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ESSENTIAL OILS AS ANTIMICROBIALS OF FISH PATHOGENS AND STUDY OF CYTOLOGICAL CHANGES INDUCED USING TEM

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ABSTRACT:
Medicinal plant oils of *Cinnamon cassia, Thymus vulgaris* and *Zingiber officinale* traditionally used in India for treating conditions likely to be associated with microorganisms were screened for antibacterial activity against some fish pathogens viz *Aeromonas hydrophila, Pseudomonas aeruginosa, Staphylococus aureus,* and *Bacillus* species using broth microdilution method. Among the essential oils tested thyme and cinnamon showed the most promising antibacterial properties as compared to ginger oil inhibiting all the strains tested with minimum inhibitory concentrations (MICs) ranging from 0.0625 % to 0.25% for thyme oil whereas in case of cinnamon oil MICs were in the range of 0.09375% to 0.1875 %. Among these bacteria *Aeromonas hydrophila* being the common fish pathogen was selected for TEM study. Electron microscopy results demonstrates that thyme and cinnamon oils changes the ultrastructure of *A. hydrophila.* The cytological changes induced in the morphology of *Aeromonas hydrophila* shows the break in a multilayered cell wall when treated with cinnamon oil in an 18 hrs extract-treated culture. This may be due to injury of the cell wall and alterations in the cytoplasmic membrane permeability, resulting in the loss of cytosol and finally in cell death. In case of thyme oil spheroplast was obtained indicating the total disintegration of the cell wall. The test results shows that the essential oils are effective against fish pathogens and would justify its further investigation for alternative to antibiotics.

Key words: - Fish pathogens, essential oil, antibacterial activity, MIC, TEM study

INTRODUCTION:

The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents have lead to the screening of several medicinal plants for their potential antimicrobial activity.

Natural products contribute to a great extent to fight pathogenic microorganisms. Many plants or their parts are used in food as spices and are thought to provide a natural preservation by inhibiting the microbial growth. Varieties of herbs and spices have been used traditionally in food preservation to extend shelf life.

The present study was designed to investigate the antimicrobial properties of some essential oils viz., *Cinnamon cassia, Thymus vulgaris, Zingiber officinale* against *Aeromonas hydrophila, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Bacillus spp.*

The use of higher plants and their extracts to treat infections is an age old practice in traditional Indian medicine. Traditional medical practice has been known for centuries in many parts of the world. It is, however, observed that these practices vary from one country to another. Numerous plants and herbs are used all over India by traditional medicine practitioners. The use of herbs is the most ancient approach to healing known. The herbal medicines may be in the form of powders, liquids, or mixtures, and incisions. Roots, barks, and leaves of various plants are employed in ethonomedicine.

Many investigators have demonstrated the antimincrobial activity of the constituents of some higher plants (Akobundu and Agyakara, 1987; Almagboul et al., 1988; Misra et al., 1992; Hablemariam et al., 1993) and quite a number of chemical compounds of plant origin have been

shown to possess antimicrobial activities (Corthout et al., 1992). In diseases of microbial origin, the plants function as a result of antimicrobial activity against the causative agents (Sofowara, 1993).

This work reports the antimicrobial effect of cinnamon, thyme and ginger essential oils on fish pathogens. This is in pursuance of the efforts to search for drugs from plants and the verification of the scientific basis of some known practices in traditional medicine.

Since the introduction of antibiotics there has been tremendous increase in the resistance of diverse bacterial pathogens due to R plasmid. This shift in the susceptibility greatly affects any treatment procedure.

In the present study we have evaluated the antibacterial efficacy of plant essential oils on fish pathogens as a substitute to age old antibiotic therapy.

MATERIALS AND METHODS

2.1 Bacterial strains

Bacterial strains were isolated from liver, kidney, abdominal fluid, gill and skin of infected fishes. The fishes were collected from six different culture ponds of Nagpur region, a part of central India. Out of 190 diseased fishes analysed 40 were found to be infected by bacterial pathogens. Total 72 bacterial isolates were recovered from infected fishes belonging to the genera of Aeromonas *hydrophila* (26), *Pseudomonas aeruginosa* (13), *Staphylococus aureus* (14), *and Bacillus* species (19).

2.2 *In vitro* antibacterial studies of essential oils against fish pathogens

The bacterial isolates were subjected to drug sensitivity test using essential oils and antibiotics.

Antibacterial agents

Thyme, cinnamon and ginger oils were kindly supplied by Dr. Urjita Jain's Forest Herbals Pvt. Ltd. Mumbai, whereas antibiotics (discs) like Cloxacilin, Tetracycline, Norfloxacin, Erythromycin, Cephalexin and Ofloxacin were supplied by Hi-Media laboratories Mumbai.

