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Phyto-chemical Therapy for Inhibition of Fish Pathogenic Bacteria in Sea-food and Aquaculture

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Abstract— In aquaculture and seafood sector, bacterial infections are major economic and biological threat to aquatic animals and humans. About 80% of the seafood consumed in the U.S. is imported from approximately 62 countries and over 40% is aquacultured seafood which often contains traces of banned and hazardous antibacterial chemical drugs. This study creates a basic foundation to design a safe and natural drug against bacteria of seafood and aquaculture which will be alternative to commonly practiced hazardous chemical drugs. Extracts of *Aegle marmelos*, *Ocimum sanctum* and *Azadirachta indica* plants posses potential antibacterial activity against *Aeromonas hydrophila*, *Bacillus cereus* and *Escherichia coli*, equivalent to the hazardous and banned chemical drug API Furan-2. Digital High PerformanceThin Layer Chromatography (HPTLC) Fingerprints of effective extracts were generated, which recorded number of phytoconstituents. They can be referred for bio-autography and drug formulation.

Index Terms— *Aegle marmelos*, *Aeromonas hydrophila*, Antibacterial activity, *Azadirachta indica*, *B. cereus*, *E. coli*, HPTLC Fingerprint, *Ocimum sanctum.*

1 Introduction

Aquaculture is the fastest growing food-production sector in the world. Worldwide, more than 38 million people are directly engaged in fishing and fish farming as a full-time or, more frequently, part-time occupation. Aquatic animal diseases are the most significant constraint to the development and management in aquaculture [1], [2]. At the global level, combined estimated losses in production value due to shrimp diseases from 11 countries for the period 1987–1994 were on the order of US$ 3019 million [3].

Antibiotics resistance of microorganisms is a global concern [4]. As the aquaculture industry continues to grow and compete with wild-caught seafood products, concerns regarding the use of prohibited and unsafe chemicals and the misuse of animal drugs in aquaculture operations have increased substantially. The use of antibiotics or chemicals, such as malachite green, nitrofurans and gentian violet during various stages of aquaculture production can result in the presence of residues of the parent compound or its metabolites in the edible portion of the aquacultured seafood. Furthermore, prolonged exposure to nitrofurans, has been shown to have a carcinogenic affect with long-term exposure in lab animals [5], [6], [7]. Nitrofuran employed for the treatment of bacterial diseases in livestock production, was banned from use in the European Union (EU) [8] and in countries such as Australia, USA, Philippines, Thailand and Brazil [9].

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But about 80% of the seafood consumed in the U.S. is imported from approximately 62 countries and over 40% is aquacultured seafood [7]. Inspection by EU authorities revealed that nitrofuran contamination in products originating from over nine countries in 2007, the highest incidences being from India (37%), China (37%), Bangladesh (10%) and Thailand (5%) in a variety of products including shrimp, honey and canned meat [10].

This present scenario has forced to search for new, safe anti-microbial substances from various natural sources, like medicinal plants. This research proposes the use of plant extracts as antimicrobial agents for pathogenic bacteria of seafood and aquaculture. This work deals with the antimicrobial activity of leaf-extracts of medicinal plants *Aegle marmelos, Ocimum sanctum* and *Azadirachta indica* on pathogenic bacteria *Aeromonas hydrophila, Bacillus cereus* and *Escherichia coli*. These bacteria are included in the first edition of the Bad Bug Book, published by the Center for Food Safety and Applied Nutrition (CFSAN) of the Food and Drug Administration (FDA), U.S. Department of Health and Human Services, as the major known agents which cause food-borne illness [11].

The active antibacterial components from plant leaves were extracted in 5 cold and hot solvents and tested *in vitro* by agar well diffusion method. Statistical analysis for equivalence of effectiveness of plant extracts against commonly practiced chemical drug is carried out. The effective extracts were subjected to HPTLC Fingerprinting and fluorescent HPTLC fingerprints of the effective compounds were generated. With the utilization of advanced instrument and data analysis system as well as optimized experimental operation, HPTLC is feasible for development of chromatographic fingerprint profiling methods to determine complex herb extracts [12]. The

corresponding digital scanning profile was generated for further reference for drug development.

