Annals of Microbiology, **59 (3)** 431-437 (2009)

Antimicrobial potential of a marine seaweed *Asparagopsis taxiformis* against *Leptospira javanica* isolates of rodent reservoirs

Kumaresan VEDHAGIRI, Aseer MANILAL, Thangavel VALLIYAMMAI, Santhanam SHANMUGHAPRIYA, Sugathan SUJITH, Joseph SELVIN, Kalimuthusamy NATARAJASEENIVASAN*

Division of Medical Microbiology, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli – 620 024, Tamilnadu, India

Received 28 April 2009 / Accepted 10 July 2009

Abstract - In this present investigation pharmacologically active compounds were isolated from red algae (*Asparagopsis taxiformis*) and their efficacy was evaluated against the *Leptospira javanica* isolates of rodent carriers. The GC-MS analysis of the purified compound revealed the presence of 4,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (56.012%), fattyacids, 14-methyl-pentadecanoic acid methyl ester (26.6%), octadecanoic acid methyl ester (8.46%), octadec-9-enoic acid 2,3-dihydroxy-propyl ester (4.11%), 9-octadecanoic acid, methyl ester (4.535%) and trace amount of chlorobenzene (0.09%). MICs and MBCs of the purified compound against pathogenic leptospiral strains belonging to 14 serovars and 11 isolates belonging to serovar *javanica* of *Leptospira borgpetersenii* were determined in a range of 100-1600 µg/ml. The antibiotics penicillin and doxycycline were used as the standards for the efficiency determination of the seaweed extract against the leptospiral reference serovars and isolates. The minimal inhibitory concentration of penicillin and doxycyline were in the range of 25-200 µg/ml. The seaweed active fraction exhibited comparable MIC and MBC values with that of the standard antibiotic doxycycline. In the present study the seaweed compound has been developed to apply for the 2nd nodal point of transmission cycle, the environment. Thus the present study draws the development of a novel drug to treat leptospires particularly in environments augmented with rodent carriers.

Key words: leptospirosis; *Asparagopsis taxiformis*; *Leptospira borgpetersenii*; MIC; MBC.

INTRODUCTION

Leptospirosis occurs in a variety of urban and rural settings, and is considered to be the most widespread zoonosis in the world. Spirochetal bacteria of the genus *Leptospira* are responsible for leptospirosis, causing both endemic and epidemic disease in human and animals (Faine *et al*., 1999). Infections may be clinically inapparent or may produce a febrile illness ranging in severity from mild to life threatening. The typical symptomatic presentation of leptospirosis is a mild febrile illness without pathognomonic findings. Definitive diagnosis relies upon either culture which has a low yield and requires incubation of 4 to 6 weeks or serological testing of acute and convalescent-phase samples. Therefore, initial therapy of leptospirosis is often empirical, based upon a broad differential diagnosis that includes leptospirosis and other etiologies of acute febrile illness in the community.

Data regarding the *in vitro* efficacy of the antibiotics or natural pharma are very scanty. *In vivo* testing for the activity of antimicrobials against *Leptospira* sp. is limited to few agents for human in epidemic situations. Most of the available data were relevant to randomized trials among the humans, which includes the evaluation of intravenous penicillin in treatment of severe or icteric disease (Edwards *et al.*, 1988; Watt *et al*., 1988). In another study, the oral doxycycline for treatment of acute febrile illness (McClain *et al.,* 1984) as well as for the prevention (Sehgal *et al.,* 2000) have been evaluated. These studies reported decreased symptoms with the used antimicrobials and have been used in small scale to examine the activities of numerous drugs against *Leptospira* isolates (Broughton and Flack, 1986; Prescott, 1991; Murgia and Cinco, 2001). A simple and effective method of testing various antimicrobial agents against *Leptospira* sp. would point out a potential alternative therapy for leptospirosis.

The bacterial diseases are usually tackled by preventing disease outbreaks or by treating the actual disease with drugs or chemicals. The use of antimicrobial agents has increased significantly and the pathogenic bacteria acquire resistance to drugs due to indiscriminate uses of antibiotics. It becomes a greater problem of giving treatment against resistant pathogenic bacteria. Moreover, the cost of the

* Corresponding Author. Phone: + 914312407082; Fax: +914312407045; E-mail: natarajaseenivasan@rediffmail.com

drugs is high and also they cause adverse effect on the host. The decreased efficacy of the antibiotics and the resistance of pathogens to various antibiotics have made necessary the development of new alternatives (Smith *et al.,* 1994). Many bio and pharmacologically active substances have been isolated from algae. Seaweeds provide a rich source of structurally diverse secondary metabolites. These secondary metabolites offers defense against herbivores, fouling organisms and pathogens. Harder (1917) was the pioneer to observe the antimicrobial potentials of seaweeds. Many algal species have been shown to have bactericidal or bacteriostatic substances (Glombitza, 1979; Michanek, 1979; Fenical and Paul, 1984; Paul and Puglisi, 2004).