Antimicrobial susceptibility studies of thyme, cinnamon and ginger oils

Inhibition of microbial growth was tested by using the paper disc diffusion method (Kirby-Bauer Method; 1966, Drago *et al.,* 1999). For this, sterilized blank Whatman filter paper discs of size 6 mm were used. These discs were impregnated with essential oils for 20 minutes and kept in slanted position so as to drain off excess oil. These discs were later weighed and amount of oil per disc was fixed at 15 mg. For sensitivity testing nutrient agar plates were seeded with each isolate separately and different oil impregnated paper discs were placed on it in the centre aseptically. All the plates were kept in refrigerator for 30 minutes to facilitate diffusion and incubated at 37°C for 24 hrs. After incubation results were noted by measuring zone of growth inhibition in mm on zone reader and average values of three replicates were calculated for each isolate and recorded, Oils showing promising results were assayed for MIC determination using broth microdilution method. Standard aseptic microbiological procedures were followed throughout this antibacterial study.

MIC By Broth Microdilution Method (Finegold and Baron1990)

A series of double dilutions of thyme oil ranging from 4% to 0.0078% was prepared in 96-well microdilution tray. Emulsifier Tween 20 was used to enhance thyme oil solubility. After the addition of 5 µl inocula of *A. hydrophila, P. aeruginosa, S. aureus* and *bacillus* sp. (corresponding cell density to 0.5 Mac Farland scale) from column 1 to 11 (A to H wells) tray was incubated at 37°C for 24 hrs. MIC was determined visually with aid of a reading mirror. MIC was determined as the lowest concentration resulting in no growth. Similar procedure was repeated for cinnamon oil

(6%), where the range of doubling dilution was from 6 to 0.0117%.

Antibiotic sensitivity

For antibiotic sensitivity test, the antibiotics used were Cloxacilin (30 mcg), Tetracycline (30 mcg), Norfloxacin (10 mcg), Erythromycin (15 mcg), Cephalexin 30 mcg), and Ofloxacin (5 mcg). (Plumb *et al,*.1995). Small paper discs (Hi-media, Mumbai) impregnated with known amount of antibiotics were placed on the surface of pre-inoculated Muller-Hinton agar plates (by different bacterial isolates) and incubated at 37°C for 18-20 hours. After incubation, the plates were observed for any zone of inhibition surrounding the disc. A zone of inhibition (a clear zone) around the disc indicated that the organism was inhibited by the drug, which diffused into the agar from the disc.

2.3 Electronmicroscopic studies of thyme and cinnamon oils treated A. *hydrophila*.

Direct examination of bacteria (control) : A few micro liters of sample was taken on the parafilm then placed the carbon coated grid over the sample for 15 min. washed the excess material with distilled water. Stained the grid with uranyl acetate for 15 min. and allowed for drying then observed in transmission electron microscope (Hitachi H-7500 model) at Ruska laboratory, Hydrabad.

Protocol for bacteria incubated/treated with thyme and cinnamon oils :

A few micro liters of bacterial sample was taken on a parafilm in a petridish and mixed evenly with a drop of oil. This mix is incubated for 18 hrs. at 37°C. Then placed the carbon-coated grid over the incubated sample for 15 min. The grid was washed with distilled water to remove the excess material. Stained the grids with 2% uranyl acetate for 15 min. Allowed for drying and observed in transmission electron microscope (Hitachi H-7500 model) at Ruska laboratory, Hyderabad.

RESULT & DISCUSSION:

3.1 *In vitro* antibacterial susceptibility assay of cinnamon, thyme and ginger oils.

A perusal of Table-1 and Fig. 1 enumerates the antibacterial effect of thyme, cinnamon and ginger oils. These results revealed that thyme and cinnamon oils have exhibited strong inhibitory action against *A. hydrophila, P. aeruginosa, S. aureus and Bacillus*, whereas ginger oil was found to be least effective. *A. hydrophila* did not show any inhibition against ginger oil (Figs. 2, 3 and 4).

3.2 Minimum inhibitory concentration (MIC) of thyme and cinnamon essential oils

MIC values of thyme oil for all bacterial isolates ranged from 0.0625 % to 0.25 % whereas in case of cinnamon oil MIC values were in the range of 0.09375 % to 0.1875 % (Table-2) (Figs. 5 and 6).

