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Statistical optimization and valuable applications of bioactive compounds produced by marine *Pseudoalteromonas piscicida*

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ABSTRACT

Pseudoalteromonas piscicida was isolated from sea water in Suez Bay and identified using 16S rRNA sequence analysis. The bacterium produced compounds active against different pathogenic microbes including *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Vibrio anguillarum, Aeromonas hydrophila* and *Candida albicans*. Plackett Burman experimental design was applied to achieve maximum production of the bioactive compounds. It achieved 1.2 fold increase when grown in medium composed of g/l: peptone, 3; beef extract, 1; inoculum size (ml), 0.5; culture volume (ml), 25 ml; sea water concentration >100 % adjusted to pH 9 and incubation period 18h at 32^{o}C. Immobilization using adsorption technique on different support materials was applied to improve the productivity. Cells adsorbed on medical pumice realized 1.4 fold increase than the free cells. Recycling of the immobilized cells caused an increase by 1.5 fold than the free cells. Moreover, the anticancer activity of the produced compounds were tested against 4 cell lines (EI-4, Caco-2, HepG2, MCF-7). The highest activity (63.8%) was detected against Caco-2 cell line while very low activity was detected towards HepG2 and EI-4 cell lines. Antiviral activity was also studied. It succeeded to inhibit HCV replication at100 µg/ml. Another aim was to test the antioxidant activity. There was a significant antioxidant activity compared with ascorbic acid as control. Gas liquid chromatography mass spectroscopy was used to determine the major constituents in the benzene extract. It revealed the presence of 3 major compounds which are benzene,1-pentyloctyl, Benzene, 1-butylheptyl and di-n-octyl phthalate.

INTRODUCTION

Bacterial overcoming of the tools with which humans have to fight them is continuous. Resistance develops as a result of spontaneous genetic mutations and has been attributed to many factors such as widespread use of antibiotics, lack of knowledge about proper use of antibiotics, production of large quantities of antibiotic waste from livestock rearing and an over confidence in human control over infectious diseases (Leekha *et al*., 2011). The emergence of resistance to antibiotics in microbial pathogens, such as Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), have caused a resurgence of interest in the discovery of natural products with unique scaffolds to combat new diseases and drug-resistant pathogens (Xiong *et al*., 2012). Natural bioactive compounds have played a significant role for discovery of new drugs over the last few decades. Some of the new chemical entities and the anticancer drugs introduced into the market can be traced back to natural products (NPs') (Newman and Cragg, 2007). The majority of microbial natural products isolated from terrestrial-borne microbes have an important role in drug discovery (Bérdy, 2005). However, the discovery and development of unique NPs' has dramatically declined over the last two decades. Recent trends in drug discovery are interested in marine microorganisms as potentially productive source of novel drugs

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which cause an increase in the number of marine NPs in clinical trials (Mikhailov *et al*., 2002; Waters *et al*., 2010; Xiong *et al*., 2012). Marine microbes have amazing metabolic and physiological features and are an important source of new biomolecules. Fungi, bacteria and cyanobacteria obtained from different marine sources secret several bioactive compounds with antimicrobial activity against a broad spectrum of pathogens and could be used to treat infections instead of the traditional drugs (Eduardo *et al*., 2015). Moreover, biologically active compounds with varying degrees of action, such as anti-bacterial, anti-cancer, anti-microtubule, as well as antifouling properties, have been isolated to date from marine sources (Bhatnagar and Kim, 2010).

Production of natural products by the marine genus *Pseudoalteromonas* has come to attention in the natural product in the last decade. Production of an array of compounds such as pyrolle-containing alkaloids, substituted phenolic cyclic peptides and a range of bromine substituted compounds with antimicrobial, anti-fouling, algicidal and various pharmaceutically-relevant activities have been reported ((Yu *et al*., 1997; Vimala, 2016). Studies have been so far limited to a relatively small subset of strains compared to the known diversity of the genus so more discoveries of novel marine natural products may remain to be made (Bowman, 2007). Production of antimicrobial agents is affected by the nutritional and environmental conditions. Statistical experimental design, such as Plackett-Burman methodology is used to eliminate the limitations of single-factor optimization process and optimizing all the affecting parameters collectively (Urvish and Gupte, 2010).

The present study was aimed to design an optimal medium for antimicrobial agent (s) production by *Pseudoalteromonas piscicida.* Moreover, improvement of the production was also carried out using immobilization technique. Other applications such as anticancer, antiviral and antioxidant will be tracked using different techniques.

MATERIALS AND METHODS

Bacterial isolation and growth conditions

Bacterial isolates were isolated from sea water samples along Suez Bay, Egypt. The bacterial isolates were cultivated and purified on nutrient agar medium which contains (g/l): peptone, 5; yeast extract, 3; agar, 20.

Pathogenic indicators

Test bacterial pathogens used in the present study included *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 6739, *Vibrio anguillarum, Aeromonas hydrophila* and *Candida albicans*. Strains were kindly provided by National Institute of Oceanography and Fisheries, Egypt.

