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In-Vitro Screening of *Phyllanthus Amarus* and *Eclipta Alba* Against *Leptospira Autumnalis*

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Abstract

Background and Study Aims: *Multi-drug resistance is one of the major public health problems especially in developing countries where relatively easy availability and higher consumption of medicines have lead to disproportionately higher incidence of inappropriate use of antibiotics and greater levels of resistance compared to developed countries. Leptospirosis is the most wide spread zoonoses in the world and recognized as an important public health problem.*

Aim and Methods: *To extract and identify some phytochemicals from the commonly available and widely studied Indian medicinal plants (Phyllanthus amarus and Eclipta alba) with anti-bacterial, hepatoprotective and renoprotective properties and also to conduct an anti-leptospiral susceptibility study on MIC of a most common Leptospira interrogans serovar autumnalis following the standard TDT.*

Results: *The present study revealed the presence of tannins, alkaloids, saponins, flavonoids, terpenes and anthraquinones in the leaves of these plants extracts thereby exhibiting anti-leptospiral activity. The drug dose concentration the extracts of P. amarus was found to be having a better active principle (160 µg/ml) than the extracts of E. alba (320 µg/ml).*

Keywords: *Leptospira autumnalis, P. amarus, E. alba, Tube dilution technique.*

Abbrevations: *TDT= Tube Dilution Technique, MIC= Minimum Inhibitory Concentration.*

Introduction

Leptospirosis a contemporary infectious disease also known as Weil's syndrome is an emerging and re-emerging life threatening zooanthroponotic disease which affects internal organs producing multiple organ dysfunctions [MOD] to multiple organ failure [MOF] (Isa et al., 2014). Leptospirosis is a notifiable disease in India for

the past three decades no accurate disease incidence figures are available. Leptospira has hit virtually all parts of urban, semiurban, semirural and rural India (Parasuraman et al., 2014). It is a serious contagious disease commonly transmitted by not only the urine of rats but also it spreads through flood waters, garbage, wet ground and contaminated plants (Calvo-Cano et al., 2014). Emergence of multiple drug resistant strains of microbial pathogens including Leptospira occurs due to indiscriminate use of antibiotics (Kumar, et al., 2013). Due to allergic problems and side effects produced by chemotheraphy today, people are showing greater interest towards alternate therapeutic methods especially of herbal medicines (Chakraborty et al., 2010). It is safer than synthetic medicines because of the presence of several anti-microbial metabolites like alkaloids, anthroquinoes, tannins, flavonoids, glycosides, essential oils, saponins, phytosterols, amides, etc in the plant extract which target the biochemical pathways of bacteria only and not on human physiology (Maity et al., 2013). However, detailed studies pertaining to the active principles of herbal drugs are very limited. Herbal extracts' of hepatoprotective and renoprotective phytochemicals from important medicinal plants are need of this hour. In the present study *Phyllanthus amarus* and *Eclipta alba* were screened for their anti-leptospiral activity especially on *Leptospira autumnalis* to overcome the side effects produced by synthetic drugs.

Materials and methods

I. Collection of plant material

The Fresh whole plant of *Phyllanthus amarus* were received from ABS medicinal plant research center, Karippatti, Salem Tamilnadu, India during the month of December 2013. Fresh plant leaves were washed with distilled water, air dried at room temperature ground into powder and stored until further use.

II. Preparation of aqueous extract

Ten grams of air-dried powder was added to distilled water and boiled on slow heat for 2 h (Sadiaque, et al., 1989). It was then filtered through8 layers of muslin cloth and centrifuged at 5000g for 10 min. The supernatant was collected and the same 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 (Parekh, et al., 2005).

III. Preliminary qualitative phytochemical analysis

The preliminary qualitative phytochemical analysis of P. amarus and E. alba was performed to screen for the presence of bio-active components in the selected plant leaves (Evans, 1989; Sofowora, 1993).