3.3 Antibiogaram of bacterial isolates

Out of six antibiotics tested against the bacterial isolates recovered from the diseased fishes, in the presents study, it was observed that *A. hydrophila, P. aeruginosa*, and *Bacillus* were sensitive to ofloxacin and Norfloxacin whereas other antiboitics like Tetracycline, Erythromycin, Cephalexin and Cloxacilin showed varying degree of sensitivity. *S. aurues* showed sensitivity to all the antibiotics followed by *P. aeruginosa, A. hydrophila* and *Bacillus*. *A. hydrophila* showed resistance to Cloxacilin, Erythromycin and Cephalexin, whereas *Bacillus* was resistant to only Cloxacilin and Cephalexin (Table-3) (Figs. 7 and 8).

3.4 Electronmicroscopic observations of thyme and cinnamon oils treated *A. hydrophila*.

Exposure of *A. hydrophila* to thyme and cinnamon oils for 18 hrs induces ultrastructural changes in the morphology of the *A. hydrophila.* The cytological changes induced in the morphology of *Aeromonas hydrophila* showed the break in a multilayered cell wall when treated with cinnamon oil. This may be due to injury of the cell wall and alterations in the cytoplasmic

membrane permeability, resulting in the loss of cytosol and finally in cell death(Fig-9 A B). In case of thyme oil spheroplast was obtained indicating the total disintegration of the cell wall(Fig. 9 C).

In the present study effects of thyme, cinnamon and ginger oils were observed against *A. hydrophila, S.aureus, P.aeruginosa and Bacillus sp.* Our data showed that there was no uniform antibacterial response against these pathogens in terms of susceptibility to these oils when compared with the antibiotics like tetracycline, cloxacilin, norfloxacin, erythromycin, cephalexin and ofloxacin. The antibiogram (Table 18 and fig: 34) of bacterial isolates indicate that maximum number of isolates were sensitive to norfloxacin followed by ofloxacin, tetracycline, erythromycin, cloxacilin and cephalexin. Our findings showed that thyme and cinnamon oils were very effective in inhibiting the growth of *A. hydrophila, P. aeruginosa, S. aureus and Bacillus* sp. These findings corroborate with the findings of other research workers. Fabio *et. al.,* (2003) reported inhibition of growth of *A. hydrophila* by cinnamon and thyme oil. Kalemba and Kunika (2003), Valero and Salmeron (2003) reported in-vitro inhibition of growth of *B. cereus* by cinnamon oil. Tabak *et. al.,* (1996) reported alcoholic extracts of cinnamon and aqueous extracts of thyme found most effective in reducing the growth of *Helicobacter pylori.* Alzoreky and Nakahara (2003) reported inhibitory activity against *E.coli* and *S.infantis* by cinnamon cassia extracts at the highest MIC of 2640 mg/ml. Nevas *et al.,* (2003) reported antibacterial activity of thyme by inhibiting the growth of *Clostridium botulinum* and *Clostridium perfringes.* Fan and Chen (2001) reported ethanol extracts of thyme, and thyme essential oil inhibit the growth of *Bacillus subtilis, S. sonnei,* and *E. coli.* Juven et al (1994) reported thyme essential oil decreases viable counts of *S.typhimurium* on nutrient agar.

The essential oil of *Z.officinale* showed antimicrobial activity against gram-positive and gram-negative bacteria (Martins *et al.,* 2001; Habsah *et al.,* 2000; Srinivasan *et al.,* 2001). Mahady *et al.,* (2003) reported the crude extract containing gingerols, inhibited the growth of all strains of *H. pylori* with an MIC range of 0.78 to12.5 µg/ml. Akoachere *et al.,* (2000) reported the extracts of ginger exhibited antibacterial activity against the pathogens *S. aureus, S pyogenes, S. pneumoniae* and *H. enfluenza.* In the present study *Aeromonas hydrophila* shown the most resistant against ginger oil, where as *P. aeruginosa, S. aureus* and *Bacillus* sp. showed least sensitivity to ginger oil. Similarly Ahmad *et.al.*, (1998) also reported that *P.aeruginosa, S. aureus* and *bacillus subtilis* were least sensitive to ginger oil. There was no antibacterial activity in extracts of *Z. officinale* against the *E. faecium, S. typhimurium* and *E. coli.* Our results are contradictory with some researchers who reported antibacterial activity of *Z. officinale* against gram positive and gram negative bacteria (Habsah *et al.,* 2000; Martins *et al.,* 2001)

From the present study it was concluded that the thyme and cinnamon oils showed greatest inhibition against all bacterial fish pathogens by disc diffusion method. The minimum inhibitory concentration of these oils was carried out by microdilution method. The observations showed that the MIC value for thyme was in the range of 0.0625% to 0.25% where as in case of cinnamon it was in the range of 0.09375% to 0.1875%.

