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Isolation and Identification of Marine Bacteria Producing Alkaloids

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Abstract
Marine bacteria were isolated from various sea samples and then they were screened for the production of alkaloids. The m that showed positive result for the production of alkaloids were then subjected to study under varying physical and chemical conditions namely: effect of shaking and non-shaking, effect of temperature, effect of pH, effect of concentration of sodium chloride and protein substrate. Following this the bacteria were subjected to antimicrobial and cytotoxicity testing and then subjected to characterization by GC- MS.

Introduction

Marine bacteria have in recent times increased the scope of study in the field of therapeutic due to various secondary metabolites that are produced by them. These secondary metabolites include wide range of compounds namely- phenols, steroids, alkaloids etc... Alkaloids in general are found to be potent anti-cancer agent and mostly are effective against various other bacterial and fungal infections. In present study, the alkaloids from these marine bacteria are isolated, characterized and tested for their antimicrobial activity and cytotoxicity against Vero cell lines are demonstrated here[1].

Isolation and characterization of bacteria

The marine water sample was collected from Chennai, Hyderabad, Pondicherry and Cuddalore. The samples were used for isolation of bacteria by spread plate technique on Nutrient agar plates and incubated in incubator for 24 hours and then were observed for colonies and the morphology of bacteria was studied by gram staining (V.A. Kinsalin, D. Prabhadevi, 2014)[2]. The biochemical tests were performed for the bacteria and thus the bacteria were predicted following these observations.

Screening of bacteria for alkaloid production

The culture medium for the bacteria was prepared and autoclaved and inoculated with the bacteria in respective culture flasks. The inoculated culture flask was then incubated for a period 4 days for a good growth. Then the Culture media was subjected to centrifugation at 10000 rpm for 10 mins and the supernatant was collected separately and measured. To the measured amount of supernatant equal volume of ethyl acetate was added mixed well and left for 24- 48 hours. 2ml of the supernatant was taken in separate test tubes and added with 0.5 ml of Dragendorff's reagent and observed for the ring formation which indicated positive result. Thus the bacteria that showed positive were used for further studies. And of the 10 well growing strains 8 strains of the bacteria showed the positive result.

Study of effect of physical and chemical condition in alkaloid production

The culture medium for the bacteria was prepared and autoclaved and then inoculated with the strains in the respective culture flasks and the alkaloid production was monitored of a period of 10 days[3].

(1) Effect of shaking and non- shaking-

The culture was prepared in 2 batches - one batch of the inoculated culture was subjected to shaking while the batch of the culture was kept stationary.

(2) Effect of pH-

The culture was prepared in 4 batches each batch was prepared with a different pH namely- 2.0, 4.0, 6.0 and 8.0

(3) Effect of temperature-

The culture was prepared in 3 batches and then the inoculated culture were kept at different temperature condition namely- 15 C, 37 °C and 45 °C

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(4) Effect of sodium chloride concentration variation:
The culture was prepared with double the concentration of sodium chloride in the culture medium.
(5) Effect of protein source variation:
The culture was prepared with double the concentration of protein source (peptone in nutrient broth).

Estimation of the alkaloid production using spectrophotometer:
1 ml of the culture was taken added with 2 ml of dragendorff's reagent and centrifuged at 10000 rpm for 10 minutes. 1 ml of this supernatant was taken and added with 3ml of 3% thiourea solution and the O.D value was taken(adapted and modified from Sreevidya & Mehrotra, 2003)[4]. If the O.D. was above 1 it was diluted and accordingly while estimation it was multiplied with the dilution factor. The concentration was calculated using a standard curve obtained by this method[5].

Bulk culturing of the bacteria
the bacteria that showed higher alkaloid production in the study was cultured in bulk where the bacteria was inoculated in 200 ml of culture medium and then incubated in a shaker and left undisturbed for 6-7 days. Also they were regularly checked for the contamination.

Downstream processing of the alkaloids: The bulk cultured bacteria was the subjected to downstream processing where the culture was subjected to centrifugation at 10000 rpm for 20 mins and the clear supernatant was collected and measured to which equal amount of ethyl acetate was added and subjected to mild shaking for about 10 minutes and transferred to a separating funnel and kept overnight on the stand to allow separation. This process was repeated for about 2-3 times for each of the sample in order to collect adequate extract for analysis. The following day the ethyl acetate layer was collected separately and poured onto sterile petri plates and allowed for evaporation. The remains on the plates the next day was scrapped off and collected in eppendorf tubes and used for analysis.