2 EXPERIMENTAL

2.1 Materials

Plant material (fresh leaves) of *A. indica*, *A. marmelos* and *O. sanctum* were collected from Thane district of Maharashtra, India . Based on external morphology, identification of plant samples was carried out by Ponkshe [13] from Sathaye College, Mumbai, India based on morphological characteristics. The plant material (leaves) of above plants were first washed with distilled water and dried separately at room temperature for two days. For next 10 days they were dried in Hot Air Oven at 42 ^{0}C. A grinder is used to convert them into a fine powder. The powder sample of each plant was then stored separately at room temperature (29± 1^{0}C) in airtight plastic containers for further use.

2.2 Preparation of plant extract

The plant powders individually were used to prepare Decoctions (individual extracts) cold extracts and hot extracts using 5 solvents- Acetone, Methanol, Petroleum Ether, Chloroform and Distilled water (D/W) [14], [15]. The hot extracts were prepared by Soxhlet extraction with concentration of 50 mg/mL [16]. For preparation of cold extracts; the individual plant powder were dissolved in respective solvents followed by vigorous shaking for 24 hrs. The Concoction (mixed extract) extract of all 3 plants together were made by composing the concentration 16.66 mg/mL of each plant [17].

2.3 Test Organisms

The pathogenic bacteria *Aeromonas hydrophila* and *Escherichia coli* were isolated from tissues of diseased fishes on isolation agar (Rimler and Schotts medium, MacConkey agar) (HiMedia, Mumbai) and their identity was confirmed by IMViC tests (HiMedia, Mumbai). All the tests were carried out at the Central Institute of Fisheries Education (CIFE), Mumbai, India. *Bacillus cereus* was obtained from culture library of Central Institute of Fisheries Education (CIFE), Mumbai, India

2.4 The Minimum Inhibitory Concentration (MIC)

MIC of the plant extracts for antibacterial activity was performed using five concentrations of each extract (0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL, 1.6 mg/mL, 3.2 mg/mL or 1 mg/mL, 2 mg/mL, 4 mg/mL, 8 mg/mL, 16 mg/mL) for each bacteria using Muller- Hinton broth (HiMedia, Mumbai) [18]. A widely used [19] standard aquaculture chemical drug API Furan-2 was used at its given concentration by manufacturer 0.052 mg/mL. The results were observed and recorded.

2.5 Agar Well Diffusion Test

The bacteria were subjected to Agar well Diffusion Test in Mueller- Hinton agar (HiMedia, Mumbai) with variable concentrations (1.65 mg/mL, 3.25 mg/mL, 6.5 mg/mL or 8.25 mg/mL, 16.25 mg/mL, 32.50 mg/mL) considering the MIC results of plant extracts. API Furan-2 was used with the concentrations 0.052 mg/mL, 0.104 mg/mL, 0.208 mg/mL. The plates were incubated at 37°C for 18- 24 hours. All tests were carried out in triplicates to minimize test error. The zones of inhibition were measured and results were recorded [20].

2.6 Statistical Analysis

The results of Agar well diffusion test were subjected to Chi-square test to check whether chemical drug and plant extracts are equivalent in activity [21].

2.7 HPTLC Fingerprinting

The effective antibacterial extracts of tested plants were subjected to HPTLC Fingerprinting. HPTLC was carried out with a Camag TLC system (Camag, Muttenz, Switzerland) fitted with WinCATS 1.2.3 software. Samples were applied with a Camag automatic TLC sampler 4 (ATS 4) (5 µL and 10 µL) on HPTLC precoated plates- silica gel-G aluminium plate -20 cm × 10 cm was used (Merck, Darmstadt, Germany) and developed in twin-trough glass chamber. Camag Visualizer: 150503 and Camag TLC Scanner3_070408 S/N 070408 (1.14.28) with documentation software (Camag, Muttenz, Switzerland) was used for the imaging, saving and spectral analysis of the chromatograms. All chemicals and solvents were of analytical grade and used as obtained [22].

Mobile phase:

The components were separated in following mobile phase for 20 minutes.

$CHCl_3$ - Toluene - Ethanol (4:4:1).

