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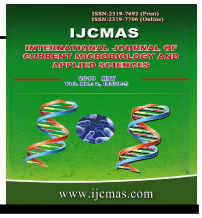


Original Research Article

Study on *Leptospira autumnalis* protein damage due to *Eclipta alba*

K. Divya[1], M. Chellakkannu[1]*, R.Saravanan[2] and P. Saradhai[3]

[1]Department of Microbiology, Adhiparasakthi college of Arts and Science, G.B.Nager, Kalavai-632 506, Tamil Nadu, India.
[2]Biomedical Engineering Research foundation, Salem- 122, Tamil Nadu, India.
[3]Assistant professor, Presidency College, Chennai, Tamil Nadu, India.
**Corresponding author e-mail: chella1980@rediffmail.com*

A B S T R A C T

Leptospirosis is a potentially fatal bacterial disease that can display a wide array of clinical presentations thus mimicking better-know illnesses. Although, leptospirosis is primarily a zoonotic disease, it frequently inflicts severe illness and death on communities around the globe. The present study was therefore carried out to find out the phenotypic conservation of the Leptospiral proteins ompl1 and LPL41, and the genetic conservation of ompL1 and LPL41 genes among the Leptospiral isolates of Andaman Islands and among the reference strains. If one dimensional SDS-PAGE the Leptospiral sample prepared from strains of various Leptospiral serovars were run and transferred on to nitrocellulose paper and probed with pooled convalescent phase human sera to find out phenotypic conservation of the protein fragment at 31 and 41 KDa. Further, the proteins were indirectly confirmed as ompL1 and LipL41 by using specific rabbit hyper immune sera. Crude extract from *Eclipta alba* plant leaves were performed by using aqueous extraction. The extracts were administered on *Leptospira autumnalis* one week culture in liquid EMJH medium. One set of the liquid culture was maintained without incorporation of the extracts as a control. The control and medicinal plant extracts treated *Leptospira autumnalis* proteins were partially purified and estimated for SDS-PAGE analysis. The aqueous extracts of *Eclipta alba* was very effective in causing *Leptospira autumnalis* proteins damage which was studied by SDS-PAGE assay.

Keywords

Phytochemicals; antioxidant; cytotoxicity assay; *Phyllanthus emblica; Eclipta alba; Leptospirosis; Leptospira autumnalis.*

Introduction

Leptospirosis is a potentially fatal bacterial disease that can display a wide array of clinical presentation thus mimicking better-known illness (Ricardo Izurieta *et al*., 2008). It is a globally important zoonotic disease (Adler *et al*., 2009) most commonly found in tropical or sub-tropical countries and may be prevalent in both urban and rural settings. It was first described by Adolf Weil in 1886 when he reported on "acute infectious disease with enlargement of spleen, jaundice and nephritis. Annual incidence is estimated from 0.1-1 per 100,000 in temperate climate (James *et al*., 2006).

Human leptospirosis is often acquired via contact with fresh water contaminated by bovine, rat or canine urine as part of occupational contact with these animals. Leptospires can survival longer in the warm and humid condition. Leptospirosis is highly prevalent in the Asia pacific region. In India, the disease has been found more commonly associated with natural disasters especially during the monsoon period at which times acute epidemics may eventuate. The predominant serovars are *copenhageni, atumnalis, pyrogens, grippotyphosa, canicola, australis, javanica, sejroe and pomona*. Outbreaks of leptospirosis have increasingly been reported from Kerala, Gujarat, Tamilnadu and Karnataka and sporadic cases have been reported from Goa, Andrapradesh and Assam (WHO, 2006).

Leptospirosis is frequently under diagnosed, because of the non-specific symptoms early in the disease. Therefore, diagnosis is based on laboratory test rather than on clinical symptoms alone (Ahmad *et al*., 2005). There are different types of diagnosis test are available. They are following, microscopic demonstration, isolation of Leptospires, Molecular diagnosis, serological diagnosis. Leptospires may be observed in clinical material under dark field microscope (CRC press, 1999).