Transmission of pathogenic leptospires occurs in a cyclic fashion from the rodent carriers to the human/ domestic animals through the environment. There is no proper remedy for the environmental control of leptospires. Therefore in the present study, we reported the efficacy of the seaweed extract of *Asparagopsis taxiformis* along with the antibiotics penicillin and doxycycline as standards for the leptospiral reference strains and the rodent isolates of *Leptospira borgpetersenii* serovar *javanica.*

MATERIALS AND METHODS

Collection of seaweeds. Field collection of healthy and matured seaweed was made from rocky intertidal region of (08°54′ N and 76°38′ E) of Kollam coast. Voucher specimens were deposited in the laboratory in 4% formaldehyde for future analysis. Immediately after the collection the seaweed thallus was gently washed with filtered seawater to eliminate epibiota and other algal contaminants. Cleaned samples were then surface dried under shade for one week at room temperature. Dried fronds of seaweed were powdered in a coffee grinder and packed in polyethylene bags and stored in moisture free place until extraction.

Extraction of crude bioactives. For the extraction of crude bioactives, 100 g of coarsely powdered algal material was soaked in methanol:water (1:10) and fermented at 120 rpm for seven days. After one week, algal material was collected by centrifugation at 8000 *x g* for 20 min and re-extracted with methanol in a 3 l capacity round bottom flask at 60 °C for 3 h. The individual crude extracts were pooled and filtered through Whatman No. 1 filter paper fitted with a Buchner funnel using suction pressure. The pooled extract was reduced to a dark green oily natured crude mass in a rotary vacuum evaporator (Yamato) at 40 °C. The resultant extracts were collected in air-tight plastic vials and stored at 4 °C and used for further analysis.

Leptospiral strains. In the present study a total of 24 *Leptospira* strains were used, out of which 13 were reference strains (received from the Leptospira Reference Laboratory, Port Blair) and 11 test strains (recovered from rodents of semi urban area of Tiruchirappalli, India and coded as TR1L, TR1R, TR2L, TR2R, TR3L, TR3R, TR4L, TR4R, TR5L, TR5R, and TR6R) (Table 1). All the isolates were identified at serovar level at Leptospira Reference Laboratory, Brisbane, Australia. All the strains used in the study were maintained in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Faine *et al.,* 1999).

Antimicrobial agents. The antimicrobial agents like penicillin G, benzylpenicillin potassium salt (60 mg/l), doxycycline hyclate,

TABLE 1 - MIC, MBC and EC_{50} of penicillin (P), doxycycline (D) and seaweed extract (SE) against leptospiral pathogenic strains and isolates

Leptospira serovar (strain)	MIC (µg/ml)			MBC (µg/ml)			EC_{50} (µg/ml)
	P	D	SE	P	D	SE	SE
australis (Ballico)	50	200	100	100	400	400	127.4
autumnalis (N2)	25	200	200	100	400	800	64.24
ballum (Mus127)	25	100	100	50	200	400	35.48
bataviae (Swart)	50	100	200	100	400	400	96.53
canicola (HondUtrechIV)	50	200	200	100	400	800	101.8
cynopteri (3522C)	25	100	100	100	200	400	163.9
grippotyphosa (MoskvaV)	25	200	200	100	400	400	64.66
icterohaemorrhagiae (RGA)	50	200	200	100	400	400	56.66
javanica (Poi)	50	200	400	100	400	1600	185.6
manhao (L60)	50	100	100	100	200	400	197.2
pomona (Pomona)	25	100	400	50	400	1600	312.1
pyrogenes (Salinem)	25	200	400	50	400	800	322.9
tarassovi (Perpelistian)	25	50	100	50	100	200	264.2
TR1L	50	200	200	100	400	800	179.1
TR1R	25	100	200	100	200	400	110.8
TR2L	50	200	200	100	400	800	133.8
TR2R	50	200	200	100	400	1600	316.7
TR3L	50	100	400	100	400	1600	183.6
TR3R	50	100	200	100	200	400	153.0
TR4L	25	200	200	50	200	400	91.91
TR4R	25	100	200	50	200	400	94.41
TR5L	50	100	200	50	200	400	128.3
TR5R	50	100	200	100	200	400	141.8
TR6R	2	50	200	50	100	400	90.61

doxycycline hydrochloride hemiethanolate hemihydrate (1 mg/ml) (Sigma, USA) were used as standards. Initial antibiotic stock was prepared as per the manufacturer's instructions, filter sterilized and stored at -20 °C until use.

