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Research Article

ANTI-BACTERIAL ACTIVITY OF ACORUS CALAMUS AND SOME OF ITS DERIVATIVES AGAINST FISH PATHOGEN AEROMONAS HYDROPHILA

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ABSTRACT
Eighteen compounds isolated from the residue of *Acorus calamus* were subjected to micro titer assay to find out their inhibitory concentrations (IC) against A. *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 IC_{90} values of 1.6 mg/ml, 0.8 mg/ml, 0.26mg/ml and 0.13mg/ml respectively. The chemical structure of the active compound in F2, derived from calamus oil was predicted through detailed spectroscopic analytical procedures like ^{1}HNMR, ^{13}C NMR, GC-MS, UV and IR spectroscopy and found related to calamus- Asarone.

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

INTRODUCTION
Natural products and their derivatives represent more than 50 % of the drugs in clinical use in the world (Sofowora, 1984). One of the paramount reasons for pursuing natural products chemistry resides in the actual or potential pharmacological activity of natural compounds found in alkaloids, terpenoids, coumarins, essential oil, flavonoids, lignans and the like; since the advent of antibiotics in 1950s, the use of plant derivatives as a source of antimicrobial has been virtually non –existent. Antimicrobial plant extracts have been recognized as a future source of new antimicrobials (Cowan, 1999).
Resistance to anti-microbial agents is recognized at present as a major global public health threat with infective diseases accounting for approximately one-half of all death in tropical regions. In industrialized nations, despite the progress made in the understanding of microorganisms and their control, incidents of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns (Iwu *et al*., 1999). Berkowitz, (1995) referred to the emergence of drug resistant bacteria as a medical catastrophe. Leggiadro, (1995) stated that effective regimens might not be available to treat some bacterial pathogens isolates; hence, it is critically important to develop new antimicrobial compounds for these and other organisms before we enter the post –antibiotic era.
In the context plants have served as a source of new pharmaceutical products and inexpensive starting material for the synthesis of some known drugs. Components with medicinal properties from plants play an important role in conventional western medicine. In 1984, at least 25 % of the western medicine issued in the US and Canada were derived from or modeled after plant natural products and 119 secondary metabolites were used globally as drugs (Farnsworth, 1994). It has been estimated that 14- 28% of higher plant species are used medically. Only 15% of all angiosperms have been investigated chemically and 74 % of pharmaceutically active plant derived compounds were discovered after following up on ethnomedical use of the plants (Farnsworth and Soejarto, 1991).
Compounds inhibiting microorganisms, such as essential oil, alkaloids, flavonoids, and other have been isolated from plants. It is possible that anti – microbial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in the treatment of

resistant microbial strains (Cox, 1994). For this reason, it is important to investigate plants as alternative sources of anti- microbial compounds.
In this back drop this work aims to perform phytochemical analysis of the chosen herb *Acorus calamus* through: a) isolation of active compounds b) testing their antimicrobial potency against *in vitro* growth of *Aeromonas hydrophila* and c) structure prediction of the active compounds.

MATERIALS AND METHODS

Microorganisms used and growth media

The reference strain *Aeromonas 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 T*ryptone soy agar* (Hi media) slopes at 28 ± 32˚C and periodically checked for their pathogenecity on the basis of ulcer occur in the animal model when infect with the same CFU value (Scharperculeus 1991; Chabot &Thune 1991).

Plant material and extraction

Acorus 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 University 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 apparatuses. 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 *Acorus calamus* was submitted to chromatography over on silica gel (32g) 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 acetate20% 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 acetate60% 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 (17.6mg), F5 (21.3mg), F6 (19.6mg), F8 (74.5mg),F9 (8.1mg), F10 (28.7mg), F11 (36.8mg), F12 (17.1mg), F13 (16.3mg), F14 (12.3mg), F15 (29.5mg), F16 (59.5mg), F17 (47.8 mg), F18 (17.3mg).

