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RESEARCH ARTICLE

Anti-mycobacterial activity of *Withania somnifera* and *Pueraria tuberosa* against *Mycobacterium tuberculosis* $H_{37}Rv$

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Abstract

Mycobacterium tuberculosis is a leading cause of morbidity and mortality worldwide. *Mycobacterium tuberculosis* infects about one third of world population and tuberculosis remains the most frequent cause of death due to a single infectious disease. Anciently, herbal plants have established home remedies for many of common alignments, diagnostic procedures and preventive measures. Using this strategy, this study was proposed to find out anti-tuberculosis activity of medicinal plants viz. *Withania somnifera* and *Pueraria tuberosa* against *M. tuberculosis* $H_{37}Rv$. *Mycobacterium tuberculosis* activity was tested using minimal inhibitory concentration method (MIC). Aqueous extract of *W. somnifera* (0.01-1.0 mg/mL) had significant effect against *M. tuberculosis* whereas, *P. tuberosa* in higher concentration showed no inhibition. The outcomes are possibly demonstrating a comprehendible evidence of the effective anti-tubercle activity of *W. somnifera*.

Keywords: *Mycobacterium tuberculosis,* anti-tuberculosis activity, *Withania somnifera, Pueraria tuberosa.*

Introduction

Tuberculosis (TB) is one of the oldest diseases known to humanity. Throughout history, TB has been among the world's most deadly epidemics (Van Wormer, 2000). Tuberculosis is like any other infectious disease, can happen to anyone, and spares no age, sex and nationality. Tuberculosis is mainly caused by infection with *Mycobacterium tuberculosis* (*M.TB*). An organ other than lungs had been observed for *M.TB* for many centuries, but was not recognized as such (Friedman, 2001). Tuberculosis also rarely caused by *M. bovis*, a bovine type that gets transmitted through the flesh or milk of the infected animal (Sailler *et al*., 2000).

Latterly, the world has gained the opportunity to rid itself of this ancient terror and most importantly, it can be completely cured-but only if the disease is diagnosis in time and treated properly (Sharma and Mohan, 2004). Of all the infectious diseases of man, none has been studied about more intensively than TB. The corner stone of the modern treatment of TB is chemotherapy. Drugs used in the treatment of TB are isoniazid, rifampicin, ethambutol, streptomycin, and pyrazinamide etc. (Sensi, 1989; Sharma and Mohan, 2004). These drugs have disadvantages of causing adverse side effects and organisms can gain easy resistance against these drugs (Mohan and Sharma, 2004). Apart from these effects, there is always a chance of "relapse tuberculosis" due to course discontinuation of chemotherapy within the first year of treatment (Swain *et al*., 2001). This results in a serious condition where by the *Mycobacterium* develops resistance to the drugs resulting in "multi-drug resistance tuberculosis" (MDR-TB). Hence, the critical need arises towards the construction of a component with a severe anti-TB activity, with easy availability and without side effects (Hoareau and DaSilva, 1999). Therefore, by extending our survey into the plant kingdom we could get a superior solution towards the procurement of the crippling disease "a new phyto-look to the old killer" (Sivarajan and Balachandran, 1994; Miller and Miller, 1998).

There are various traditional plants which have anti-TB activity, but so far, very few plants have been tested against *Mycobacteria* and showed anti-TB activity (Babbar *et al*., 1970; Tan *et al*., 1996; Ulubelen *et al*., 1997; Pietro *et al*., 2000; Gernaey *et al*., 2001). Pharmacologically important prospects of medicinal plants should be studied and among two of a kind which are *Withania somnifera* and *Pueraria tuberosa* in beneficiating human diseases. *Withania somnifera* is distributed in dry places of India, used as a highly esteemed rasayana drug which is capable of imparting long life, youthful vigor and good intellectual powers; cures ulcers, fever, cough, dyspnoea, consumption dropsy, impotence, rheumatism, toxicosis and leucoderma (Bhakuni *et al*., 1969; Delaha and Garagusi, 1985; Varier, 1995; Kirtikar and Basu, 1999).

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Pueraria tuberosa is distributed throughout India. It promotes strength and complexion, improves voice, promotes breast milk and semen, cures burning sensation, the drug is recommended in all cases of general debility and rheumatism (Dhar *et al*., 1968; Asolkar and Chakre, 1992; Rastogi and Mehrotra, 1995; Varier, 1995). Hence, with use of these medicinal plants, the present study is carried out to exploit the anti-tuberculosis activity of *Withania somnifera* and *Pueraria tuberosa* aqueous extracts against susceptible strain *M.TB* $H_{37}Rv$.

Materials and methods

Test organism and chemicals: Strain $H_{37}Rv$; bovine serum albumin (BSA); distilled water (DDH_2O); fungizone; glucose; glycerol; oleic acid; penicillin (5000 Units/mL); sodium chloride (NaCl); sodium hydroxide (NaOH).

