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In vitro antimycobacterial activity of acetone extract of *Glycyrrhiza glabra*

[Actividad antimicobacteriana *in vitro* del extracto de acetona de *Glycyrrhiza glabra*]

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Abstract

Context: Glycyrrhiza glabra (licorice) has been used since ages as expectorant, antitussive and demulcent. *G. glabra* has been indicated in Ayurveda as an antimicrobial agent for the treatment of respiratory infections and tuberculosis.

Aims: To evaluate the antimycobacterial activity of acetone extract of *G. glabra* by *in vitro* techniques.

Methods: The anti-tubercular activity of acetone extract of *G. glabra*, obtained by Soxhlet extraction, was evaluated against *Mycobacterium tuberculosis* $H_{37}R_v$ (ATCC 27294). The *in vitro* anti-tubercular activity was determined by Resazurin Microtiter Plate Assay (REMA) and colony count method. Further, the anti-tubercular activity of acetone extract of *G. glabra* was determined in human macrophage U937 cell lines and was compared against that of the standard drugs isoniazid, rifampicin and ethambutol.

Results: G. glabra extract showed significant activity against *Mycobacterium tuberculosis*, when evaluated by REMA/colony count methods and in U937 human macrophage cell lines infected with *Mycobacterium tuberculosis* $H_{37}R_v$. The activity of the extract was comparable to those of standard drugs. It was observed that the extract showed time and concentration dependent antimycobacterial activity.

Conclusions: The present study reveals that *G. glabra* extract has promising anti-tubercular activity by preliminary *in vitro* techniques and in U937 macrophage cell line. Therefore, it has the definite potential to be developed as an affordable, cost-effective drug against tuberculosis.

Keywords: Infections; licorice; macrophage; *Mycobacterium tuberculosis* $H_{37}R_v$.

Resumen

Contexto: La especie *Glycyrrhiza glabra* (regaliz) ha sido usada desde la antigüedad como expectorante, antitusiva, y demulcente. *G. glabra* ha sido indicada en la medicina ayurvédica como un agente antimicrobiano para el tratamiento de infecciones respiratorias y tuberculosis.

Objetivos: Evaluar la actividad antimicobacteriana del extracto de acetona de *G. glabra* por técnicas *in vitro*.

Métodos: La actividad anti-tuberculosa del extracto de acetona de *G. glabra*, obtenido por Soxhlet, fue evaluada contra *Mycobacterium tuberculosis* $H_{37}R_v$ (ATCC 27294). La actividad anti-tuberculosa *in vitro* fue determinada por Ensayo de Placa de Microtitulación *Resazurin* (REMA) y el método de conteo de colonias. Además, la actividad anti-tuberculosa de este extracto fue determinada en células de macrófagos humanos U937 y fue comparada contra aquella de fármacos de referencia como isoniacida, rifampicina y etambutol.

Resultados: El extracto de *G. glabra* mostró una actividad significativa contra *Mycobacterium tuberculosis* cuando fue evaluado por los métodos REMA/conteo de colonias y en macrófagos U937 infectados con *M. tuberculosis*. La actividad del extracto fue comparable a aquella observada con los fármacos de referencia. El extracto mostró una actividad antimicobacteriana dependiente de la concentración y el tiempo.

Conclusiones: El presente estudio revela que el extracto en acetona de *G. glabra* tiene actividad anti-tuberculosa prometedora, demostrada por técnicas preliminares *in vitro* y en la línea de macrófagos U937. De esta manera, esta especie tiene un potencial definido para desarrollar un producto asequible contra la tuberculosis.

Palabras Clave: Infectiones; regaliz; macrófago; *Mycobacterium tuberculosis* $H_{37}R_v$.

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INTRODUCTION

Tuberculosis is a highly infectious disease with about one third of the world's population estimated to be infected by it. The side effects and long treatment duration of commonly used anti-tubercular drugs have further complicated the problem of tuberculosis control (WHO 2008). Medicinal plants that produce secondary metabolites that may be potential anti-tubercular agents provide hope for developing new drugs with fewer side effects (Gautam et al., 2007).

