



RESEARCH ARTICLE

**SDS- PAGE STUDY ON LEPTOSPIRA AUTUMNALIS PROTEIN DAMAGE
DUE TO PHYLLANTHUS AMARUS AND ECLIPTA ALBA**

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ABSTRACT

Leptospirosis is a potentially fatal infectious disease which is neglected and very widely prevalent in India causing a major public health problem. The present leptospiral research is focused on *Leptospira autumnalis* protein damage due to *Phyllanthus amarus* and *Eclipta alba*. SDS – PAGE study on *Leptospira autumnalis* protein damage by both extract treated sample damaged 65 kDa region, a major outer protein (Sphingomyelinase). Interestingly *Phyllanthus amarus* treated leptospira protein additionally damaged 25 kDa region, inner membrane protein. Protein damage were reported at various levels in *Phyllanthus amarus* than *Eclipta alba*. Further in-depth analysis of phytochemical plant compounds against the leptospiral proteins will reduce the side effects produced by synthetic drugs.

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INTRODUCTION

Leptospirosis is a zoonosis of worldwide distribution, caused by infection with pathogenic spirochaetes of the genus *Leptospira* (Fentahun and Alemayehu, 2012). The organisms are maintained in nature by chronic renal infection of carrier mammals, which excrete the organisms in their urine. Humans become infected through direct exposure to infected animals or their urine or through indirect contact via contaminated water or soil (Kamnath and Joshi, 2003). The adhesion of *Leptospira* to host tissue components is thought of as an initial and necessary step for infection and pathogenesis. Attachment to host cells and ECM components is likely to be necessary for the ability of *Leptospira* to penetrate, disseminate and persist in mammalian host tissues (Breiner *et al.*, 2009). In *Silico* analysis and experimental techniques employed to identify leptospiral surface-exposed proteins that might have potential roles in *Leptospira* adhesion and pathogenesis (Pinne and Haake, 2009). It is known that *Leptospira* can alter their biosynthetic mechanism for the production of the lipopolysaccharides in their outer membrane thus allowing them to adapt to new hosts (Nascimento *et al.*, 2004). Penicillin, cephalosporins, tetracyclines and macrolides have been widely used in the treatment of human leptospirosis. However, when these antimicrobial agents are used for the treatment of leptospirosis, long-term therapy with large doses may be required from the early stage of the disease until the appearance of antibodies (Misao *et al.*, 1955). Due to side effects produced by chemotherapy today, people are showing greater interest towards herbal medicines. It is safer than synthetic medicines because the phytochemicals in the plant extract target the biochemical pathway. To overcome the side effects produced

by synthetic drugs, herbal based therapeutics had been used in treating leptospirosis (Emmanouilides *et al.*, 1994).

Phyllanthus amarus (L.) belongs to the family Euphorbiaceae commonly called as Bahupatra in India. Traditionally, these plants are ayurvedic herb used in southern India for the treatment of liver diseases (Portilo *et al.*, 2001). *Eclipta alba* belongs to the family Asteraceae grows in tropical and subtropical countries. It is commonly known as Karishalenganni in Tamil (Sureshkumar *et al.*, 2005). Traditionally, these plants are ayurvedic herb used in southern India for the treatment of liver related diseases (Nirmaladevi and Periyannayagam, 2011). The herb contains wedelolactone as coumestan derivatives, the first one has been responsible as major anti-hepatotoxic compounds of this plant. It is an active ingredient of many herbal formulations prescribed for liver ailments and shows effect on liver cell generation (Portilo *et al.*, 2011). Herbal extract researches on protein damage of *Leptospira autumnalis* are not documented. Keeping these drawbacks the current research work was designed as SDS-PAGE study on *Leptospira autumnalis* protein damage due to *Phyllanthus amarus* and *Eclipta alba*.

MATERIALS AND METHODS

Collection of Plant material

The commonly available medicinal plants of our region *P. amarus* and *E. alba* were received from ABS medicinal plant research center, Karippatti, Salem Tamilnadu, India during the month of December 2011. Fresh plant material were washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles.

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Preparation of aqueous extract

Ten grams of air-dried powder was added to distilled water and boiled on slow heat for 2 h. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000g for 10 min. The supernatant was collected. This procedure was repeated twice. After 6 h, the supernatant collected at an interval of every 2 h, was pooled together and concentrated to make the final volume one-fourth of the original volume (Sadique *et al.*, 1989).