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). Inocutilon 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 hr 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 (0.2mm 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

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chamber and overlaid with 10ml molten nutrient agar seeded with 0.2ml 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 minutes after which they were incubated at 32˚C for 24hrs. The cultures were sprayed with an aqueous solution of a dehydrogenate indicator - 2.5mg/ml thialzolyl 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 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 ßow 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} HNMR, ^{13} C NMR, GC-MS, UV and IR spectroscopy (Plate 2).

RESULTS

A. calamus fractions were isolated on the basis of standardized Eluent profile determined by solvent polarity scale (Dandiya1959, Dandiya 1959b). Of the 18 isolated compounds subjected to bioautographic assay F2, F3, F8, F9, and F15 compounds showed zones of inhibition (Plate 3&4); of these F2 had the highest inhibitory zone (Plate 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 I). At the least concentration of $0.13 \times 10\ ^{4}$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 (Robert & Fiancis1981) it as essential oil (plate.1). The oil was further fractionated to obtain a pale yellow β- Asarone (Rf 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.

Structure Determination

Mass spectrum gave molecular weight (ion) - 280 ion molecule at m/e208(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 .-1HNMR (CDCl. Sub.3, 300 MHz) signals showed add at. Delta.6.50 (1H, J = 15.8 Hz and 1.5 Hz, H- 1 1) , 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).

Figure 1: *Calamus* Oil

Table 1: Physicochemical properties of Calamus Oil

Physicochemical properties	
Appearance	Slightly viscous liquid
Color	Brownish yellow
Odor	Fragrant /aromatic smell
Solubility	Soluble in MeoH

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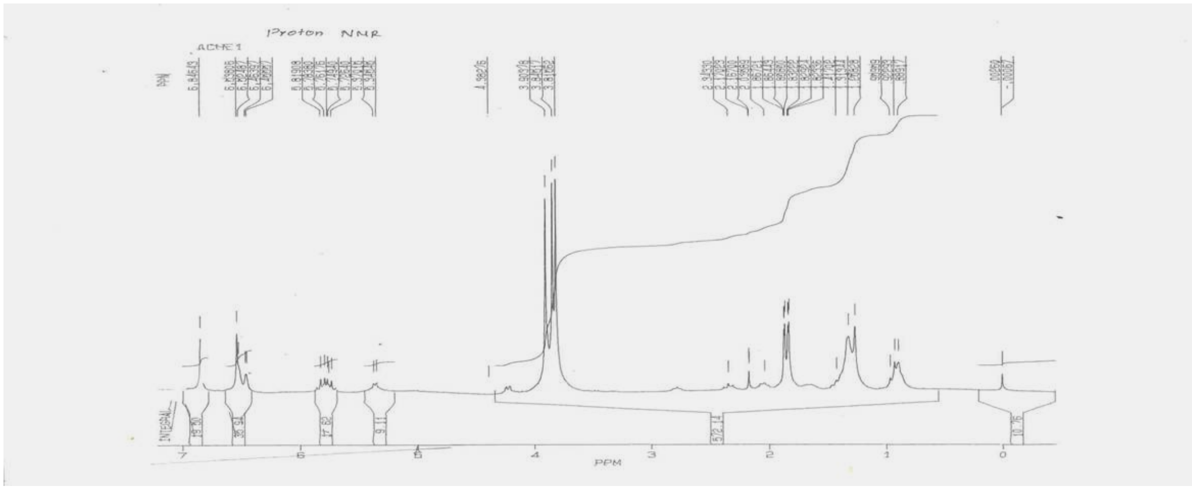
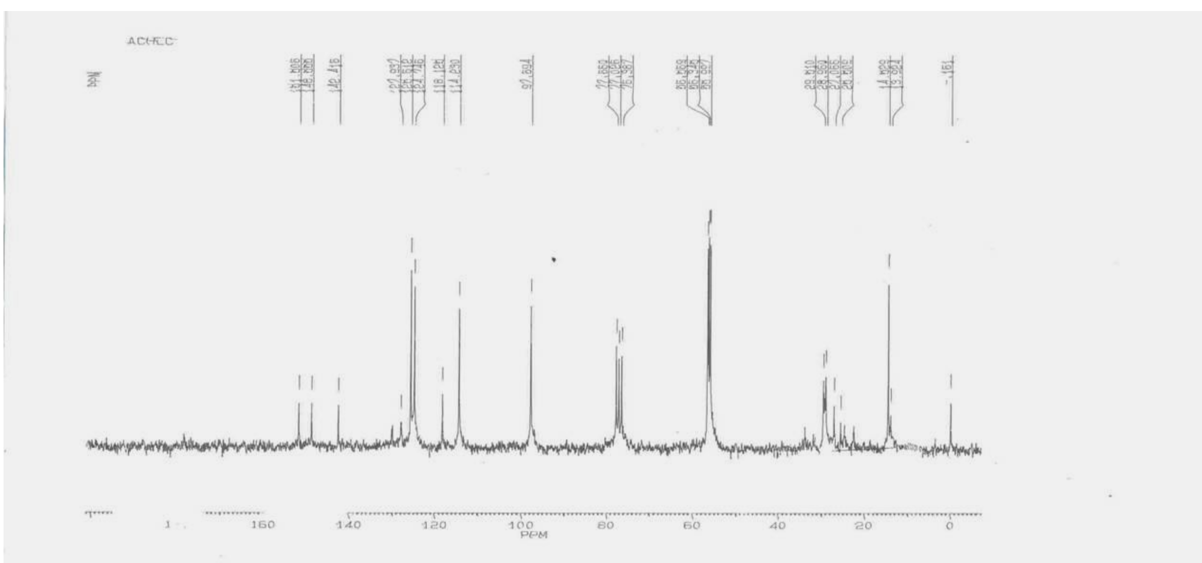
Figure 2: Spectra for calamus oil – Asarone