IV. Anti-leptospiral susceptibility testing (Tube Dilution Technique)

The efficacy of *Phyllanthus amarus* extract (PAE) and *Eclipta alba* extract (EAE) against Leptospira interrogans serogroup autumnalis was investigated by Minimum Inhibitory Concentration (MIC) study of the extracts following the standard method of Tube Dilution Technique (TDT) (Oie et al., 1983; Mathew, 2001). 1ml of various concentrations of plant extracts ranging from 5, 10, 20, 40, 80, 160, 320 and 640 µg/ml was added in the Ellinghausen, McCullough, Johnson and Harris (EMJH) liquid medium. Sterility checking of the medium was done by placing it in the room temperature for 48h, the *Leptospira autumnalis* were inoculated with syringe filter. The tubes were incubated at room temperature for 8 days. The inhibition patterns in different concentrations were observed under darkfield microscope by placing a loopful of suspension on the clear glass slide and observed under different magnifications of the darkfield microscope. The inhibition profile of the extracts were tabulated and compared with control. Experiments were carried out in duplicates and repeated for three times. The

results of both the extracts were described to improve the therapeutic values of the leptospirosis by the predominant serogroup. The antimicrobial standardization of the spirochaetal member was studied in Tube dilution technique and screened under darkfield microscope and the results were impregnated as percentages.

Results

(a)Preliminary qualitative screening of PAE and EAE

The preliminary screening tests may be useful in detection of bioactive principles and subsequently may lead to drug discovery and development. Several workers use different solvents a system for the extraction of bioactive compounds in the current study water was utilized as a solvent system, traditional healers use primarily water since it is a safe solvent compared to other solvents for preparing ayurvedic formulations. The phytochemical analysis of the current study revealed the presence of tannins, alkaloids, saponins, flavanoids, terpenes and anthraquinones in the leaves of P. amarus and E. alba.

(b) Anti-leptospiral susceptibility testing (Tube Dilution Technique)

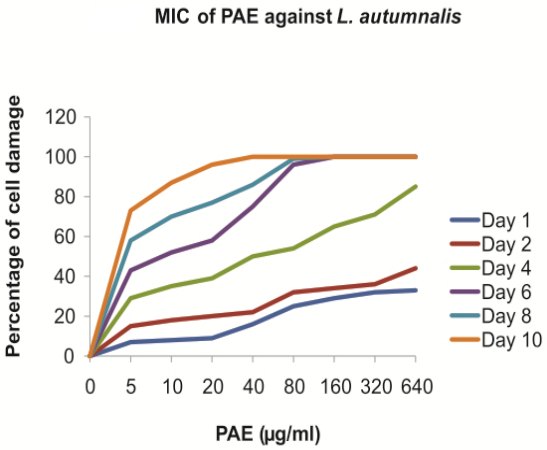
The tube dilution technique was followed for the conduction of MIC of chosen medicinal plant extracts. The aqueous extracts of both the selected plants showed satisfactory leptospiral inhibitory property. The MIC value was observed from 5µg/ml to 640µg/ml; the maximum inhibition (100%) for PAE was found to be ≥ 160 µg/ml. For EAE the complete inhibition was at ≥320 µg/ml. Thus the MIC effect of PAE extract was found to be comparatively superior to the extracts of E. alba. Standard antibiotic doxycyline (Control) exhibited the MIC at 200µg/ml. The results are given in detail.

MIC of PAE

P. amarus extract (PAE) of known quantities were dissolved in each 1 ml of sterile phosphate buffered saline (PBS) 7.2 pH and used for the anti-leptospiral activities. A negative control of plain sterile saline was used simultaneously along with the test vials. Starting from 5µg/ml double fold concentration of up to 640 µg/ml were prepared and treated with approximately 2 x 10^4 / ml of Leptospira. The results were observed on 1^{st}, 2^{nd}, 4^{th}, 6^{th}, 8^{th} and 10^{th} day of the PI (Post inoculation). On 1^{st} and 2^{nd} day of observation PAE exhibited least inhibition (33% and 44%) even at 620 µg/ml concentration.