Antimicrobial activity

The bacterial isolates were inoculated in 250 ml flasks containing 50 ml of nutrient broth medium. The inoculated flasks were incubated on a rotary shaker for 1 d. the cultures were harvested at the end of the incubation period, filtered using 0.22 µm sterilized filters. The antimicrobial effect of the bacterial supernatants was investigated using the agar diffusion method (El-Masry *et al*., 2002). Nutrient agar plates were seeded with the target pathogenic bacteria and wells of 10 mm diameter were made in the plates using a sterile cork borer. 50 µl of each supernatant was transferred into each well under aseptic conditions. Plates were then incubated for 1 d at 30°C. Clear inhibition zone around each well was detected as the antimicrobial activity and was linearly measured in mm.

Effect of growth medium on production of bioactive compounds by *P. piscicida*

Cultures of the selected bacterial strain were grown using nutrient broth (g/l): peptone 5, yeast extract 3 and Luria Bertani (g/l) peptone 10, yeast extract 5 and minimal medium (g/l): glucose, 3; $(NH_4)_2SO_4$, 1; K_2HPO_4; 1; Mg $SO_4.7H_2O$, 0.1; NaCl, 5 at 30°C for 24 hrs. Cell free supernatant of each culture was tested against the previously chosen target pathogens using well cut diffusion technique aiming at obtaining the highest antimicrobial activity (Aassila *et al*., 2003).

Optimization of nutritional factors

Optimization of the culturing conditions was investigated in order to reach to a maximum production of the antimicrobial agents. Plackett-Burman statistical design (Plackett and Burman, 1946; Yu *et al*., 1997) was applied to reflect the relative importance of various factors involved in the production of these agents by *P. piscicida*.

For each variable a high (+) and low (-) levels were tested. Eight different trials were performed in duplicates. Table 2 represents the high and low concentration of the different Independent variable (factors) affecting the production of antimicrobial agents. The rows in Table 3 represent the different trials (row no. 9 represents the basal control). The main effect of each variable was determined with the following equation:

$$\mathbf{Exi = (Mi+ – Mi-) / N}$$

where Exi is the variable main effect, and Mi+, Mi- are the radius of the clear zone around each well in the trials, where the independent variable was present in high and low concentrations, respectively, and N is the number of trials divided by 2. Statistical t-values for two samples assuming unequal variances were calculated using Microsoft Excel to determine the variable significance.

Verification experiment

To verify the results obtained from the statistical analysis of Plackett-Burman design, a verification tests were performed in duplicates using the predicted optimized media against the basal condition media. The production of the

antimicrobial agents was determined by measuring the average of inhibition zones diameter (mm).

Immobilization technique

Immobilization was performed by adsorption of bacterial cells on different solid porous supports. 0.5 ml ($625x10^3$ CFU/ml) bacterial suspension were added to each Erlenmeyer flasks (250 ml capacity) containing 25 ml of the optimized culture medium and the porous support materials (Luffa pulp, pumice, medical pumice**,** ceramic, coral reefs, and sponge) were cut to small pieces, washed several times with water before use. The flasks were then shaken slowly at 120 rpm. After 18 h incubation, the production of bioactive compounds was estimated using the well-cut diffusion technique (Ivanova *et al*., 1998).

The fermentation medium containing 20 cubes of medical pumice was supplemented with 0.5 ml of bacterial inoculum, the pH adjusted to 9 and incubated at 32 °C for 18 h. The reuse of the adsorbed cells was carried out by removing the medium after 18 h, and then a new sterilized medium (25 ml) was added, and new cycle was run. This process was repeated several times. At the end of each cycle, the antimicrobial activity was estimated.

Statistical analysis

Data analysis was performed with the soft package Microsoft Excel, version 2003. Statistically significant difference was determined using analysis of variance (ANOVA) and $p<0.05$ was used as the limit to indicate statistical significance.

Electron microscopy

For electron microscopy, the optimized medium was inoculated with the strain and incubated for 24-48 h. at 32°C. Glutaraldehyde (2.5 %, v/v) was used to fix the culture and post-fixed in osmium tetroxide (1 %, w/v) for 1h.The sample was washed twice with water and dehydrated in ascending ethanol (30 %, 50 %, 70 %, 90 % and 100 %), and finally coated in gold and examined at 15-20 KV in JEOL JSM 5400 LV, scanning electron microscope, Japan at the electron microscope unit of Assuit University.

Extraction of the active antimicrobial compounds

Culture of *P. pisicida* grown under the optimized conditions was centrifuged at 8000 rpm for 20 min. at 4°C. Equal volume of n-hexan, n-butanol, ethyl acetate, benzene or chloroform were used for extraction of the bioactive compounds. The organic phase was collected and evaporated using rotary evaporator, and each crude extract was dissolved in the appropriate solvent for further purification and characterization.

Each crude extract was tested for its antibacterial activity against *E.coli* using well cut diffusion technique. The antibacterial activity was detected as inhibition zone diameter (mm).

Thin layer chromatography

The dissolved crude extract was chromatographed on silica gel preparative slides using different solvent systems: ethyl acetate:Hexane (1:10 and 1:20) and hexane only as mobile phases. Migration and separation of the starting crude spots using each mobile phase were observed and the retarding factor of each solute (R_f) was estimated.

The active band (s) were gathered and dissolved in ethyl acetate and concentrated to dryness in the vacuum. Bioactivity of the selected bands was examined against *E.coli* using well-cut diffusion method. The antibacterial activity was detected as the inhibition zone diameter (mm).