The resultant chromatogram was then illuminated at UV 254nm and UV 366 nm cabinet for the characteristic fluorescence. The HPTLC fluorescence image was documented. The plates were derivatized for the detection of the compounds.

Derivatization Solution:

Following most common non-specific derivatization reagents were used-

Anisaldehyde - Sulphuric acid- Methanol

Preparation of Solution: 10 mL sulphuric acid is carefully added to an ice-cooled mixture of 170 mL methanol and 20 mL acetic acid. To this solution, 1 mL Anisaldehyde is added. The plate is immersed in this reagent for 1 second and then heated at 100 ^{0}C for 2-5 minutes or until the bands started becoming visible. The plate was observed immediately at UV 366 nm and 580 nm cabinet and the

HPTLC fluorescence image was documented. The corresponding digital scanning profile was generated [23].

3. RESULTS

3.1 The Minimum Inhibitory Concentration (MIC)

MIC for *A. hydrophila* and *E. coli* was recorded at 1.6 mg/mL by chloroform cold extracts of *A. marmelos*, *A. indica* and concoction (mixed plant extracts) extract while (MIC) for *B. cereus* was recorded at 8 mg/mL by hot petroleum ether concoctions extract. Other extracts showed no activity against the bacteria.

3.2 Agar Well Diffusion Test

Among the plant extracts, the most pronounced activity against *A. hydrophila* was noticed by the cold chloroform decoction (individual plants) extract of *A. indica* which recorded a zone of maximum 30 mm at 6.5 mg/mL, whereas a zone of maximum 29 mm was observed at 6.5 mg/mL by cold chloroform decoction extract of *A. marmelos*. The cold chloroform concoction (mixed plant extracts) extract of all 3 plants recorded a zone of 22 mm at 6.5 mg/mL. The *B. cereus* was actively inhibited by the hot petroleum ether concoctions extract which recorded a zone of maximum 18 mm at 32.5 mg/mL. The cold chloroform decoction extract of *A. marmelos* showed maximum antibacterial activity against *E. coli* at 6.5 mg/mL recording maximum 17 mm zone of inhibition. Hot chloroform decoction extract of *A. indica* recorded a zone of 16 mm at 32.5 mg/mL while the cold chloroform concoction extract of all 3 plants recorded a zone of maximum 14 mm at 6.5 mg/mL.

API Furan-2 showed very less inhibiting activity at 0.104 mg/mL concentration.

0.208 mg/mL concentration recorded antibacterial activity against *A. hydrophila* recording maximum zone of inhibition of maximum 12mm while *B. cereus* recorded 13mm and *E. coli* recorded maximum 11 mm zone of inhibition. (Fig. 1 to Fig. 4)