Licorice (*Glycyrrhiza glabra* Linn.) commonly known as mulethi or yashtimadhu is an age old plant used in traditional medicine for its ethnopharmacological values to cure various ailments from simple cough to hepatitis to more complex ones like SARS and cancer. In folk medicine, it is used as laxative, emmenagogue, contraceptive, anti tussive, anti asthmatic, galactagogue and antiviral agent (Saxena et al., 2005). Friis-Moller et al. (2002) reported that a flavonoid licochalcone A isolated from Chinese licorice roots showed anti-tubercular activity against *M. bovis, M. tuberculosis, M. kanasii* and *M. marinum* species. Gupta et al. (2008) reported that glabridin, the major flavonoid present in *G. glabra* could be a potential antimycobacterial agent due to the presence of two free phenolic groups at 1,3- positions. Quan-min et al. (2010) reported that antimycobacterial activity of flavonoid glabridin from *Glycyrrhiza uralensis* by Microdilution Alamar Blue Assay against *Mycobacterium tuberculosis* $H_{37}R_v$ and $H_{37}R_a$ was observed at 25 µg/mL.

The objective of this study was to assess anti-tubercular activity of the acetone extract of *G. glabra* by various *in vitro* methods. *In vitro* activity of acetone extract of *G. glabra* was tested by Resazurin Microtiter Plate Assay (REMA) method and colony count method. The extract showed significant activity by these techniques. Hence, the activity of the extract was evaluated in macrophages infected with *Mycobacterium tuberculosis* $H_{37}R_v$. Human macrophage U937 cell lines were selected as the model to further evaluate the anti-tubercular activity since *Mycobacterium tuberculosis* primarily remains housed within the macrophages during the early stages of infection (Smith, 2003).

MATERIAL AND METHODS

Plant material

Roots of licorice (*Glycyrrhiza glabra* Linn.) were purchased from Yucca Enterprises, India and were authenticated from G. N. Khalsa College, India (voucher specimen SN/080312). The roots were powdered using a hammer mill and the powder (60-80 mesh) was extracted by Soxhlet extraction method for twelve hours using acetone as the solvent. After completion of extraction, the solvent was recovered by distillation at 40°C and the dry extract was stored at 4°C.

Bacteria

In vitro antimycobacterial activity of the extract and standard anti tubercular drugs was checked using *Mycobacterium tuberculosis* $H_{37}R_v$ (MTBH) (ATCC 27294) which was obtained from National Institute for Research in Tuberculosis, Chennai, India.

The cultures were preserved on Middlebrook 7H11 agar (Himedia Laboratories, India) and one colony was subcultured in Middlebrook 7H9 broth (Himedia Laboratories, India). After seven days of subculturing, the turbidity of bacterial suspension was adjusted to match the turbidity of McFarland no. 1 solution. This was further diluted in 1:10 proportion to obtain a suspension containing approximately 10^6 bacterial Colony Forming Units (CFU)/mL (Martin et al., 2003).

Antimycobacterial agents

Antimycobacterial activity of the extract was compared with standard anti-tubercular drugs rifampicin (RIF), ethambutol (ETH) and isoniazid (INH), which were obtained as gift samples from Lupin Laboratories Ltd., Mumbai, India.

Stock solutions of extract and RIF were prepared in 10% ethanol and 10% methanol, respectively. Stock solutions of INH and ETH were prepared in sterile distilled water. Further dilutions to obtain working solutions of extract

and standard drugs were made in sterile distilled water. The drugs and extracts were sterilized by filtering through 0.2 µm nylon membrane filter.

Minimum Inhibitory Concentration determination by Resazurin Microtiter Plate Assay (REMA) method

REMA was performed as described by Banfi et al. (2003) with minor modifications. Briefly, 100 µL of Middlebrook 7H9 broth was dispensed in each well of the microtitre plate. Serial two-fold dilutions of extract and standard antimycobacterial drugs were made in the plate. Bacterial suspension (100 µL) containing approximately 10^6 CFU/mL was added in all the wells. Sterility control and growth control (including controls with 10% ethanol and 10% methanol) were also included. The plate was wrapped in aluminium foil and incubated at 37°C for seven days. After completion of the incubation period, 30 µL resazurin solution (100 µg/mL) was added to each well and plate was again wrapped in aluminium foil and incubated overnight. The plate was then observed for change in color. The color change from blue to pink or colorless indicated growth of the bacteria. The lowest concentration of drug or extract that prevented color change from blue to pink was taken as the upper limit for Minimum Inhibitory Concentration (MIC) range; the highest drug/extract concentration that showed change in color from blue to pink was considered the lower limit.

Minimum Bactericidal Concentration and Minimum Inhibitory Concentration determination by colony count method

Minimum bactericidal concentration (MBC) of the extract and standard anti-tubercular drugs was determined by colony counting on agar plate method as described by Mor et al. (1993) with minor modifications. Middlebrook 7H11 agar was seeded with different concentrations of the extract and standard drugs. 0.1 mL of a bacterial suspension containing approximately 10^6 CFU/mL was spread on top of the seeded agar. The plates were incubated at 37 ± 0.5°C in 5% CO_2 condition for twenty one days. MBC was defined as the lowest concentration that effectively reduced the viable counts in drug-containing plates by at least 95% as compared to the positive control experiments. MIC was defined as the lowest concentration at which growth was inhibited i.e. same number of colonies was observed in test plates as in growth control.