As *Aeromonas hydrophila* is the most commonly found pathogen of all the fishes, more detailed study was carried out to investigate ultrastructural changes induced by thyme and cinnamon oils in it. This is the first report of such activity. Our results demonstrates that thyme and cinnamon oils changes the ultrastructure of A. hydrophila. The cytological changes induced in the morphology of *Aeromonas hydrophila* showed that the normal trilaminated cell wall structure

became irregular. There was break in a multilayered cell wall when treated with cinnamon oil. This may be due to injury of the cell wall and alterations in the cytoplasmic membrane permeability, resulting in the loss of cytosol and finally in cell death. In case of thyme oil spheroplast was obtained indicating the total disintegration of the cell wall. Similar observation were recorded by Carson. *et al.,* (2001) while studying the action of *Melaleuca alternifolia* oil (Tea Tree oil) on *Staphylococcus aureus.* He observed membrane damage by the appearance of mesosomes and the loss of cytoplasmic material and Daud *et al.,*(2005) while studying antimicrobial properties of *Phrygilanthus acutifolius* on *Staphylococcus aureus.* He observed alterations in the cytoplasmic permeability, resulting in the loss of cytosol and finally in cell death.

CONCLUSIONS:

Data from this study support the hypothesis that essential oils of cinnamon and thyme exert antibacterial action against *Aeromonas hydrophila, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus* sp.

TEM studies carried out on *A. hydrophila* treated with thyme and cinnamon oils showed not only disintegration of cell wall but also formation of spheroplast thus confirming its antibacterial efficacy.

In vitro studies prove the notion that plant essential oils may have a role as pharmaceuticals and preservatives.

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TABLE-1: Antibacterial effect of essential oils against fish pathogens.

Fish pathogens	Essential oils Zone of inhibition in mm		
	Thyme oil	Cinnamon oil	Ginger oil
A. hydrophila	31	27	R
P.aeruginosa.	27	25	11
S. aureus	29	22	11
Bacillus.sp.	29	28	10