Fig. 1. Effect of Plant extracts on *A. hydrophila*

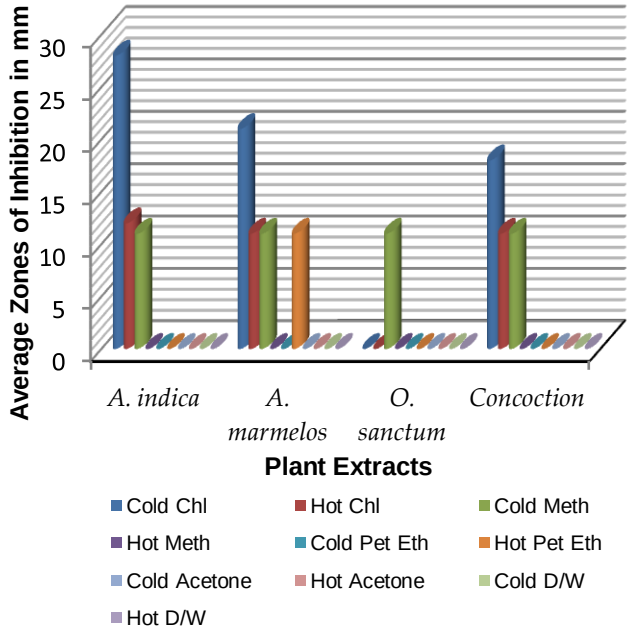
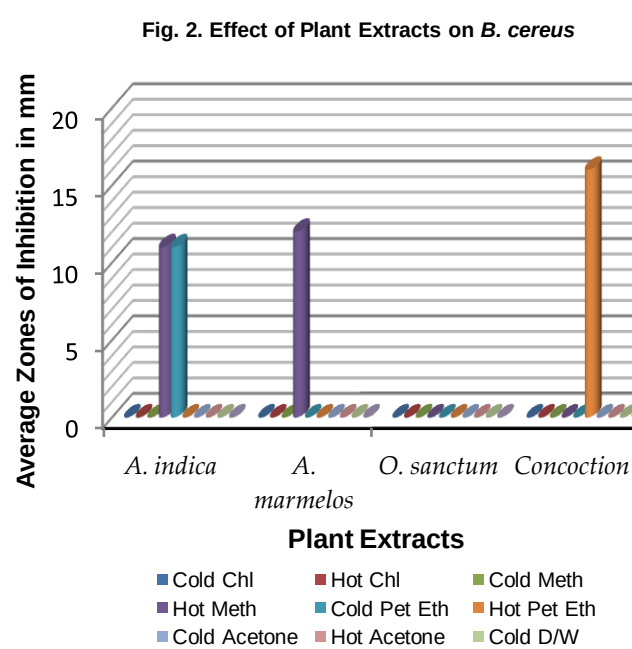
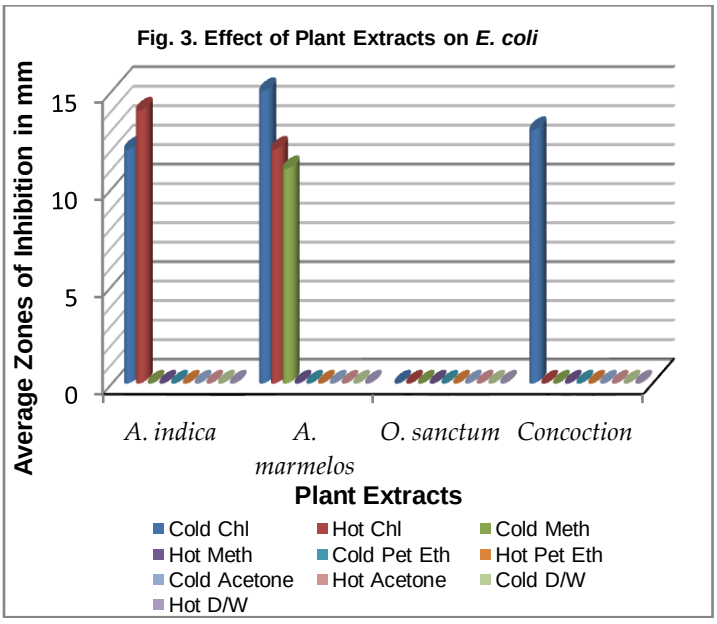
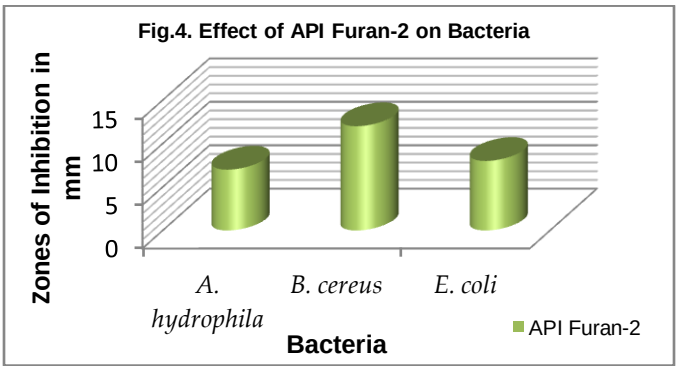