Anticancer activity

Several steps were carried out to estimate the anticancer activity: lyophilization, cytotoxicity test and effect of IC50 on three different tumor cell lines. Lyophilization included three steps. Freezing, primary drying and secondary drying.10 ml of filtrate were lyophilized using a Telsonlyophilizer (Spain). Different concentrations (5, 25, 50, 75, 100 µg/ml) of the powder were prepared and used in the second step (cytotoxicity test). The second step is the cytotoxicity test which represent a rapid sensitive and inexpensive standardized test. to determine the toxicity of the test substances. To measure cytotoxicity of the examined compounds, $5x10^4$ lymphocyte cells were seeded per well in 96 well plates and incubated in RPMI media containing different concentrations (5, 25, 50, 75, 100 µg/ml) of the tested compounds and incubated for 24,48 and 72 hrs in 5% CO_2 incubator. Next, the media were removed and wells were washed with HBSS. The fraction of viable lymphocytes was measured by the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide)

In this assay, the yellow MTT is reduced to purple formazan in the mitochondria of viable cells. 100 µl of the MTT working solution (0.5 mg/ml) were added to each well and incubated at 37 °C for 4 h. and media were removed, wells were washed with phosphate buffer saline, and 100 µl of dimethyl sulfoxide (DMSO) were added to solubilize the formazan crystalline product. The absorbance was measured with a plate reader at 570 nm and normalized to the absorbance at 630 nm. Fraction of viable cell was determined by dividing the normalized absorbance of the test well to that of the control well (El-Hawash *et al*., 2006). Cytotoxicity assay was performed on different human cell line using the previously described method **.**

The anticancer activity of the substance was tested using different human cell lines (EI-4, Caco-2, HepG2, MCF-7). Cells (2×10^4) were seeded per well in 96 well plates and incubated in Ham's F-l2, RPMI and DMEM, respectively for 24 h in 5% CO_2 incubator for cell attachment. Next, the media were changed with media containing 10% of IC50 of the tested substances and cells were incubated in that media for 24, 48 and 72 h. Media without any chemical was used as the negative control. At each time point,

the media were removed, wells were washed with PO_4 buffer Saline (PB) and fractions of viable cells were measured by the MTTassay.

Antiviral activity

HepG2 cells were washed twice in RPMI1640 media supplemented with 200 µM L-glutamine and 25 µM HEPES buffer; N-[2-hydroxyethyl] piperazine-N9-[2-ethanesulphonic acid] (all chemicals and media, Cambrex). The cells were suspended at 2 $X10^5$ cells/mL in RPMI culture media (RPMI) supplemented media, 10% fetal bovine serum (FBS); Gibco-BRL). The cells were left to adhere on the polystyrene 6-well plates for 24 h in an incubator (37 °C, 5% CO_2, 95% humidity). The cells were washed twice from debris and dead cells by using RPMI supplemented media.

Cytotoxicity assay was performed on PBMC cell line using the previously described method .HepG2 cell culture were prepared as discussed before (El-Awady *et al*., 1996). then infected with 2% HCV-infected serum in RPMI culture medium containing 8% FBS. Tested compound was added at its safe dose. Positive and negative control cultures were included. After 96 h of incubation at 37 °C, 5% CO_2, and 95% humidity a second dose of the test compound was added.

The cells were incubated for a further 96 h followed by total RNA extraction using the method described by El-Awady *et al*. (1996). The positive strand and its replicating form (negative strand) were detected by RT- PCR using HCV specific primers.

Antioxidant activity

Total phenolic content of the artichoke samples was determined using Folin-Ciocalteu assay (AOCS, 1990). A 0.5 ml extract was added to 2.5 ml of Folin-Ciocalteu reagent followed by addition of 2 ml sodium carbonate (Na_2CO_3) (75 g/l). The sample was then incubated for 5 min at 50 °C. The absorbance was then measured at 760 nm. The phenolic content was expressed as mg gallic acid equivalents per gram of extract (mg GAE/g). The DPPH stable free radical scavenging assay was performed as previously described by Hu and Kitt (2000) with some modifications. Sample extract was dissolved in a methanol at different concentrations ranging from 6.25 to 100 µg/ml and then of the extract (2 ml) was incubated with 0.5 ml (0.2 mM) DPPH in 100% methanol. Sample absorbance at 519 nm was recorded after 30 min of incubation at room temperature. Inhibition of the DPPH stable free radical was calculated as follows:

$$\% \text{ Inhibition} = (\text{AB control} - \text{AB sample}) \div (\text{AB control} - \text{AB Blank}) \times 100$$

Where:

Abs control = absorbance of 0.1 mM DPPH alone in methanol; Abs sample = absorbance of 0.1 mM DPPH + sample extract in methanol; and Abs blank = absorbance of methanol solvent control.

Spectral analysis

Identification of constituents of the crude extract was performed using high performance 5890 gas liquid chromatography (GLC) (Hewlett Packard) coupled with 5989 B series mass spectrometer (MS) (Shimadzu (EI), Japan). The gas liquid chromatography mass spectrometer (GC-MS) peaks were identified using WILEY MASS SPECTRAL DATA BASE Library (Kaoutar *et al*., 2010).