Fractionation and purification of bioactive compounds from *Asparagopsis taxiformis.* The concentrated methanolic extract of *A. taxifomis* (1 g) was loaded in a silica gel (60-120 mesh) (Merck) column equilibrated with petroleum ether. An isocratic elution with petroleum ether and ethyl acetate (9:1 to 1:9 and 100% ethyl acetate) followed by ethyl acetate and methanol (9:1 to 1:9 and 100% methanol) was carried out to yield seven fractions. Individual fractions were collected and tested for bioactivity against the *Leptospira* isolates used in the present study. The active antileptospiral fraction was further purified by preparative TLC using silica gel G as stationary phase with 1% methanol in dichloromethane as mobile phase. After the development of chromatogram, the resolved spots were analyzed by spraying with 50% sulphuric acid for detecting the lipophilic compounds. The TLC resolved spot were recovered by scrapping off the adsorbent at the appropriate place and eluted with methanol, centrifuged at 10000 *x g* for 5 min. The supernatant with bioactivity was subjected to HPLC (Shimadzu) and GC-MS analysis (Hewlett Packard).

Antileptospiral assay. The Minimal Inhibitory Concentration (MIC) was determined by the broth dilution method (Hospenthal and Murray, 2004). Antibiotics and seaweed extract dilutions were made in EMJH medium in order to reach the final concentration in the range of 12.5, 25, 50, 100, 200, 400, 800 and 1600 µg/ml. Seven-day-old culture of *Leptospira* was used as inoculums and the concentration were determined under dark field microscopy (Natarajaseenivasan and Ratnam, 1997). Inoculums were added to make a final concentration of 1 x 10^6 organisms/ml and the tubes were incubated at 30 °C for 7 days. All the experiments were performed in triplicates. The EMJH medium tubes without any antimicrobial additives were inoculated with the leptospires and served as a control. The MIC was recorded as the lowest concentration that did not show any presence of live leptospires. For the Minimum Bactericidal Concentration (MBC) all the tubes were sub cultured in fresh EMJH media without any antimicrobial agents and incubated at 30 °C for 7 days. Further, the lowest concentration that showed 90% reduction in the growth of the leptospires was considered as MBC.

EC_{50} and statistical analysis. Frequency of inhibition (EC_{50}) at each concentration of antibiotic and bioactive compounds was determined by counting the numbers of live leptospires in tested and control group and graphs plotted using graph pad software Prism (version 5.0). The statistical analysis was performed using student's t-test for two independent variables using SPSS (version 11.0) and the significance between the antibiotics and seaweed extracts were studied.

RESULTS AND DISCUSSION

Antileptospiral activity of *Asparagopsis taxiformis*

The methanolic extract of *A. taxiformis* showed maximum inhibition against 20% (*Leptospira tarassovi, Leptospira australis, Leptospira ballum, Leptospira cynopteri, Leptospira manhao*) and moderate activity against 80% of the reference and recovered strains. In our preliminary screening experiments it has been proved that the marine seaweed has antimicrobial potential through their variety of secondary metabolites against a wide range of multidrug resistance pathogens (Shanmughapriya *et al.*, 2008). The diverse groups of marine seaweed from members of Chlorophyta, Rhodophyta and Phaeophyta with a seasonal variation in the distribution of the seaweeds both at the genus and species level in Kollam, the south west coast of India have also been studied. But the results obtained in the present study threw light on the dark edge of the bioactive potential of seaweeds viz., the antileptospiral activity which have seldom been explored or studied.

Fractionation and purification o*f Asparagopsis taxiformis bioactives*

The highly active methanolic extract of *A. taxiformis* was fractionated through column chromatography. The active fraction was eluted with petroleum ether: ethyl acetate (1:1) and it was fractionated using preparative TLC to obtain a single spot with Rf value of 0.487. The active TLC resolved spot was again purified with reverse phase HPLC (Shimadzu chromatographic system Kyoto, Japan) at 254 nm with methanol at a flow rate of 1 ml/min; head pressure at 25 kgf/cm^2. The whole setup was maintained at room temperature (25 °C) and three peaks with retention time (min) of 3.307, 3.863 and 4.730 respectively were attained at 254 nm (Fig. 1). The eluted HPLC peak that retained antimicrobial activity was chosen for GC-MS analysis.