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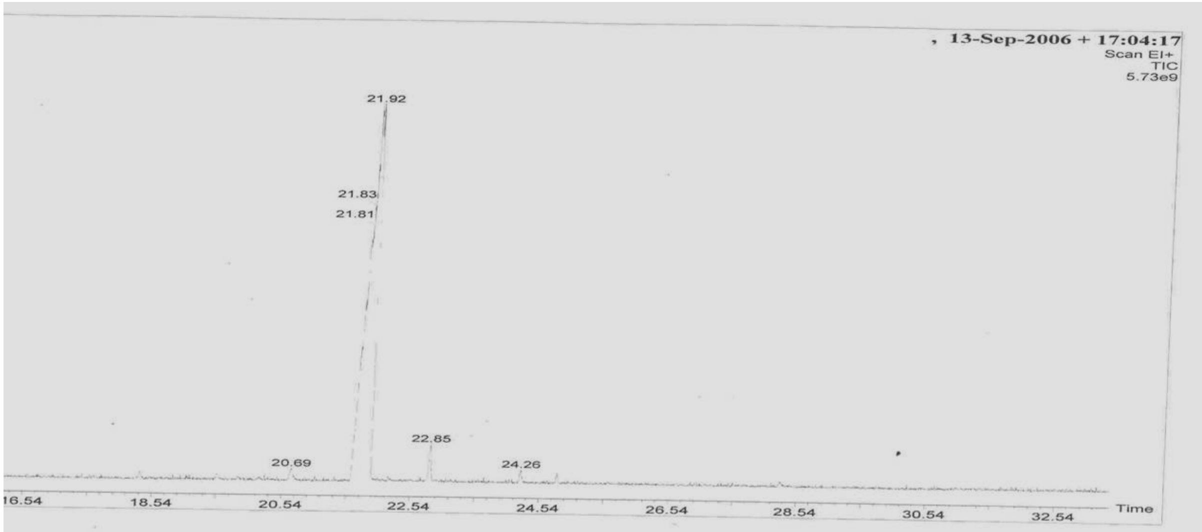

