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Original Article

In vitro anti leptospiral activity of chloroform extract of *Piper betle* L

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ABSTRACT

The chloroform extract of the leaf of *Piper betle* L., (*Piperceae*) was investigated for its antileptospiral activity against 10 serovars of selected *Leptospira*. The leaves of this plant have been long in use tropical countries for the preparation of traditional herbal remedies. The minimum inhibitory concentration (MIC and minimum leptospiricidal concentration (MLC) of the chloroform extract were determined by using broth microdilution method. Time kill curve studies, post antileptospiral effects and mutation prevention concentrations were determined against *L. australis* and *L. patoc*. The membrane permeability was measured by the uptake of propidium iodide. The extract exhibited the inhibitory effect on leptospiral serovars of clinical significance, with the MIC ranging from 17.5 to 500µg/ml for the serovars whereas the MLCs were found to be similar or two fold greater than the MICs. There was concentration dependent killing of leptospires. There was increased uptake of propidium iodide by leptospires cells when exposed to chloroform extract thus indicating that the membrane disruption could be the probable mode of action of the chloroform extract of *Piper betle*. The antileptospiral activity exhibited by this extract warrants its use as a potent antileptospiral agent particularly for liver disorder and dysfunction.

Keywords: *Piper betle*, Chloroform extract, anti leptospiral activity

INTRODUCTION

Leptopsirosis is a zoonotic infection with a worldwide distribution that is associated with both endemic and epidemic diseases with the incidence of disease being highest in tropical climates. Infection can range in severity from clinically inapparent to life threatening with an epidemic case fatality rate as high as 15%. Limited studies have been examined the *in vitro* and *in vivo* effects of antibiotics against *Leptospira*. And further the *in vitro* anti leptospiral activity using herbal extracts are very less. The usage of antibiotics like benzyl pencillin and doxycycline are very effective against leptospiral members but observation of Jarish Harishmer reaction is high in liver and kidney damaged patients. Therefore it is necessary to search for more effective and less toxic novel antileptospiral agents that would overcome these disadvantages. The best solution for this issue is the use of herbal medicines which have been used since the time of immemorial in Indian villages. The World Health Organization has adopted a major policy change wherein most of the developing nations have to make use of more traditional medical practices for health care [1]. Several medicinal plants have been extensively studied in order to find more effective and less toxic compounds [2].

Piper betle L (*Piperaceae*) has been extensively used in traditional herbal remedies in India, China, Taiwan, Thailand and many other countries. It is reported for various pharmacological activities such as antimicrobial, antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory [3]. It also acts

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as a stimulant, a breath freshener, a carminative, a sialagogue, a cardiac tonic, a pain killer in joint pain, an aphrodisiac, an astringent, an antiseptic [4], a digestive and pancreatic lipase stimulant [5], wound healing [6]. Leaves are very nutritive and contain substantial amount of vitamins and minerals. The leaves also contain the enzymes like diastase and catalase besides a significant amount of all the essential aminoacids except lysine, histidine and arginine, which are found only in traces [7].

The main objective of this present investigation is to find out the effect of chloroform extract of the leaf of *Piper betle* L., (Piperceae) for its antileptospiral activity against 10 serovars of selected *Leptospira*.

MATERIALS AND METHODS

Plant material: Fresh and healthy leaves of *Piper betle* were collected from the Tiruchirapalli market and were brought to laboratory after proper identification. Leaves were washed with tap water followed by sterile water and dried. The dried leaves were ground by the help of kitchen blender and the powder was used for the extraction of bioactive compounds.

Chloroform extract: The powdered betel leaves of 5gms were soaked in 50ml of chloroform and kept in dark for 4 days so that all the secondary metabolites get dissolved. It was then filtered in petridish with the help of Whatman filter paper No.1. After filtration, the filtrate in petridish was kept in oven at 50^{o}C so that all chloroform gets evaporated. Dried metabolite was dissolved in double volume of 100mm Tris HCl, thus giving the final concentration of the extracted metabolite to 500mg/ml.

