation that organic solvents provide a higher efficiency in extracting compounds for antimicrobial activities compared to water-based methods (Lima-Filho et al. 2002). Similarly, Lima-Filho et al. (2002) also reported that the highest activity

against *B. subtilis* of the macroalga, *Gracilaria* sp. is in the n-Hex extract. In addition, the antibacterial activity exhibited by the n-Hex extract against the four test microorganisms indicates that the bioactive components could be non-polar since as the solvent becomes more polar, as in the aqueous extract, the activity was lost. The result of this study is consistent with the study done previously on two species of *Vibrio* and *B. subtilis* (Seraspe et al. 2007).

The partially purified TLC fractions which were from the n-Hex extract further showed that all pooled fractions, C1, C2, C3 and C4 exhibited notable antibacterial activities (Table 5). The C2 pooled fraction which is non-polar had the highest antibacterial activity and was significantly as effective as the positive control, Amoxicillin. This result ascertains the non-polar characteristics of the bioactive compounds.

As to the class of bioactive compounds responsible for the antibacterial properties of the species, the prevailing colours of the spots produced during TLC in all the pooled fractions was blue violet. These colours can be indicative of the presence of terpenes which are typical secondary metabolites of algae (Garson 2001). They prevent bacterial growth by inhibiting biosynthesis of folate (Bugni et al. 2004) thereby resulting in the disruption of a wide range of bacterial primary metabolic processes (Agger et al. 2008). The compounds to which terpenes belong (the terpenoids) have a wide range of biological functions as anti-feedants, pollinator attractants and antimicrobial, antifungal, molluscicidal activities (Paul 2007; Popova et al. 2009). Analysis of the FT-IR spectra of the C2 pooled fraction corroborated the TLC profile that the component of the antibacterial bioactive compounds could be terpenes.

Conclusion

This study confirmed the presence of active antibacterial compounds in extracts of the microalgae, *C. calcitrans* which could be useful in the treatment of bacterial diseases of shrimp/fishes. Partial purification and characterisation of the extracts showed that its antibacterial activity was in the non-polar fraction and the bioactive compounds could be terpenes.

It is recommended that more sophisticated instrumentation techniques be used to elucidate the structure of the compounds present in the different isolated fractions responsible for the antibacterial properties of the sample. Moreover, other bioassays should be employed to evaluate other bioactivities of the microalgae sample, e.g., anti-inflammatory, analgesic, anti-tumor and anti-viral tests. The results will have significant industrial applications in aquaculture and pharmaceuticals.

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