Chemical characterization of the bioactive principle

A high resolution mass spectrometer equipped with a data system in combination with Gas Chromatography (Hewlett Packard) was used for the chemical analysis of active fraction. The active fraction on the basis of spectral data by GC-MS was found to be a mixture of fatty acids with volatile compounds. The active fraction showed the presence of 5 prominent peaks corresponding to chlorobenzene (MW 112), 14-methyl-pentadecanoic acid methyl ester (MW 270), octadec-9-enoic acid 2,3-dihydroxy-propyl ester (MW 365), 9-octadecanoic acid, methyl ester (MW 298), octadecanoic acid methyl ester (MW 296), 4,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (MW 167).

The active fraction was composed of volatile metabolites and fatty acids. The relative percentage and chromatogram of identified compounds is summarized in Table 2 and Fig. 2. It was found that the main constituent of the purified fraction correspond to 4,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (56.012%), fattyacids, 14-methyl-pentadecanoic acid methyl ester (26.6%), octadecanoic acid methyl ester (8.46%), octadec-9-enoic acid 2,3-dihydroxy-propyl ester (4.11%), 9-octadecanoic acid, methyl ester (4.535%) and trace amount of chlorobenzene (0.09%). The antimicrobial properties of the seaweed extract against the leptospires are first of its kind and it has showed significance feat against *Leptospira* reference strains and isolates.

Mechanism of antibiosis

The MIC of both antibiotic and purified compound for the 24 leptospiral strains of the genus *Leptospira* are shown in Table.1. The MIC of penicillin against the leptospiral strains were in the range of 25-50 µg/ml and it was 50-200 µg/ml for doxycycline. In comparison with the antibiotics the purified compound has increased MIC in the range of 100-400 µg/ml. Even though the antimicrobial potential of penicillin was found to be superior against all the reference strains as well as for the rodent isolates the antibiotic doxycycline and the purified compound displayed comparable MIC values. The purified compound showed effective antileptospiral activity against the freshly isolated rodent isolates. Moreover the purified compound showed a better efficacy for the