Testing for the antimicrobial activity: The antibacterial activity was determined by well diffusion methods (Holder and Boyce 1994). About 25 mL of molten Mueller Hinton agar was poured into a sterile Petri plate (Himedia, Mumbai, India). The plates were allowed to solidify, after which 18 h grown (OD adjusted to 0.6) 100 µl of above said pathogenic bacteria were transferred onto plate and made culture lawn by using sterile L-rod spreader. After five min setting of the pathogenic microbes, a sterile cork borer was used to make 5 mm well on the agar. The test samples were dissolved in sterile saline and loaded in to wells with 100 µg/well. The solvent saline loaded well served as negative control and Streptomycin (30 µg/ml) well served as positive control. The plates were incubated at 37°C in a 40 W florescent light source (~ 400 nm) for 24 h. The antibacterial activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale (Himedia, Mumbai, India)[3].

Cyto toxicity testing
Cells (1 × 105/well) were plated in 24-well plates and incubated in 37°C with 5% CO2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells .The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The % cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells / A570 of control cells × 100

Characterization of alkaloid by GC-MS:
● GC-MS information
Make : Perkin Elmer
GC model : clarus 680
Mass Spectrometer: clarus 600 (EI)
Software : Turbo Mass ver 5.4.2
Library ver : NIST-2008
● Inst ACQUISITION PARAMETERS
Oven : Initial temp 60°C for 2 min, ramp 10°C/min to 300°C, hold 6 min,
Total Run Time : 32.00 mint
InjAauto = 260°C,
Volume = 1 µL,
Split = 10:1,
Flow Rate : 1 mL/mint
Carrier Gas = He,
Column = Elite-5MS (30.0m, 0.25mmID, 250µm df)
● MASS CONDITION (EI)
Solvent Delay = 2.00 min,
Transfer Temp = 240°C,
Source Temp = 240°C,
Scan : 50 to 600Da,

The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min−1; and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

Results and discussion

Isolation and characterization of bacteria:

NA-1	Gram positive cocci
NA-2	Gram negative rod
NA-3	Gram positive cocci
NA-4	Gram negative rod
NA-5	Gram positive cocci
NA-6	Gram positive cocci
NA-7	Gram positive cocci
NA-10	Gram positive cocci

Culture	Indole test	Methyl red test	Voges-Proskauer test	Citrate utilization test	Urease test	Triple sugar iron test	Catalase test	Oxidase test
NA 1	-	+	+	+	+	-	+	+
NA 2	-	-	+	-	-	-	+	+
NA 3	+	+	-	+	-	-	-	+
NA 4	+	+	+	+	-	-	-	+
NA 5	+	+	+	+	-	-	+	+
NA 6	+	+	+	+	-	-	+	-
NA 7	+	-	+	-	+	-	-	+
NA 10	+	-	+	-	-	-	-	+

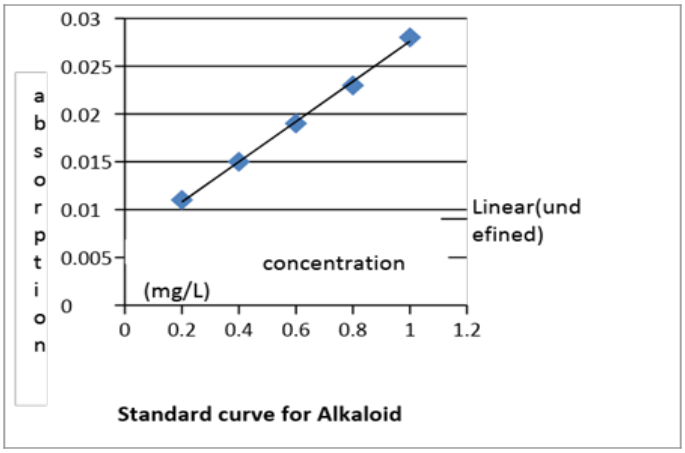
Screening of the bacteria for alkaloid production

The bacterial strains that produced a band on addition of Dragendorff's reagent were considered positive for alkaloid production and the positive strains were used for further studies.