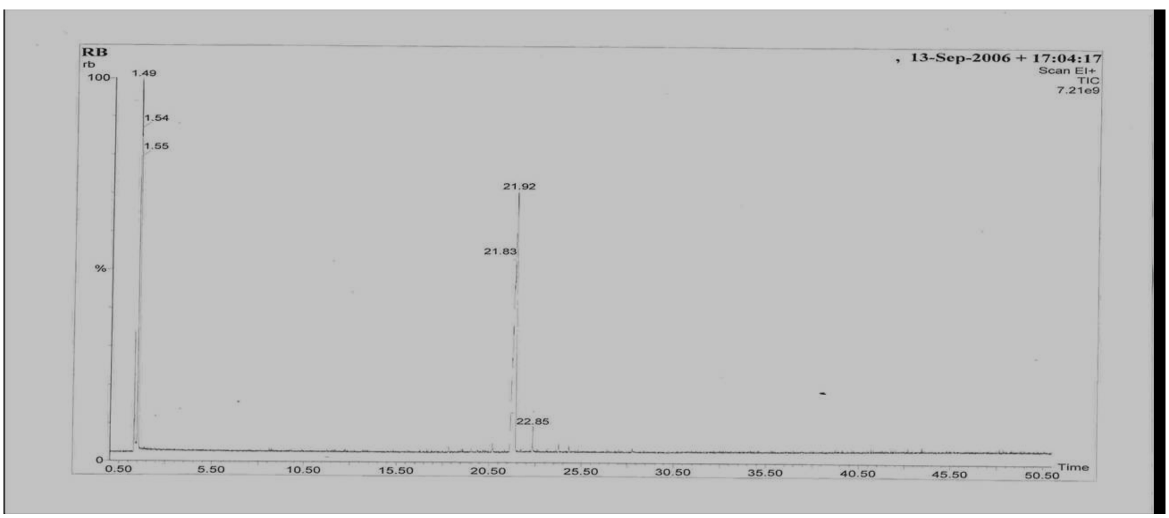

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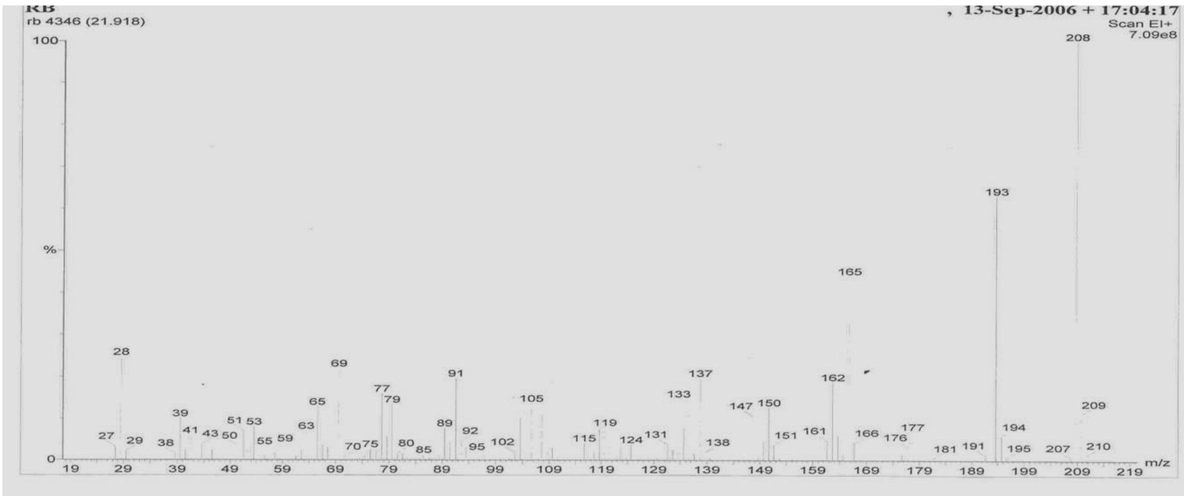