R- Resistant

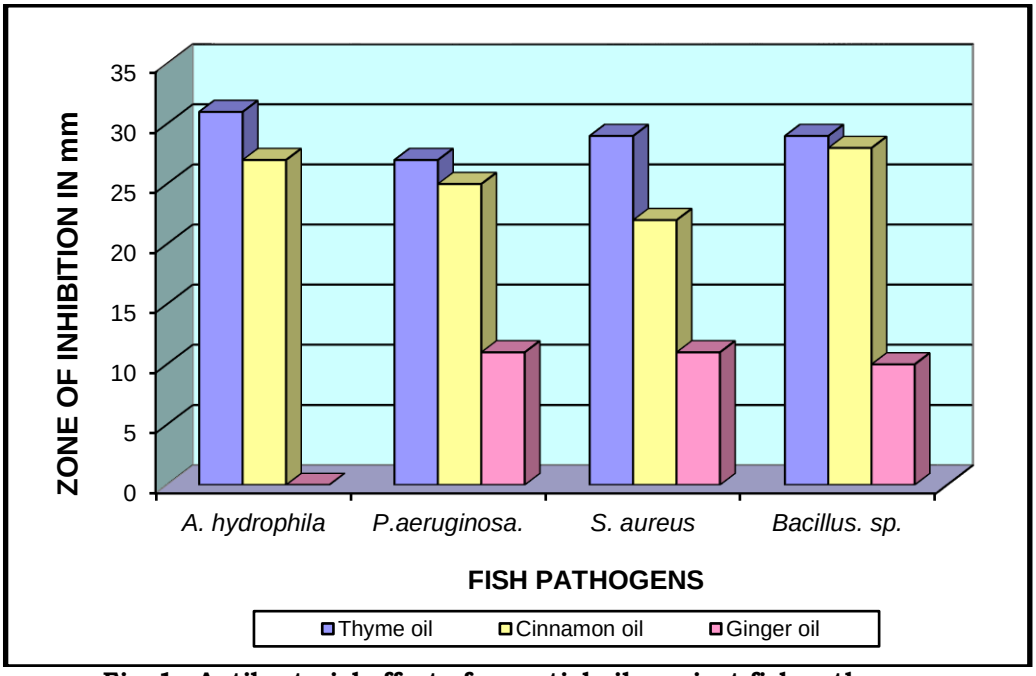

Fig. 1 : Antibacterial effect of essential oils against fish pathogens

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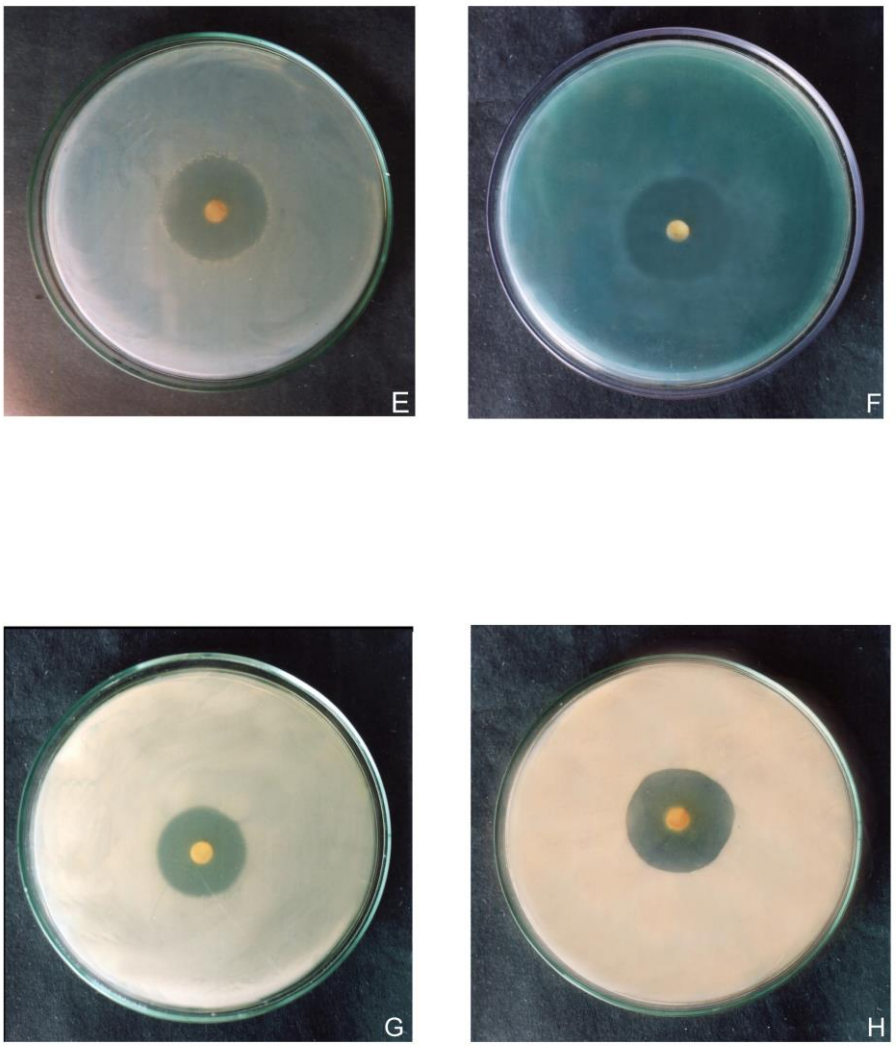


Antibacterial activity of cinnamon oil on-
E-*A.hydrophila*
F-*P.aeruginosa*
G-*S.aureus*
H-*Bacillus sp.*

FIGURE : 2

TABLE 2 : Broth microdilution assay of thyme and cinnamon oils

Fish pathogens	MIC(%v/v)	
	Thyme oil	Cinnamon oil
S. aureus	0.125	0.09375
P.aeruginosa.	0.0625	0.1875
A. hydrophila.	0.0625	0.1875
Bacillus sp.	0.25	0.09375