(i) 4^{th} day observation

Interesting results were observed only from the 40 µg/ml concentration with 54% of reduction for the pathogen's growth inhibition potentiality. Up to 20 µg/ml concentration the PAE was giving up to 39% of anti-leptospiral inhibition only. In the 80 µg/ml concentration the reduction was 54% but in the next higher concentration (160 µg/ml) the inhibition was to a tune of 61%. Interestingly in the next higher concentration (320 µg/ml and 640 µg/ml) 71% and 85% inhibition of the Leptospira was able to be observed (Figure: 1).



(ii) 6^{th} day observation

During the 6^{th} day observations for up to 80 µg/ml extract concentrations, the L. autumnalis growth was observed to be declining sharply. On the 6^{th} day approximately 43% of decline in leptospiral density was noted when compared with that of the negative control. The Leptospira growth reduction of 58% in 20 µg/ml with the P. amarus treatment was noted. Interestingly in 80 µg/ml around 96% of reduction was noted. From the next

concentration of 160 µg/ml, no live Leptospira (100% inhibition) was observed.

(ii) 8th day observation

On the 8th day observation a sharp decline in growth of the Leptospira was noted up to a concentration of 80 µg/ml extract treatment. On this day 5 µg/ml treatment, approximately 58% growth reduction was noted when compared with that of the negative control. While doubling the concentration (10 µg/ml) a gradual increase in inhibition of 70% was noted. Similarly for the next concentration (20 µg/ml) the inhibition in growth was noted as 77%. But with the same extract at 80 µg/ml a reduction of 99% was noted. While doubling this concentration complete (100%) inhibition was observed.

(iii) 10th day observation

On the 10th day of PI with 5 µg/ml the Leptospira were showing a reduction in their growth potentiality up to 73%. Interestingly when the concentration was doubled a very good inhibition of up to 87% was observed. No live Leptospira (100% inhibition) was observed on this 10th day for the concentration of 40 µg/ml and above. Based on the data obtained from this study, the PAE were found to be effective inhibitor of Leptospira interrogans autumnalis (Figure: 1).

MIC of EAE

Similarly the effect of EAE on the Leptospira was tested with the same concentration as that of the PAE. On 1st and 2nd day of observation EAE exhibited least inhibition (19% and 40%) even at 620 µg/ml concentration.

(i) 4th day observation

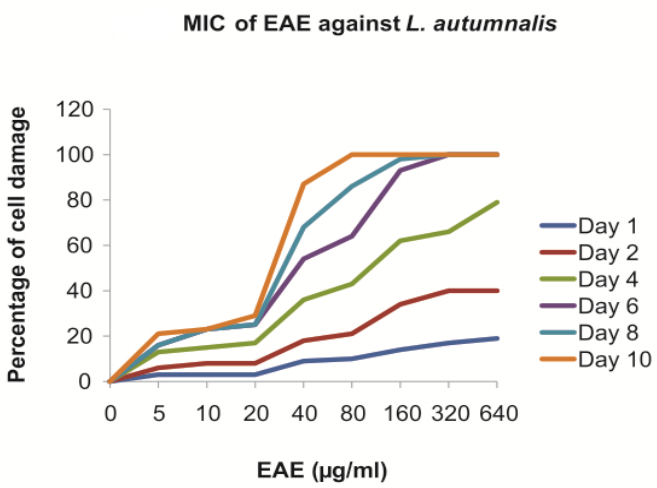
L. autumnalis growth was observed on the 4th day after treatment with EAE extract dilutions of 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, 160 µg/ml, 320 µg/ml and 640 µg/ml; the decline in growth was noticed to be approximately 13%, 15%, 17%, 36%, 43%, 62%, 66% and 79% respectively, in comparison with that of the control on the same day.

