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Anti tuberculosis activity of a Siddha herbal formulation for treating tuberculosis in children

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Abstract

Siddha system of medicine has rich collection of herbs for the treatment of various acute and chronic ailments. Childhood tuberculosis is a major killer disease for children. It is the main cause of morbidity in modern era and came to existence many decades ago and has emerged as pandemic disease. The use of allopathic medicine in complex disease like TB is associated with the problem of cross resistance and herbal drugs has proven to be most effective in this context. So, the author of this paper, enlights the anti tubercular activity of the drug which is cost, effective and less invasive.

Keywords: Siddha, tuberculosis, morbidity, infective, mycobacterium.

Introduction

TB causes by Mycobacterium tuberculosis is an infectious deadly disease that spread through the air sneezes. It can be easily spread within the home, especially from a family member to a child. Children can also get TB from an infected person at school or any other place. Infants and young children are at special risk of having severe and often fatal form of TB, such as TB Meningitis, which can leave the child blind, deaf, paralyzed or mentally disable. Globally it is estimated that up to 140,000 children die of TB each year and over half a million are infected, according to WHO. The actual evidence of TB, along children is possibly high; the disease is difficult to diagnose the children.

Symptoms of childhood TB:

Chronic Cough:

A variety of durations of cough have been proposed as a significant sign of TB. The WHO Policy document (WHO, 2006 B), suggests that chronic cough, that is not approving and has been present for more than 21 days (3weeks) with or without wheezing begin present.

Fever:

The WHO policy document (WHO 2006 b) propose that fever for the purpose of susceptive TB for a child should be defined as " fever greater than 38oc for 14 days after common causes like malaria or pneumonia have been excluded.

Loss of weight & failure to gain weight:

The WHO policy document (WHO 2006 b) adds the important consideration that weight loss or failure to gain weight are of particular significant especially after being treated a nutritional rehabitation programme.

Materials and Methods

Method and Preparation of Extracts*

The freshly collected Sample materials were kept on the laboratory bench, air driedand pulverized in to coarse powder. A micro-scale extraction was carried out by packing 6.0 g of the Sample material into a 10ml sterile pipette; defatting with Hexane and successively extracting with Dichloromet hane (DCM), ethylacetate(EtoAc) and methanol (Me OH). Pasteur pipettes were used to transfer solvent int othe pipette and pipette bulb to force the solvent through the packed Sample Material.

The Formulation were collected in 50ml flasks, evaporated using a Buchi rotary evaporator at 37°C. The concentrated Formulation were completelyairdr ied under air stream at room temperature and wei ghed. The process affordedsoluble fractions of HE X (160 mg), DCM (140 mg), EtOAc (20mg) and MeOH (520mg) with yields of 2.7%,2.3%, 0.33% and 8.7% respectively. Samples of 2.0 mg of the each of the successiveFormulationwere transferred into sterile 1-ml vials for anti-TB bioassay.

Following the promising results obtained from the pilot study (micro-scale extraction) of Sample, 0.7 kg of the pulverized leaves was packed into a 2000 ml separating funnel. The separating funnel was connected to a tank (2.5 L bottle) withTeflon tubing and rubber stopper. The tank is suspended above the funnel with the help of clamps to make a percolation set-up.

The material was first defatted with hexane (2.5Lx 2),and the solvent removed in vacuo using Buchi-RotoVap at 37 °C.The defatted material was then successivelyand exhaustively extracted with dichlor omethane followed by ethylacetate.

TheFormulationwere combined and completely eva porated and dried in a dessicator to afford 41.2g of the crude extract (yield =5.8%). 40.0 gm of the crude extract was loaded dry by adsorption on silica gel and fractionated by vacuum liquid chromatography (VLC, 800 g, 60 M, 0.04-0.063 mm/230-400 mesh silica gel), eluting with a gradient of hexane-ethyl acetate methanol (5% stepwise increase).