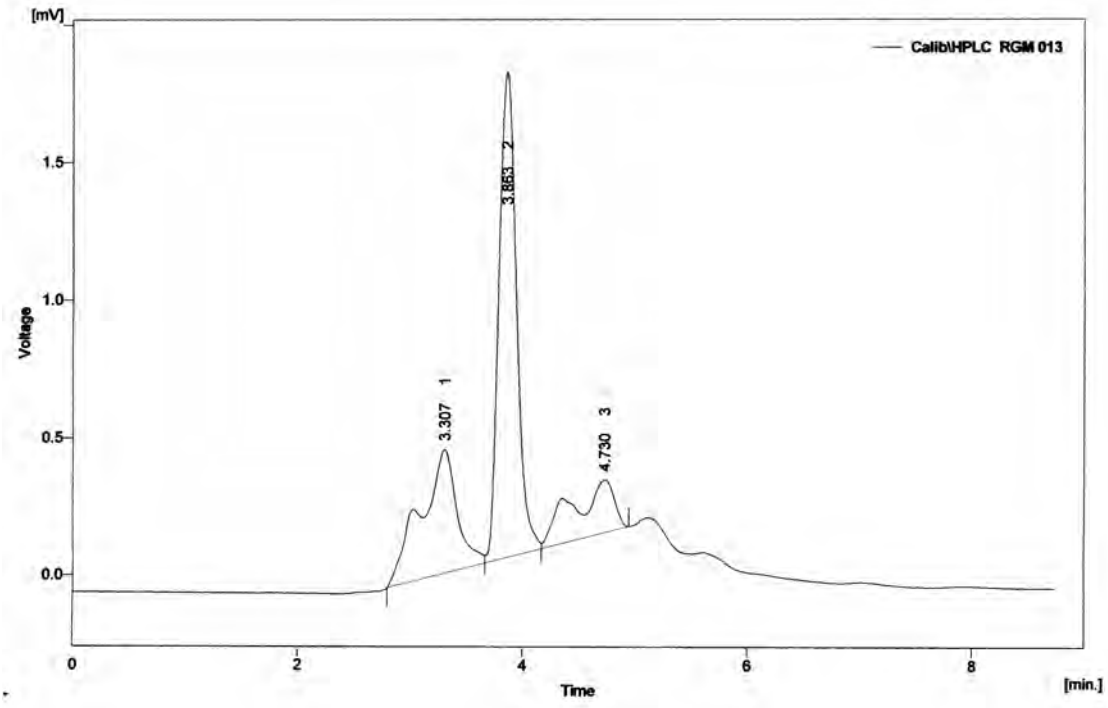

FIG. 1 - HPLC profile of active TLC fraction of *Asparagopsis taxiformis.*

TABLE 2 - GC-MS data of active fraction of *Asparagopsis taxiformis*

Systematic name	Retention time	Overall (%)	Fragmentation
Benzene, chloro	3.276	0.09	112 (M^+), 77
14-Methyl-pentadecanoic acid-methyl ester	27.930	26.683	270(M^+), 227, 213, 143, 129, 74
Octadec-9-enoic acid-2,3-dihydroxy-propyl ester	30.552	4.1195	265(M^+), 211, 209, 151,111, 73, 55
9-Octadecenoic acid methyl ester	30.886	4.537	255(M^+), 241, 209, 191, 143, 101, 55
Octadecanoic acid methyl ester	32.379	8.648	209(M^+), 191, 151, 149, 73
4,5-Dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester	34.899	56.012	167(M^+), 121

locally predominant leptospiral strains of human and of animals like Autumnalis, Grippotyphosa, Icterohaemorrhagiae, Pomona and Javanica.

Invariably all the tubes tested for MIC were sub cultured in fresh EMJH media to find out the MBC. For penicillin MBC was observed in the range of 50-100 µg/ml and for doxycycline it was in the range of 100-400 µg/ml. Similar to the results of MIC, MBC for penicillin was attained at a comparatively lesser concentration than that of doxycyline and purified compound. But still a comparable MBC value existed between doxycycline and purified compound. For the strains of Autumnalis, Canicola, Javanica, Pomona, TR1L, TR2L, TR2R, TR3L the MBC obtained was in the range of 800-1600 µg/ml. Thus the result obtained clearly depicts that the final working concentration of 1600 µg/ml of the purified compound was required for the environmental applications against the *Leptospira*. Penicillin and doxycyline are the drugs of choice in the treatment of human leptospirosis (Hospenthal and Murray, 2003). This is based chiefly on the fact that they are the only agents that have been studied widely in randomized controlled clinical trials. Considering this fact these two antibiotics were used as the standards for the comparison of the results of the seaweed extract. Our study is in accordance with the prior *in vitro* efficacy testing of penicillin and doxycline (Hospenthal and Murray, 2003) and the MIC and MBC values observed were in homogeny among the strains from various serovars and isolates. There is no much disparity among the various leptospiral serovars in the MIC and MBC as reported earlier. Most of the earlier reports one or two isolates of interest only has been utilized (Murgia and Cinco, 2001). In the present investigation, leptospiral serovars of various genomospecies were incorporated and considering the environmental control a wide number of rodents isolates also included to find out the efficacy of the purified seaweed compound.

The frequency of inhibition (EC_{50}) has varied from one serovar to another and also in strains; it implies that the antigenic nature, surface protein moieties and lipopolysaccharide of the *Leptospira* sp. have a wide variation among different serovars. Further the EC_{50} level ranged from 35.48-322.9 µg/ml, clearly