Effect of *Phyllanthus amarus* and *Eclipta alba* on *Leptospira autumnalis* protein damage

1ml of *Leptospira autumnalis* culture was treated with 50µl of plant extracts and incubated at room temperature for 24 hours (Nirmaladevi and Periyannayagam, 2011). The treated samples were further analyzed for the protein damage by SDS PAGE.

Leptospira Culture

Leptospira autumnalis cultures in EMJH semisolid medium (Difco, Sparks, UK) were received from the WHO Center for Reference and Research on leptospirosis, Brisbane, Australia.

Whole cell lysate antigen

The above mentioned medium with the bacterial growth was used for lysis. The whole cell culture with and without exposure to the chosen herb extracts were 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 petroleum ether wash and was reconstituted with very little quantity of the above mentioned buffer. The protein samples were cryopreserved until use (Biswas *et al.*, 2005).

Estimation of antigenic protein

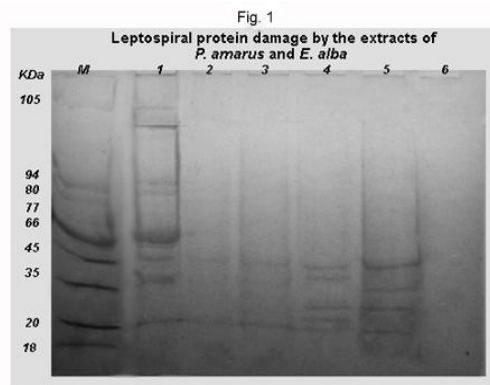
The partially purified leptospiral whole cell lysate protein was subjected for protein estimation study following the standard procedure (Lowry *et al.*, 1951).

SDS-page

Preparation of whole-cell lysate proteins was performed by using the following lysis solution: 60 mM Tris-HCl (pH 6.8)-10% glycerol-2% SDS-5% mercaptoethanol-0.1% bromophenol blue. Whole cell lysate proteins were boiled for 10 min, respectively, in the lysis buffer. Discontinuous SDS-PAGE was performed by the method of Laemmli (Laemmli, 1970) in a vertical electrophoresis apparatus (Biotech, Yercaud) with some modifications. Resolving gel used was 12 per cent acrylamide over hanged with 5 per cent of stacking gel. Twenty micrograms of whole-cell protein lysate of detergent-soluble protein of the strain prepared in 2X gel loading buffer was added to the appropriate well along with molecular weight marker. The samples were first run at a constant voltage of 50 V, till the dye front crossed the stacking gel. Then the voltage was increased to 100V till the dye front reached the end of the gel. Thereafter, the gel was subjected to the coomassie brilliant blue R- 250 (Sigma, USA) staining for 1 h and then destained for 6-8 h with several changes of destaining solution.

RESULT

The interesting results of SDS-PAGE study on *P. amarus* and *E. alb* aexposed *L. autumnalis* protein profile are as follows: Some deeply stained and faintly stained protein bands were observed during the analysis. Low molecular weight protein bands observed were 22, 25, 41 and 45 kDa. These protein bands were almost intact in both the control and *E. alba* treated samples. A deeply stained 65 kDa band which was reported to be a major immunogenic protein was intact in the control (untreated *Leptospira*). The present study gives us hope in the management of Indian Leptospirosis. Thus in spite of many repeated trials the extracts treated *Leptospira* was evincing sphingomyelinase protein damaged.



1 = Untreated *Leptospira* 2 & 3 = *P. amarus* treated 4 & 5 = *E. alba* treated 6 = Blank

Proteins damage were noted at higher molecular weight regions namely 94 and 105.5 kDa of both extract treated samples however, no such damage was observed for the untreated (control) *Leptospira* protein samples. The major protein band at 65 kDa region which was reported as Sphingomyelinase protein was clearly resolved in the present SDS – PAGE study using the bacterial whole cell lysate antigens. (Fig. 1). The virulent protein was found intact in the control however, a mild damage in *E. alba* exposed *Leptospira* and a clear damage in *P. amarus* exposed *Leptospira* was very interesting to be noted. Moreover the other important inner membrane protein damage was observed only for *P. amarus* at 25 kDa regions. However, the other important outer membrane protein did not show any damage at 45 kDa region in spite of the repeated exposure by both the extracts on the *Leptospira* samples.