Fig. 2. Effect of Plant Extracts on *B. cereus*





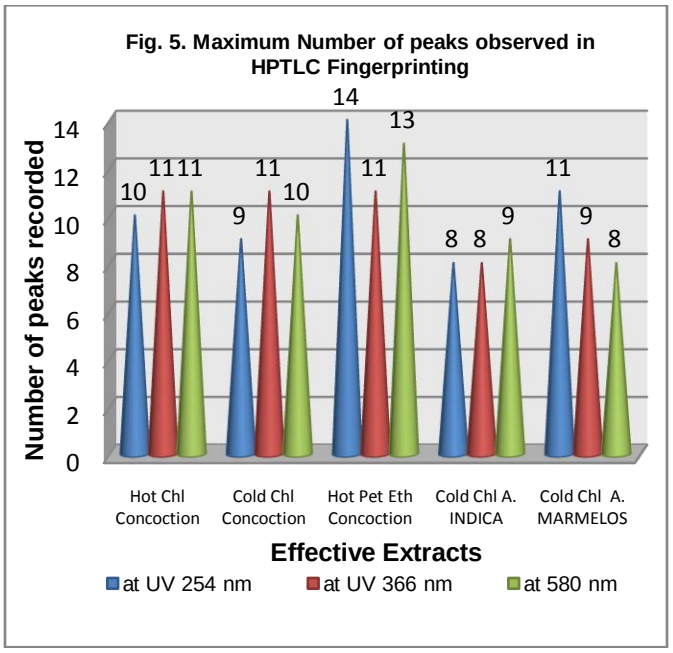


3.3 Statistical Analysis

The results of the Chi-square tests indicated that the chemical drug API Furan-2 and most effective plant extracts are equivalent in antibacterial activity at P< 0.05 level of significance.

3.4 HPTLC Fingerprinting

UV illuminated chromatogram detected varying number of peaks at UV 254 nm, UV 366 nm and UV 580nm (visible) at 10 µL application concentration (Fig. 5). At UV 254 nm, 10 peaks were detected in the hot chloroform concoction extract, 9 peaks in cold chloroform concoction extract and 14 peaks in hot petroleum ether concoction extract. While 8 peaks were observed in cold chloroform extract of *A. indica* plant and 11 peaks were detected in cold chloroform extract of *A. marmelos* plant.



After derivatization of the plate, at UV 366 nm, 11 peaks were detected in the hot chloroform concoction extract, 11 peaks in cold chloroform concoction extract and 11 peaks in hot petroleum ether concoction extract. While 8 peaks were detected in cold chloroform extract of *A. indica* plant and 9 peaks were detected in cold chloroform extract of *A. marmelos* plant. (Fig. 6 to Fig. 11)