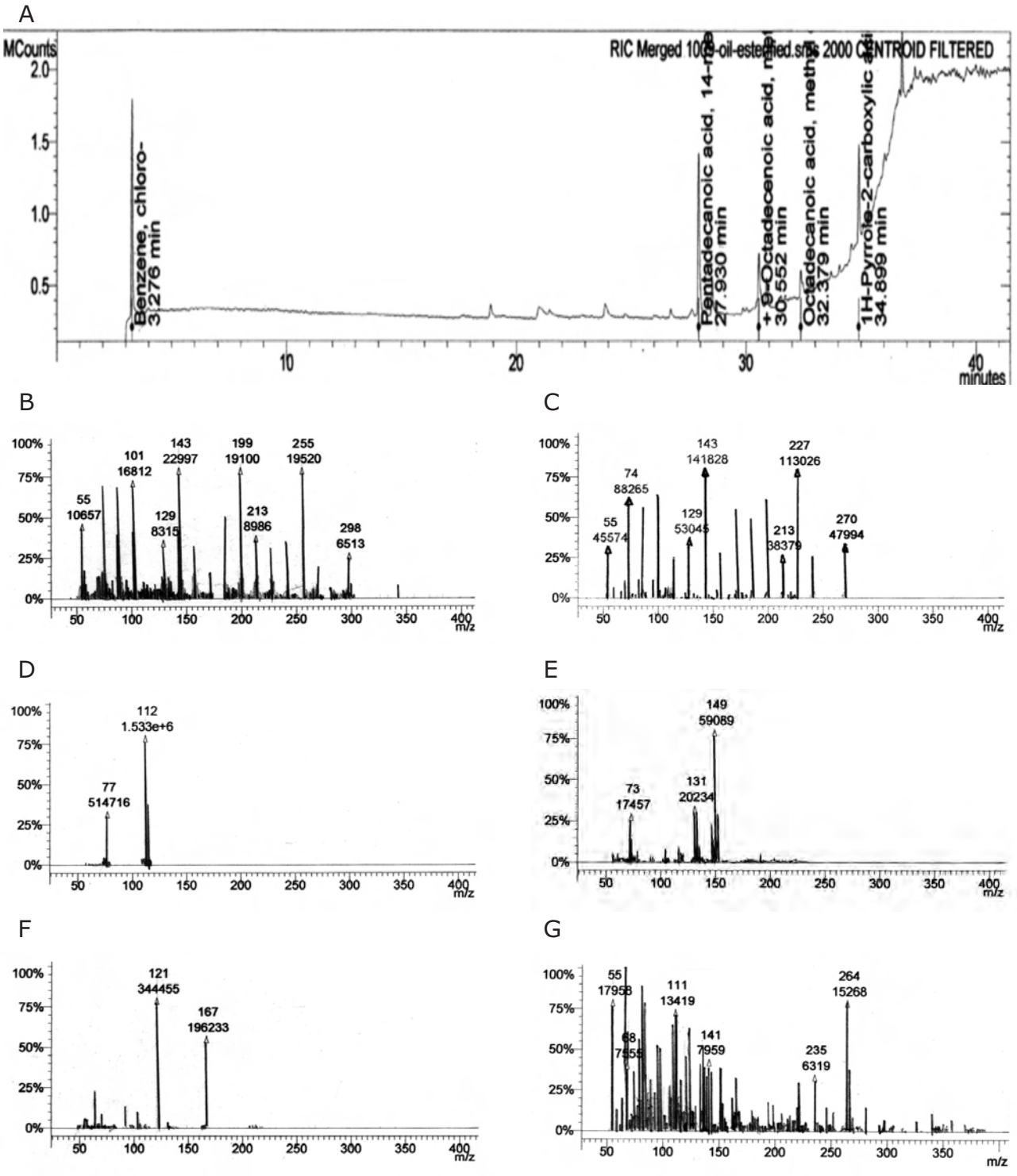

FIG. 2 - A: GC-MS chromatogram of the active fraction of *Asparagopsis taxiformis.* B to G: MS spectrum of the individual compounds analyzed from NIST library.

indicating the efficiency of the compound is not similar for all the serovar tested. In rodent strains the EC_{50} value was in the range of 126.3 ± 55 µg/ml (Table 1, Fig. 3) showing significant activity against locally isolated strains. Significant difference ($P < 0.001$) was observed for MIC and MBC values of the antibiotic penicillin, doxycycline and seaweed extract at 95% confidence interval.

Control strategies for leptospirosis are generally targeted at any of the three nodal points in the transmission cycle of the disease, i.e. the animal carriers, the environment or humans. Traditional strategies that target rodents, the reservoir hosts, farm and pet animals, the carrier hosts and the environment are not practical in developing countries like India. Control strategies that advocate use of protective kit at the workplace are impractical, as such measures are difficult to implement because of the economic overheads and the lack of community acceptance in underdeveloped countries. Vaccination and chemoprophylaxis are two of the alternative measures that target humans. Due to the difficulty in designing vaccine candidates against the large number of circulating serovars of leptospires, chemoprophylaxis appears as the only option for the prevention of this disease.