DISCUSSION

Thus successfully the major immunogenic protein at 65KDa region (Sphingomyelinase) was isolated from the leptospira using their whole cell lysate antigens. The Sphingomyelinase protein was found intact as that of the control and showing damage by the *P. amarus* and *E. alba* treated leptospira. It was suggested that the SPhH as a novel leptospira haemolysin identified as a pore-forming protein among the pathogenic leptospira. Direct membrane damage of sheep erythrocyte and mammalian cells caused by the SphH in vitro studies indicated its potential role in pathogenesis of leptospirosis (Seoung *et al.*, 2002). It was further stated that the proteins of 67, 41, 35, 32, 28 and 22 kDa were the major outer membrane protein reported in the commonly circulating leptospira serogroups in India, while 94, 32, 25 and 18 kDa proteins were of inner membrane (Biswas *et al.*, 2005). In the present study the inner membrane protein 25 kDa was completely damaged in *P. amarus* treated sample where as not damaged in *E. alba* treated sample. 25 kDa inner membrane

protein was designated as LfhA (Leptospiral factor H-binding protein) and also found to bind human factor H related protein 1 (FHR-1). The capacity of pathogenic leptospira to bind host extra cellular matrix and factor H depends on virulence property (Verma *et al.*, 2006). In the present study those major immunoreactive proteins reported by Biswas *et al.*, (Biswas *et al.*, 2005), were also reported with some minor positional changes respectively at 65, 45 and 31.0 kDa regions. This could be due to serogroup difference. In the present study *L. autumnalis* was used but in the previous study *L. pomona* was used. Thus it is evident by the present study that sphingomyelinase could be demonstrated in between 66 kDa and 77 kDa region. Sphingomyelinase damage observed by the treatment of both sample is very interesting to note because the protein is in the major virulent factor of leptospira (Volina *et al.*, 1986).

In the present study some minor bands were also observed at 22, 25, 41 and 45 kDa, this observation is similar to the report of Galayanee and his coworkers who have reported similar pattern of multiple reactive bands, ranging from 10 to 90 kDa (Galayanee *et al.*, 2007). In an earlier study it was reported that a protein with an apparent molecular weight of 47 kDa was observed but in the present study this band was reported at 45 kDa. It was included in a group of outer membrane proteins which was described in the leptospire of different serogroups (Cullen *et al.*, 2002). The antigenic and genetic conservation of the two proteins OMPL1 and Lip41, were indicated and it was further stated that these could be potential candidates for the development of diagnostic test system for leptospirosis (Natarajaseenivasan *et al.*, 2005). This present study is going hand with the above study. Faint bands were reported at 45 kDa in both extract treated samples. A novel 45 kDa OMP from *L. kirschneri* was reported to induce antibody response in hamsters indicating that LipL45 was expressed during infection (Matsunga *et al.*, 2002). An earlier study which reported the antileptospiral activity of *E. alba* by both tube dilution technique and microdilution technique and the results showed showed better inhibitory action against various serogroups of *Leptospira interrogans* (Prabhu *et al.*, 2008). In the present study protein damage was observed at various levels *P. amarus* treated sample than *E. alba*. The present study gives a clear insight on the Sphingomyelinase and inner membrane protein damages by evincing either distorted protein bands or absence of the bands at specific region in comparison with untreated (control) leptospiral protein bands. The herbal extracts exposed *L. autumnalis* after 24 hours were showing clear protein damage at 65 kDa, as observed in the slab gel protein profile.