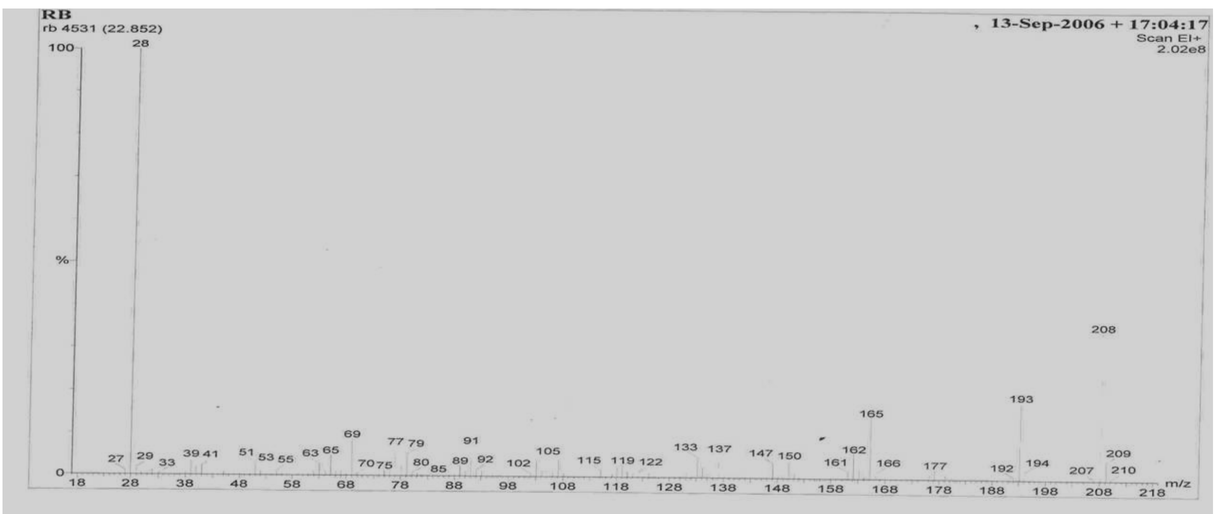

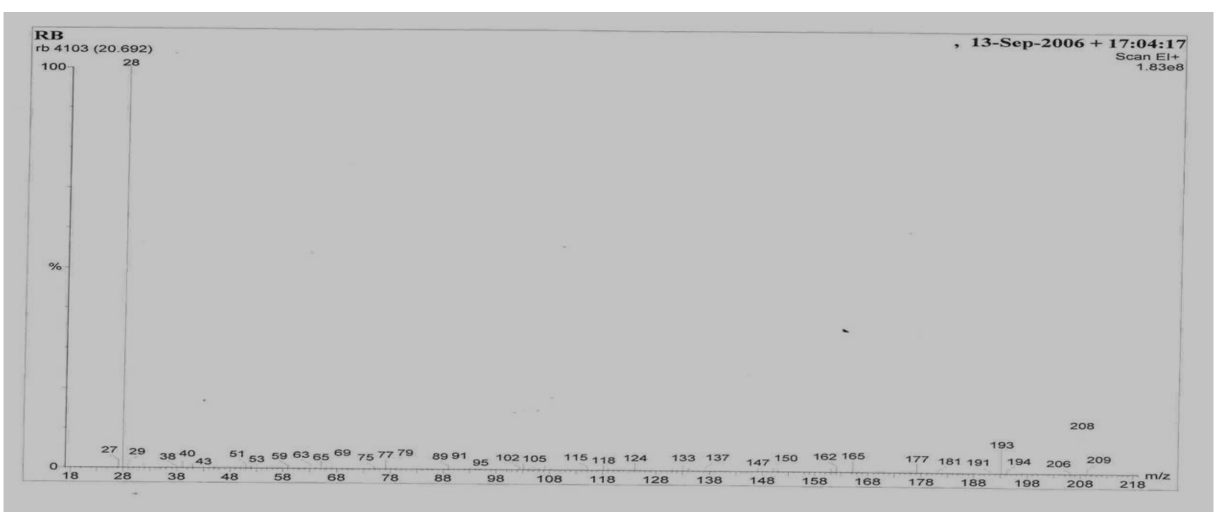

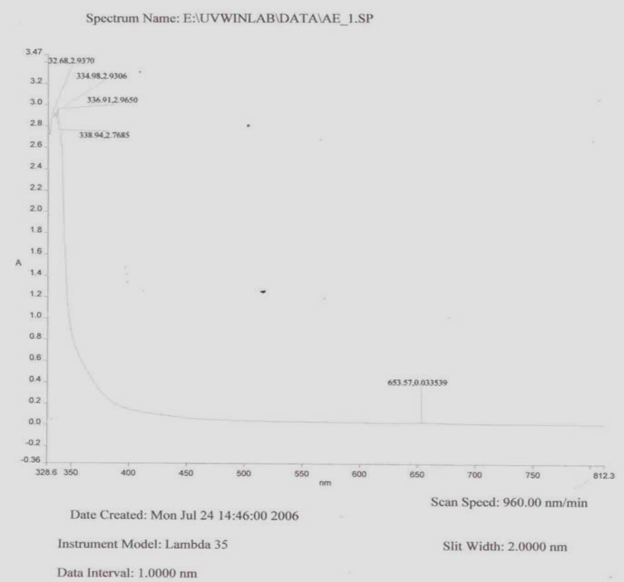
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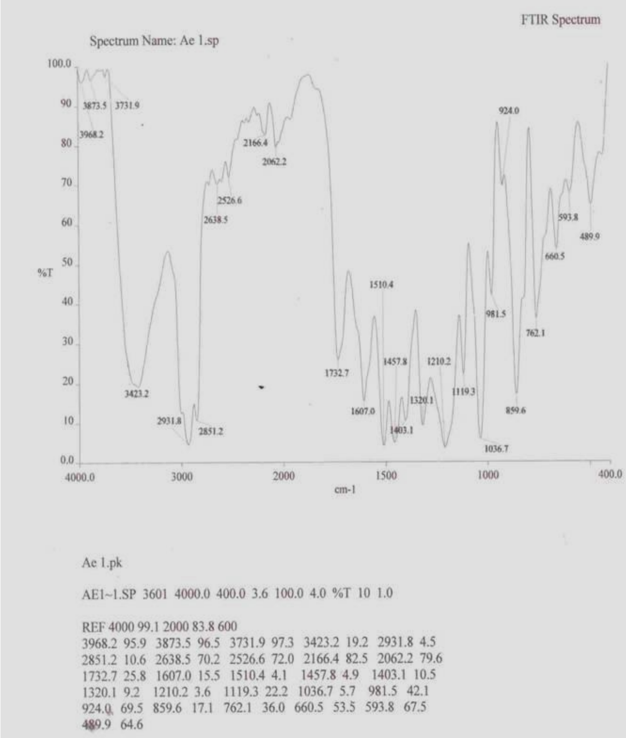
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3968.2 95.9 3873.5 96.5 3731.9 97.3 3423.2 19.2 2931.8 4.5
2851.2 10.6 2638.5 70.2 2526.6 72.0 2166.4 82.5 2062.2 79.6
1732.7 25.8 1607.0 15.5 1510.4 4.1 1457.8 4.9 1403.1 10.5
1320.1 9.2 1210.2 3.6 1119.3 22.2 1036.7 5.7 981.5 42.1
924.0 69.5 859.6 17.1 762.1 36.0 660.5 53.5 593.8 67.5
489.9 64.6

