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Original Research Article

In-Vitro Cytotoxicity Assay of Crude Extract of Ethnobotanical mixtures used in Indigenous Treatment of Tuberculosis

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Abstract

Introduction: Use of ethno botanical therapies is more vigorously being explored in combating diseases. Studies published mentioned that crude essential oil (EO) extract of herbal formulary (HS) indigenously used in inhalational & oral treatment of Tuberculosis (TB), is a promising natural product with potential for new drug development in the treatment of TB. Cytotoxicity studies of HS on cell lines are not available in published literature. **Materials and Methods:** Extraction of essential oil (EO) from the mixture was done by hydro distillation and chemical characterization was done by Gas Chromatography and mass Spectroscopy (GCMS). Cytotoxic effect of two different ethnobotanical mixtures (HS1 and HS2) was studied by MTT assay using human monocyte THP-1 cell line. **Statistical Analysis:** The IC50 of HS1and HS2 was determined by constructing a dose-response curve of the effect of different concentrations of HS1 and HS2. IC50 values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, San Diego, CA, USA)**.** **Results:** HS1 showed an IC_{50} value of 454.7µg/ml in THP-1 cells. HS2 did not show significant cytotoxicity. **Conclusion:** This study proves that the HS1 and HS2 with potent in-vitro anti TB effect are non-cytotoxic on THP-1 cell line, thus proving biological safety of use of these ethno botanical mixtures, traditionally used in TB treatment. This is a milestone development towards novel anti TB drug development.

Keywords: MTT Assay, THP-1cell lines, IC50, Ethnobotanical, Antitubercular

Introduction

Use of ethno botanical therapies is more vigorously being explored in present health scenario. Treatment of TB using oral and inhalational administration (*Hawan*) of a mixture of herbs (*hawan samagri*, HS) has been practiced in India since ages and scientific evidence on its efficacy using modern research methodologies are increasingly being reported[1,2,3]. In vitro anti-tubercular effect of extract of HS against H37Ra and clinical strains of Mycobacterium *tuberculosis* (MTB) was recently reported[2,3]. Identification of an array of compounds known to have immune enhancing and multi-system health benefits supplementing the potent in-vitro anti-tubercular effect highlights the

promising potential of this formulation HS, in novel TB drug discovery and management[3]**.** Cytotoxicity is one of the most important indicators for biological evaluation in in-vitro studies[4,5]**.** To the best of our knowledge there is no previous report on cytotoxicity of HS on any human cell line. A MEDLINE survey on PubMed for "*hawan samagri* crude extract and cytotoxicity" (October 2021) retrieves zero results in last 10 years. This suggests that the studies in this field have not yet been initiated despite the fact that crude extract of HS was used as oral medicine for treatment of tuberculosis since ancient time apart from use of HS as oblation in inhalation therapy of TB[3]. Hence this study was done to evaluate the cytotoxicity of this potent anti-TB formulation in a step towards novel anti-TB drug development, an area of priority research.

Materials and Methods: Two different mixture of herbs HS1 and HS2 were used. HS1 was prepared as per standards mentioned in literature as used in studies by Rastogi et al.[3] and HS2 was obtained from "Rishi Udhyan" Arya Samaj, Ajmer, where *hawan* is being performed daily since ages using the fixed formula mixture. Extraction of essential oil (EO) from both herbal mixtures was done by hydro distillation using Clevenger apparatus by standard technique[3].

Cytotoxic effect of both HS1 and HS2 EO on living cells was done by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay using human monocyte THP-1 cell line. The absorbance was measured using a micro plate reader at a wavelength of 590 nm, and result was analyzed.

Extraction of EO from HS (HS1 and HS2): The extraction of essential oil from HS1 and HS2 mixtures was done by hydro distillation technique. Water constituents in the oil collected after hydro distillation was removed by using anhydrous sodium sulphate. Obtained oil was stored at -4°C in a dark colored bottle[6,7]**.** The extraction yield and the physical properties like density, refractive index, solubility of HS1 and HS2 was determined as per standard protocol[8]**.**

MTT Assay using THP-1 cell line:

Preparation of test solutions:

Cell lines used: THP-1, TIB-202, (ATCC, USA), (Homo sapiens), peripheral blood, monocyte

Sample preparation

For cytotoxicity studies, 50mg/ml EO stocks were prepared using DMSO. Serial two fold dilutions were prepared from 500µg/ml to 7.81µg/ml using RPMI plain media for treatment[9,10]**.**

Cell lines and culture medium

THP-1 cell line was procured from ATCC, stock cells was cultured in RPMI supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37ºC until confluent. The cell was dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells was checked and centrifuged. Further, 50,000 cells/well were seeded in a 96 well plate and incubated for 24hrs at 37ºC, 5% CO_2 incubator[11].