Study of effect of physical and chemical condition in alkaloid production:

The effect of shaking, pH, temperature, Sodium chloride and nutrient concentration variation was studied using the standard graph plotted by the method of estimation of the alkaloids using Dragendorff's reagent[5].

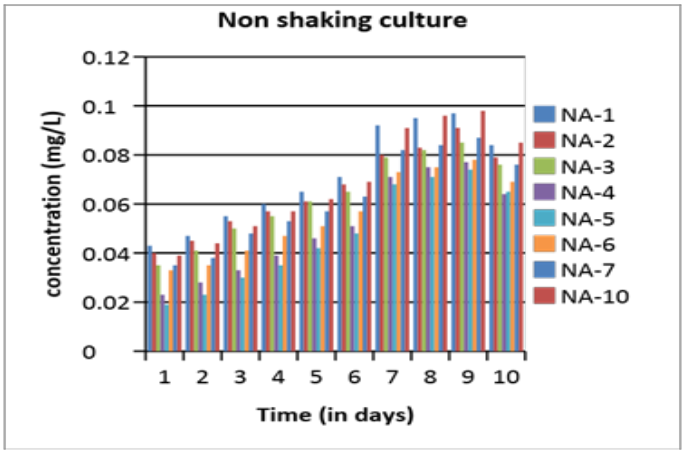
In the present study, the effect of shaking, pH, temperature, NaCl, and the nutrient concentration variation was studied and the optimal pH was found to be 6.0 for bacteria, the optimal temperature was found to be 37°C for bacteria, the optimal NaCl concentration was 0.6% for bacteria and the concentration variation did not show significant effect on metabolite production. The effect of various parameters like incubation time, temperature, pH, carbon and nitrogen sources and sodium chloride concentration on metabolite production were studied by varying single parameter at a time (Das et al., 2016)[3]. It was found that the metabolite production by the isolate was greatly influenced by various culture conditions.

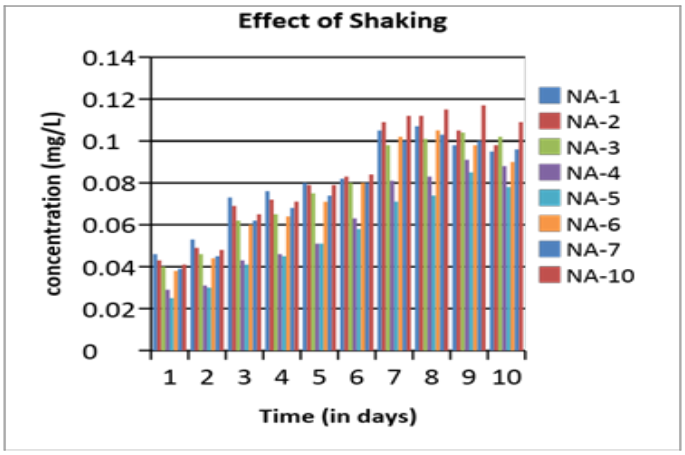


Standard curve for Alkaloid

Effect of shaking and non- shaking condition

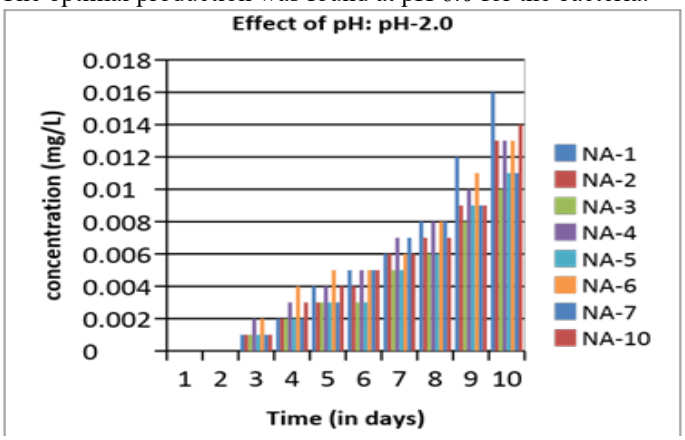
The effect of shaking was compared with simultaneous study of non-shaking condition for the culture.