(ii) 6th day observation

Interesting results were observed only from the 40 µg/ml concentration with 54% of reduction for the pathogen's growth inhibition potentiality. In the 5 µg/ml concentration only 16% of inhibition was observed. A very minor difference was noted in the next concentration i.e., 10 µg/ml with a result of 23% decline in growth of the organisms was observed. In the next concentration (20 µg/ml) the reduction was 25% but in the next higher concentration (160 µg/ml) the inhibition was to a tune of 93%. Interestingly in the next higher concentration (320 µg/ml) 100% inhibition of the Leptospira was able to be observed.

(iii) 8th day observation

On the 8th day observation a gradual reduction in the growth potential was noted up to the 20 µg/ml concentration and a drastic drop in the growth was noted in the 40 µg/ml dilution. The lower concentrations were showing respectively growth reductions of 16%, 23% and 25% for the 5, 10 and 20 µg/ml dilutions respectively. The sudden drop in the cell count at the 40 µg/ml was to a tune of 68% reduction. On an average 86% of inhibition was observed with 80 µg/ml treatment on this day of observation. Still the growth was dwindling to give a result of 98% in the next concentration of 160 µg/ml. Beyond this concentration no live Leptospira (100% inhibition) was observed.



(iv) 10th day observation

For the 10th day observation of PI only a marginal reduction of growth at low concentration (up to 20µg/ml) of the extract treatment and an excellent result from the 40 µg/ml were noted. The MIC results were 21%, 23% and 29% respectively for the extract concentrations of 5, 10 and 20 µg/ml. A sudden drop in growth to a tune of 87% visibly was noted for the 40 µg/ml on the eighth day with this extract. Interestingly above than this concentration no Leptospira (100% MIC) were able to be observed (Figure: 2).

Discussion

The phytochemical analysis of PAE and EAE revealed the presence various secondary metabolites like tannins, alkaloids, saponins, flavanoids, terpenes and anthraquinones in the aqueous extracts of the medicinal plants. Tube dilution technique (TDT) was followed in the present study to have a better understanding about the efficacy of drugs against *Leptospira autumnalis* by adding various concentrations (5µg/ml double fold concentration of up to 640 µg/ml) of the compound in the EMJH liquid medium. Darkfield microscope was utilized and the results were impregnated as percentages. Utility of darkfield microscope for the TDT was recommended for Leptospira by some early researchers (Ben- yaacov, et al., 1994).

Studies are available on many Indian medicinal plants such as Ocimum sanctum, Herpestis monneria, Azardirachtha indica, Curcuma longa, Aegle marmelos and Nelumbo nucifera that exhibited maximum inhibition against Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus and Vibrio cholera (Dahanukar et al., 2000). However, anti-bacterial effects of plants such as P. amarus and E. alba are poorly studied. Moreover, such studies on Leptospira are still scanty. Thus in the present study anti-leptospiral activity of P. amarus and E. alba was carried out and the interesting MIC data obtained during the study are discussed below:

PAE of known quantities were dissolved in sterile phosphate buffered saline (PBS) 7.2 pH and used for the study. A negative control of plain sterile saline was used simultaneously along with the test vials. Starting from 5µg/ml double fold concentration up to 640µg/ml were prepared and treated with approximately 2 x 10^4/ml of Leptospira. When the results were observed on 4th day from the 40 µg/ml concentration with 54% of reduction for the pathogen's growth inhibition potentiality. Up to 20 µg/ml concentration the PAE was giving up to 39% of anti-leptospiral inhibition only. In the 80 µg/ml concentration the reduction was 54% but in the next higher concentration (160 µg/ml) the inhibition was to a tune of 61%. Interestingly in the next higher concentration (320 µg/ml and 640 µg/ml) 71% and 85% inhibition of the Leptospira was able to be observed.

