

Full Length Research Paper

Anti-bacterial activity of *Acorus calamus* and some of its derivatives against fish pathogen *Aeromonas hydrophila*

R. Bhuvaneswari and Chellam Balasundaram*

Department of Animal Science, Fish Disease Diagnostic Laboratory, School of Life Science, Bharathidasan University, Tiruchirappalli, Tamilnadu, India.

Accepted 21 June, 2007

Eighteen compounds isolated from the residue of *Acorus calamus* were subjected to micro titer assay to find out their inhibitory concentrations (IC) against *Aeromonas hydrophila*. The antimicrobial potency of the substances was ranked through bioautographic assay. The second compound (F2) obtained in the third fraction, on serial dilution, exhibited the maximum antimicrobial property with IC₉₀ values of 1.6, 0.8, 0.26 and 0.13 mg/ml respectively. The chemical structure of the active compound in F2, derived from calamus oil was predicted through detailed spectroscopic analytical procedures like ¹HNMR, ¹³C NMR, GC-MS, UV and IR spectroscopy and found related to β Asarone.

Key words: *Acorus calamus*, compounds isolation, *in vitro* screening, *Aeromonas hydrophila*, structure determination, essential oil fraction.

INTRODUCTION

Aquaculture, through having a great potential to augment the increasing demand for aqua products, is traversed by the problems in fish disease management. This is evidenced by a host of diseases like motile aeromonad septicemia (MAS) and epizootic ulcerative syndrome (EUS) with a cosmopolitan distribution that have sliced down the harvest size in captive and feral fisheries. In aquaculture industry the intensification of fish farming often leads to the emergence of infectious and parasitic diseases (Thampuran et al., 1995) that renders disease management a critical factor hampering the development of fish culture in many countries. Among the various diseases, those caused by the pathogenic bacteria represent the gravest threat to aquaculture (Davis and Hayasaka, 1983). Fishes are vulnerable to a wide variety of bacterial diseases like hemorrhagic disease, erythrodermatitis, enteric redmouth disease and epizootic ulcerative syndrome (Jeney and Jeney, 1995) of these ulcerative dermatitis caused by the ubiquitous gram negative bacteria *Aeromonas hydrophila* (Thune et al., 1993) is of prime importance either as a primary (Palumbo et al., 1992) or

secondary pathogen (Grizzle and Kirya, 1993) in several countries.

In fish disease management synthetic chemicals and antibiotics have been used for the last 30 years or so at least with partial success (Stoskopf, 1993). Of the drugs and antibiotics used in the disease management malachite green, formalin, terramycin, potassium permanganate and Romet 30 are widely recommended (Rukyani, 1994) though complete disease control remains elusive to the fish farmer. These therapies will remain as one of the main means of controlling transmissible diseases in aquaculture. Today, pharmaceutical companies produce more than 160 different antibiotics to combat bacterial infection (Service, 1996). However, bacteria have developed resistance, either by mutations or by acquiring new genes from other bacteria, to these molecular targets of macromolecular biosynthesis targets. Hence many existing antibiotics have been modified to yield new and more potent derivatives. Introducing analogues of existing drugs, however, provide only temporary solutions to the problem of resistance because existing resistance mechanisms often rapidly adapt to accommodate the new derivatives (Bax et al., 2000; Hancock et al., 1998; Breithaupt, 1999). Besides most of the drugs and chemicals used in aquaculture have residual effects on the aquatic environment, while some of them used are known to accumulate and

*Corresponding author. E-mail: bal213@rediffmail.com. Tel.: +91-431-2407045 or +91-431-2407040.

get magnified in higher trophic levels (Mitchell and Plumb, 1980). Few vaccines are presently available as a panacea in disease prevention; but their efficacy is questioned in several cases, at least under practical field conditions (Knowles, 1997). However till date no natural medicine to protect farmed fish against *A. hydrophila* infected are commercially available (Moral et al., 1998; Santos et al., 1996) a few studies report the efficacy of treating with herbs.

Since origin of human's life, plants continue to play a curative and therapeutic role in preserving human health against disease and decay. The widespread use of herbal remedies and healthcare preparations, such as those described in ancient texts like the Vedas and the Bible have been traced to the occurrence of natural products with medicinal properties (Nair et al., 2004; Stuffness and Douros, 1982). In this context, India being a subtropical country is a good repository of plants that are widely used in the preparation of herbal therapies. Application of phytotherapy in fish disease management is gaining importance since plant byproducts are ecofriendly, biodegradable and little side effects.

Among the Indian herbs plants like *Azadirachta indica*, *Oscimum sanctum*, *Curcuama longa* have been widely investigated for their antimicrobial properties with reference to fishes and animals (Harkrishan et al., 2005); while there is a need to identify new herbs that could be gainfully applied in fish disease management, the isolation of active compounds with reference to their antimicrobial properties also has to be attempted. *Rhizomes of Acorus calamus* Linn used for the present study are utilized extensively by the Chinese, Indians and American Indians as well as by other cultures including in Thai tradition (Anonymous, 2000) even to day (Motley, 1994). The ethanol extract of this plant possesses very strong antibacterial activity against *A. hydrophila* (Bhuvanewari and Balasundaram, 2006). Therefore, the antibacterial property of the rhizome oil of *Acorus calamus* tested against the in vitro growth of the fish pathogen *A. hydrophila* is reported.

MATERIALS AND METHODS

Microorganisms used and growth media

The reference strain *A. hydrophila* (MTCC code no-646) was purchased from the Institute of Microbial Technology, Chandigarh, India and maintained in the laboratory under standard conditions. Subcultures were maintained on *Tryptone soy agar* (Hi media) slopes at $28 \pm 32^{\circ}\text{C}$ and periodically checked for their pathogenicity on the basis of ulcer occur in the animal model when infect with the same CFU value (Scharperculus, 1991; Chabot and Thune, 1991).

Plant material and extraction

A. calamus Linn is commonly known as sweet flag is an aromatic medicinal plant belonging to the Araceae family. The fresh rhizomes of *Acorus calamus* were collected from Bharathidasan Univer-

sity campus, Trichirappalli, Tamil Nadu India. A part of the plant material was washed under running tap water. Small hairs of *A. calamus* were removed and could be chopped and dried at low temperature. And then homogenized to fine powder and stored in airtight bottles. Powder of rhizome extracted with 90% w/w ethanol using a Soxhlet apparatus. The ethanol was removed under pressure using a rotary evaporator. The dried residue of the crude extract was stored in a dark bottle at 4°C in airtight bottles for further studies (Nair et al., 2005).