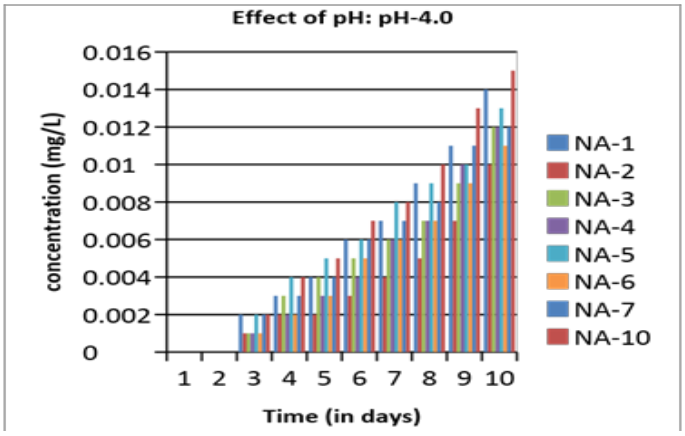


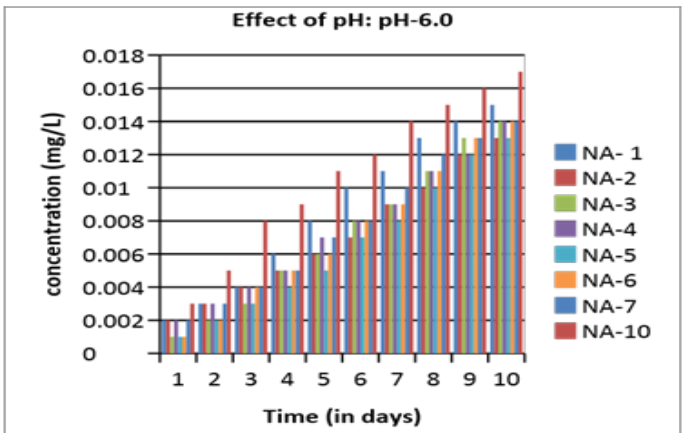


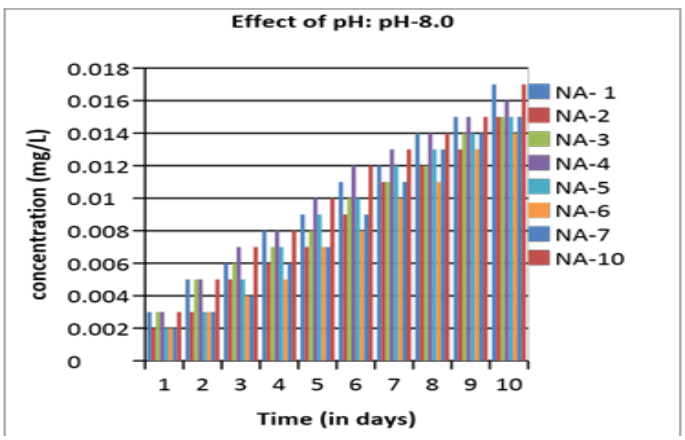
Effect of pH

The cultures were cultured under varying pH condition such as at pH 2.0, 4.0, 6.0 and 8.0. The concentration of alkaloids produced by the cultures at varying pH conditions was compared. The optimal production was found at pH 6.0 for the bacteria.