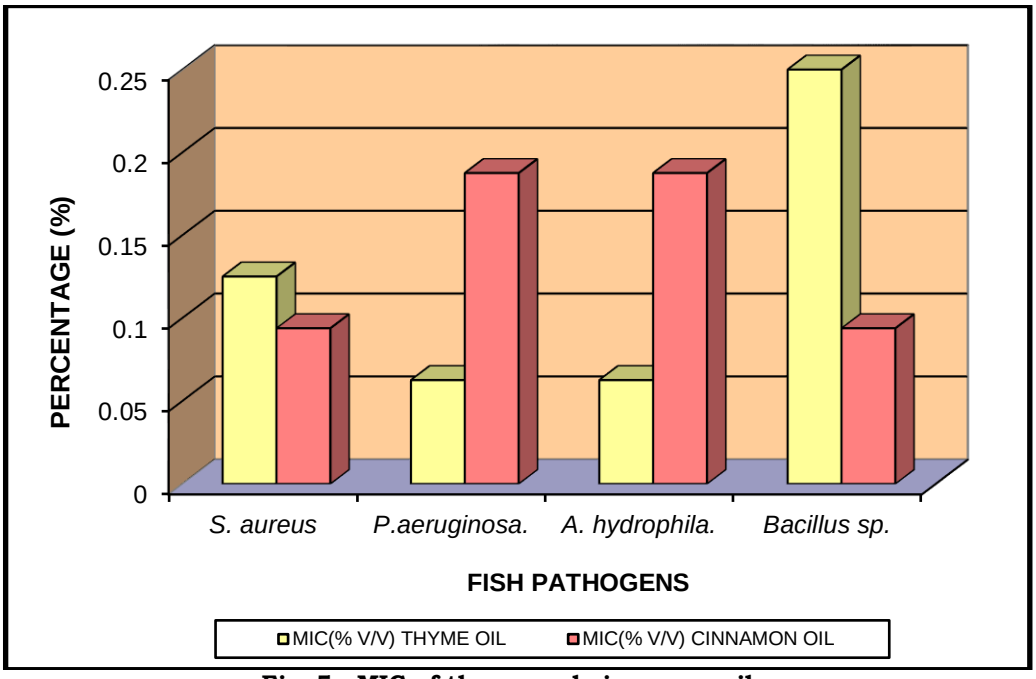
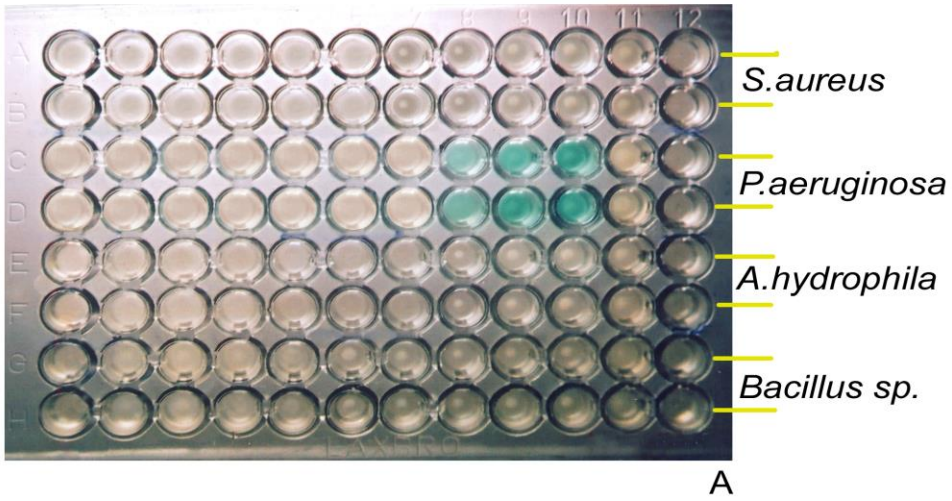
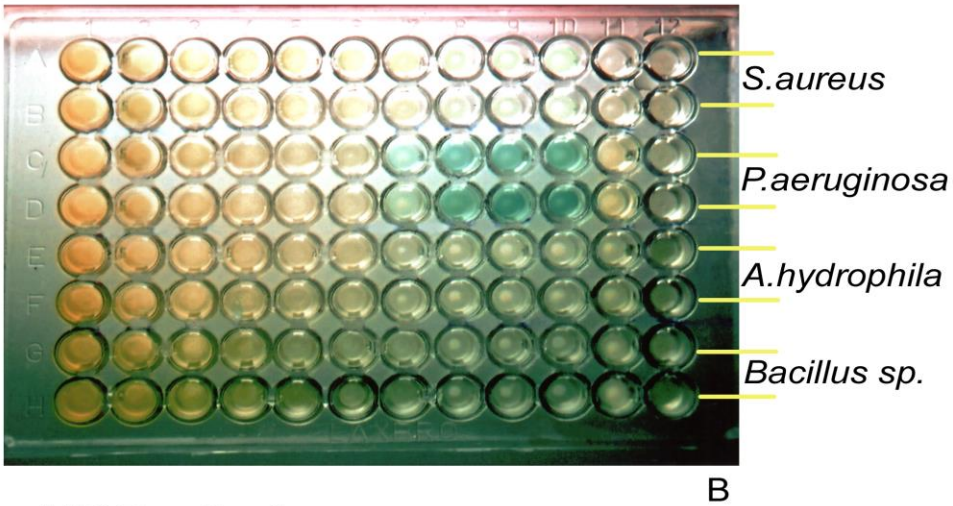


Fig. 5 : MIC of thyme and cinnamon oils.