Characterization of the purified bioactive compounds

IR-spectral analysis was investigated at the Faculty of Pharmacy, Alexandria University. Three milligram of the crude extract was taken and mixed well with potassium bromide (KBr) and pressed into a disc shape. The sample was placed into the sample holder and FTIR spectra were recorded at the range 400–4000 cm^{-1} (Kaoutar *et al*., 2010). The purified bioactive compounds were characterized by recording the absorption peak using Ultraviolet-visible spectrophotometer analysis.

RESULTS

Antimicrobial resistant microorganisms cause killing of about 3 million people each year in the world (Navarri *et al*., 2016). This threat requires renewal of the traditional antibiotics Underexplored marine environment may represent an untapped reservoir of novel antimicrobial agents (Blunt *et al*., 2015).

In the present study, among 22 tested bacterial isolates coded (AB1-AB22), the marine bacterial isolate AB12 which was previously identified as *Pseudoalteromonas piscicida* using 16Sr RNA sequence analysis and has GenBank accession number of JX861209.1 exhibited broad spectrum of antimicrobial activity against the tested pathogens recording the highest antimicrobial activity (17 mm) against *E.coli* ATCC 8739. Study concerned with antimicrobial screening using nine target marine isolates against *Staphylococcus aureus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli, Micrococcus luteus and Candida albicans* was previously carried out (Wekky *et al*., 2009). The antimicrobial activity of *P. piscicida* was previously proven in different studies (Bowman, 2007; Secun, 2015).

Effect of growth medium type on production of the bioactive compounds

Secondary metabolites affect by variation in the growing environment which in turn can have significant impacts on the diversity and quantity of secondary metabolites. Such optimization becomes challenging in a drug-discovery screening situation (VanderMolen *et al*., 2013).

Pseudoalteromonas piscicida was examined for the antagonistic activity using three different media; nutrient broth (NB), luria broth (LB) and minimal media (MM). As shown in Table 1, broad spectrum of antimicrobial activity against the tested pathogenic bacteria and also the highest inhibition zone (19 mm) was observed up on using nutrient broth (NB) and thus nutrient broth was used for further investigation. NB and LB were

documented as production media for antimicrobial agents by the marine bacterium *Enterococcus faecium* (Wefky *et al*., 2009).

Table 1: Effect of different media on production of the antimicrobial agent(s) by *P.piscicida.*

Pathogen	Diameter of inhibition zone (mm) produced by *P. piscicida* using different media		
	LB	NB	MM
E.coli	15	17	10
V. anguillarum	13	19	-
A. hydrophila	-	13	-
P. aeruginosa	-	15	-
S. aureus	13	-	-
C. albicans	-	13	-

Optimization of culture conditions

Statistical experimental designs are powerful tools for rapid searching of the key factors from a multivariable system and error minimization in determining the effect of parameters and the results are achieved in an economical manner (El-Sersy, 2007; Abou-Elela *et al*., 2009; Shabbiri *et al*., 2012; Sharma *et al*., 2013; Saraniya and Jeevaratnam, 2014. El-Sharouny *et al*., 2015; Zeinab *et al*., 2015). The medium components in addition to some physical factors (Table 2) were examined using the design matrix (Table 3).

Table 2: Independent variables affecting production of the antimicrobial agent(s) and their levels in the Plackett- Burman design.

Factor	Symbol	Level		
		-1	0	1
Peptone (g/l)	P	3	5	7
Beef extract (g/l)	B	1	3	5
Inoculum size (ml)	IS	0.5	1	1.5
Seawater concentration (%)	S.W	50	100	150
pH	pH	5	7	9
Incubation period (h).	IP	18	24	48
Culture volume (ml)	C.V	25	50	75

All experiments were performed in duplicates and the averages of results (diameter of inhibition zone in mm) are presented as the response. Results indicated that the highest inhibition zone (20 mm) was against *E. coli*.

Table 4 and Figure 1 show the main effect of each variable on the production of the antimicrobial agents against *E.coli* (ATCC 8739) as well as t-values presented in Table 4. These results indicated that pH and sea water concentration have positive main effects while the other tested variables exhibited negative main effects on the antagonistic agents production. Statistical analyses of the results (t-test) showed that variations in sea water concentration and beef extract in the tested ranges had the most significant effects on the production of antimicrobial agents by *P. piscicida* (Table 4).

Table 4: Statistical analysis of the Plackett-Burman experimental design for the production of antimicrobial agents by *P. piscicida* against *E. coli* (ATCC 8739).

Variables	Symbol	Main effect	t-value
Peptone	P	-9	1.9
Beef extract	B	-5.5	**2.0**
Inoculum size	IS	-1	1.9
Sea water concentration (%)	SW	2.5	**2.0**
pH	PH	7.5	1.9
Culture volume	CV	-9	1.9
Incubation Period	IP	-1	1.9

t-value significant at the 1% level = 3.70.

t-value significant at the 5% level = 2.45.

t-value significant at the 10% level = 1.94.

t-value significant at the 20% level = 1.37.

Standard t-values are obtained from statistical methods (Cochran and Snedecor, 1989).