Table 1. Effect of serially diluted concentrations of *Acorus calamus* fractions against in vitro growth of *Aeromonas hydrophila* after 48 hours 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	1.6×10^{1}*	0.8×10^{2}*	0.26×10^{3}*	0.13×10^{4}*	0.06×10^{5}*
F3	1.7	0.85×10^{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	1.45×10^{1}*	0.72×10^{2}	0.36×10^{3}	0.18×10^{4}	0.09×10^{5}
F9	3.6	1.81×10^{1}*	0.90×10^{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	1.21×10^{1}*	0.60×10^{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}	01×10^{5}

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

DISCUSSION

A number of herbs have been assessed for their antimicrobial property with reference to *Aeromonas hydrophila*. Of the five herbs tested Acorus calamus extract has the highest inhibitory activity in vitro (Bhuvaneswari & Balasundaram, 2006). In koi carp infected with *A. hydrophila* short bath treatment heals the lesion and restores the 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,C^{13} 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 & Bos, 1979). The triploid *A. calamus* is distributed throughout Europe, temperate India and the Himalayan region, whereas the tetreploid one, also is found in castern and tropical southern Asia (Rost, 1979). The Mungkorrubrum (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 *C. albicans* and *C. 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 0.13mg/ml than those of Thirach *et al.* 2003.

REFERENCES

Anonymous (1975) Herbal pharmacology in the people's *Republic of China, National Academy of Science Washington DC*(5).

Anonymous (2000) Thai Herbal Pharmacopocia volume B. Department of Medicinal sciences, Ministru of public health, Thailand . *Prachachon Co., Ltd ., Bangkok* (13).

Balasundaram C, P Mariappan and M Ravichandran (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 and Verhoef J (2000). Ten millennium drugs – the need for and developed new antibacterial agent. *Journal Antimicrobials* (16) 51-56.

Bhuvaneswari R and Balasundaram C(2006). Traditional Indian herbal extracts used In Vitro against growth of the pathogenic bacteria *Aeromonas hydrophila*. *Islamic Journal of Aquaculture* (58) 89-96.

Breithaupt H(1999). The new antibiotics. *Nature Biotechnology* (17) 1165- 1169.

Chopra IC, Khajuria BN and Chopra CL (1957). Antibacterial properties of volatile principles from *Alpinia galanga* and *Acorus calamus. Anti. Chemistry* (1) 378-383.

Dandiya PC and Cullumbine H (1959). Studies on *Acorus calamus*. III. Some pharmacological actions of the volatile oil, *Jour. Pharm. Expt.* (125) 353-359.

Dandiya PC, Cullumbine H and Sellers EA (1959). Studies on *Acorns calamus*. IV. Investigations on mechanism of action in mice, *Jour. Pharm. Expt.* (126) 334-337

Dandiya PC and Menon MK (1963). Effects of asarone and beta-asarone on conditioned responses, fighting behavior and convulsions, *British Journal of Pharmacy*(29) 436-442

Dandiya PC (1959). Studies on *Acorns calamus*. II. Investigation of volatile oil, *Journal of Pharmacy* (11)163-168

Davis JF, Hayasaka SS (1983). Pathogenic bacteria associated with cultured american eels *Angilla rostrata le sueur*. *Journal of Fish Biology* (23) 557-564.

Grosvenor PW, Suptino A and Gray DO (1995. Medicinal plants from Rian province, sumatra, Indonesia, Part 2; antibacterial and antifungal activity. *Journal in Ethnopharmacy* (45) 97-111.

Grizzle JM and Y Kirya (1993). Histopathology of gill, liver and pancreas and serum enzyme levels f channel catfish infected with *Aeromonas hydrophila* complex. *Journal of Aquatic Animal Health* (5) 36–50.