Propagation of human macrophage U937 cell line

Human macrophage U937 cell line was obtained from National Centre for Cell Sciences, Pune, India.

Propagation and maintenance of human U937 macrophage cell lines were carried out as per the method of Okoko et al. (2007). Briefly, the macrophage cell line was propagated in RPMI- 1640 medium with L-glutamine and 25 mM HEPES buffer (HiMedia Laboratories, India), supplemented with 2.0 g/L sodium bicarbonate, 10% heat inactivated fetal bovine serum and 1 mg/mL streptomycin, at a subculture interval of 2 days. The cells were incubated at 37^{0}C in 5% CO_2 atmosphere.

Infection of U937 macrophage cells with *Mycobacterium tuberculosis* $H_{37}R_v$

U937 macrophage cells were cultured as described above and were centrifuged, washed and the cell pellet was resuspended in RPMI medium without antibiotics and adjusted to a cell density of 2 x 10^6 viable cells/mL by Trypan blue dye exclusion technique (Patel et al., 2009).

Infection of macrophages with MTBH was carried out by the process described by Park et al. (2006), with slight modifications. Four hundred µL U937 cell suspension containing approximately 2 x 10^6 cells/mL was inoculated with four hundred µL bacterial suspension of MTBH containing approximately 10^6 CFU/mL and incubated for 1, 3, 6, and 24 h at 37 ± 0.5°C in 5% CO_2 atmosphere. After completion of the incubation period, internalization of bacteria into macrophages was checked by colony counting and Ziehl-Neelsen staining method. It was observed that the optimum number of bacilli was internalized into all the macrophages after 6 h of incubation. Hence, in the rest of the study, infection of

macrophages with MTBH was carried out by incubating the macrophages with bacteria for 6 hours. After internalization of bacteria into macrophages, the extracellular bacilli were removed by washing thrice with RPMI – 1640 medium followed by washing with phosphate buffered saline (PBS).

Antimycobacterial activity of extract and standard drugs in U937 macrophages infected with *Mycobacterium tuberculosis* $H_{37}R_v$

The test and standard compounds were added to the infected macrophages in various concentrations and incubated. Untreated infected cells were used as a control for each time point. After completion of the incubation period, the cells were washed thrice with PBS to remove the extracellular drug. The cells were lysed using 0.06% sodium dodecyl sulphate (SDS) (Barrow et al., 1998). Ten µL of the lysate was placed on Middlebrook 7H11 agar and were incubated at 37 ± 0.5°C in 5% CO_2 atmosphere for twenty one days. The number of CFUs was counted after completion of the incubation period. The study was conducted in triplicate on three different days.

Statistical analysis

MIC and MBC values obtained for extract and standard drugs by REMA and colony count method were statistically analyzed by Kruskal-Wallis test and results of CFU counts of control and drug/ extract for cell-line studies at each time point were compared by two-way analysis of variance (ANOVA) at 95% confidence interval using Graphpad Prism 6.0 software. Statistical significance was reached when the *p*- value was <0.05.

RESULTS

MIC determination by Resazurin Microtiter Plate Assay (REMA) method

In this study, MIC of acetone extract of *G. glabra* against MTBH was found to be 0.97 - 1.95 µg/mL. MIC of extract and standard drugs against MTBH by REMA method is given in Table 1.

Table 1. MICs of acetone extract of *G. glabra* and standard drugs against MTBH, determined by REMA method.

Extract/drug	MIC (µg/mL)
Acetone extract	0.97 - 1.95 ± 0.56[b]
RIF	0.009 - 0.019 ± 0.05[a]
INH	7.8125 - 15.625 ± 0.09[c]
ETH	15.625 - 31.25 ± 0.15[d]

Different letters symbolize significant differences ($p < 0.05$) by mean of the nonparametric Kruskal-Wallis test. Data represent the mean ± SD of at least n = 9.
MIC: Minimum inhibitory concentration; MTBH: *Mycobac-terium tuberculosis* $H_{37}R_v$; RIF: Rifampicin; INH: Isoniazid; ETH: Ethambutol.