During the 6h day of the PI approximately 43% of decline in growth was noted. Interestingly in 80 µg/ml around 96% of reduction was noted. From the next concentration of 160 µg/ml, no live Leptospira (100% inhibition) was observed. However, on the 8th day observation approximately 58% of growth reduction was noted at the 5 µg/ml but in10 µg/ml a gradual increase in inhibition of 70% was noted. But with the 80 µg/ml a reduction of 99% was noted. Beyond this concentration100% inhibition was observed. Interestingly on the 10th day just for 20 µg/ml a very good inhibition of up to 87% was observed. 100% inhibition was observed on this day observation for the concentration of 40 µg/ml and above. Thus the aqueous extract had a good MIC over the Leptospira tested. In some earlier studies pertaining to MIC, investigations were made on the anti-microbial activity of P. amarus tested against various bacteria including human intestinal facultative anaerobic flora. The aqueous extract of P. amarus controlled the growth of P. aeruginosa, S. aureus, E. fecalis, E. coli, P. mirablis and K. Pneumonia at 50mg/ml (Babatunde et al., 2014). However, in the present study antimicrobial activity of PAE was tested

against *Leptospira autumnalis* and the extract showed greater inhibitory activity (100%) in 80 µg/ml concentration of the extract.

Several workers use different solvents systems for the extraction of bioactive compounds like Tannins, alkaloids, flavonoids, flavonones, terpenoids, saponins, lignans, sterols, anthraquinones, etc from medicinal plants. Okolo and his coworkers analyzed the efficacy of various solvents such as aqueous, chloroform and ethanol in extracting the bioactive compound from *Phyllanthus amarus*. Their study clearly revealed that only aqueous extraction showed the presence of highest number of bioactive compounds like alkaloids, anthraquinone, balsam, flavonoids, saponins, steroids, tannins and terpenoids than chloroform and ethanol (Okolo et al., 2012). They also compared the presence of these bioactive compounds from various parts of the plant such as leaves, stem, seeds and roots. Leaves showed the highest number of bioactive compounds. The current study goes in full agreement with the above findings in utilizing aqueous as a solvent system for the extraction of bioactive compounds and leaves were chosen. The extracts of both the plant P. amarus and E. alba exhibited anti-leptospiral activity.

In another study four types of solvents namely aqueous, benzene, ethanol and methanol were used for the extraction of bioactive compound from A. vasica. Aqueous and methanol extracts were able to inhibit the motility of Leptospira interrogans at a concentration of 10 to 1000 mg/ml. The ethanol extract extracts of A. vasica was able to control leptospiral activity in all the concentrations tested ranging from 0.1 to 1000 mg/ml. In the same study the plant extract treated L. interrogans did not show the presence of inclusion body (surface vacuole) on the outer envelope of the fresh isolate. Inclusion body in L. interrogans is normally associated with the virulence of this pathogen (Nelson, et al., 2013). In contrast to the above finding in the present study a much lesser concentration of 160 µg/ml (PAE) and 320 µg/ml (EAE) 100% inhibition in leptospiral growth was observed which is encouraging in the management of this disease.

Four crude and five purified xanthones namely alpha-mangostin, gamma-mangostin, garcinone C, garcinone D and 8-deoxygartanin of Garcinia mangostana were active against the L. interrogans serovars such as bataviae, autumnalis, javanica and saigon and L. biflexa serovar patoc. All four crude extracts had greater MICs ranging from 200 to ≥ 800 µg/ml. The anti-leptospiral activity of five purified xanthones was variable in the range of 100 to ≥ 200 µg/ml (Seesom, et al., 2013). The above finding is in agreement with the present study that for PAE 20 to ≥ 160 µg/ml is giving 50 to 100% inhibition respectively.