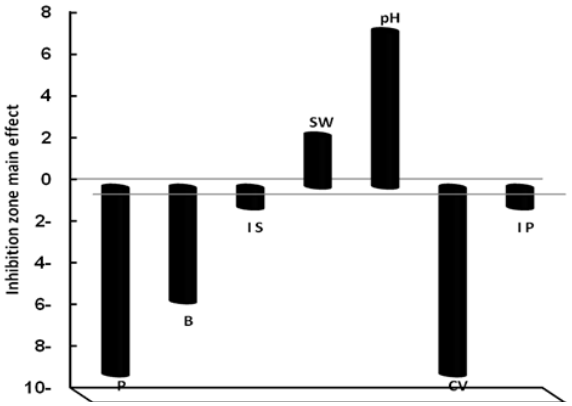


Fig. 1: Elucidation of the cultured factors that affect the production of antimicrobial agent(s) by *P. piscicida* against *E. coli* ATCC 8739.

Table 3: Experimental results of the Plackett-Burman design.

Trials	Factors symbols							(Response) Diameter of inhibition zone (mm)					
	P	B	pH	I.S	I.P	S.W	C.V	*P. aeruginosa*	*A. hydrophila*	*V. anguillarum*	*E. coli*	*C. albicans*	*S. aureus*
1	-1	-1	-1	1	1	1	-1	0	0	0	16	10	0
2	1	-1	-1	-1	-1	1	1	0	0	0	0	0	0
3	-1	1	-1	-1	1	-1	1	0	0	0	0	0	0
4	1	1	-1	1	-1	-1	-1	0	0	0	0	0	0
5	-1	-1	1	1	-1	-1	1	0	13	0	13	12	0
6	1	-1	1	-1	1	-1	-1	17	13	10	13	0	0
7	-1	1	1	-1	-1	1	-1	0	14	0	20	0	15
8	1	1	1	1	1	1	1	0	0	0	0	13	0
9	0	0	0	0	0	0	0	15	13	19	17	13	0

Verification experiment

In order to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was applied to compare between the predicted optimum levels of independent variables and the basal condition settings. Results in Table 5 showed that growth of *P. piscicida* on the optimized medium with the following composition: (g/l) peptone, 3; beef extract, 1; inoculum size (ml), 0.5; culture volume (ml),25; sea water concentration 150 % adjusted to pH 9 and incubation period 18h. at 32^{o}C increased the production of the antagonistic agents (expressed in mm diameter of inhibition zone) to 21 mm with 1.2 fold increase when compared to cells grown under the basal conditions.

Abd-Elnaby *et al*. (2016) reported that increasing levels of pH, inoculum size caused an increase in the antagonistic activity by about 1.3 fold for *Streptomyces parvus*. Wefky *et al*. (2009) stated that peptone, beef extract, sea water concentration and culture volume were negatively affected the antagonistic activity by *Enterococcus faecium.* The data were also examined and compared to anti-optimized medium (Table 5).

Table 5: A verification of the Plackett-Burman experimental results that carried out using *P. piscicida* against *E.coli* (ATCC 8739).

Response	Basal medium	Optimized medium	Anti-optimized medium
Diameter of inhibition zone (mm)	17	21	0

Cell immobilization

The majority of bacteria in the aquatic systems are organized in biofilms. This structure provides the bacteria present in biofilms with several advantages compared to the free cells (Donlan and Costerton, 2002). Immobilized cells in the production of metabolites by microbial cultures is one of the most interesting techniques proposed for development of fermentation process. Immobilized cell particles are about 1,000 times larger than free cells so they can easily be handled and packed in fermentation system for industrial processes (Darah *et al*., 2011). Living cells of *P.piscicida* were subjected to immobilization using adsorption technique with luffa pulp, ceramic, coral reefs, pumice, medical pumice and sponge as supporting materials. Results in Table 6 showed that the antimicrobial activity of immobilized *P. piscicida* on medical pumice (Figure 2) was superior than free cells and raised the activity to 23 mm which realized 1.4 fold increase followed by immobilized cells on pumice, coral reefs ceramic with inhibition zone diameter of 22, 21 and 20 mm respectively. On the other hand, the lowest antimicrobial activity was detected up on using immobilized cells on luffa pulp which reduced the antimicrobial activity by 0.94 folds. This result could be attributed to poor mechanical stability of the support (Lunestad and GoksQy, 1990). The second factor is the individual characteristics of the bacterium (Pursel *et al.*, 1995). The present results coincide with Wefky *et al*. (2009) who stated that biofilm of *Enterococcus faecium* on pumice raised the antimicrobial activity with 1.2 fold while luffa pulp exhibited the lowest antimicrobial activity. Abd-Elnaby *et al*. (2016) also reported the potentiality of the immobilized cells in raising the antimicrobial activity of *Streptomyces parvus* compared to free cells. The same feature was reported by Ivanova *et al*. (2000-2002).

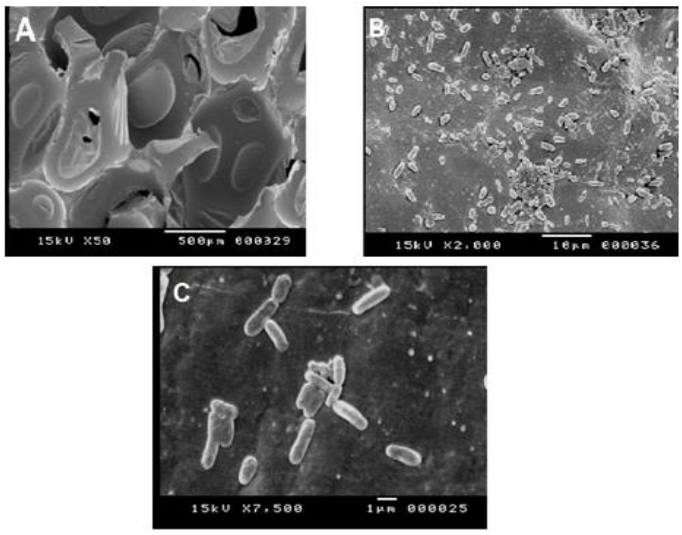


Fig 2: Scanning electron micrographs showing (A) control medical pumice; (B) *P. piscicida* (AB12) adsorbed on medical pumice and (C) free cells of *P. piscicida*

Table 6: Effect of immobilization on production of the antimicrobial agents by *P. piscicida*.