A



B

MIC Results of
A - Thyme oil
B - Cinnamon oil

FIGURE : 6

TABLE-3 : Antibiogram of fish pathogens

Antibiotic		Fish pathogens Zone of inhibition in mm			
		A.hydrophila.	***P.aeruginosa***	***S.aureus.***	***Bacillus.sp.***
Cloxacilin	30 mcg	R	R	40	R
Tetracyclin	30 mcg	17	R	36	18
Norfloxacin.	10 mcg	19	17	10	26
Erythromycin.	15 mcg	R	R	29	20
Cephalexin.	30 mcg	R	R	14	R
Ofloxacin.	5 mcg	20	10	10	22

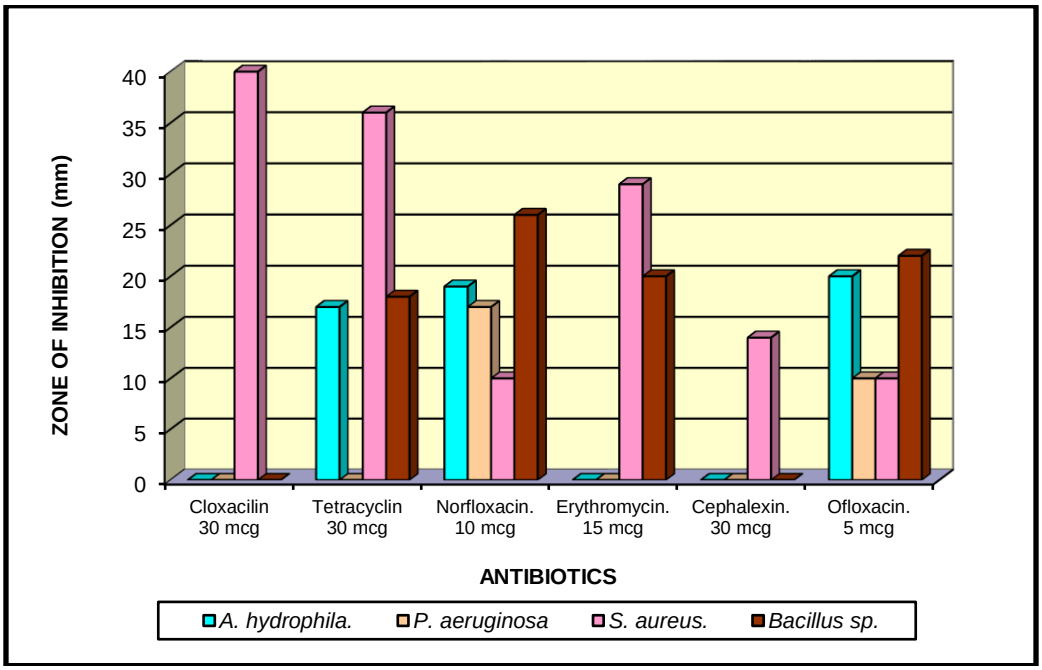
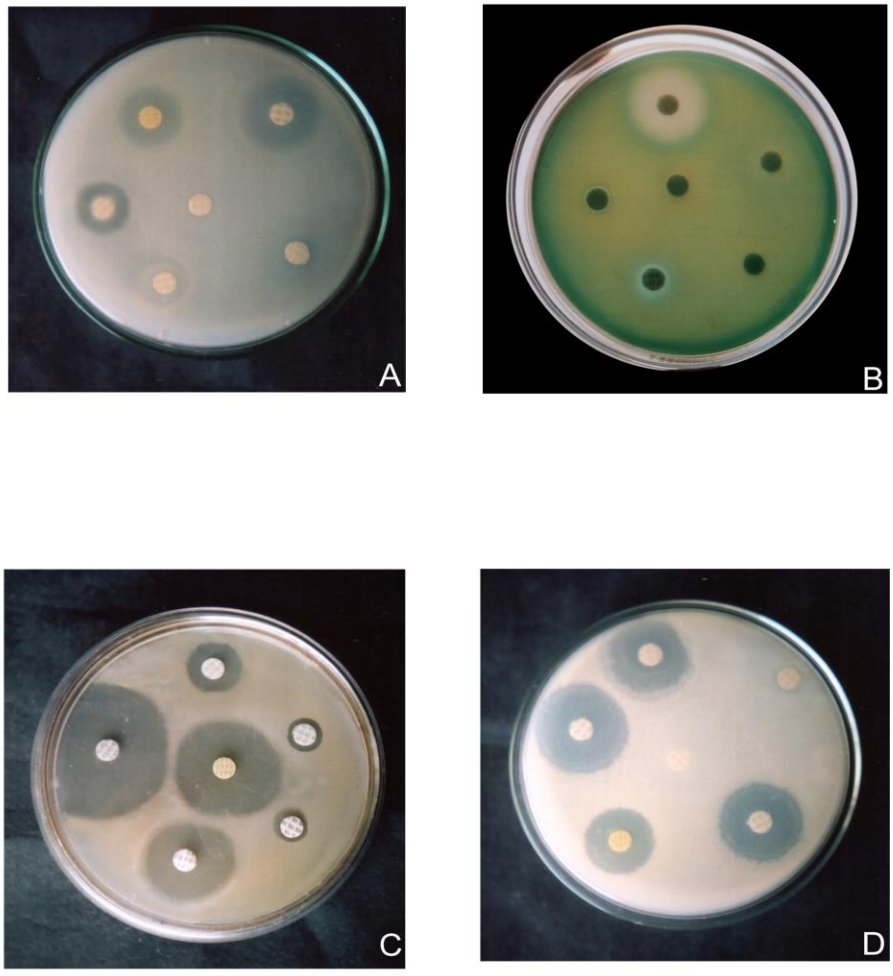