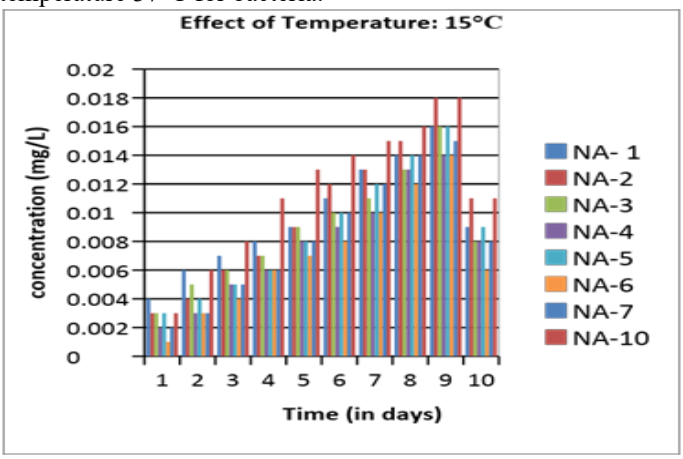


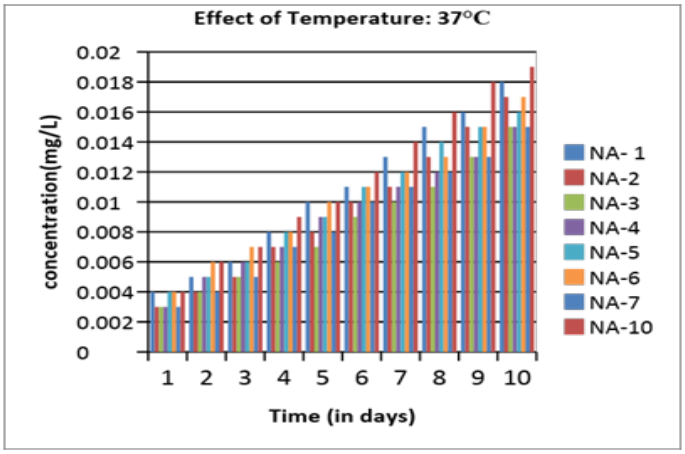


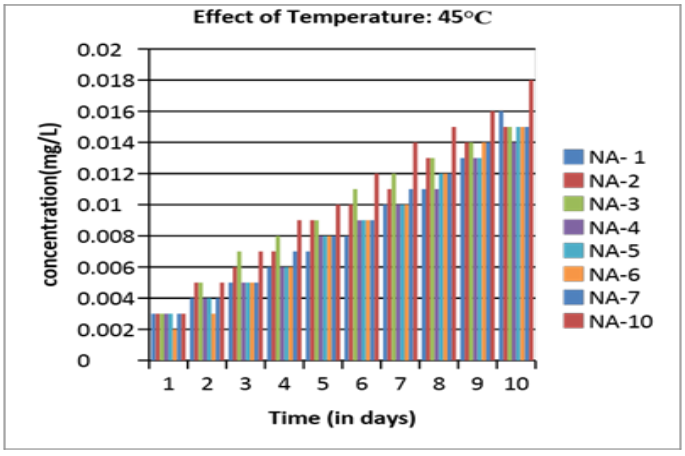


Effect of temperature

The cultures showed varying production of alkaloids with varying temperature conditions. They showed optimal production at temperature 37°C for bacteria.

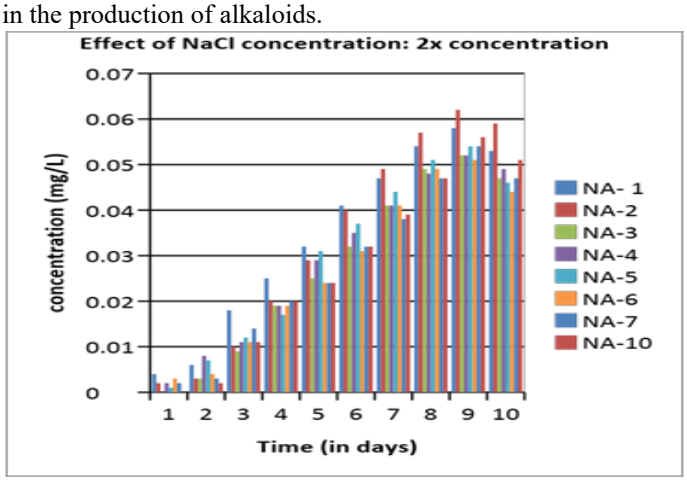






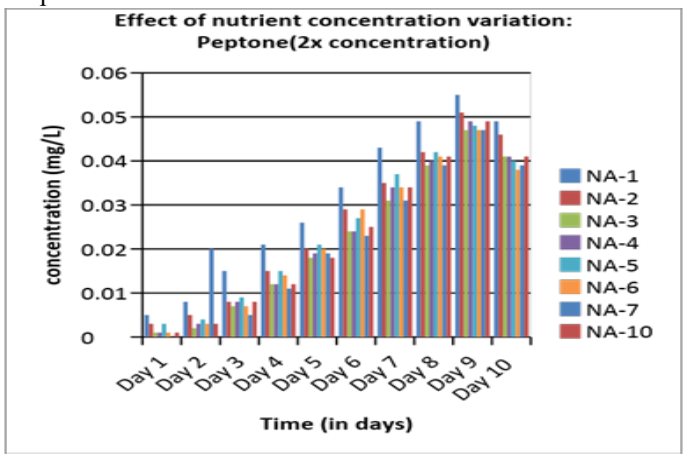
Effect of NaCl

The culture was grown in medium containing twice the concentration of sodium chloride. Significant difference was observed in the production of alkaloids.