RIF showed significantly better activity as compared to acetone extract of *G. glabra*, whereas, acetone extract of *G. glabra* showed significantly greater activity than INH and ETH ($p < 0.0001$), when evaluated by REMA. The growth controls with and without 10% ethanol and 10% methanol, respectively showed change in color from blue to pink indicating that use of these solvents for preparation of extract and RIF, respectively did not have any bearing on the antimycobacterial activity of the extract and RIF.

MIC and MBC determination by colony count method

MIC and MBC of extract and standard drugs against MTBH were determined by colony count method. The results for MIC and MBC by colony count method are summarized in Table 2.
It was observed that MIC and MBC of acetone extract of *G. glabra* was significantly lower than that of INH and ETH ($p < 0.0001$), when evaluated by colony count method.

Since acetone extract of *G. glabra* showed promising anti-tubercular activity by *in vitro* test methods, the activity of the extract was checked in cell lines.

Table 2. MBC and MIC of acetone extract of *G. glabra* and standard drug against MTBH, determined by colony count method.

Drug/ extract	MBC (µg/mL)	MIC (µg/mL)
Extract	15[b]	5.0 ± 0.528[b]
INH	25[c]	12.5 ± 0.697[c]
ETH	60[d]	35.0 ± 1.527[d]
RIF	<1[a]	<1.0 ± 0.557[a]

Different letters symbolize significant differences ($p < 0.05$) by mean of the nonparametric Kruskal-Wallis test. Data represent the mean ± SD of at least n = 9.
MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration; MTBH: *Mycobacterium tuberculosis* $H_{37}R_v$; RIF: Rifampicin; INH: Isoniazid; ETH: Ethambutol.

Antimycobacterial activity of extract and standard drugs in U937 macrophages infected with *Mycobacterium tuberculosis* $H_{37}R_v$

A two-log increase in the number of CFUs/mL was observed in the positive control at day seven as compared to day 0.125 for MTBH. Two-way ANOVA comparisons were done between control experiments and each drug experiment. The activities of extract and standard drugs were expressed as $\log_{10}$ CFU/mL count observed on each day compared with the positive control experiments.

Antimycobacterial activity of extract in U937 macrophages infected with *Mycobacterium tuberculosis* $H_{37}R_v$

The $\log_{10}$ of number of colonies of MTBH after incubation with extract for various time intervals is shown in Fig. 1.

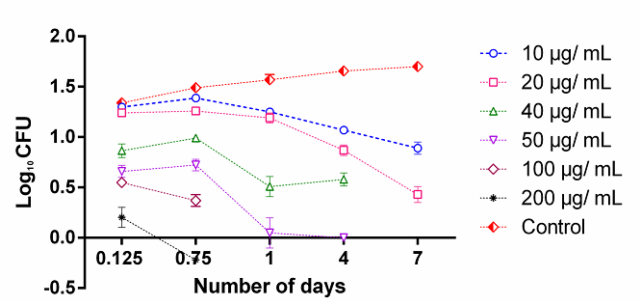

Figure 1. $\log_{10}$ number of CFUs of MTBH after incubation with various concentrations of acetone extract (10 - 200 µg/mL) of *G. glabra* for various time intervals. The activity is expressed as $\log_{10}$ CFU/mL observed at each day compared to the control. Data represent the mean ± SEM of n= 9 sets of replicate performed in triplicate on three different days.

As evident from Fig. 1, it was observed that extract showed time and concentration-dependent activity. The difference in $\log_{10}$ CFUs at each concentration and each time interval was statistically significant ($p < 0.05$) except on days 4 and 7. Extract (20 µg/mL) reduced CFU counts significantly after just 18 h of incubation with the infected macrophages ($p < 0.05$) as compared to the control. Complete inhibition was observed at 100 µg/mL after 1 day incubation of extract with macrophages infected with MTBH.

Antimycobacterial activity of extract and standard drugs in U937 macrophages infected with *Mycobacterium tuberculosis* $H_{37}R_v$

The results obtained for extract were compared with the antimycobacterial activity of standard drugs used. The result for the antimycobacterial activity of extract in comparison with standard drugs is summarized in Fig. 2.

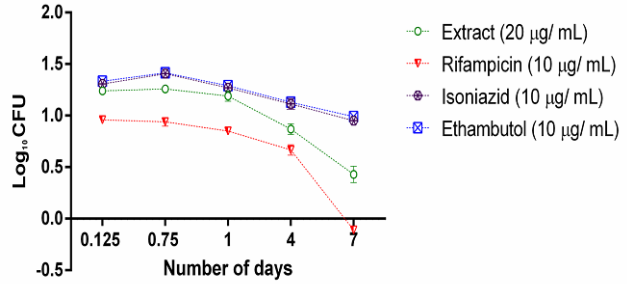

Figure 2. Activity of acetone extract of *G. glabra* in comparison to those of standard drugs against MTBH at different time points. The activity is expressed as log10 CFU/mL observed at each day compared to the control. Data represent the mean ± SEM of n= 9 sets of replicate performed in triplicate on three different days.