Response	Antagonistic activity using different support materials						
	Free cells	Luffa pulp	Ceramic	Coral reefs	Pumice	medical Pumice	Sponge
IZ.	17	16	20	21	22	23	15

IZ. = Inhibition Zone (mm).

Moreover, the cells on medical pumice was recycled for four successive cycles aiming for enhancing the antimicrobial agent(s) production. As shown in Table 7 the antagonistic activity increased during 1st , 2nd, 3rd and 4th cycles compared to free cells to reach (24±2.33, 24±2.61, 25±2.71, 24±2.31 mm.), respectively with significant difference (ANOVA, $p<0.05$) compared to the free cells (17±1.52) and there was 1.5 fold increase in the antagonistic activity during the third cycle.

Table 7: Immobilization cycles of antimicrobial agent(s) production on medical pumice.

Immobilization cycle	Inhibition zone (mm) against *E.coli*
1st cycle	24±2.33b
2nd cycle	24±2.61 b
3rd cycle	25±2.71 b
4th cycle	24±2.31 b
Free cells	17±1.52a

Antibacterial activity of the extracted compounds

The crude extracts of *P.piscicida* produced up on using benzene, chloroform and ethyl acetate were tested for their

antibacterial activity against *E.coli*. Results in Figure 3 revealed that benzene extract recorded the highest antibacterial activity (25 mm) while the lowest antibacterial activity (22 mm) was detected upon using ethyl acetate extract which prove the efficiency of benzene for extraction of the bioactive metabolites. *Pseudoalteromonas* strains are known as prolific producers of bioactive secondary metabolites with antimicrobial activity (Vynne and Gram, 2011). Marine bacteria of the genus *Pseudoalteromonas* are recognized as major producers of various biologically active metabolites such as halogenated compounds, cyclic compounds, enzymes (Secun *et al*., 2015). Kim *et al*. (2016) reported the production of various secondary metabolites from the genus *Pseudoalteromonas* against *E.coli.*

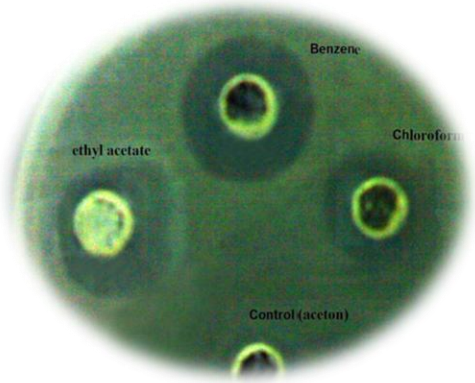


Fig. 3: Effect of solvents extracts on antibacterial activity against *E.coli* ATCC 8739.

Thin layer chromatography

Partial purification of the crude extract using TLC showed 3 separated spots with R_f values 0.1, 0.3 and 0.8 and they were visible under UV lamp. The antimicrobial activity of the separated spots were investigated against *E. coli*. As shown in Table 8.

Table 8: The bioactivity of the three major components compared to the crude extract using *E.coli* ATCC 8739.

Crude extract & TLC spots	Inhibition zone diameter (mm) against *E.coli*
Crude extract	25
Band 1	11
Band 2	15
Band 3	9

Anticancer activity

Data in Table 9 showed the effect of culture supernatant concentration on (EI-4) cell line (Figure 4A, B) using the MTT assay which causing 38% inhibition after 72h. Upon using tumour cell line (Caco-2) (Figure 4 C, D), the supernatant of tested compound showed a reasonable degree almost 63.8 % inhibition respectively after 72 h using the MTT assay. On the other hand, using the supernatants of the tested compound showed very low activity towards EI4 (Figure 4 A,B), (HepG2) (Figure 4 E,F) cell line and (MCF-7) (Figure 4 G,H) as detected by MTT assays where they showed only 33 % and 44.6% inhibition respectively after 72 h. Noble *et al*. (2016) reported that secondary metabolites from the marine *Pseudoalteromonas sp*. inhibited the proliferation of A549 lung carcinoma cells. Wang *et al*. (2012) stated that the extracted compounds from *Pseudoalteromonas* sp. 1020R, isolated from the Pacific coast of Japan exhibited different extents of cytotoxicity against U937 leukemia cells. In similar study, Zheng *et al*. (2006) reported the cytotoxicity of secondary metabolites from *P. piscicida* towards both the HeLa cervical-cancer cell line and the BGC-823 stomach-cancer cell line, with an IC_{50} of 5 µg/ml

Table 9: IC50 and the percentage of inhibition of different cell line growth using the MTT assay.