Isolation of components

The residue of *A. calamus* was submitted to chromatography over silica gel (32 g) eluted with a gradient system of increasing polarity (hexane, dichloromethane, ethyl acetate and methanol). Such as ethyl acetate 10% in Hexane (10:90)-F1, ethyl acetate 20% in Hexane (20:80)-F2, ethyl acetate 30% in Hexane (30:70)- F3, ethyl acetate 40% in Hexane (40:60) - F4, ethyl acetate 50% in Hexane (50:50) -F5, ethyl acetate 60% in Hexane (60:40)-F6, ethyl acetate 80% in Hexane (80:20)-F7, ethyl acetate 90% in Hexane - F8, ethyl acetate - F9, Dichloromethane 20% in ethyl acetate (20:80)-F10, Dichloromethane 40% in ethyl acetate (40:60) - F11, Dichloromethane 45% in ethyl acetate (45:55) -F12, Dichloromethane 60% in ethyl acetate (60:40)- F13, Dichloromethane 85% in ethyl acetate (85:15)-F15, Methanol 25% in Dichloromethane 75% (25:75) F-15, Methanol 35% in Dichloromethane 65% (35:65)-F16, Methanol 95% in Dichloromethane (05:95)-F-17 and Methanol-F18 to gave Eighteen fraction F2 (8.1mg),F3 (32.1mg),F4 (17.6 mg), F5 (21.3 mg), F6 (19.6 mg), F8 (74.5 mg),F9 (8.1mg), F10 (28.7mg), F11 (36.8mg), F12 (17.1mg), F13 (16.3mg), F14 (12.3mg), F15 (29.5mg), F16 (59.5 mg), F17 (47.8 mg), F18 (17.3 mg).

In vitro screening of isolated compounds from *Acorus calamus* determination of the antibacterial activity

The Minimal inhibitory concentrations (MICs) of all compounds were determined by micro dilution techniques in Mueller- Hinton broth (Hi Media). Inoculum was prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard [2.7×10^3 colony forming units (CFU)/ml] and diluted 1:5 for the broth micro dilution procedure. Micro titer plates were incubated at 30°C and the MICs were recorded after 24 h of incubation. Two susceptibility endpoints were recorded for each isolated. The MIC was defined, as the lowest concentration of compounds at which the microorganism tested did not demonstrate visible growth.

Thin layer chromatography and bioautography

Chromatography plates used were pre-coated with silica gel (0.2 mm Merck) and glass - backed. The samples were loaded on the plates in bands. They were developed in duplicates in selected solvent system for each of the *A. calamus* extract. After air-drying overnight, each of the plates was placed in a humid chamber and overlaid with 10 ml molten nutrient agar seeded with 0.2 ml of *A. hydrophila*. Adequate humidity was maintained by placing moist cotton buds at the corners of the plates. The overlaid plates were left for 30 min after which they were incubated at 32°C for 24 h. The cultures were sprayed with an aqueous solution of a dehydrogenate indicator - 2.5 mg/ml thiazolyl blue (methyl thiazoyl tetrazolium chloride). The plates were further incubated at 32°C for four hours and thereafter sprayed with absolute ethanol to kill the test organisms in order to conserve the bioautography plates. The plates were allowed to air dry and covered with plastic plates. A reference plate (not overlaid with the culture medium) sprayed with



Figure 1. Physicochemical properties of calamus oil.

Physicochemical properties: Appearance = Slightly viscous liquid; Color = Brownish yellow; Odor = Fragrant /aromatic smell; Solubility= Soluble in MeOH.

vanillin / sulphuric acid was compared with each of the bioautography plates to ascertain the location of the active constituents of the extracts. The next day the inhibition zones were noted (Rohalison et al., 1991).

Chemical analysis of essential oils

The obtained essential oil was washed with NaCl solution, dried on sodium sulfate and evaporated under vacuum in a rotary evaporator. Gas chromatography coupled with mass spectrometry was used to identify the main volatiles released by each essential oil. GC-MS analysis was performed using a Perkin-Elmer Turbomass system with a split-split less PSS injector and a fused-silica capillary column (30 m by 0.32 mm i.d.) with a thick methylsilicone coating (4 m). The carrier gas was 99.999% helium at 1.5 ml/min flow for the 10 m column length. The column temperature program was 5°C/min from 70 to 250°C. Total ion chromatograms and mass spectra were recorded in the electron impact ionization mode at 70eV. The transfer line and the source temperature were maintained at 150°C.

Isolation of Beta - Asarone

Stream distillation of *Acorus calamus* rhizomes gave calamus oil (1.7% w/w), which after column chromatography on a silica gel column with hexane/ethyl acetate (99:1 to 90:10) provided 1 (82% w/w) as pale yellow liquid (R_f 0.39 on silica gel TLC plate in 4% ethyl acetate in hexane) and its spectral data agreed well with reported literature value.

Structure determination

It was determined by detailed spectroscopic analysis viz $^1\text{H NMR}$, $^{13}\text{C NMR}$, GC-MS, UV and IR spectroscopy.

RESULT

A. calamus fractions were isolated on the basis of stand-

ardized Eluent profile determined by solvent polarity scale (Dandiya, 1959; Dandiya, 1959b). Of the 18 isolated compounds subjected to bioautographic assay F2, F3, F8, F9, and F15 compounds showed zones of inhibition (Figures 3 and 4); of these F2 had the highest inhibitory zone (Figure 3). All the serially diluted 18 compounds tested for the antimicrobial activity except F2 exhibited growth of *A. hydrophila* in the micro titer assay. The F2 compound showed antimicrobial activity up to fourth dilution (Table 1). At the least concentration of 0.13×10^4 mg/ml, F2 effectively inhibited the growth of the pathogen *in vitro*. We isolated F2 compound using column chromatography, characterized it using its R_f values (R_f 0.39 on silica gel TLC plate in 4% ethyl acetate in hexane) and identified (Fiancis, 1981) it as essential oil (Figure 1). The oil was further fractionated to obtain a pale yellow β -Asarone (R_f value 0.63 on silica gel TLC plate). Spectrum analysis study was carried out to determine the structure of the fraction; the structure obtained was analyzed (soft ware – Chem.draw ultra 6.0) to predict the structure.