Leptospiral serovars and growth conditions: A total of 10 leptospiral serovars were tested for their susceptibility to chloroform extract of *Piper betle* leaves. The details of the strains and its respective serovars are depicted in Table 1. All the listed strains were obtained from National leptospirosis Reference Centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar islands and maintained by periodic sub culturing in EMJH semisolid medium. The medium used for the leptospiral study is highly sensitive due to the addition of bovine serum albumin and vitamin fractions and this get contamination very easily if there is no proper precautions to be taken during the preparation and processing [8,9].

Inoculum preparation: The dark field microscopy confirmed leptospiral strains maintained in the EMJH semisolid medium was transferred to the same medium without agar (EMJH broth). The inoculated broth was examined properly after incubation at room temperature for 7 days in dark condition and the leptospiral inoculum has the final concentration of 10^6 organisms/ml [10].

MIC and MFC determination: Susceptibility testing was used to determine the minimal amount of drugs which inhibit the maximum leptospires (MIC) or kill in vitro (MLC). Broth microdilution testing was standardized with 96 well round bottom microtiter plates [11]. The Twelve strains under ten different leptospiral serovars in EMJH broth of 150µl were poured in all wells (each column contain separate serovars). Each row is designated for various concentrations of chloroform extract of *Piper betle*. In each row, 50µl of various concentrations of leaf extract (2, 4, 6, 8, 10, 15, 20, 25% of extract) were added and incubated for one hour at room temperature in dark condition. After incubation, from each well, a drop of sample was placed in the slide and observed under dark field microscope for checking the motility inhibition by comparing with the control culture serovars.

Propidium iodide uptake assay: The disruptive effect of crude compound of the chloroform extract of *Piper betle* on *Leptospira australis* cell membrane was assessed by using phytochemical-mediated propidium iodide uptake. One ml volume of 5×10^6 cell suspensions per ml of *L. australis* in sterile MilliQ water were incubated with two to eight times the MIC (500 to 2000 µg/ml) of crude extract at 35°C for 60 minutes under agitation in the dark chamber. Fifteen minutes prior to the completion of incubation, 10µl each of propidium iodide and sodium deoxycholate solution were added at a final concentration of 25µg/ml and 2.5mg/ml respectively. Doxycycline at six times the MIC (4.0µg/ml) was used as the positive control and the cells without crude extract served as the negative (growth) control, treated in similar fashion. After incubation, 50µl aliquot was transferred into fluorescence activated cell sorting (FACS) tube (Life Technologies) containing 950µl of sterile MilliQ water. Each tube was analyzed using a FACScan flow cytometer with Cell Quest Pro software for data acquisition and analysis.

RESULTS

The MICs and MLCs of the crude chloroform extract of the Piper betle were evaluated in vitro against ten serovars of *Leptospira interrogans* and all the values are listed in Table 2. Among the serovars, the *L. autumnalis*, *L. javanica*, *L. sejroe* *L. icterohaemorrhagiae*, and *L. patoc* were found to be the most susceptible to chloroform extract of

Piper betle compared with other serovars included in this study. The killing activities of crude chloroform extract of leaves of *Piper betle* against *L. autumnalis* and *L. icterohaemorrhagiae* are shown in Figure 1 and 2. The antileptospiral activity against the leptospiral serovars and the reduction in the percentage of cells and movement were noted and was described as greater than 3 log units (99.9%). The leptospiricidal endpoint of *L. autumnalis* was achieved after 10 hours and 1 hour at 4 X MIC (4 X 200µg/ml) and 8 X MIC (8 X 200µg/ml) of leaf extract of *P. betle.* In *L icterohaemorrhagiae*, killing was observed at a lower concentration (200µg/ml) due to its lower MIC. There was concentration dependent killing observed in case of *L. autumnalis* and *L. icterohaemorrhagiae* with two, four and eight times the MIC exhibited leptospiricidal activity.