Most cases of Leptospirosis diagnosed by serology. The MAT is considered the "gold standard" method for Leptospirosis serology **(**Ahmad *et al*., 2005). ELISA can easily accommodate a large number of samples and gives a less subjective result than MAT **(**Levett *et al*., 2001). A commercially available slide agglutination test(SAT) for the diagnosis of human Leptospirosis was evaluated by comparing it to an Immunoglobulin M(IGM) Enzyme-linked immunosorbent assay(ELISA) and to the Microscopic agglutination test (MAT) (Galton *et al*.,1958). Latex agglutination test depends on the sensitization of commercially available latex particles with Leptospiral antigen. Direct demonstration of leptospires in human samples is diagnosis based on polymerase chain reaction. Many primers were reported for detection leptospires.

Different types of PCR used for diagnosis of Leptospirosis detection (Kositanontv *et al*., 2007). An indirect haemagglutination test for the diagnosis of leptospirosis is described. The sensitivity of commercial IHA for the detection of acute leptospirosis was 100% and the specificity was 94% **(**Levett *et al*., 1998). Sodium dodecyl sulphate (SDS - PAGE) (discontinuous) was originally described Laemmli, (1970). It is the most commonly used system in which proteins are fractionated strictly by their size. The separation of proteins by electrophoresis is based on the fact that the charged molecules will migrate through a matrix upon application of an electric field usually provided by immersed electrodes (Laemmli, 1970).

In this study *Eclipta alba* plant is used for detection of protein damage of *Leptospira* strain. *Eclipta alba* grouped under the family Asteraceae that is of herbaceous type and spreads on ground or partly ascending with its stem and small leaves are succulent and are found mostly in tropical and subtropical regions where water logging condition is very high. Traditionally, this plant was used for curing the liver related problems. In this study, an attempt was made to screen *Eclipta alba* to formulate anti Leptospiral

drug. In spite of modern developments medicinal facilities, about 80% of Indian population are depend on traditional system of medicines because of severe adverse reactions by western medicines. Herbal medicines are still the mainstay of about 75.80% of the world population, mainly in the developing countries, for primary health care (Prabhu *et al.,* 2008). To overcome the adverse reaction by the above drugs, herbal-based therapeutics had been used in treating leptospirosis. Hence in their study medicinal plant (*Eclipta alba*) used as an alternative choice of chemotherapy.

Materials and Methods

Samples used

Leptospira autumnalis cultures were received from BMERF Salem in EMJH liquid medium and *Eclipta alba* plants were collected from Mathanoor, Vellore district, Tamil Nadu, India.

Plant aqueous extraction method

After drying at 37°c for 24hours the plant material was ground in a marter and pestle. Exposure to direct sunlight was avoided to prevent the loss of active components.

Preparation of leaf aqueous extraction

Fifty grams of selected fresh leaf materials was macerated with 50ml of sterile distilled water in a grinding machine (Marter and Pestle) for about 10-15 minutes. The macerate was first filtered through double layer muslin cloth then centrifuged at 3500rpm for 30min (Fig: 3). The supernatant was filtered through what man no.1 filter paper. And sterilized at 120°c for 30 minutes. The extracts were preserved aseptically at 5° for further use (Girish and Sathish, 2008).

Preparation of test sample (medicinal plant treated culture)

50 µl of plant extraction was added to the 1ml of *Leptospira autumnalis* culture named as Test.

Whole cell lysate antigen

The above mentioned medium with the bacterial growth was used for lysation. The whole cell culture was sonicated in a chilled atmosphere with the frequency of 20Hz, 3 times for a duration of 15seconds giving a few seconds interval. This sonicated culture was centrifuged at 4°c, in a refrigerated centrifuge with a revolution of 10,000 rpm for 20mts. The pellet was reconstituted in a very little quantity of Tris Hcl buffer, PH6.8. The supernatant was added with equal amount of 10% TCA and centrifuged at a gyration of 2000rpm for 10minutes. The pellet was given 5 to 6 times either wash and was reconstituted with very little quantity of the above mentioned buffer. These two preparations (Pellet and supernatant) were lyophilized.