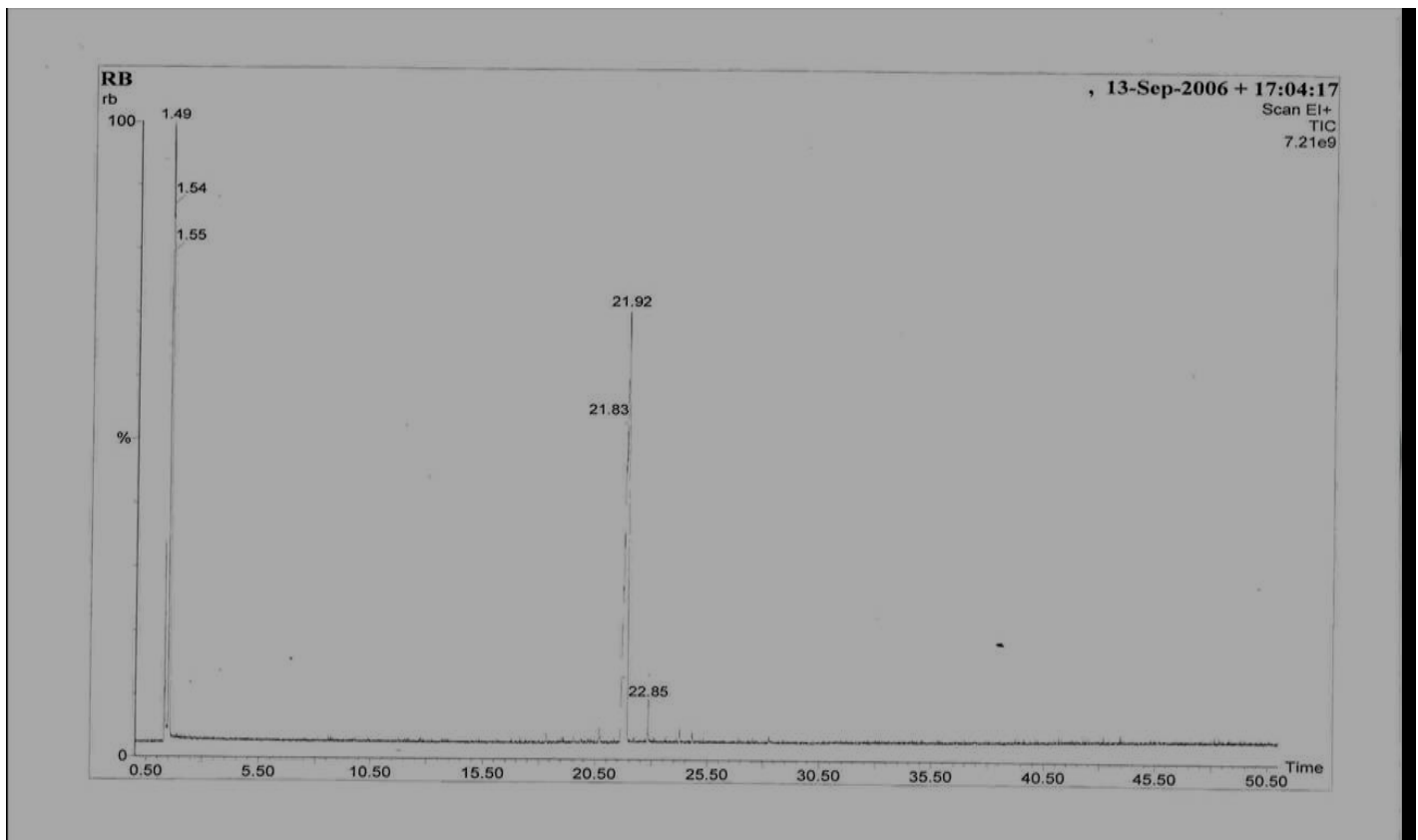
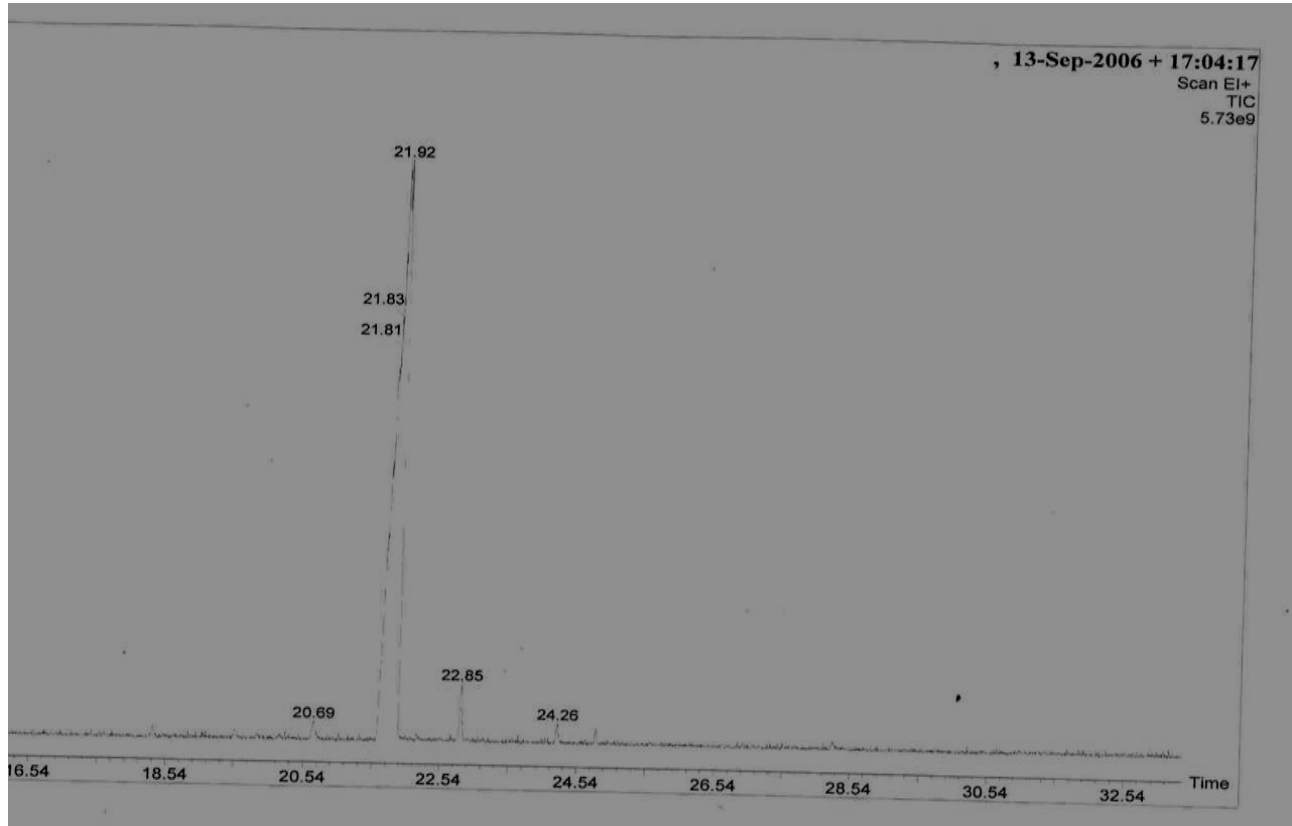
Figure 1 shows the chromatographic plate revealed by the Vanillin / sulphuric acid color reagent. The microbial inhibition zones exhibited by the *A. calamus* are shown in Figure 2.