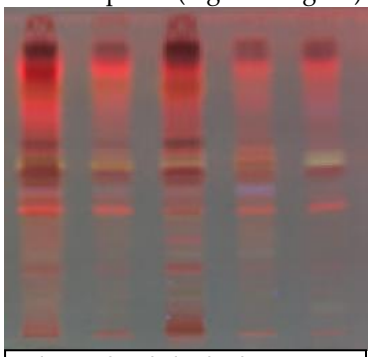


Fig. 6. After derivatization HPTLC Fingerprint at 366nm

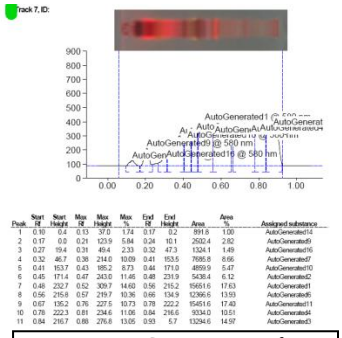


Fig. 7. HPTLC Fingerprint of Hot Chloroform Concoction

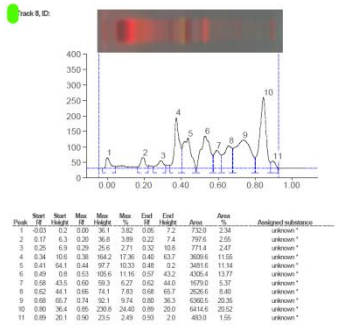


Fig. 8. HPTLC Fingerprint of Cold Chloroform Concoction

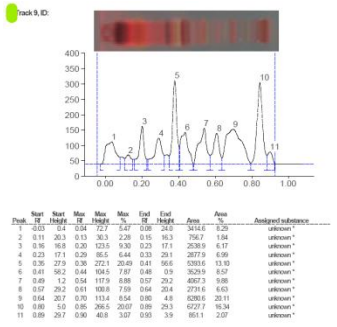


Fig. 9. HPTLC Fingerprint of Cold Pet Eth Concoction

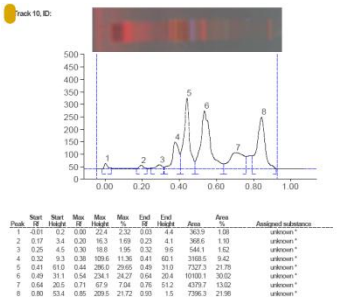


Fig. 10. HPTLC Fingerprint of Cold Chloroform *A. indica*

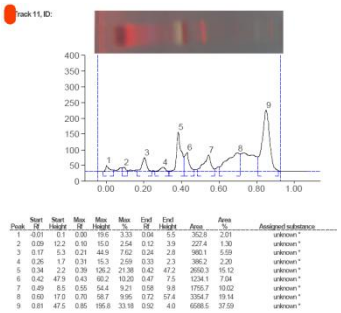


Fig. 11. HPTLC Fingerprint of Cold Chloroform *A. marmelos*

After derivatization of plate, at 580 nm, 11 peaks were detected in the hot chloroform concoction extract, 10 peaks in cold chloroform concoction extract and 13 peaks in hot petroleum ether concoction extract. While 9 peaks were detected in cold chloroform extract of *A. indica* plant and 8 peaks were detected in cold chloroform extract of *A. marmelos* plant. (Fig. 12 to Fig. 17)

Fig. 12. After derivatization HPTLC Fingerprint at 580nm

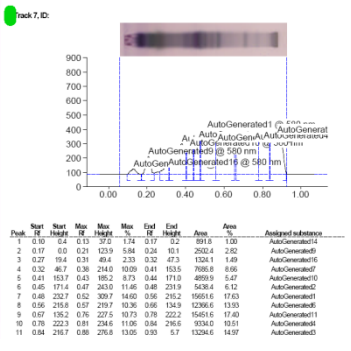


Fig. 13. HPTLC Fingerprint of Hot Chloroform Concoction

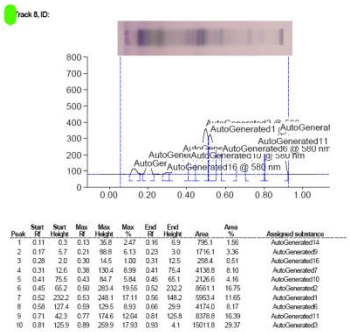


Fig. 14. HPTLC Fingerprint of Cold Chloroform Concoction

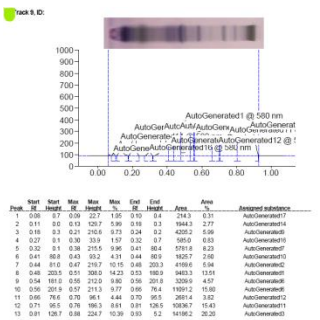


Fig. 15. HPTLC Fingerprint of Cold Pet Eth Concoction

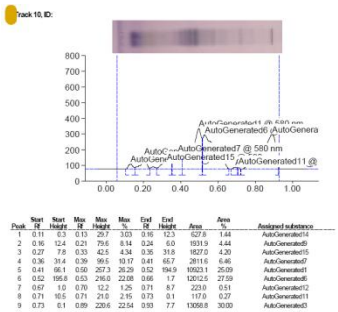


Fig. 16. HPTLC Fingerprint of Cold Chloroform *A. indica*

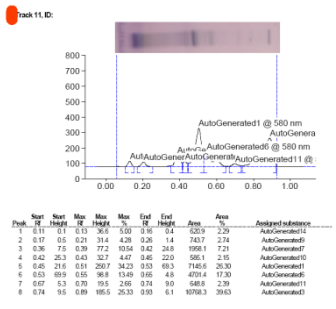


Fig. 17. HPTLC Fingerprint of Cold Chloroform *A. marmelos*

All detected peaks were introduced to spectrum scan. Spectral analysis of the effective extracts indicated presence of multiple phyto-constituents. (Fig. 18)