Apparatus: Autoclave units; binocular microscope; homogenizer; laminar air flow chamber; McCartney bottles; millipore filtration unit; mortar and pestle; Thoma counting chamber; universal containers; vortex mixer; weighing balance.

Collection of plant material: The fresh leaves and roots of *Withania somnifera* (Linn.) Dunal (winter cherry) and *Pueraria tuberosa* DC (Indian kudzu) were collected from Ashtanga Ayurvedic clinic, Trichy, India free from toxic and no drug/nutrient interactions reported (Varier, 1995). The chemical constituents of these plants used are alkaloid and steroidal compounds (Ashwagandholine; Withaferin A; pterocarpan- hydroxy-tuberosin; hydroxy-isoflavone; tuberostan and puerarone).

Preparation of plant aqueous extracts: Thoroughly washed, dried leaves and roots of *W. somnifera* and *P. tuberosa* were weighed (25 g each) and pulverized using blender separately. Approximately 100 mL water was added to each powdered samples and homogenized for 1 h and filtered using whatmann paper No. 1. Filtered solutions of 50 mL were filled in empty crucibles and heated until a thick paste was obtained. The amount of concentrated *W. somnifera* and *P. tuberosa* paste present in the crucibles were diluted accordingly with sterile water and different concentrations of 0.0, 0.01, 0.1, and 1.0 mg/mL medicinal plant aqueous extracts were prepared and maintained in triplicates. Prior to use the prepared samples were preserved at 5°C in an airtight bottle.

Preparation of OADC (Oleic albumin dextrose catalase) medium: About 675 mg of NaCl and 2 g of glucose were dissolved in 5 mL of N/20 NaOH. To this 95 mL of sterile water was added, mixed and autoclaved. Mixture was cooled and layered with 5 g of BSA and allowed to dissolve. Penicillin, fungizone and oleic acid (60 µL) were added and filtered under sterile condition.

Preparation of $_7H_9$ broth base: In 4.7 g of Dubos broth base, 2 mL of glycerol and 900 mL of sterile water were added; the mixture was autoclaved and cooled. Then, 100 mL of OADC was added and aliquoted to 4.5 mL of broth each in McCartney bottle.

Preparation of $_7H_{11}$ agar base: About 6.5 g of agar base was added to 900 mL sterile water, boiled, cooled and autoclaved. It was then poured on to the disposable petri dish containing 12 mL base.

Preparation of inoculum: The *M.TB* $H_{37}Rv$ strain was inoculated using solid Lowenstein Jensen medium (Vasanthakumari, 1990) under ambient conditions of 37°C, and pH 6.4-7. The colony appears dry, rough and wrinkled surface in approximately 8 weeks. From the slope, a loop-full of colonies were taken and inoculated in bottles containing $_7H_9$ broth and incubated at 37°C. The 6^{th} d old culture was taken and homogenized using a vortex mixer; 20 µL of it was transferred into a Bijou bottle and heat-killed at 60°C for 1 h. To this 20 µL of Hank's balanced salt solution (HBSS) was added, mixed and spread evenly on Thoma counter slide. The slides were then spread with cover-slip and bacilli's were counted using microscope. The final concentration of *M.TB* obtained was approximately $3x10^6$ cells/mL. *Cell count:* No. of bacilli/mL = No. of bacilli counted**x**2**x**10^7***/***256

Infection of M.TB $H_{37}Rv$ for anti-mycobacterial studies: The 6^{th} d old bacterial culture of *M.TB* $H_{37}Rv$ inoculums was obtained from TRC, TN, India and maintained on nutrient broth at 37°C and used for infection and antimicrobial activity.

Anti-mycobacterial activity: Inoculum of 0.05 mL *M.TB* $H_{37}Rv$ was mixed with 4 mL of broth base $_7H_9$ and equally transferred to the Nunc vials using MIC method. Each vial was treated with different concentration of 0.0, 0.01, 0.1, and 1.0 mg/mL plant aqueous extracts and correspondingly labeled as $_W1$, $_W2$, $_W3$ and $_W4$ for *W. somnifera* and $_I1$, $_I2$, $_I3$ and $_I4$ for *P. tuberosa*. The aliquoted dilutions of *M.TB* treated with medicinal extracts plated on a petri dish containing $_7H_{11}$ agar medium in the basis of 0, 3 and 7 d were incubated at 37°C for 15 d. At the end of 15^{th} day, each plate of 0, 3 and 7 d cultures were counted for the colony developed in the control and plant extracts and colony forming units (CFU) in log Units/mL were calculated.

Statistical analysis: Data's were expressed as mean $\pm$ SD and significant changes was assessed by student *t* test.