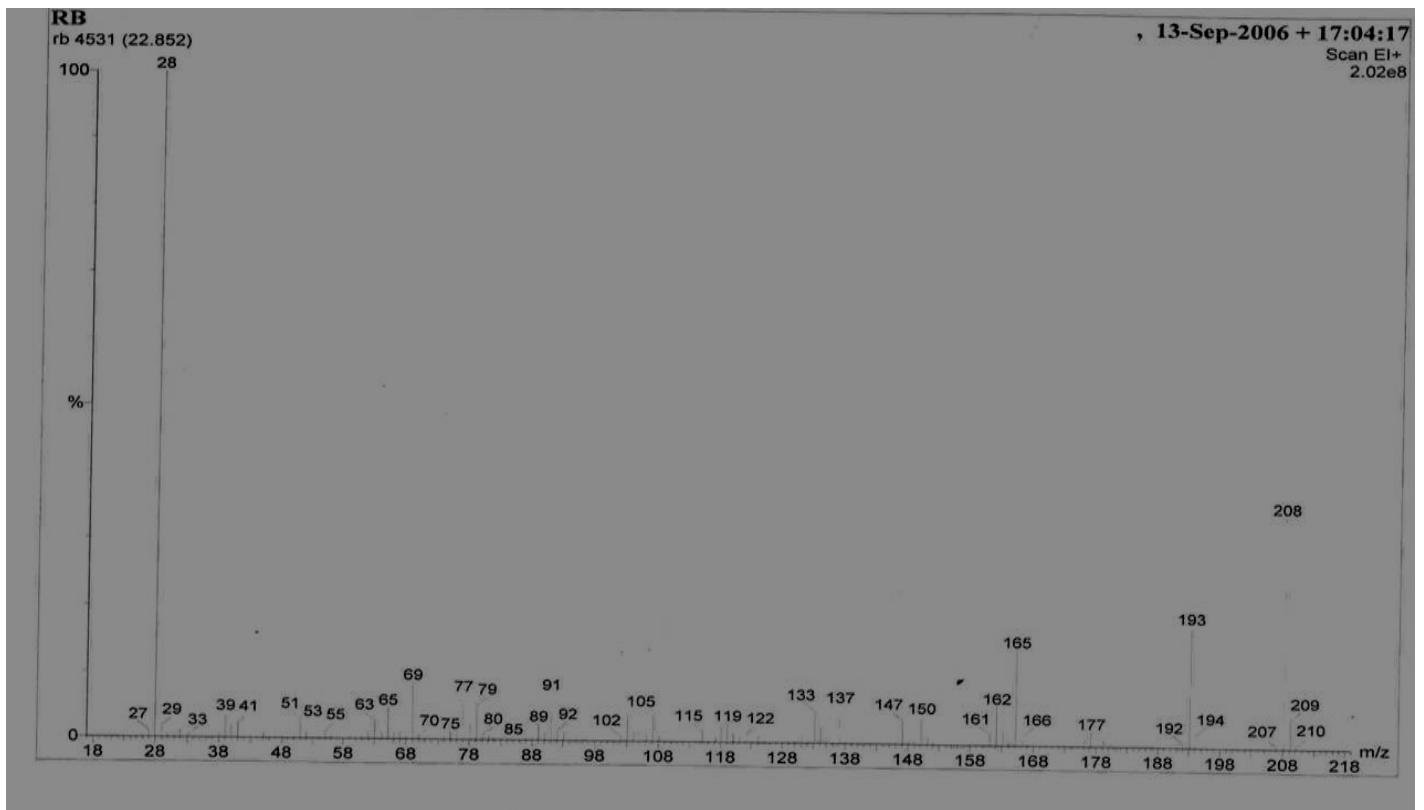
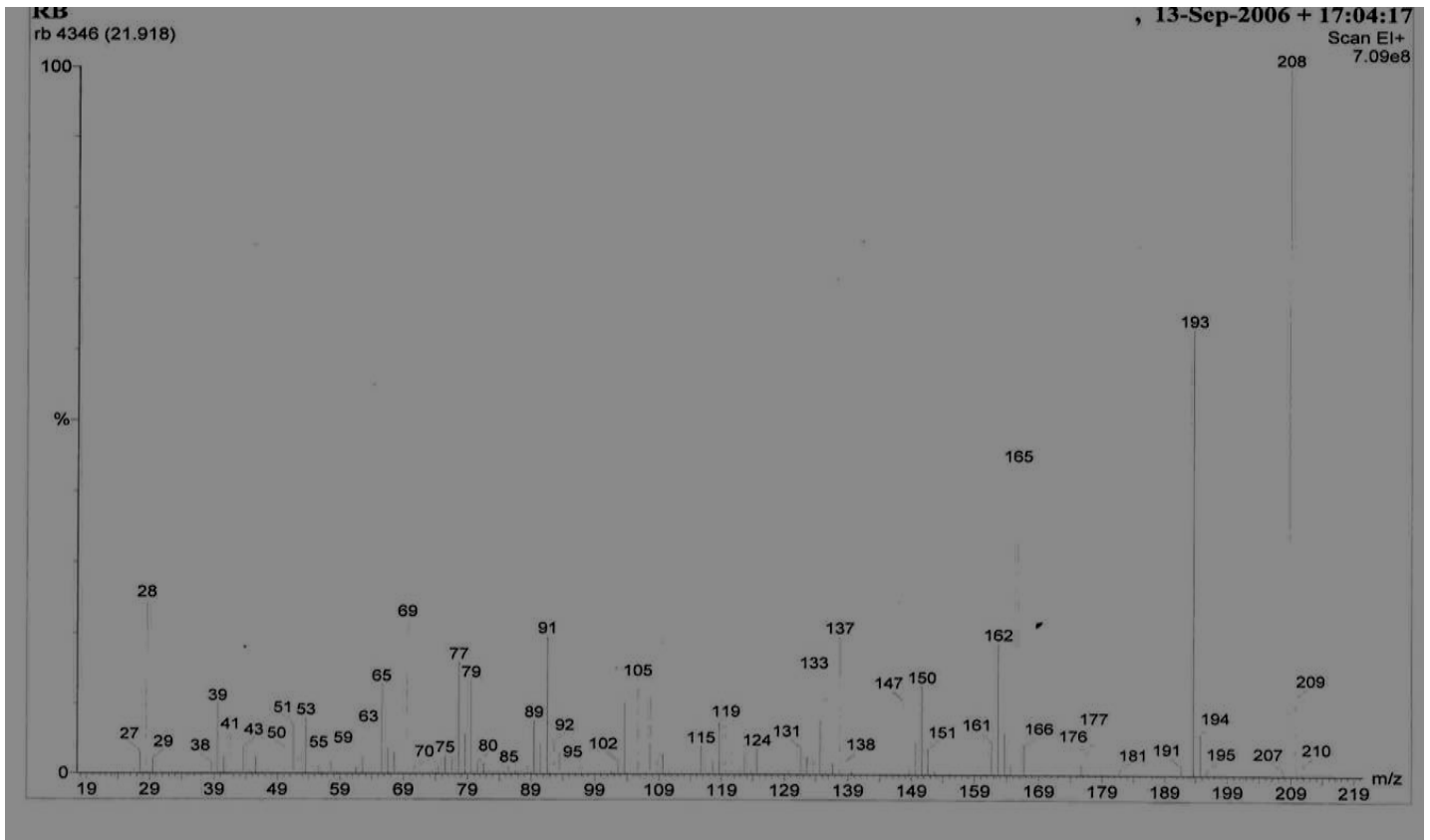
Structure determination