Exposing the leptospiral cell suspensions to two to eight times (800 to 2500 µg/ml), the MIC of crude chloroform extract of *P. betle* for 60 minutes increased the cell permeability to the propidium iodide due to the disruption of membrane integrity. This resulted in the increase in fluorescence in comparison to untreated control and was depicted in Figure 3. This increase in fluorescence was proportional to the increase in the crude chloroform extract of *P. betle.*

DISCUSSION

Leptospirosis is a potentially fatal infection that is often treated empirically due to the lack of rapid and accurate diagnostic testing. This lack necessitates therapeutic decisions that may cover a broad range of infectious diseases as part of the differential diagnosis. Treatment options are limited, as very few drugs have been evaluated in randomized human trials. For assessing the antileptospiral activity broth microdilution technique was standardized [11]. Compared to traditional broth macrodilution However, a rapid and convenient *in vitro* screening tool to assess the activity of numerous antimicrobial agents against various serovars of *Leptospira* could more expeditiously assess potential antimicrobial agents for clinical utility in humans and animals. By using broth microdilution technique, we performed the *in vitro* antileptospiral activity of chloroform extract of *Piper betle*.

Leptospirosis is most commonly involving zoonotic infections in the tropical countries like India during rainy season with increasing morbidity that range from multiple organ dysfunction to multiple organ failure. The role of host parasite chain breakage is very important to prevent such infections annually. The prophylactic measures should be followed as per the guidelines so that the infection rate may reduce.

The solvent extracted *Piper betle* was effective in inhibiting the *Leptospira interrogans* serovars that cause severe hepato-renal issues. There is no such report published in the antileptospiral activity of *P. betle*. Recently some reports highlighted the *P. betle* extracts have wide antibacterial and antifungal activities with average IC_{50} and IC_{90} values. Eventhough the phytochemicals present in the *P. betle* has broad bactericidal action [12,13], this study is the first time reported the antileptospiral potential.

Propidium iodide is a fluorescent nucleic acid stain that is unable to penetrate the cell membrane structures of healthy cells. However, cells with damaged or permeabilised cell membranes do not exclude propidium iodide. Therefore, propidium iodide staining of cells indicates cytoplasmic membrane (bacteria) damage [14]. Sodium deoxycholate was used in this study as it is reported to enhance the diffusion of propidium iodide across the cell wall to pass through the damaged leptospiral cell membranes. Interestingly, the growth controls did not show dye uptake in the presence of deoxycholate as the deoxycholate is nontoxic to Leptospiral serovars. The increased uptake of propidium iodide in the *P. betle* extract treated cells of L. interrogans in our study, further confirmed the earlier findings that the phytochemical alters the cell membrane structure, resulting in the disruption of the permeability barrier of microbial membrane structures [15]. The clinical applications of crude compound of the *P. betle* were challenging to interpret in this study due to a lack of pharmacokinetic and safety studies. However its comparable cytotoxicity profile with that of thymol widely used natural phenolic as food preservative and oral care agent in the earlier study is indicative of the safety of this compound.

CONCLUSION

The findings interpreted in this study are the first information of *P. betle* for antileptospiral activity. In general the phytochemicals in *P. betle* exhibited a broad range antimicrobial activity against clinically significant human pathogenic species. Further studies are therefore warranted in order to explore of this natural compound for therapeutic use in leptospiral infections.

Table 1: Details of leptospiral strains included in this study

Serogroup	Serovar	Strain
Australis	Australis	Ballico
Autumnalis	Bangkinang	Bangkinang
Canicola	Canicola	Hond Utrech IV
Grippotyphosa	Grippotyphosa	Moskva V
Hebdomadis	Hebdomadis	Hebdomadis
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
Javanica	Poi	Poi
Pomona	Pomona	Pomona
Sejroe	Hardjo	Hardjoprajitno
Semaranga	Patoc	Patoc I

Table 2: MICs and MLCs of crude chloroform extract of *P. betle* for 10 leptospiral serovars (14 strains) by broth microdilution method

Leptospira serovars	No. of strains tested	Antileptospiral activity in µg/ ml	
		MIC range	MLC range
L. australis	2	400 - 600	400 - 600
L. autumnalis	1	200	200
L. canicola	1	400	400
L. grippotyphosa	1	400	400
L. hebdomadis	1	800	800
L. icterohaemorrhagiae	1	200	200
L. javanica	2	200	200
L. pomona	1	400	400
L. sejroe	1	200	200
L. patoc	1	200	200