Cell line	IC50	Inhibition %	Time (h)
EI-4	40.55	38	72
Caco-2	54.38	63.8	72
HepG2	70.83	33	72
MCF-7	64.22	44.6	72

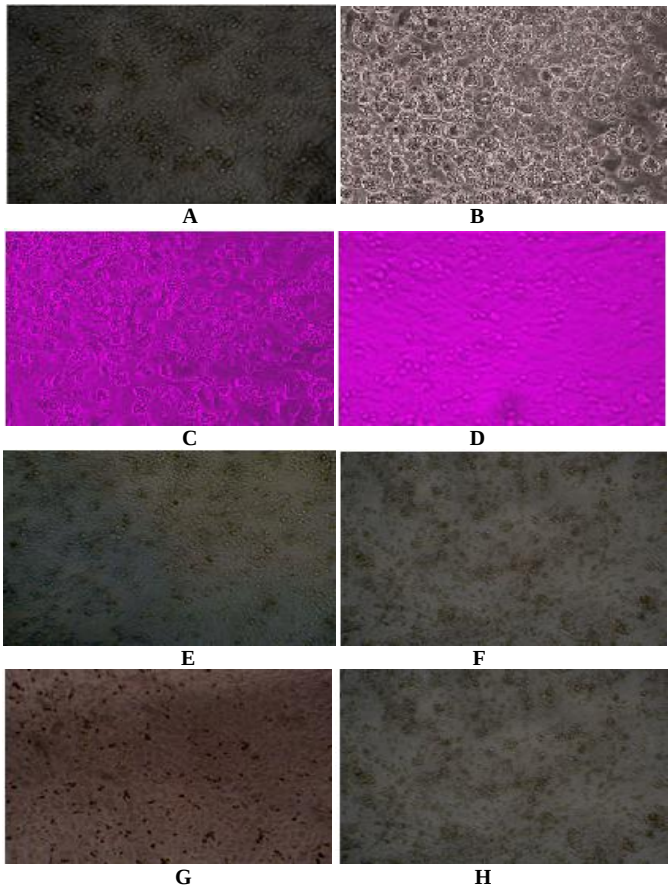


Fig. 4: Photographs illustrating the difference between the tested compound (B) on the growth inhibition of EI-4 tumor cell line compared to control (A), difference between the tested compound (D) on the growth inhibition of CaCo-2 tumor cell line compared to control (C), difference between the tested compound (F) on the growth inhibition of HepG2 tumor cell line compared to control (E), difference between the tested compound (H) on the growth inhibition of MCF-7 tumor cell line compared to control (G).

Antiviral activity

The supernatant of the bacterial strain *P. piscicida* succeeded to inhibit HCV replication at 100 µg/ml as shown in Figure 5. Zhou *et al*. (2016) found that the secondary metabolites

produced by *Pseudoalteromonas* sp. exhibited antiviral activity on Bombyx mori nucleopolyhedro virus (BmNPV)-infected cells in vitro, with specific modes of action and selectively killed virus-infected cells, inhibited viral gene transcription. In another study, Elnahas *et al*. (2011) reported the antiviral activity of secondary metabolites produced by *Pseudoalteromonas* sp. against herpes simplex (HSV-I) activities. In that context, the antiviral activity of sulfated EPSs was attributed to their ability to inhibit virus particle adsorption to host cells (Llamas et al., 2010).

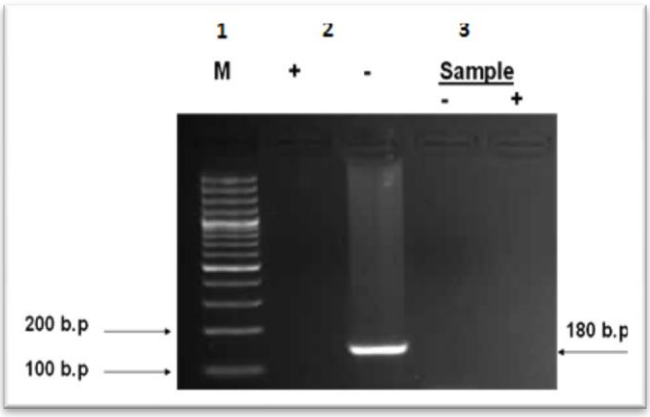


Fig. 5: PCR amplification picture of HCV RNA (+) and (–) strands in the presence of supernatant of the bacterial strain *P. piscicida*. Lane 1: contains the molecular weight marker (M) 100bp ladder, lane 2: control, and lane 3: effect of the supernatant of the bacterial strain *P. piscicida* (100µg/ml.) on HCV RNA (+) and (–) strand after 8 days of treatment.

Antioxidant activity

As shown in table 10, the sample has a significant antioxidant activity compared with ascorbic acid as control. The percent of inhibition were (4.92, 24.36, 32.51, 35.59 and 39.13%) respectively for the sample and (10.64, 28.86, 39.76, 48.56 and 60.32%) respectively for ascorbic acid, which proves that there is linear relationship between antioxidant capacity and phenolic content. This result was almost in accordance with that reported by Nurul *et al*. (2011). The bacteria belonging to genera *Pseudoalteromonas* were reported for their antioxidant activity (Velho-Pereira *et al*., 2015).

Table 10: DPPH stable free radical scavenging activity of sample %.