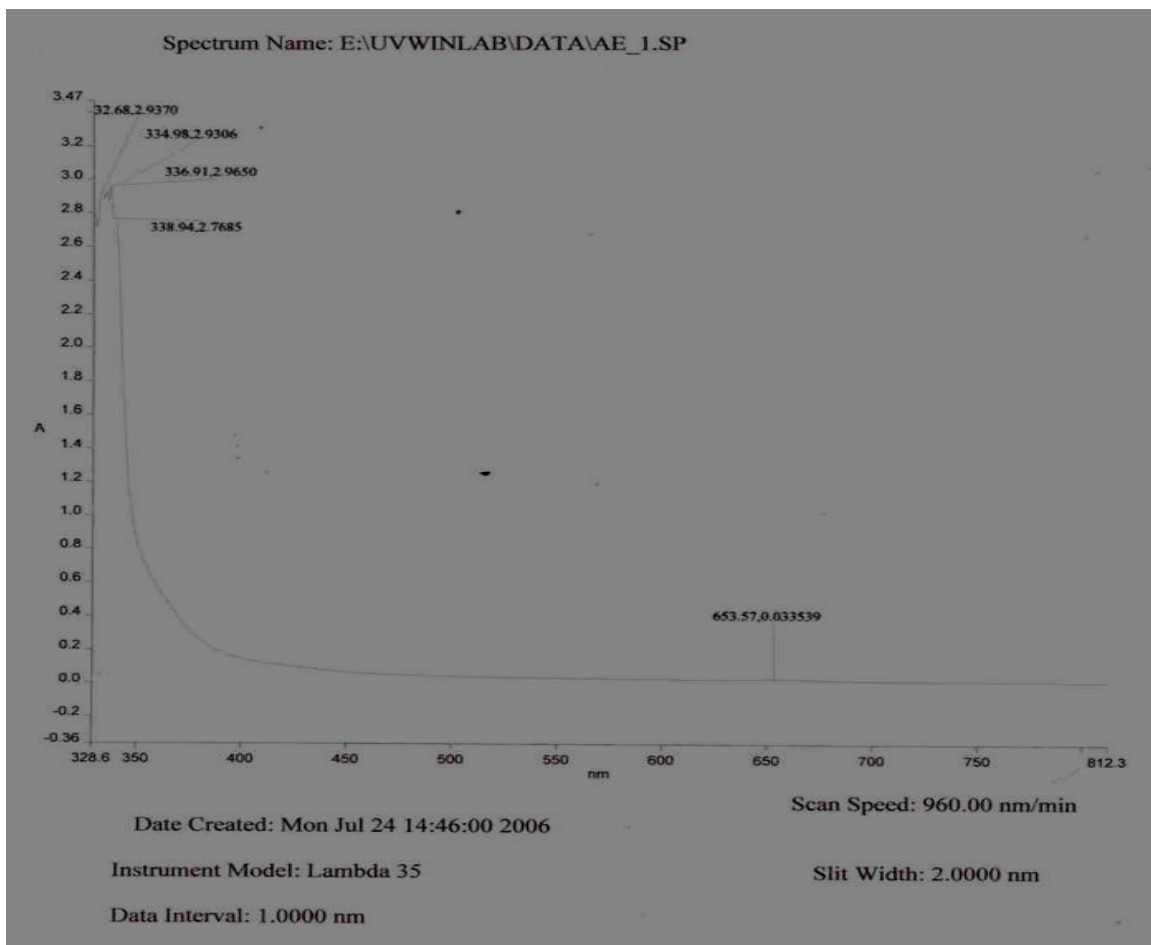
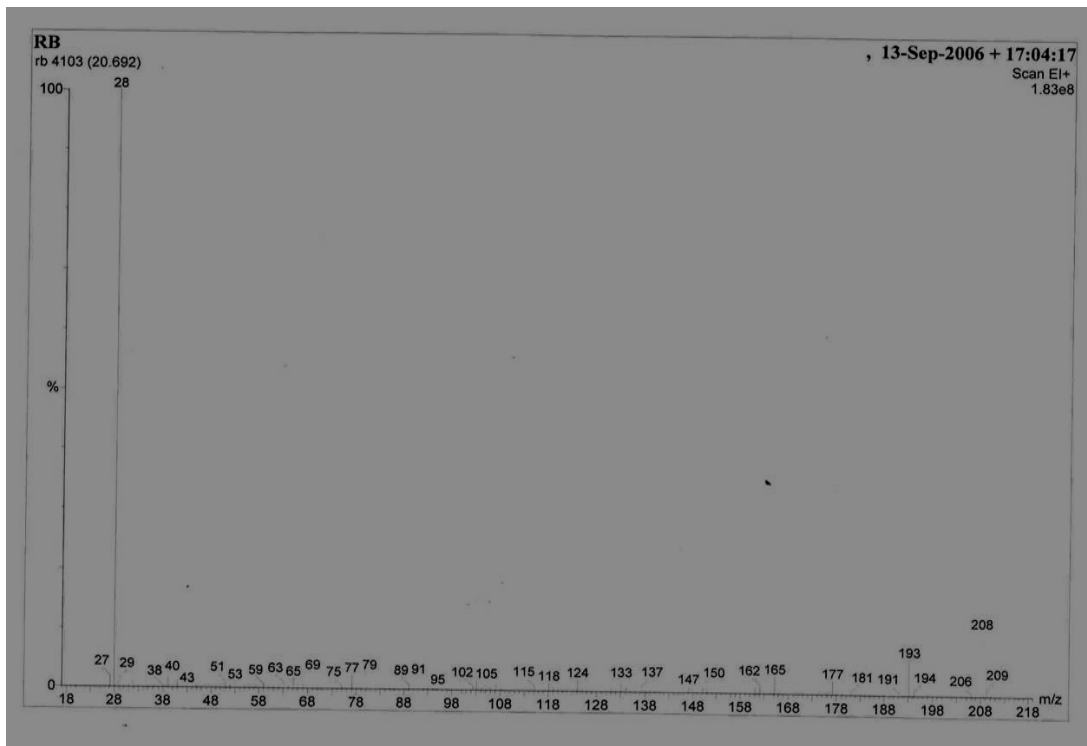
Mass spectrum gave molecular weight (ion) - 280 ion molecule at m/e 208(M+,100),193(M.sup.+Me,46),165 (M.sup.+ - C₃H.sub.7,24) - suggesting the molecular formula C sub .12 H sub 16 O sub 3 in good agreement of with the observation of 12 signals with peak at Delta 125.5 (C - 1'), 124.7 (C - 2') and 14.5 (C - 3') for side propenyl group and rest carbons at 151.4 (C - 2), 148.5 (C - 4), 142.3 (C - 5), 118.0 (C - 1), 114.1 (C - 6), 97.6 (C - 3) and 56. 5, 56.2 and 55.9 (3 - OCH .SUB.3) on the Sup.13 C NMR (CDCl sub.3 .75.4 MHz). The IR spectrum showed strong absorption at 1608 cm. sup. - 1, indicating the presents of an olefinic group and rest are at 2937, 2835, 1583,1512, 1465, 1321, 1213, 1119, 1037, 965 and 859 cm sup. -1H NMR (CDCl. Sub.3, 300 MHz) signals showed add at. Delta.6.50 (1H, J = 15.8 Hz and 1.5 Hz, H- 11) , dq at 5.78 (1H, J = 6.5 Hz and 15.8 Hz, H- 21) , and 1.85 (3 H ,dd, J= 6.5 Hz and 1.5Hz, H - 3 1 due to proton of propenyl side chain and rest protons at 6.84 (1H, s, H- 6), 6,53(1 H,s,H-3), and 3.88, 3.83 and 3.79 (s, 3H, each,3-OCH.sub.3).UV.Lambda. Max MeOH nm (log.epsilon,); 269 (2.36), 301 (4.01).