Figure 1: Killing effect of crude chloroform extract of *Piper betle* against *L. autumnalis*

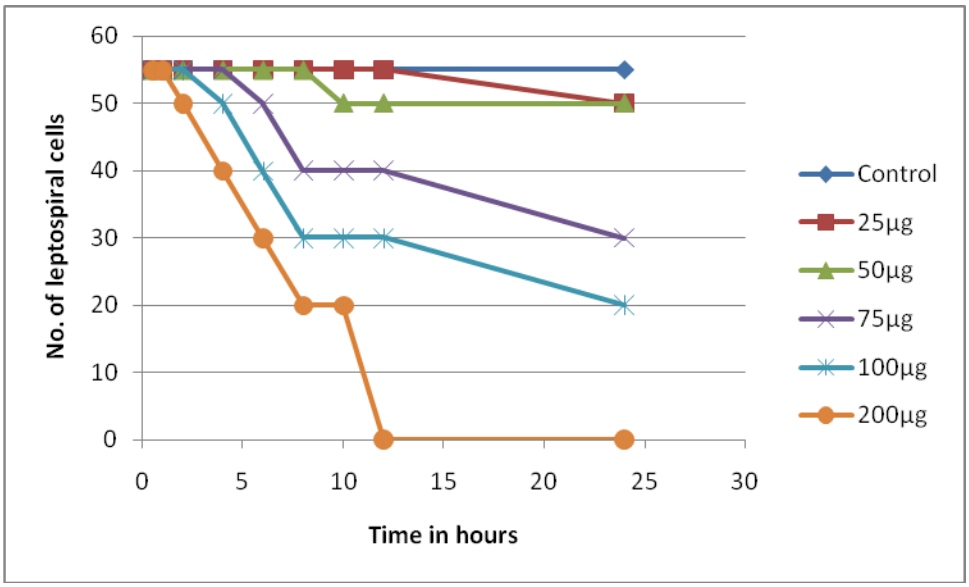


Figure 2: Killing effect of crude chloroform extract of *Piper betle* against *L. icterohaemorrhagiae*

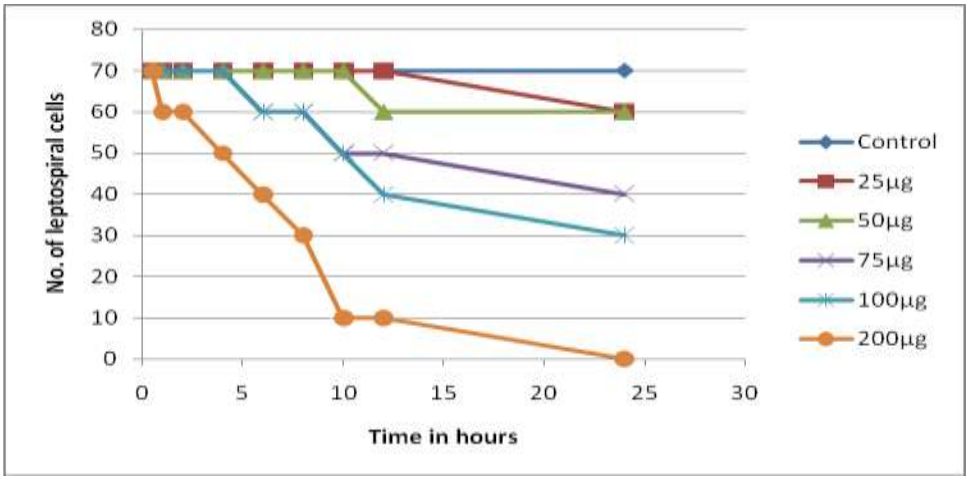
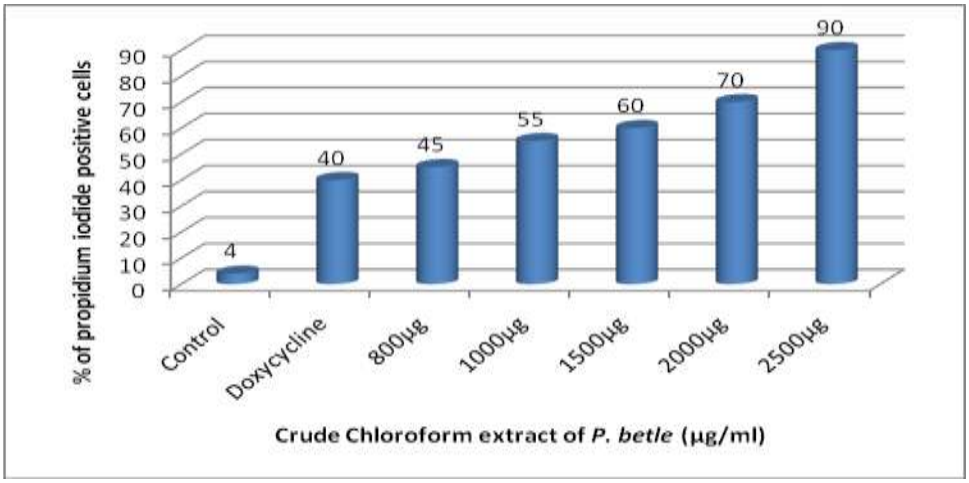