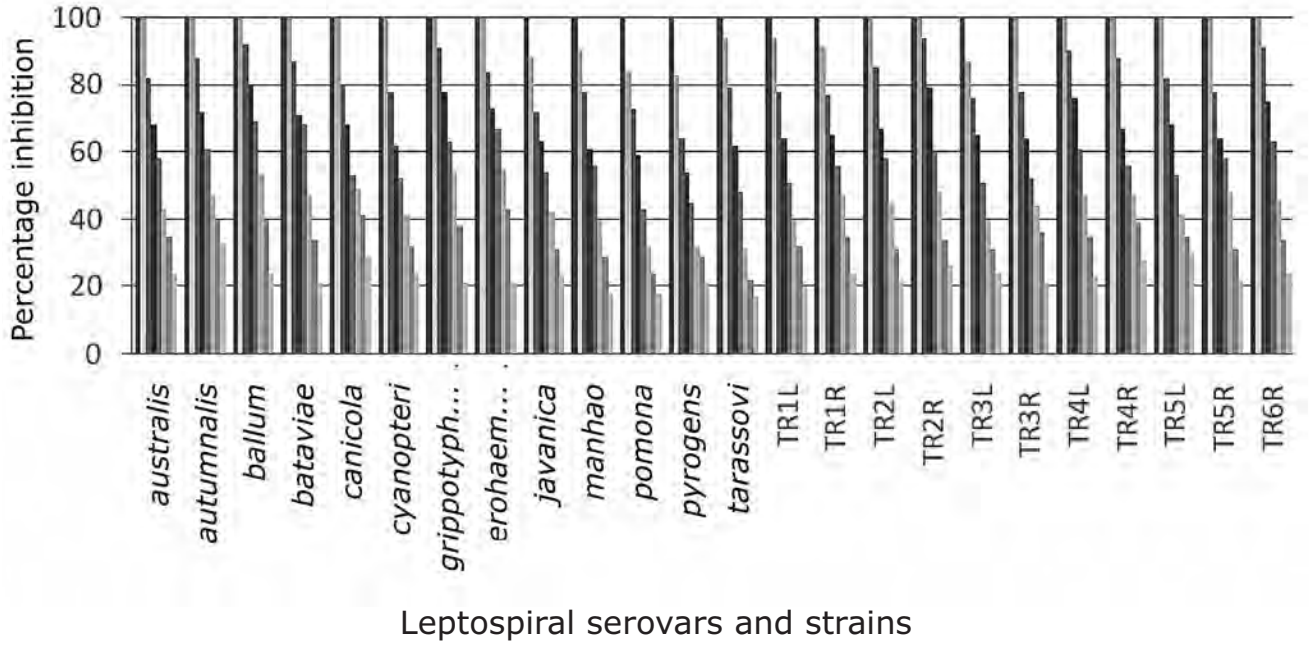

FIG. 3 - Percentage inhibition of leptospires against different concentrations (1600–12.5 µg/ml) of seaweed extract.

With this argument, Sehgal *et al.* (2000) evaluated the efficacy of doxycycline chemoprophylaxis as a possible control measure in preventing infection and disease by conducting a randomized control trial. Alternatively in this investigation the efficacy of the seaweed extract was developed to apply for the second nodal point the environment, because it plays a key role in the transmission of leptospires in rodent manifest areas.

CONCLUSION

Infection by pathogenic *Leptospira* species is an important and frequently life-threatening cause of human disease which is characterized by hematogenous dissemination to multiple organs like brain, aqueous humor, liver, lungs, and kidneys (Vijayachari *et al.,* 2004). It is caused by heterologous *Leptospira* sp. belonging to the genus *Leptospira*. More than 300 serovars have been described in *Leptospira interrogans* sensu lato whereas *Leptospira biflexa* sensu lato contains 45 serovars (Kmety and Dikken 1993). The pathogenic leptospires are mainly responsible for the human/animal infection. The early and accurate diagnosis of leptospirosis is obligatory to start the treatment and prevent to lead up further complications like pulmonary haemorrhages and renal failure (Vijayachari *et al.,* 2008). The present findings have developed a seaweed based extract for the applications of controlling the leptospires. It has been proved based on the determination of the MIC and MBC for the seaweed extract *Asparagopsis taxiformis* in comparison with the routinely administered antibiotics like penicillin and doxycycline for the treatment of clinical leptospirosis. The seaweed extract has showed comparative MIC and MBC with that of the antibiotic doxycycline, proving it to be an environmental controlling agent for the leptospires and its transmission may be routinely applied.

Acknowledgment

The financial support of Department of Science and Technology (DST), Government of India, New Delhi, is gratefully acknowledged. KV is thankful to University Grants Commission (UGC) for his fellowship. The authors thank the Vice-Chancellor, Bharathidasan University for the facilities. We also thank Dr. L.D. Smythe, WHO Reference Center for Leptospirosis, Brisbane, Australia for serovar level identification of the *Leptospira* isolates.