Lyophilization procedure

Lyophilization (freeze-drying) is an invaluable method for concentrating small molecular weight peptides and for obtaining a dry powder of protein. The solution to be lyophilized was placed in a freeze-drying flask is and shell frozen by slowly rotating the flash in a bath of dry ice and methanol or liquid nitrogen. Automated freeze-drier VIRTIS VACUOSPIN was used for Lyophilization of Leptospiral whole cell extracts. The lyophilized preparations were used for the protein partial purification.

Protein partial purification
(Hussain *et al.,* 2000)

The whole cell lysate antigen *L.autumnalis* serogroup was grown in 25ml of EMJH medium. Then added equal volume of 10% TCA (Trichloro acetic acid). It was centrifuged at 6000 rpm for 10minutes. Then precipitated proteins were collected. Then 20ul of petroleum either was added for removing lipids and then it was air dried.

Estimation on antigenic protein

To estimate the antigenic protein content, evaluate its purification and quantification of it, Lowry *et al.,* (1951) procedure was followed. This method is sensitive enough to give constant volume and hence routinely followed. 0.2, 0.4, 0.6, 0.8 and 1 mg/ml of working standards (bovine serum albumin) were pipetted out into a series of test tubes. 25ul of *Leptospira autumnalis* antigenic proteins were transferred to fresh sterile test tubes. Volume was made up to 1ml in all the test tubes with the use of sterile distilled water. Another tube with 1ml sterile distilled water served as the blank. 5ml of reagent c was added to each tube including the blank. Mixed well and allowed to stand for 10 minutes. 5ml of reagent d was added, mixed well and allowed incubation at room temperature in the dark for 30 minutes. Blue color was developed. Readings were taken after 30 minutes at 660nm. Standard graph was drawn and the amount of protein in the sample was calculated. The estimated proteins are analysed by SDS-PAGE.

Proteins analysis by SDS-PAGE
(Laemmli, 1970)

There are two components to the gel used for SDS-PAGE; a separating gel (12%) and stacking gel (5%). Separating gel was prepared and poured carefully into gel cassette and allowed to polymerize in about 30 minutes. After polymerization, stacking gel was prepared and poured down along the side of the gel apparatus near one of the spacers. It was poured slowly and carefully to avoid the formation of bubbles. The comb was placed at a slant between the two glass plates and lowered gradually. The gel was allowed to polymerize in 20 to 40 minutes. During polymerization, the prepared samples were taken for loading.

The samples and molecular weight markers were heated at 100°c in the water bath for 3 minutes just before loading. It was rinsed with buffer to remove any non-polymerized acrylamide. Then the comb was removed by pulling it straight up, slowly and gently. The wells were rinsed with distilled water at the sink using a syringe. The gel assembly was removed from the casting stand and snapped into the cooling core assemblage of the gel in the tank, so that the two gels were in one unit. The tank was filled with running buffer. Samples were loaded. The lid was placed on top of the lower buffer chamber.

The electrical leads were attached to the gel apparatus. The leads were attached to the power supply and power was turned on. The power was set at 50 volts. The gel was run for about 45 minutes or until the dye just began to run out from the bottom. The voltage was turned to zero and then the power supply was tuned off. After removing the gel lid, the inner cooling core was pulled out of the lower chamber. The upper buffer was poured into the sink and the gel was flushed with running water. The units were disassembled to draw glass sandwich. The gel was

carefully ripped out grabbing two corners. These gels were very thin and identification was easy. One edge was nicked to the separating gel. The coomassive blue stain was placed. The gel was stained for 30 minutes. The gel was transferred from the stain to the destaining solution. The container was covered. Coomassive brilliant blue was transferred back into its original bottle using a funnel. The gel was in the destaining solution on the shaker for 30 minutes to 3hrs. After destaining, the gel was taken for observation. The destain was poured into the "used destain bottle" for recycling or disposal. The bands was observed using transilluminator and then measured.