Gudding R, A Lillehaug and O Evensen (1999). Recent developments in fish vaccinology. *Vet. Imm. Immunopathology*. (72), 203–212.

Hancock REW and Knowles D (1998). Are we approaching the end of the antibiotic era? Editorial over view. *Current Opinion in Microbiology* (1) 493-494.

Jeney Z and Jeney G (1995). Recent achievements in studies on diseases of common carp(*Cyprinus carpio* L). *Journal in Aquatics* (129) 397-420.

Knowles DJ (1997). New strategies for antibacterial drug design. *Tre. Microbiology* (5), 379-383.

McGaw L, Jager AK and Van Staden J (2002). Isolation of Beta Asarone, an antibacterial and antihelmintic compound, from *Acorus calamus* in South Africa. *Journal of Botany* (68) 31-35.

Mitchall and plumb (1980). Toxicity and efficacy of Furance on chanal cat fish *Ictalurus punctatus* infected experimentally with *A. hydrophila*. *Journal in Fish Diseases*(5), 93-99.

Moral CH, Castilla FD, AV Castillo and JA Soriano AC, Salazar MS, Peralta BR and 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. *Journal of Immunology* (66) 1813-1825.

Motley TJ (1994). The ethnobotany of sweet flag, *Acorus calamus* (Araceae). *Journal of Economic Botany* (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 and Chanda S (2005). Antibacterial activity of some selected Indian medicinal flora. *Turkey Journal Biology* (29),41-47.

Palumbo S, Auya C and Steima G (1992). *Aeromonas hydrophila* group, In compendium of methods for microbiological Examination of food *.In;Vanderzant, cand splitstoesser.F.(eds)Washington* APHN(14).497-515.

Rani AS, Satyakala M, Devi VS and Murty US 2003. Evaluation of antibacterial activity from rhizome extract of *A. calamus*. *Journal of Scientific and Industrial Research* (5) 621- 623.

Rohalison L, Hamburger M, Hosttetman K, Monod M and Frank E (1999). A bioautography agar overlay method for the detection of antifungal compound from higher plants. *Phytochemical Analysis.* (2) 199-203.

Robert MS and Fiancis XW (1981). spectrometric identification of organic and inorganic compounds. *Stat.Univ.NewYork* (3) 214-221.

Rukyani A (1994). Status of epizootic ulcerative disease in Indonesia. *Fish Pathol*ogy (32).47-62.

Rukyani A, Cambell B and Mac rea TH (1995). Proceedings of ODA Regional seminar on epizootic ulcerative syndrome. *Aquatic Animal health research Institute, Bangkok.* (13). 25- 27.

Santos Y, Bandin I and Toranzo AE(1996). Immunological analysis of extracellular products and cell surface components motile Aeromonas isolated from fish . *Journal of Applied Bacteriology.* (81), 585-593.

Service RF(1995). Antibiotics that resist resistance. *Sci.*(270), 724-727.

Scharperculeus W (1991). *Text. Fish dis..*(1). 75-102.

Stoskopf MK(1993). Fish medicine. *W.B.Saunders Co., Philadelphia, P.A.*473- 474.

Stiffness M, Douros J (1982). Current status of the NCL plants and animals product program. *Jour. Natl. Prodt.*(45), 1-45.

Thampuran N, Surendran PK, Mukundan MK and Gopal Kumar K (1995). Bacteriological studies on fish affected by epizootic ulcerative syndrome (EUS) in Kerala. India. *Asi.Fishs. sci.*(8), 103 -111.

Thirach S, Tragoolpua K, Punjaisee S, Khamwan C, Jatisatiennr C and 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), 217 -221.

Thune RL, Stanley LA and Cooper K (1993). Pathogenesis of Gram –negative bacterial infection in warm water fishes. *Anl. Rev. Fish Dis.* (3), 37- 68.