Extract concentration of 20 µg/mL was selected for evaluating comparative activity of extract versus standard drugs at different time points as this was the lowest extract concentration that gave almost complete growth inhibition after seven days of incubation. Standard drug concentration of 10 µg/mL was used for evaluating the

comparative activity, as the plasma C_{max} of the standard drugs after oral administration of standard dose is approximately 10 µg/mL (Acocella, 1978; Peloquin et al., 1999a; 1999b).

RIF (10 µg/mL) showed significantly greater antimycobacterial activity than the extract (20 µg/mL) at all time intervals of incubation ($p < 0.05$). Extract (20 µg/mL) showed significantly ($p < 0.05$) greater intracellular antimycobacterial activity as compared to standard drugs ETH and INH (10 µg/mL). The results obtained by cell line studies correlate with the anti-tubercular activity data of acetone extract of *G. glabra* obtained by REMA and colony count methods.

DISCUSSION

Current tuberculosis therapy, also known as DOTS consists of treatment with a combination of drugs. This combination therapy causes hepatotoxicity as the major side effect (Ramappa et al., 2013). Medicinal plants can act as potential anti-tubercular agents that can be used in combination with the standard anti-tubercular drugs (Gautam et al., 2007). This would reduce the dose of conventional anti-tubercular agents, thus reducing the dose related side-effects. In this experiment, acetone extract of *G. glabra* was tested for antimycobacterial activity by various *in vitro* methods.

MIC of the extract was checked by REMA method and colony count method. As evident from the Tables 1 and 2, the extract showed lower MIC by REMA method than by colony count method. It has been reported that the MIC shown by a compound depends on the technique used for determination. MICs obtained in a liquid medium are lower than those obtained in solid medium as the drug has to diffuse through the matrix in the solid medium in order to exert activity (Pellegrin et al., 1996). It was observed that acetone extract of *G. glabra* showed significantly lower MIC (0.97 - 1.95 µg/mL) against MTBH as compared to standard drugs INH (7.81 - 15.62 µg/mL) and ETH (15.62 - 31.25 µg/mL) ($p < 0.0001$), whereas, MIC of RIF was significantly lower than MIC observed for acetone extract of *G. glabra* ($p < 0.0001$). MBC of the extract was determined by colony count method. Lowest concentration of drug which reduced the number of CFUs by 95% was considered as MBC. MBC of the extract against MTBH was found to be 15 µg/mL, whereas MBC of INH was 25 µg/mL and that of ETH was 60 µg/mL. It was observed that acetone extract of *G. glabra* showed significantly better activity than standard drugs INH and ETH by *in vitro* tests ($p < 0.0001$).

To validate the results obtained by REMA and colony count methods, the activity of acetone extract of *G. glabra* was checked in human U937 macrophages infected with MTBH. As demonstrated in Fig. 1, it was observed that the number of CFUs decreased with increase in time of exposure of the infected macrophages to extract, with the extract showing highest degree of activity on day seven, at all concentrations. It was also observed that the number of CFUs reduced with an increase in the concentration of extract. Therefore, it was concluded that activity of extract increased with time and concentration. However, on day four and day seven, extract and standard drugs did not show concentration-dependent activity.

As evident from Fig. 2, extract (20 µg/mL) showed greater reduction in number of CFUs than ETH and INH (10 µg/mL) at all-time intervals, with statistically significant difference in activity observed at all time points, except day four and day seven ($p < 0.05$). RIF showed significantly better activity than acetone extract of *G. glabra* ($p < 0.05$).

By *in vitro* studies, acetone extract of *G. glabra* showed better antimycobacterial activity as compared to the standard drugs INH and ETH. Hence, acetone extract of *G. glabra* can be developed as a potential antimycobacterial agent with activity better than or equal to INH and ETH as evident from *in vitro* and cell line studies.

CONCLUSIONS

Acetone extract of *Glycyrrhiza glabra* showed antimycobacterial activity comparable to the standard drugs, though not as potent as rifampicin when evaluated by *in vitro* method and in cell lines infected with *Mycobacterium tuberculosis* $H_{37}R_v$. The activity was found to be significantly better than that of isoniazid and

ethambutol. The results of the study indicate that *G. glabra* has the potential to be developed as a mainline anti-tubercular drug or as an adjunct to the existing antimycobacterial agents.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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