Results

The dose effects of *Withania somnifera* and *Pueraria tuberosa* on the growth of *M.TB* are shown in Table 1.

Table 1. Anti-mycobacterial activity of 0.01-1.0 mg/mL concentrations of plant extracts against *M. tuberculosis* $H_{37}Rv$.

Days	*Withania somnifera* (mg/mL)				*Pueraria tuberosa* (mg/mL)			
	$_W1$	$_W2$	$_W3$	$_W4$	$_I1$	$_I2$	$_I3$	$_I4$
0	30+2.1	30+4.2	29+2.8	29+1.4	29+2.8	29+1.4	29+2.8	29+1.4
3	238+12.7	193+10.9	166+16.2	131+11.3	234+4.20	221+14.2	209+11.3	191+1.13
7	429+14.1	344+2.1	261+19.7	155+5.6	438+4.2	411+5.6	396+12.0	344+16.9

The effects of aqueous extracts (leaves and roots) on inhibition of *M.TB* were compared with control. When tested by MIC method, aqueous extracts of different concentrations of *W. somnifera* showed consistent reduction in activity against *M.TB*. The increase in anti-mycobacterial activity by *W. somnifera* was dose dependent and the increase in anti-mycobacterial activity was significant in 3rd d and remained increasing in 7th d. Aqueous extract of *P. tuberosa* showed insignificant activity against *M.TB* even in higher concentration and longer period of treatment. Likewise in higher dilutions of *M.TB* also shows no potential activity against *M.TB* in *P. tuberosa* tested. *Withania somnifera* extract showed substantial inhibitory antibacterial activity against *M.TB*.

Extracts of 0.0 to 1.0 mg/mL concentrations treated with *M.TB* were tabulated using colony forming units (CFU) in log Units/mL. Hence, the colony counts were equated by nth dilution of *M.TB* $H_{37}Rv$ and CFU were calculated. Percent inhibitions of medicinal extracts were calculated using control CFU values. The obtained results of CFU in log units for *W. somnifera* showed a potential significant activity in CFU observed when analyzed with *M.TB*. At the same time, *P. tuberosa* didn't show any definite reductions in CFU when compared to control values. Table 2 shows the percentage inhibition of different concentrations of *W. somnifera* and *P. tuberosa* extracts. The highest antibacterial activity of *M.TB* was found in $_W4$ (1.0 mg/mL) which is 64.47% inhibition and least activity inhibits in lower dose of 0.01 mg/mL which observed 17.88%. The percent of different concentrations of *P. tuberosa* had some inhibition ranging from 2.95% to 17.95%, but they didn't show any significant inhibition in incubated days. Therefore, it could be deduced that *W. somnifera* has a coherent inhibitory effects and *P. tuberosa* has no anti-mycobacterium activity.

Discussion

The view of percent inhibition for aqueous extracts showed that *W. somnifera* has exposed substantial reduction in the growth of *Mycobacterium* activity and is a prospect for future curative intrusions. Simultaneously, *P. tuberosa* has clearly indicated that there was no explicit reduction in *M.TB* growth. Earlier reports using medicinal plants soluble in methanol extracts confirmed bacteriostatic effects of *W. somnifera* and *P. tuberosa*. Proposed study doesn't confirm antibacterial activity in *P. tuberosa* may be because of non-polar extracts of aqueous based studies or else may be due to species specificity. Moreover there may be many other components in the extracts, which may have an inhibitory or activating effect on the bacilli and a solid method of isolation of components has to be established using gel electrophoresis or chromatographic technique to purify them. Thus, by this means it might suggest that the high polar molecule in the isolated components of soluble extracts may be responsible for anti *M.TB* activity. Appropriate doses of the extracts may act against the most deadly disease in the humankind can be battled in near future.

Conclusion

From the examined results, it could be seen that *W. somnifera* has shown considerable activity against *M.TB* and no change in *P. tuberosa* treated *M.TB*. Aqueous extract of *W. somnifera* (0.01-1.0 mg/mL) had significant effect against *M. tuberculosis* whereas, *P. tuberosa* in higher concentration showed no inhibition. Main disadvantage of *P. tuberosa* being potent anti-tuberculosis component of the plant could be water insoluble and so other ether extract of the plant could give better results.

Table 2. Percent inhibition of 0.01-1.0 mg/mL concentrations of plant extracts against *M. tuberculosis* $H_{37}Rv$.

Days	*Withania somnifera* (% inhibition)				*Pueraria tuberosa* (% inhibition)			
	$_W1$	$_W2$	$_W3$	$_W4$	$_I1$	$_I2$	$_I3$	$_I4$
0		1.323	1.95	1.695		1.34	1.32	1.39
3	-	12.51	29.74	43.53	-	4.33	13.45	19.81
7		17.88	38.12	64.47		2.95	11.10	17.95

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