Fig. 7 : Antibiogram of fish pathogens



Photograph shows antibiotic sensitivity on-
A-*A.hydrophila*
B-*P.aeruginosa*
C-*S.aureus*
D-*Bacillus sp.*

FIGURE : 8

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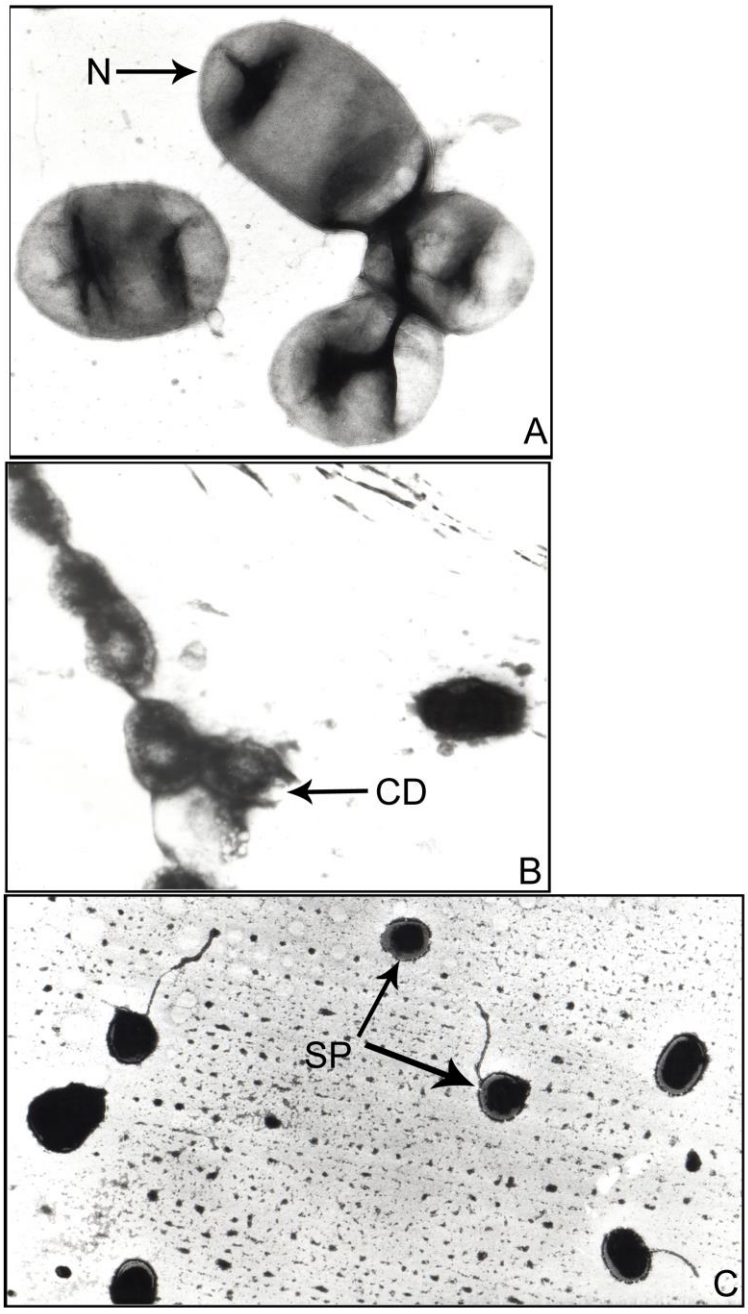

N-Normal,CD-Cellwall disintegration,SP-Spheroplast

Transmisson electron micrograph of
A-Normal (Control) *A.hydrophila* (15000x)
B-Cinnamon oil treated *A.hydrophila* (12000x)
C-Thyme oil treated *A.hydrophila* (3500x)

FIGURE : 9