DISCUSSION

A number of herbs have been assessed for their antimicrobial property with reference to *A. hydrophila*. Of the five herbs tested *Acorus calamus* extract has the highest inhibitory activity *in vitro* (Bhuvaneshwari and Balasundaram, 2006). In koi carp infected with *A. hydrophila* short bath treatment heals the lesion and restores the







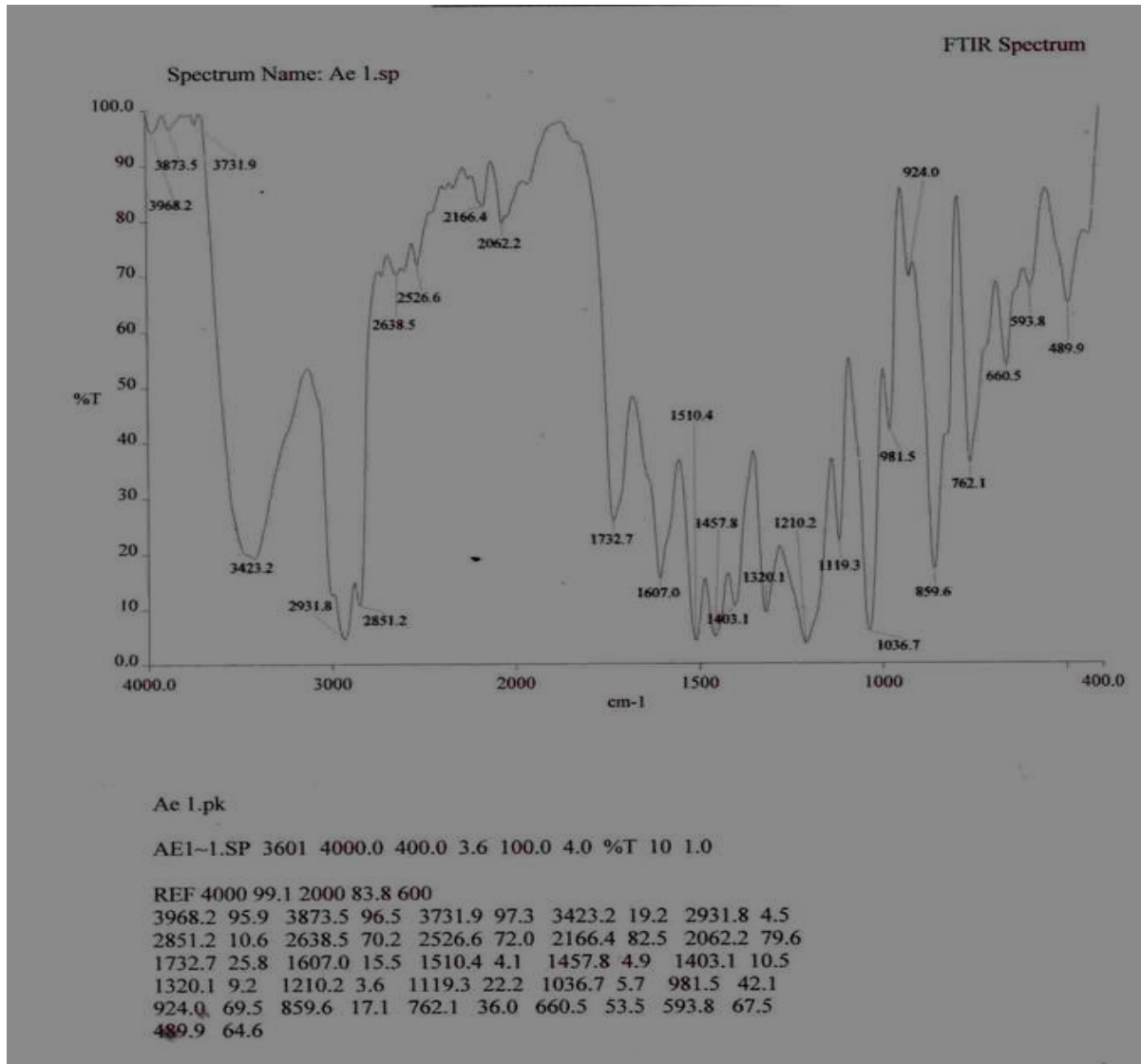


Figure 2. Spectra for calamus oil - β -Asarone.

hematological and biochemical parameters in 15 days (MS under preparation).

The *A. calamus* rhizomes are considered to possess anti-bacterial, anthelmintic properties and also used for treatment of chronic diarrhea, dysentery, bronchial catarrh, intermittent fevers and tumors (Chopra, 1957; Baxter, 1960). The extracts of *A. calamus* have been found to possess an antibacterial activity (Grosvenor et al., 1995; Rani et al., 2003). It is listed as an insecticide, an antifungal agent, an antibacterial agent (Anonymous, 1975). As part of a search for antibacterial compounds from plants, we found that one of purified fraction obtained from the crude ethanol extract of *A. calamus* rhizomes showed antibacterial activity. Therefore, we report here the antibacterial properties of this fraction which contained β -asarone as a major component according to NMR, C¹³ NMR, GCMS, IR and UV.