Effect of nutrient concentration variation

The concentration of the peptone was added twice the concentration in nutrient broth. Significant difference was observed in the production of alkaloids.



Bulk culturing of the microorganisms

Of the 13 strains the strains that showed relatively higher production of alkaloids were cultured in bulk for extraction of the alkaloids. The strains are NA 1 and NA 10. The bulk culture was done and the supernatant was filtered out separately and subjected to extraction. In the present study, the fermentation broth 170 mL of the nutrient broth was prepared and inoculated, and incubated at 30°C at 180 rpm for 7 days.

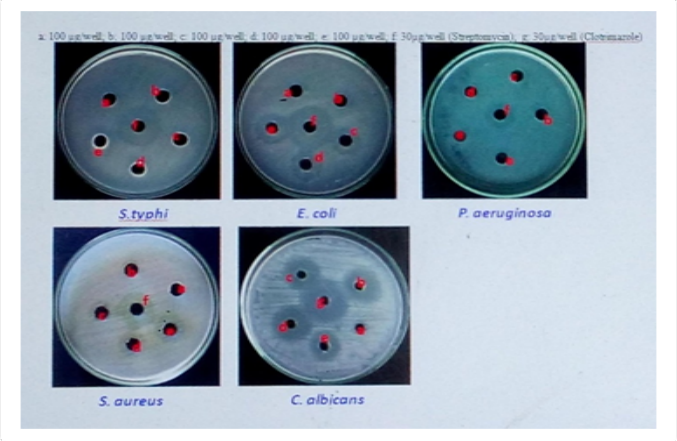
Downstream processing of the alkaloids

The filtered supernatant was measured and added with equal volume of ethyl acetate and kept for 24hrs and the ethyl acetate layer was collected separately. The ethyl acetate was poured onto petri plate for evaporation and then the remains on the plate was scrapped off using blade and transferred into an eppendorf for further analysis.

Testing for anti-microbial activity:

The extract was tested for its antimicrobial activity against test pathogens. They were found to be reactive against the pathogens namely: S.typhi, E.coli, S.aureus,P.aerogenosa, and C.albicans [6].

Name of the organisms	ZOI(mm) Test Extract					ZOI (mm) Standard
Concentration	100 µg/Well Compound a	100µg/Well Compound b	100µg/Well Compound c	100µg/Well Compound d	100µg/Well Compound e	30µg/well
Salmonella typhi#	-	9	11	14	10	25
Escherichia coli#	-	8	10	13	14	24
Pseudomonas aeruginosa#	-	8	10	12	9	16
Staphylococcus aureus#	6	7	9	10	11	19
Candida albicans*	13	15	18	19	16	24



Cyto toxicity testing

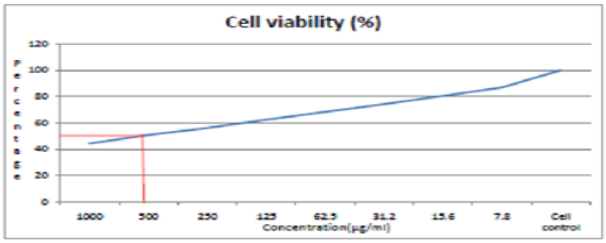
The extract collected was subjected to cyto toxixcity testing on vero cell lines successfully and was shown to be favourable at IC 50 concentration and thus is safe to be used in edible form. In the present study the cytotoxic activity and IC 50 value was found at 500µg/ml and 1000µg/ml on the vero cell lines(Jiao, Zhang, Zhao, Hu, & Suh, 2013).