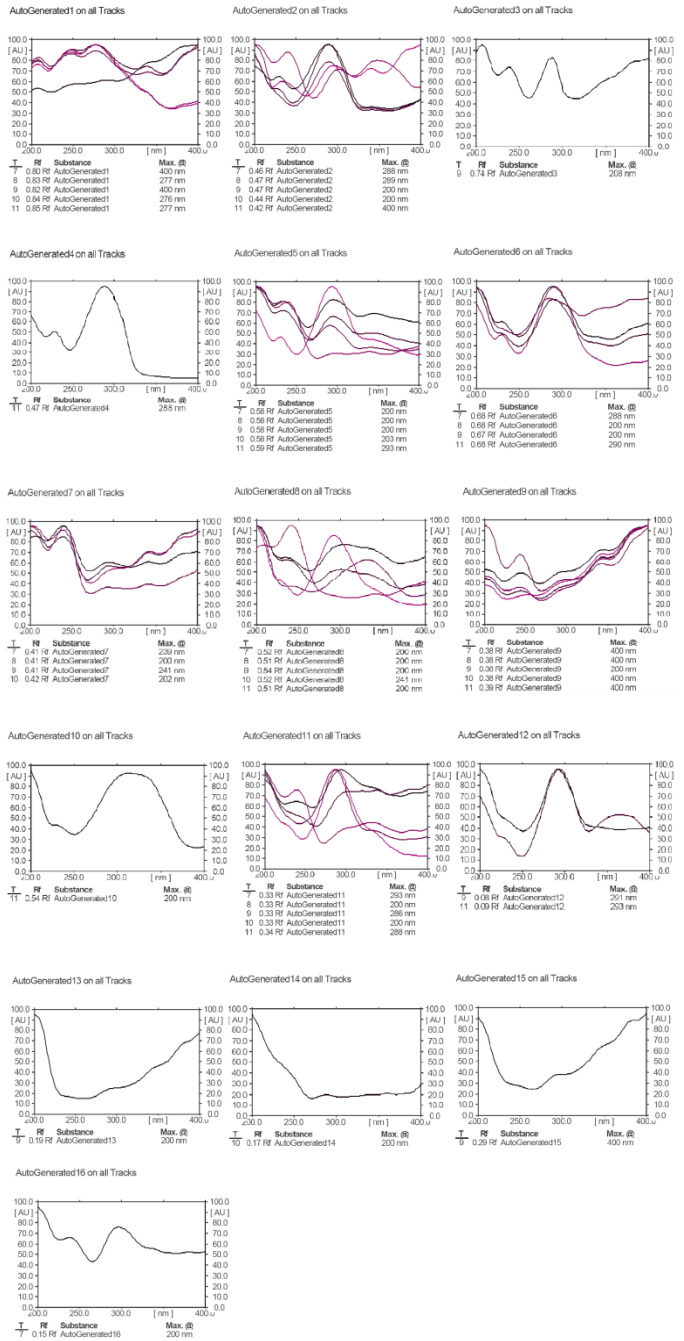


Fig. 18. Spectrum scans of all detected peaks

4. CONCLUSION

This present investigation creates a basic foundation to design a safe and natural drug against seafood and aquaculture bacteria which will be alternative to commonly practiced hazardous chemical drugs. The study showed that commonly available plants *Aegle marmelos, Ocimum sanctum* and *Azadirachta indica* posses potential antibacterial

activity against *A. hydrophila, B. cereus and E. coli* equivalent to the hazardous and banned chemical drug API Furan-2. Digital HPTLC fingerprints of effective extracts were generated which recorded number of phytoconstituents. Since digital scanning fingerprint was intuitively converted from HPTLC image, all the peak intensities are in accord with fluorescent bands and their brightness, so from the results of Fingerprints, the record of R_f values and λmax of each separated band (component) can be used for identification, and characterization of the active phytochemical in the effective extract and can be used in future for: (a) Further Research: The HPTLC fingerprints can be useful in future for further reference for identification, isolation and detection of effective phytochemicals for specific antibacterial activity by Bio-autography.

(b) Commercial production of drug: The effective phytochemicals can be modified and treated so as to develop a commercial product as a safe antibacterial in the aquaculture and seafood industry.

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