The result obtained from this study show that the Beta - Asarone fraction has stronger antibacterial activity. Beta -Asarone in *A. calamus* rhizome was demonstrated to have antibacterial activity (Macgaw et al. 2002). However, Beta- Asarone concentrations vary markedly among the oil from *A. calamus* varieties. The tetraploid and triploid plant oil is high in β -Asarone and the diploid plants lack Bete-asarone (Rost and Bos, 1979). The triploid *A. calamus* is distributed throughout Europe, temperate India and the Himalayan region, whereas the tetraploid one, also is found in eastern and tropical southern Asia (Rost, 1979). The Mungkorubrum (2000) demonstrated the antifungal activity of crude dichloromethane extract of *A. calamus* rhizomes by TLC bioassay using *Cladosporium cladosporioides* and Asarone was found to be the main compound. Thirach et al. 2003 reported that the ethanol extract of *A. calamus* inhibited clinical isolates of

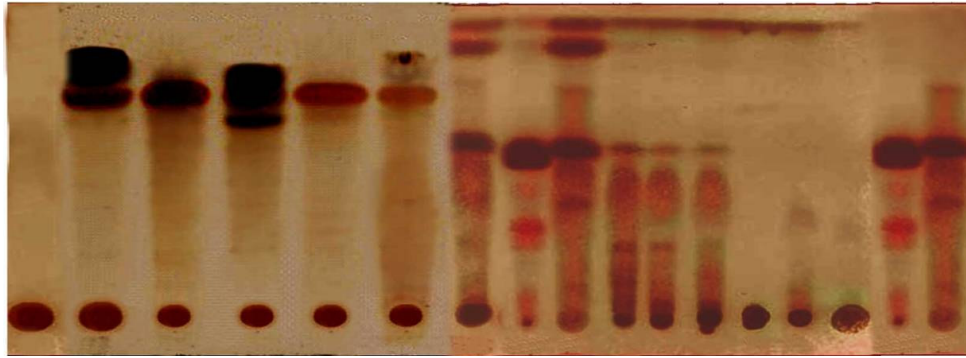


Figure 3. Chromatic profile of fractions.

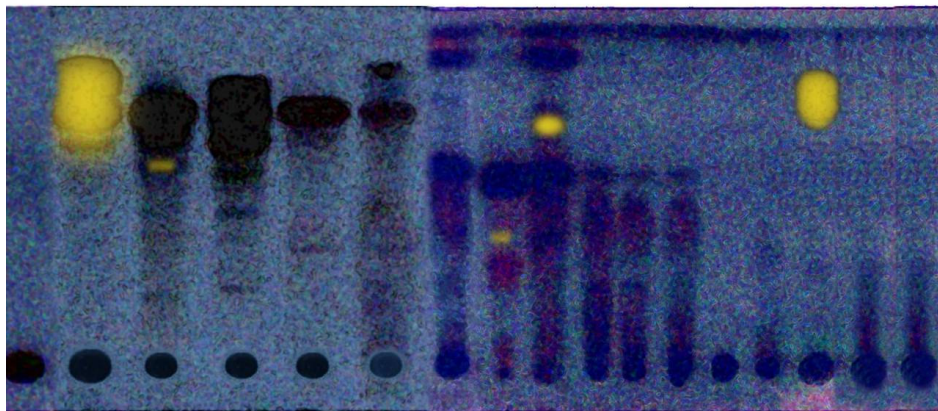


Figure 4. Bioautogramas.

Table 1. Effect of serially diluted concentrations of *Acorus calamus* fractions against *in vitro* growth of *Aeromonas hydrophila* after 48 h of incubation.

Fraction	Concentration	I	II	III	IV	V
F1	0.98	0.49×10^1	0.24×10^2	0.08×10^3	0.02×10^4	0.10×10^5
F2	3.2	$1.6 \times 10^{1*}$	$0.8 \times 10^{2*}$	$0.26 \times 10^{3*}$	$0.13 \times 10^{4*}$	$0.06 \times 10^{5*}$
F3	1.7	$0.85 \times 10^{1*}$	0.425×10^2	0.141×10^3	0.070×10^4	0.03×10^5
F4	7.1	3.55×10^1	1.78×10^2	0.88×10^3	0.44×10^4	0.22×10^5
F5	1.9	0.95×10^1	0.47×10^2	0.23×10^3	0.11×10^4	0.50×10^5
F6	2.2	1.1×10^1	0.55×10^2	0.27×10^3	0.13×10^4	0.06×10^5
F7	0.8	0.4×10^1	0.2×10^2	0.1×10^3	0.05×10^4	0.02×10^5
F8	2.9	$1.45 \times 10^{1*}$	0.72×10^2	0.36×10^3	0.18×10^4	0.09×10^5
F9	3.6	$1.81 \times 10^{1*}$	$0.90 \times 10^{2*}$	0.45×10^3	0.22×10^4	0.13×10^5
F10	0.7	0.36×10^1	0.18×10^2	0.09×10^3	0.04×10^4	0.02×10^5
F11	2.6	1.35×10^1	0.67×10^2	0.33×10^3	0.16×10^4	0.08×10^5
F12	1.3	0.64×10^1	0.32×10^2	0.16×10^3	0.08×10^4	0.04×10^5
F13	5.9	2.95×10^1	1.47×10^2	0.79×10^3	0.37×10^4	0.18×10^5
F14	6.2	3.11×10^1	1.55×10^2	0.77×10^3	0.38×10^4	0.19×10^5
F15	2.4	$1.21 \times 10^{1*}$	$0.60 \times 10^{2*}$	0.30×10^3	0.15×10^4	0.07×10^5
F16	1.3	0.65×10^1	0.32×10^2	0.16×10^3	0.08×10^4	0.04×10^5
F17	3.2	1.60×10^1	0.80×10^2	0.40×10^3	0.2×10^4	0.1×10^5

*Inhibitory concentration that arrested the growth of *A. hydrophila*. At all the other concentrations the medium became turbid indicating the bacterial growth.

of *Candida albicans* and *Cryptococcus neoformans* with the MIC / MFC value of 28.8/75 and 3.02 / 30.8 mg/ml. The MIC values of the Beta Asarone fraction in our study lower 0.13 mg/ml than those of Thirach et al. 2003.