Sample concentration (mg/ml)	% Inhibition	Ascorbic acid
6.25	4. 92	10.64
12.5	24.36	28.86
25	32.51	36.76
50	35.59	48.56
100	39.13	60.32

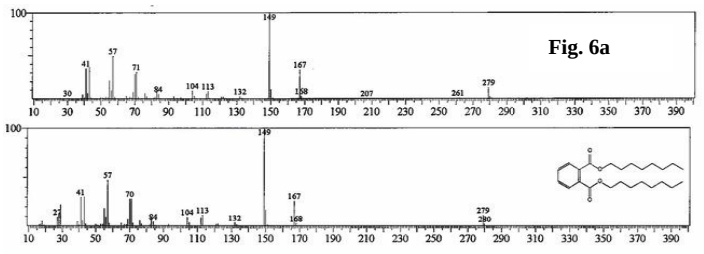
Chemical characterization of the benzene extract

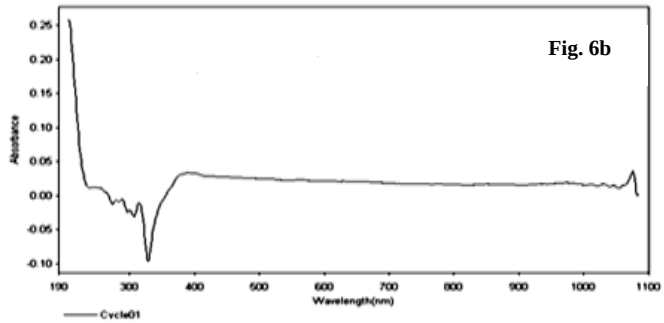
Chemical composition of the benzene extract was determined using GC-MS. The chemical constituents in the crude extract are shown in Table 11.

Results indicated that the major components in this extract are Di-n-octyl phthalate representing 22.05 % afforded (0.20 g/l) of the extract, Benzene,1-butyheptyl (8.4 %) afforded (0.0848 g/l) of the extract and Benzene, (1-pentyloctyl) (8.2 %) afforded (0.0828 g/l) of the extract with molecular weight 390, 232 and 260 respectively. Mass spectrum of the most active compound (Di-n-octyl phthalate) is illustrated in Figure 6a. Chemical characterization including UV and IR analysis were carried out as illustrated in Figure 6 b,c. IR spectra of the active compound showed peaks at 3448 , 300.15 cm^{-1} (O-H stretching), 2135.67 cm^{-1} (C-H alkane), 1714, 1640.38 cm^{-1} (C=O stretching), 1361.90, 1422.08 cm^{-1} (C= C aromatic), 1221,.28 1092.22 cm^{-1} (Aromatic C-O).

Table 11: Chemical composition of the three major compounds of the benzene crude extract obtained from *P.piscicida* and fractionated using GC/MS analysis.

Compound	Molecular formula	Retentionti me	Molecular weight	Area%	Similarity %
Benzene, (1-butylheptyl)	$C_{17}H_{28}$	13.769	232	8.4	96
Benzene, (1-pentyloctyl)	$C_{19}H_{32}$	15.205	260	8.2	93
Di-n-octyl phthalate	$C_{24}H_{38}O_4$	25.717	390	22.05	97





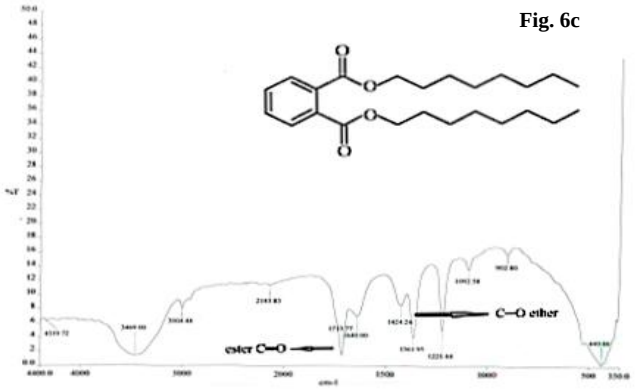


Fig. 6: Characterization of the purified Di-n-octyl phthalate a) Mass spectrum using GC-MS, b) UV spectrum and c) IR spectrum.

The major chemical component Di-n-octyl phthalate was proven to have pharmaceutical uses as was reported by Kim *et al.* (2004) who stated that phthalates which include butyl benzyl phthalate (BBP), di(n-butyl) phthalate (DBP), and di (2 ethylhexyl) phthalate (DEHP) had been demonstrated anticancer activity. Moreover, phenolic compounds exhibits antimicrobial activity (Patra and Baek, 2016; Rodríguez-Pérez *et al*., 2016; Marín *et al*., 2016; Tuberoso *et al*., 2016) and may reduce the risk of cardiovascular disease and stroke In these ways, phenolic compounds may also confer protection against chronic diseases such as atherosclerosis and assist in the management of menopausal symptoms. Thus, phenolic compounds have been referred to as semi essential food components.

CONCLUSIONS

Marine environment is an exploding source of microorganisms with potentiality of their bioactive compounds. The aim of the present study was to search for marine bacteria as an ecofriendly source of bioactive compounds with various uses. The findings of the present approach suggested that the bioactive compounds produced by marine *P. piscicida* could be used as antibiotics, and might have future biomedical applications as anticancer, antiviral and antioxidant agents. Complete identification of the extracted bioactive compounds will be tracked in the future study.

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