EAE was tested against Leptospira with the same concentration as that of the PAE. The interesting results obtained are discussed below: L. autumnalis growth was observed on the 4th day after treatment with EAE extract dilutions of 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, 160 µg/ml, 320 µg/ml and 640 µg/ml; the decline in growth was noticed to be approximately 13%, 15%, 17%, 36%, 43%, 62%, 66% and 79% respectively, in comparison with that of the control on the same day. During the 6th day observation at 40 µg/ml concentration around 54% of reduction for the bacteria was observed but in the concentration of 160 µg/ml the inhibition was to a tune of 93%. Interestingly in the next concentrations ≥ 320 µg/ml 100% MIC of the Leptospira was able to be observed. On the 8th day observation a gradual reduction in the growth was noted up to the 20 µg/ml concentration but in the 40 µg/ml dilution a drastic drop (68%) in the growth was noted. On an average 86% of inhibition was observed with 80 µg/ml treatment for the 8th day observation. Still the growth was dwindling to give a result of 98% in the next concentration of 160 µg/ml. Beyond this concentration no live Leptospira (100% inhibition) was observed. For the 10th day observation of PI only a marginal reduction of growth at low concentration (up to 20 µg/ml) of the extract treatment and an excellent result from

40 µg/ml were noted. A sudden drop in growth to a tune of 87% visibly was noted for the 40 µg/ml on the eighth day with this extract. Interestingly above than this concentration no Leptospira (100% inhibition) were able to be observed. In an earlier study on *Eclipta alba*, bioactive compounds were extracted using various solvents like acetone, ethanol, petroleum ether and water. Extracted compound was diluted by using double distilled water. Various concentrations of those compounds were made ranging from 50, 100, 150, 200 and 250 µg in 1 ml and were used for anti-leptospiral activity. Aqueous extracts were found to be superior to all other solvent extracts. At the lowest concentration level of 50µg/ml MIC was observed for aqueous extracts (80% inhibition), where as the next better solvent was found to be acetone to get an MIC at 100 µg/ml against L. australis, L. autumnalis, L. grippptyphosa and L. icterohaemorrhagiae than other solvents utilized in their study (Prabhu, et al., 2008).

The above study is very much encouraging and going hand in hand with the results of the present study in getting excellent result with aqueous extracts (PAE and EAE) against the pathogen. In a previous study the efficacy of *Eclipta alba* against L. interrogans serogroups was investigated by evaluating the MIC of the extracts using solvents such as acetone, water and saponified lipid. They compared standard tube dilution with micro dilution technique. Tube dilution technique was reported to be a better tool in the evaluation of MIC by the authors (Mathew, 2001). In the present study tube dilution technique for leptospiral MIC was giving convincing result. This technique could increase the pathogen's survival rate and the results of growth inhibition could be because of the active principle of medicinal plants and not because of any stress or other abiotic and biotic factors.

Conclusion

Multidrug resistance, antibiotic sensitivity and allergic complications including many side effects have made Indian medicine especially from herbal sources as a very important infectious diseases management tool. Though P. amarus and E. alba have also proven to be important among medicinal plants with anti-microbial, anti-oxidant, hepato-renal protective activities. The present study revealed the presence of tannins, alkaloids, saponins, flavonoids, terpenes and anthraquinones in the leaves of these plants extracts thereby exhibiting anti-leptospiral activity. By observing the drug dose concentration the extracts of P. amarus was found to be having a better active principle (160 µg/ml) than the extracts of E. alba (320 µg/ml). Thus the study has given hope of possible Indian medicinal treatment for the human leptospirosis with these Indian medicinal plants.