REFERENCE

- Anonymous (1975). Herbal pharmacology in the people's Republic of china, National Academy Sciences, Washington DC.
- Anonymous (2000). Thai Herbal Pharmacopocia volume B. Department of Medicinal Sciences, Ministru of public health Thailand. Prachachon Co., Ltd., Bangkok.
- Balasundaram CP, Mariappan MR (1998). Social impacts of shrimp farming, Social welfare 45: 28-29.
- Baxter RM (1960). Separation of the hypnotic-potentiating principles from the essential oil of *Acorus calamus* L. of Indian origin by liquid-gas chromatograph, Nature 185: 466-467
- Bax R, Mullan N, Verhoef J (2000). Ten millennium drugs – the need for and developed new antibacterial agent J. Antimicrob. Agents 16: 51-56.
- Bhuvaneswari R, Balasundaram C (2006). Traditional Indian herbal extracts used In Vitro against growth of the pathogenic bacteria *Aeromonas hydrophila*. *Isirle J. Aquac.* 58: 89-96.
- Breithaupt H (1999). The new antibiotics. *Nat. Biotechnol.* 17: 1165-1169.
- Chopra I. C Khajuria, BN, Chopra CL (1957). Antibacterial properties of volatile principles from *Alpinia galanga* and *Acorus calamus*. *Antibiot. Chemother.* 1: 378-383.
- Dandiya PC, Cullumbine H (1959). Studies on *Acorus calamus*. III. Some pharmacological actions of the volatile oil. *J. Pharmacol. Exp. ther.* 125: 353-359.
- Dandiya PC, Cullumbine H, Sellers EA (1959b). Studies on *Acorns calamus*. IV. Investigations on mechanism of action in mice, *J. Pharmacol. Exp. ther.* 126: 334-337
- Dandiya PC, Menon MK (1963). Effects of asarone and beta-asarone on conditioned responses, fighting behavior and convulsions, *Br. J. Pharmacol.* 29 : 436-442.
- Dandiya PC (1959a) Studies on Acorns calamus. II. Investigation of volatile oil, *J. Pharmacol.* 11:163-168
- Davis JF, Hayasaka SS (1983). Pathogenic bacteria associated with cultured American eels *Angilla rostrata le sueur*. *J. Fish Biol.* 23: 557-564.
- Grosvenor PW, Suptino A, Gray DO (1995). Medicinal plants from Rian province, sumatra, Indonesia, Part 2; antibacterial and antifungal activity. *J. Ethnopharmacol.* 45: 97-111.
- Grizzle JM, Y Kirya (1993). Histopathology of gill, liver and pancreas and serum enzyme levels f channel catfish infected with *Aeromonas hydrophila* complex. *J. Aquat. Anim. Health* 5: 36–50.
- Gudding R, Lillehaug A, Evensen O (1999). Recent developments in fish vaccinology. *Vet. Immunol. Immunopathol.* 72: 203–212.
- Hancock REW, Knowles D (1998). Are we approaching the end of the antibiotic era? Editorial over view. *Curr. Opin. microbiol.* 1: 493-494.
- Jeney Z, Jeney G (1995). Recent achievements in studies on diseases of common carp (*Cyprinus carpio* L). *J. Aquac.* 129: 397-420.
- Knowles DJ (1997). New strategies for antibacterial drug design. *Trends in Microbiol.* 5: 379-383.
- McGaw L, Jager AK, Van Staden J (2002). Isolation of Beta Asarone, an antibacterial and antihelmintic compound, from *Acorus calamus* in South Afr. *J. Bot.* 68: 31-35.
- Mitchall, Plumb (1980). Toxicity and efficacy of Furance on chanal cat fish *Ictalurus punctatus* infected experimentally with *A. hydrophila*. *J. Fish Dis.* 5: 93-99.
- Moral CH, Castilla FD, AVCastillo JA, Soriano AC, Salazar MS, Peralta BR, Carrasco GN (1998). Molecular characterization of the *Aeromonas hydrophila* aro gene and potential use of an auxotrophic and aro A mutant as a Live attenuated vaccine. *J. Immunol.* 66: 1813-1825.
- Motley TJ (1994). The ethnobotany of sweet flag, *Acorus calamus* (Araceae). *J. Econ. Bot.* 68: 31-35.
- Mungkornasawakul P (2000). Fungicide from *Acorus calamus*, *Eugenia caryophyllus* Bullock Et Harrison and *Mammea siamensis* Kosterm and their Residues after application M.Sc. Thesis. Chiang Mai University, Chiang Mai.
- Nair RT, Kalariya J, Chanda S (2005). Antibacterial activity of some selected Indian medicinaln flora. *Tuky J. Biol.* 29 : 41-47.
- Palumbo S, Auya C, Steima G (1992). *Aeromonas hydrophila* group, In compendium of Methods for microbiological Examination of food .In; Vanderzant, cand splitstoesser F. (eds) Washington APHN. pp: 497-515.
- Rani AS, Satyakala M, Devi VS, Murty US (2003).Evaluation of antibacterial activity from rhizome extract of *A. calamus*. *J. Sci. Ind. Res.* 5: 621- 623.
- Rohalison L, Hamburger M, Hosttetman K, Monod M, Frank E (1999). A bioautography agar overlaymethod for the detection of antifungal compound from higher plants. *Phytochem. Anal.* 2: 199-203.
- Robert MS, Fiancis XW (1981). Spectrometric identification of organic and inorganic compounds. *StateuniversityofnewYork*.
- Rukyani A (1994). Status of epizootic ulcerative disease in indonesia; Roberts, RJCambell, B Mac, rea TH (Eds). Proceedins of ODA Regional seminar on epizootic ulcerative syndrome. Aquatic Animal health research Institute, Bangkok 13: 25- 27.
- Santos Y, Bandin I, Toranzo AE (1996). Immunological analysis of extracellular products and cell surface components motile *Aeromonas* isolated from fish. *J. Appl. Bacteriolo.* 81: 585- 59
- Service RF (1995). Antibiotics that resist resistance. *Sci.* 270: 724-727.
- Scharperculus W (1991). Textbook of Fish diseases 1: 75-102.
- Stoskopf MK (1993). Fish medicine. W.B. Saunders Co., Philadelphia, P.A pp. 473- 474.
- Stiffness M, Douros J (1982). Current status of the NCL plants and animals product program. *J. Nat. Prod.* 45: 1-45.
- Thampuran N, Surendran PK, Mukundan MK, Gopal, Kumar K (1995). Bacteriological studies on fish affected by epizootic ulcerative syndrome (EUS) in Kerala. *India. Asian Fish. Sci.* 8: 103 -111.
- Thirach S, Tragoolpua K, Punjaisee S, Khamwan C, Jatisatiennr C, Kunyanone N (2003). An Antifungal activity of some medicinal plant extracts against of some medicinal plant Extracts against *Candida albicans* and *Cryplococcus neoformans*. *Acta Hort. (ISHS)* 597: 21 217-21221.
- Thune RL, Stanley LA, Cooper K (1993). Pathogenesis of Gram – negative bacterial infection in warm water fishes. *Annu. Rev. Fish Dis.* 3: 37- 68.