REFERENCES

Broughton E.S., Flack L.E. (1986). The susceptibility of a strain of *Leptospira interrogans* serogroup Icterohaemorrhagiae to amoxycillin, erythromycin, lincomycin, tetracycline, oxytertracycline and minocycline. Zentbl. Bakteriol. Mikrobiol. Hyg. Ser. A., 261:425-431.

Edwards C.N., Nicholson G.D., Hassell T.A., Everard C.O.R., Callender J. (1988). Penicillin therapy in icteric leptospirosis. Am. J. Trop. Med. Hyg., 39: 388-390.

Faine S.B., Adler B., Bolin C., Perolat P. (1999). *Leptospira* and leptospirosis. 2nd edn., MediSci, Melbourne, Australia.

Fenical W., Paul V.S. (1984). Antimicrobial and cytotoxic terpenoids from tropical green algae of the family *Udoteaceae*. In: Bird C.J., Ragan M.A., Eds, 11th International Seaweed Symposium. Dr. W. Junk Publishers, Dordrccht, Boston, Lancaster, pp. 135-140.

Glombitza K.W. (1979). Antibiotics from algae. In: Hoppe I.L.A., Ed., Marine Algae in Pharmaceutical Science, Waiter de Gruyter, Berlin, New York, p. 303.

Harder R. (1917). Ernahrungphysiologische untersuchughen an Cyanophyceen, Hauptsac hlich an endophytischen *Nostoc punctiforme*. Z. Bot., 9: 145.

Hospenthal D.R., Murray C.K. (2004). Broth microdilution susceptibility testing for *Leptospira* spp. Antimicrob Agents and Chemother., 48: 1548-1552.

Kmety E., Dikken H. (1993). Classification of the species *Leptospira interrogans* and history of its serovars. University Press, Groningen, The Netherlands.

McClain B.L., Ballou W.R., Harrison S.M., Steinweg D.L. (1984). Doxycycline therapy for leptospirosis. Ann. Intern. Med., 100: 696-698.

Michanek G. (1979). Seaweed resources for pharmaceutical uses. In: Hoppe I.L.A., Ed., Marine Algae in Pharmaceutical Science, Waiter de Gruyter, Berlin, New York, pp. 203-235.

Murgia R., Cinco M. (2001). Sensitivity of *Borrelia* and *Leptospira* to quimupristin-dallforpristin (Synercid) *in vitro*. New Microbiol., 24: 193-196.

Natarajaseenivaasan K., Ratnam S. (1997). Expermental leptospirosis in laboratory mice and rats. J. Commun. Dis., 29: 291-293.

Paul V.J., Puglisi M.P. (2004). Chemical mediation of interactions among marine organisms. Nat. Prod. Rep., 21: 189-209.

Prescott J. (1991). Treatment of leptospirosis. Cornell Vet., 81: 7-12.

Sehgal S.C., Sugunan A.P., Murhekar M.V., Sharma S., Vijayachari P. (2000). Randomised controlled trial of doxycycline prophylaxis in an endemic area. Int. J. Antimicrob. Agents, 13: 249-255.

Shanmughapriya S., Manilal A., Sujith S., Selvin J., Seghal Kiran G., Natarajaseenivasan K. (2008). Antimicrobial activity of seaweeds extracts against multiresistant pathogens. Ann. Microbiol., 58: 535-541.

Smith P., Hiney M.P., Samuselsen O.B. (1994). Bacterial resistance to antimicrobial agents used in fish farming. Annu. Rev. Fish Dis., 4: 273-313.

Vijayachari P., Sugunan A.P., Sharma S., Roy S., Natarajaseenivasan K., Sehgal S.C. (2008). Leptospirosis in the Andaman Islands, India. Trans. R. Soc. Trop. Med. Hyg., 102: 117-122.

Vijayachari P., Hartskeerl R.A., Sharma S., Natarajaseenivasan K., Roy S., Terpstra W.J., Sehgal S.C. (2004). A unique strain of *Leptospira* isolated from a patient with pulmonary haemorrhages in Andaman islands: a proposal of serovar Portblairi of serogroup Sehgali. Epidemiol. Infect., 132: 663-673.

Watt G., Padre L.P., Tuazon M.L., Calubaquib C.J., Santiago E., Ranoa C.P., Laughlin L.W. (1988). Placebo-controlled trial of intravenous penicillin for severe and late leptospirosis. Lancet, 1: 433-435.