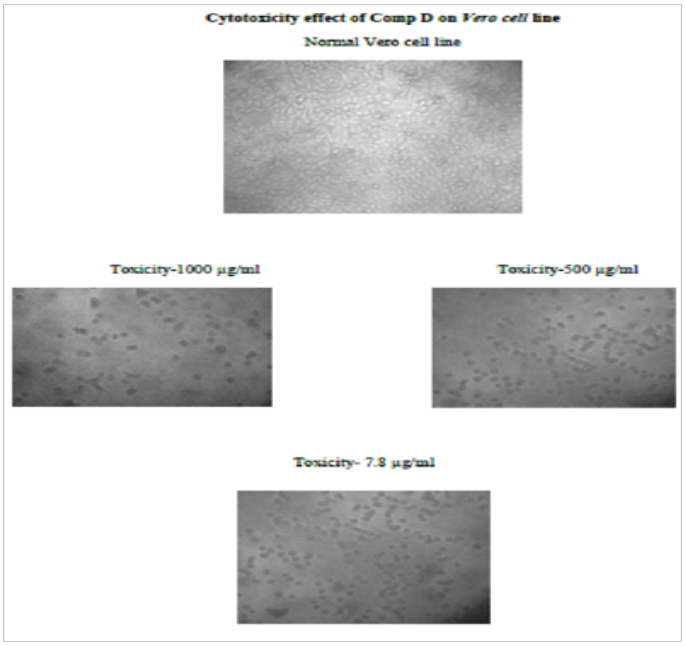
Characterization of alkaloid by GC- MS:

The extract crude mixture was subjected to gas column mass spectrometry in order find the compound present in it. The mixture was found to possess a nitrogen containing compound and it is assumed to be an alkaloid. The present study is done by GC-MS and many works have been reported using LC-MS[7], FT-IR for structure elucidation[8,9], HRMS[2]. No reports have been found to best of knowledge for characterization using GC-MS.

Cytotoxicity effect of Comp E *on Vero* cell line

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.456	44.35
2	500	1:1	0.524	50.97
3	250	1:2	0.578	56.22
4	125	1:4	0.642	62.45
5	62.5	1:8	0.704	68.48
6	31.2	1:16	0.764	74.31
7	15.6	1:32	0.828	80.54
8	7.8	1:64	0.894	86.96
9	Cell control	-	1.028	100





*Comp D ref to NA 1

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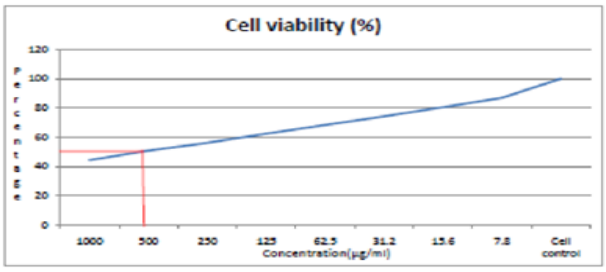

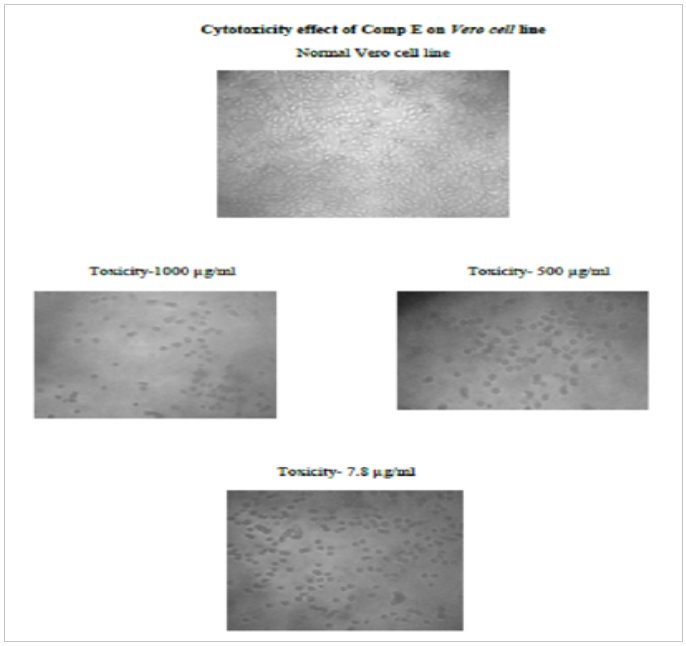

*Comp E ref to NA 10

Conclusion

Thus the extracted alkaloid from the marine microorganisms i.e., marine bacteria showed varying production of alkaloids with varying physical and chemical conditions. These showed variation in their growth and also in the production of alkaloids. They were screened for their production using Dragendorff's reagent at every stage of the work. From the performed experiments it is concluded that the isolated and screened marine bacteria possesses alkaloids that showed anti-bacterial and anti-fungal activity. Also since they were tested for the cytotoxicity at IC 50 concentration as a result they can be used in the edible forms.

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