Reference

1. Biswas, D., Roy, S., Vijayachari, P., Sugunan A. P., Natarajaseenivasan K. and Sehgal, S. C. 2005. Comparison of immunoreactive proteins of commonly circulating serogroups of *Leptospira* in Andaman Islands, India. *Indian J. Med. Res.* 121: 151-158.
2. Breiner, D., Fahey, M., Salvador, R., Novakova, J. and Coburn J. 2009. *Leptospira interrogans* binds to human cell surface receptors including proteoglycans. *Infect Immun.* 77: 5528-5536.
3. Cullen, P. A., Cordwell, S. J., Bulach, D. M., Haacke, D. A. and Adler, B. 2002. Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. *Infect. Immunol.* 70: 1211-1218.
4. Emmanouilides, C. E., Kohn, O. F. and Garibaldi, R. 1994. Leptospirosis complicated by a Jarisch-Herxheimer reaction of adult respiratory distress syndrome: Case report. *Clin. Infect. Dis.* 18: 1004-1006.
5. Fentahun, T. and Alemayehu, M. 2012. Leptospirosis and its Public Health Significance. *European J. Appl. Sci.* 4: 238-244.
6. Galayanee, D., Worachart, S., Albert, I. K., Suraphol, K., Pimjai, N. and Visith, T. 2007. Use of immunoblotting as an alternative method for serogrouping *Leptospira*. *J. Med. Microbiol.* 56: 587-592.
7. Kamath, S. A. and Joshi, S. R. 2003. Re-emerging of infections in urban India - Focus leptospirosis. *J. Assoc. Physicians India.* 51: 247.
8. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature.* 227: 680-685.
9. Lowry, O. H., Rosebrough, N. J., Farm, A. I. and Randall R. J. 1951. Protein measurement with the folin-phenol reagent. *J. Biol. Chem.* 193: 265-275.
10. Matsunaga, J., Young, T. A., Barnett, J. K., Barnett, D., Bolin, C. A. and Haake, D.A. 2002. Novel 45-kilodalton leptospiral protein that is processed to a 31-kilodalton growth-phase-regulated peripheral membrane protein. *Infect. Immunol.* 70: 323-334.
11. Misao, T., Hiroyoshi, S., Katsuta, K., Nishihara, Y., Kuwashima. K. and Kobayashi Y. 1955. Antibiotics therapy of leptospirosis. *Clinics.* 1: 274-285.
12. Nascimento, A. L., Ko, A. I., Martins, E. A., Monteiro-Vitorello, C. B., Ho, P. L. and Haake, D. A. 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J. Bacteriol.* 186: 2164-2172.
13. Natarajaseenivasan, K., Vijayachari, P., Sharma, S., Sugunan, A. P. and Sehgal S. C. 2005. Phenotypic and genotypic conservation of ompL1 and LipL41 among leptospiral isolates of Andaman Islands, India. *Indian J. Med. Res.* 122: 343-347.
14. Nirmaladevi, K. and Periyannayagam, K. 2011. Nephroprotective effect of *Plectranthus ambonicus* spreng on glycerol induced acute renal failure. *Herbal Tech Industry.* 8: 15-16.
15. Pinne, M. and Haake, D. A. 2009. A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of *Leptospira interrogans*. *PLoS One.* 4: 6071.
16. Portillo, A., Vila, R., Freixa, B., Adzet, T. and Canigueral, S. 2001. Antifungal activity of Paraguayan plants used in traditional medicine. *J. Ethnopharmacol.* 76, 93-98.
17. Prabhu, N., Joseph, P. I., Chinnaswamy, P., Natarajaseenivasan, K. and Lakshmi, S. 2008. *In-vitro* evaluation of *Eclipta alba* against serogroups of *Leptospira interrogans*. *Indian J. Pharm. Sci.* 70: 788-791.
18. Sadique, J., Rqobab, A. L., Bughaith, M. F. and Gindey, A. R. 1989. The bioactivity of certain medicinal plants on the stabilization of RBC membrane system. *Fitoterapia.* 1: 525-532.
19. Seoung, H. L., Sangduk, K., Seung, C. P. and Min, J. K. 2002. Cytotoxic Activities of *Leptospira interrogans* Hemolysin SphH as a Pore-Forming Protein on Mammalian Cells. *Infect. Immunol.* 70: 315-322.

20. Sureshkumar, S., Sivakumar, T., Chandrasekar, M. J. N. and Suresh, B. 2005. Evaluation of Anti – Inflammatory Activity of *Eclipta alba* in Rats. *Ancient Science of Life*. XXIV : 112 – 118.
21. Verma, A., Hellwage, J., Artiushin, S., Zipfel, P. F. and Kraiczky, P. 2006. LfhA, a novel factor H-binding protein of *Leptospira interrogans*. *Infect. Immunol.* 74: 2659–2666.
22. Volina, E. G., Levina, L. F. and Soboleva, G. L. 1986. Phospholipase activity and virulence of pathogenic leptospirae. *J. Hyg. Epidem. Microbiol. Immunol.* 30: 163-169.