A total of forty fractions were collected and concentrated in vacuo. 2.0 mg of each of the fractions were transferred into sterile micro tube for anti-Tbassay. Based on the normal phase TLC profile of the fractions and the results of an ti-Tb activity, similar fractions were pooled together to afford six main fractions (A, B, C, D, E & F).

Fraction E (1000 mg) was further fractionated by repeated NP VLC eluting withgradient solvent syst em-hexane-ethyl acetate-methanol (10% stepwise increase).

Twenty three fractions collected were spotted and developed on normal phase TLC, like fractions were pooled together to obtain five main fractions -E1.1(3.3 mg), E1.2 (2mg), E1.3 (682 mg), E1.4 (35.8 mg) & E1.5 (113 mg). 2.0 mg of each of these fractions were transferred into sterile microtube for anti-Tb assay.

Purification of Fraction

A sample (300 mg) of fraction E1.3 was purified by reverse phase medium pressure liquid chromate graphy (RP MPLC) using FMI pump at1.6 ml/min

The sample (300mg) was dissolved in 2.5 ml 98% methanol and injected into 3ml sample loop using a 3ml syringe) and loaded onto an equilibrated R P C18 prepacked column (Grobe B(310-25), 40-63 µM, The sample was eluted with gradient solv ent system of methanol-water, 50%, 60%, 70%, 80%, 85%,90 and 98%). Based on the TLC profile, fractions collected at the various gradient steps were combined and designated fractions.

Further Purification of E1.3.4&5

Based on the observations of the chromatogram for fractions obtained at 80-85% methanol-water (280-370), fractions were recombined and purified further on a smaller pre-packed RP C18 column (GrobeB (240-10),40-63 µM, product of Merck, Darmstadt Germany); eluted with a smaller gradient of methanol –water (75%, 77%, 80%, 85%, 90% and 98%). A total of 306 fractions were collected.

The fractions were spotted and developed on NP TLC using chloroform-methanol-TFA (80:19:1). Like fractions were pooled to obtain eighteen sub-fractions. 1mg samples of each fraction were transferred in to micro-tube for anti-TB assay.

Bacterial Strains for anti-TB biological assays

For the preparation of the inoculum, a virulent str ain of M. tuberculosis, H37Rv bacteria was grown in100 ml of Middlebrook 7H9 Broth (Difco, Detro it, MI), supplemented with 0.2% (v/v) glycerol (Si gma Chemical Co., St Louis, MO), 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase; Difco), an 0.05% (v/v) Tween 80 (Sigma).

Microplate Alamar Blue Assay (MABA)

Anti-TB susceptibility testing of Formulation and isolates was determined in the fluorometric Micro plate Alamar Blue Assay (MABA) as described previously .

The Formulation MICs against MTB H37RV (ATCC27294) were assessed by the MABA using rifampin and isoniazid as positive controls.

Sample stocks were prepared in DMSO at concent ration of 12.8 mM, and the final test concentrations range from 128µM to 0.5 µM. Two fold dilutions of compounds were prepared i n Middlebrook 7H12 medium (7H9 Broth containi ng 0.1% w/v casitone, 5.6 µg/mL palmitic acid, 5 mg/mL bovine serum albumin, 4 mg/mL catalase, filter-sterilized) in a volume of 100 µL in 96-well Microplates (BD Optilux, 96-well Microplates, black/clear flat bottom). MTB cultures (100 µL inoculum of 2 x 105 cfu/ mL) were added, yielding a final testing volume of 200 µL. The plates were incubated at 37 0C. On the seven thday of incubation 12.5 µL of 20% Tween 80, and 20 µL of Alamar Blue (Invitrogen BioSource TM) were added to the wells. After incubation at 37℃ for 16-24 hrs, fluorescence of the wells were measured (ex 530, em 590 nm).

The MIC was determined as the lowest concentrat ion effecting a reduction in fluorescence of ≥ 90% relative to the mean of replicate bacteria-only controls.