References

1. Isa SE, Onyedibe KI, Okolo MO, Abiba, AE, Mafuka JS, et al., (2014). "A 21-Year-old student with fever and profound jaundice". PLoS Negl Trop Dis. 2014; 8: e2534.
2. Parasuraman B, Pichai K, and Karumana GK. "Study on the Prevalence of Leptospirosis among fever cases reported from private clinics in the urban areas of Villupuram District, Tamil Nadu, India". Osong Pub. Health Res. Perspectives. 2014; 5: 54–67.
3. Calvo-Cano A, Aldasoro E, Ramírez MF, et al "Two cases of laboratory-confirmed leptospirosis in travellers returning to Spain from Thailand, September 2013". Euro Surveill. 2014; 19: 1-3.
4. Kumar SG, Adithan C, Harish BN, Sujatha S, Gautam R and Malini A. (2013). "Antimicrobial resistance in India: A review". J Nat Sci Bio Med. 2013; 4: 286–91.
5. Chakraborty A, Satoshi M, Sharon YAM, et al. "In-vitro sensitivity and resistance of 46 Leptospira strains isolated from rats in the Philippines to 14 antimicrobial agents. Antimicrob Agents Chemother. 2010; 54: 5403-05.

6. Maity T, Pahari N, Bera K and Chakraborty M. "Recent development in herbal drug research for global health care. World J Pharmceutical Res. 2013; 2: 838-54.
7. Sadiaque J, Rqobab NA, Bughaith MF and Gindey AR. "The bioactivity of certain medicinal plants on the stabilization of RBC membrane system". Fitoterapia. 1989; 1: 525-32.
8. Parekh J, Nair R and Chanda S. "Preliminary screening of some folklore medicinal plants from western India for potential antimicrobial activity". Indian J Pharmacol. 2005; 37: 408-09.
9. Evans WC. "Textbook of Pharmacognosy". 13th ed., Cambridge University Press, London.1989.
10. Sofowora A. "Medicinal plants and Traditional medicine in Africa". Spectrum Books Ltd., Ibadan, Nigeria.1993.
11. Oie S, Hironaga K, Koshiro A, Konishi H and Yoshii Z. "In-vitro susceptibilities of five leptospira strains to 16 antimicrobial agents". Antimicrobl Agents and Chemother. 1983; 24: 905-08.
12. Mathew LM. "Comparative analysis of Acetone, Water and Saponified extracts of *Phyllanthus niruri* against *Leptospirainterrogans* sero groups in- vitro (Dissertation)". Periyar University, Salem, Tamilnadu, India.
13. Ben-yaacov R, Knoller S and Pierard GM. "Drug resistance capability of the pathogenic microbes in-vitro". Antimicrob Agents Chemother. 1994; 38: 422-26.
14. Dahanukar SA, Kulkarni RA, Rege RE. "Pharmacology of medicinal plants and natural products. Indian Jour of Pharmacol. 2000; 32: 81-118.
15. Babatunde SK, Abubakare AA, Abdulraheem, YJ and Ajiboye, EA. "Antimicrobial activity of *Phyllanthus amarus* on some human intestinal facultatively anaerobic flora. Internl. J. Med. Biomed. Res. 2014; 3: 52-7.
16. Okolo SC, Okoh-Esene RU, Ikokoh PP, Olajide OO and Anjorin ST. "Phytochemicals, mineral content and antimicrobial screening of *Phyllanthus amarus* in Abuja, Nigeria". J Microbiol Biotech Res. 2012; 2: 17-22.
17. Nelson J, Chairman K, RanjitSingh AJA, Padmalatha C and Hepsibah B. "Cytomorphological Changes and Inhibition of Inclusion Body Formation in Leptospira interrogans on Treatment with the Extracts of Adhatoda vasica". Adv Tech Biol Med. 2013; 1: 1-4.
18. Seesom W, Jaratrungtawee A, Suksamrarn S, Mekseepralard C, et al.,. "Anti-leptospiral activity of xanthones from Garcinia mangostana and synergy of gamma-mangostin with penicillin G". BMC Compl Altern Med. 2013; 13: 182.
19. Prabhu N, Innocent JP, Chinnaswamy P, Natarajaseenivasan K and Sarayu L. "*In-vitro* evaluation of *Eclipta alba* against serogroups of *Leptospira interrogans"*. Indian J Pharma Sci. 2008; 70: 788-91.