Separation of proteins under denaturing condition

SDS is anionic and denatures proteins by "wrapping around" the polypeptide backbone. It binds to proteins specifically in a mass ratio of 1:4:1. in so doing, SDS confers a negative charge to the polypeptide in proportion to its length. The denatured polypeptide becomes "rods" of negative charge and clouds with equal charge densities. It is usually necessary for separation by size. This is done with β-mercaptoethanol or dithiothreitol. In the denaturing SDS-PAGE separation, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

Results and Discussion

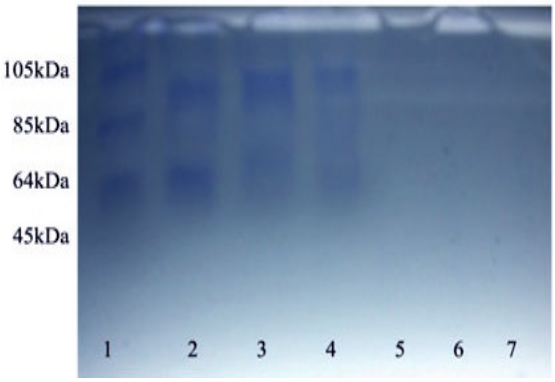
Protein content by Lowery's method

The antigenic protein content estimated by Lowery *et al*., (1951) was found to be 13.2 mg/ml. After plant treated antigenic protein content estimated by Lowery et al was found to be 24.71 mg/ml.

SDS-PAGE analysis

SDS-PAGE protein profile of *Leptospira autumnalis* before and after the treatment with the *Eclipta alba* (medicinal plant). A careful analysis of the protein profiles of the selected Leptospiral serovar revealed the following interesting results. A protein profile ranging from 45kDa to 105kDa was observed in the SDS-PAGE analysis for *Leptospira autumnalis*. Some deeply stained and faintly stained proteins were observed during the analysis. In the protein profile deeply stained band was observed at 64kDa as a major band. Some minor bands were also noted at the lower and higher molecular region of this major band. They were at 45kDa and 105kDa. Interesting difference was noted between the medicinal plant untreated and treated *Leptospiral* protein bands (Figure.1). The test samples (medicinal plant extract treated) were not showing any clear band. However, the samples were giving 24.71mg/ml as estimated in a standard biochemical procedure.

Figure.1 SDS-PAGE analysis of *Leptosira* proteins.



1.Marker Protein, 2-4. *Leptospira* without extract treatment, 5-7. *Leptospira* after extract treatment

In this study the deeply stained protein band observed at 64Kda as a major band and some minor bands observed at 45Kda and 105Kda similar to in earlier studies

Biswas *et al* ., (2005) reported 67,65,45,43,35,32 and 18Kda major proteins in the whole cell lysate were common among all the serogroups of *Leptospira.* The 67, 41,35,32,28 and 22Kda were the major outer membrane proteins, while 94, 32, 25 and 18Kda proteins were in inner membrane. Among several immunoreactive proteins, three (67,45 and 32Kda) were recognized as major antigens by both rabbit hyper immune sera and patients sera while the 32Kda proteins was recognized as the major immunoreactive protein by homologous and heterologous patient sera.

The protein band observed at 64Kda as a major band and some minor bands observed at 45Kda and 105Kda contrast to in one dimensional SDS-PAGE the leptospiral samples prepared from strains of various leptospiral serovars were run and transferred on to nitrocellulose paper and probed with pooled convalescent phase human sera to find out the phenotypic conservation of the protein fragments at 31 and 41Kda. The antigenic and genetic conservation of the two proteins, OMPL1and LiP41, indicated that these could be potential candidates for development of diagnostic test systems for leptospirosis (Natarajaseenivasan *et al*., 2005).

In this study the deeply stained protein bands observed at 64KDa named as major band. Some minor bands are observed at 45KDa and 105KDa. The test samples (*E. alba* Medicinal plant treated) were not showing any band. Similar to an early studies carried out by Prabhu *et al.,* (2008) reported the antileptospiral activity of *Eclipta alba* (L) was well studied by both tube dilution and microdilution technique and the result showed better inhibitory action against various serogroups of *Leptospira interrogans*. The aqueous extracts of *Eclipta alba* was very effective in causing *Leptospira autumnalis* proteins damage which was studied by SDS-PAGE assay.

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