Cytotoxicity Assay

Evaluation of the cytotoxicity activity of isolates in Vero cells was performed as described Previously using the Cell Titer 96 aqueous radioactive cell proliferation assay. The IC50 was defined as the reciprocal dilution resulting in 50% inhibition of the Vero cells. The cytotoxicity was determined by exposing the Vero cells to different concentrations of the samples. Stock solutions of the samples were prepared at 12.8 mg/mL.The pos itive control rifampin (RMP) at 100 mg/ML in DMSO.

Geometric six fold dilutions were performed in growth medium MEM containing 10% fetal bovine serum . Final DMSO concentrations did not exceed 1% v/v. Drug dilutions were distributed in duplicate in 96 -well tissue culture plates at a volume of 50 µL per well. An equal volume containing 5x105 Vero cells was added to each well and incubated at 37℃ in an atmosphere of5% CO2 in air. After 72 hours, cell viability was measured using the Cell Titer 96 aqueous nonradioactive cell proliferation assay according to the manufacturer's instructions. Absorbance at 490nm was read in a victor II rea der. The IC50 was determined using a curve-fitting program.

Results and Discussion

The result of anti-TB assay of the Sample Formulation shows a remarkable activity against virulent strain *Mycobacterium tuberculosis* (H37RV) in three out of the four crude Formulation tested. The hexane, dichloromethane and ethyl acetate fractions inhibited the growth of the bacterium significantly, comparable to the inhibition observed using the reference drug (rifampin). The activity of the sample formulation concentration dependent with 62 µg/ml and 128µg/ ml concentrations exhibiting greater inhibition (Table 1). The potency of the Sample extract is more remarkably consistent in the dichloromethane fraction (DCM), exhibiting MIC of 62.51 µg/ml and the hexane fraction (MIC 84.34 µg/ml) while the EtOAc fraction (128µg/ml) showed less inhibitory activity than the DCM fraction.

The lipophilic extractives exhibited greater activity than the Ethylacetate and Methanol (Table). Further purification of the active subfraction E (E 1.3.4&5) led to simple fractions with high activity (MICs 31.43 µg/ml, 29.68 µg/ml and 15.13 µg/ml (Table 2). The in vitro cytotoxicity values (IC50) of

102.4 µg/ml and 99.35 µg/ml for subfractions (E1.3.4&5.7 and (E1.3.4&5.8) respectively as well as 81.08µg/ml and 81.69 µg/ml indicates non cytotoxic and probably a good indicator for the candidature of this Sample extract as possible source of drug candidate Preliminary examination of the isolates suggests long chain fatty acid with a glycoside unit.

Table 1. ANTI TB – Micro Scale Ext of Sample Formulation

code	solvent	% inhibition at tested concn					
		8mg	16mg	32mg	64mg	128mg	MIC Mg/ml
CRC or SSE 1.	Hex (hexorne)	35	56	48	83	93	84.34
CRC or SSE 2.	Dcm (di ethyl chloro methane)	75	78	75	91	90	62.51
CRC or SSE 3.	Etoac (ethyl acetate)	45	57	69	77	90	128
CRC or SSE 4.	Meoh (methanol)	35	11	12	33	39	128
RMP	Dmso	96	98	99	100	100	0.06

Table. 2 Result of Anti TB Array of Sub Fractions(E) of Sample Formulation

CODE	MIC mg/ml
E1.1	128
E1.2	28.65
E1.3	55.52
E1.4	54.84
E1.5	63.01
RMP	0.05

Strain: H37 RV stock conc: 12.8mg/ml test conc:128.8mg/ml

Conclusion

However,the information on the therapeutic effect of the Sample Formulation in the treatment of tuberculosis seems to be an age- long secret known . The Sample of the Formulation was recently reported to have insignificant inhibitory activity against clinical isolates of M. tu berculosis and a strain of *Mycobacterium bovis*. The findings in this study have provided scientific support for the ethnomedical anti –TB activity of Formulation.

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