Figure 3: Uptake of propiodium iodide in cell suspension of *L. autumnalis*



REFERENCES

1. Khan JA, Naveen K. Evaluation of antibacterial properties of extracts of *Piper betal* leaf. J Pharma Biomed Sci 2011; 11: 1-3.
2. Nakamura CV et al. *In vitro* activity of essential oil from *Ocimum gratissimum* L. against four *Candida* species. Res Microbiol 2004; 155: 579-86.
3. Singh M et al. The n-hexane and chloroform fractions of *Piper betle* L. trigger different arms of immune responses in BALB/c mice and exhibit antifilarial activity against human lymphatic filarid *Brugia malayi*. Int Immunopharmacol 2009; 9: 716-28.
4. Norton SA. Betel: consumption and Consequences. J Am Acad Dermatol 1998; 38: 81-8.
5. Prabhu MS et al. Effect of orally administered betel leaf (*Piper betle* leaf Linn) on digestive enzymes of pancreas and intestinal mucosa and on bile production in rats. Ind J Expt Biol 1995; 33: 752-6.
6. Bhattacharya S et al. Healing properties of the *Piper betle* phenol, allylpyrocatechol against indomethacin induced stomach alceration and mechanism of action. Wld J Gatroenterol 2007; 21: 3705-13.
7. Tarun A et al. Comparative analysis of antibacterial activity of four *Piper betle* varieties. Adv Appl Sci Res 2012; 3: 698-705.
8. Johnson RC et al. Cultivation of parasitic leptospires: effect of pyruvate. Appl Microbiol 1973; 26: 118-9.
9. Ratnam S et al. Prevalence of leptospiral agglutinins among conservancy workers in Madras city, India. J Trop Med Hyg 1993; 96: 41-5.
10. Prabhu N et al. *In vitro* evaluation of *Eclipta alba* against serogroups of *L. interrogans*. Ind J Pharma Sci 2008; 70: 788-91.
11. Murray CC, Hospenthal DR. Broth microdilution susceptibility testing for *Leptospira* spp. Antimicrob Agents Chemother 2004; 48: 1548-52.
12. Sharma S et al. Evaluation of the antimicrobial, antioxidant and anti-inflammatory activities of hydroxychavicol for its potential use as an oral care agent. Antimicrob Agents Chemother 2009; 53: 216-22.
13. Ramji N et al. Phenolic antibacterials from *Piper betle* in the prevention of halitosis. J Ethnopharmacol 2002; 83: 149-52.
14. Cox SD et al. Determining the antimicrobial actions of tea tree oil. Molecules 2001; 6: 87-91.
15. Nalina T, Rahim ZHA. The crude aqueous extract of *Piper betle* L. and its antibacterial effect towards *Streptococcus mutans*. Am J Biotechnol Biochem 2007; 3: 10-15.