Procedure:

The suspension cell culture was centrifuged, and the cell count was adjusted to 1.0 x 10^5 cells/ml using respective media containing 10% FBS. To each well of the 96 well micro titer plates, 100 µl of the diluted cell suspension (50,000 cells / well) was added. After 24 h, 100 µl of different test concentrations of test drugs (EO) were added into the suspension in micro titer plates. The plates were then incubated at 37ºC for 24 hrs in 5% CO_2 atmosphere. After incubation 10µl of MTT (5 mg/ml of MTT in PBS) was added to each well. The plates were incubated for 4h at 37º C in 5% CO_2 atmosphere. Then supernatant was

collected, centrifuged and to the pellet 100 µl of DMSO was added. This solution was then transferred to their respective wells and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC50) values was generated from the dose-response curves for each cell line[12,13,14].

Statistical evaluation:

IC50 value

The half maximal inhibitory concentration (IC50) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of HS1 and HS2 is needed to inhibit THP-1 Cells by half. The IC50 of HS1 and HS2 was determined by constructing a dose-response curve and the effect of different concentrations of HS1 and HS2 was examined. IC50 values were calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the test samples HS1 and HS2. IC50 values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, San Diego, CA, USA).

Nonlinear regression

In statistics, nonlinear regression is a form of regression analysis in which observational data are modeled by a function which is a nonlinear combination of the model parameters and depends on one or more independent variables. The data are fitted by a method of successive approximation concentrations of antagonist on reversing agonist activity. IC50 values were calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist. IC50 values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, San Diego, CA, USA).

Calculating percent inhibition:

% Inhibition = ((OD of Control – OD of sample) / OD of Control) x 100

Result and discussion:

MTT Assay is far good because it is easy to use, safe, has a high reproducibility, and is widely used to determine both cell viability and cytotoxicity. HS1 has shown an IC50 value of 454.7µg/ml in THP-1 cells, which is negligible and insignificant taking into account 100% inhibition of clinical strains of MTB at MIC of 400µg/ml, as reported in an earlier study[3]. HS2 did not show significant cytotoxicity (**Table 1**, **Figure 1).** Phase contrast images also demonstrate no cytotoxic effect of HS1 and HS2 (**Figure 2)**. So far there is no study that can give a clear insight on the mode of action of EO. Each constituent of EO has its own effect and also influenced by presence of other constituents[15]**.** In study of in-vitro anti-tubercular MIC of HS1 and HS2 by Rastogi et al., dilution range used was 400µg/ml to 3.125µg/ml. Hence, it is inferred that both HS1 and HS2 have no cytotoxic effects in this MIC range. HS1 nevertheless is a more potent anti-TB formula **(Figure 1)[3].** Using Gas Chromatography Mass Spectroscopy (GC-MS) Analysis of HS1 and HS2 EO, Rastogi et al.,[3] also reported an array of bioactive chemical compounds of high therapeutic attributes, corroborating with the observed anti-tubercular activity[2]. In addition to this chemical fingerprinting of HS1 and HS2 by GC-MS analysis showed presence of Myristicin which is known to have hepatoprotective, antibacterial, anti-inflammatory, antioxidant, psychoactive and anti- cholinergic effects. Beta–

elemene identified in both HS1 and HS2 is known to have muscle relaxing, anti-inflammatory properties, and is also able to pass the blood–brain barrier[3]. HS1 and HS2 are reported to have anti-inflammatory activity as demonstrated with LPS induced inflammatory THP-1 cell based model[16]. These attributes of the HS1 and HS2 may explain the non-cytotoxic nature of these ethno botanical mixtures proving their biological safety.

Table 1: Optical density and percent inhibition of THP-1 cells at various dilutions of HS1 and HS2

THP-1 Cell line				
Compound Name	**Conc. µg/ml**	**OD @ 590nm**	**% Inhibition**	**IC50 µg/mL**
Control	0.0	0.612	0.00	
HS1	7.81	0.596	2.61	**454.7**
	15.63	0.574	6.21	
	31.25	0.534	12.75	
	62.5	0.502	17.97	
	125	0.453	25.98	
	250	0.376	38.56	
	500	0.248	59.48	
HS2	7.81	0.601	1.80	**IC50 was not calculated due to lesser inhibition**
	15.63	0.574	6.21	
	31.25	0.551	9.97	
	62.5	0.523	14.54	
	125	0.457	25.33	
	250	0.398	34.97	
	500	0.333	45.59	

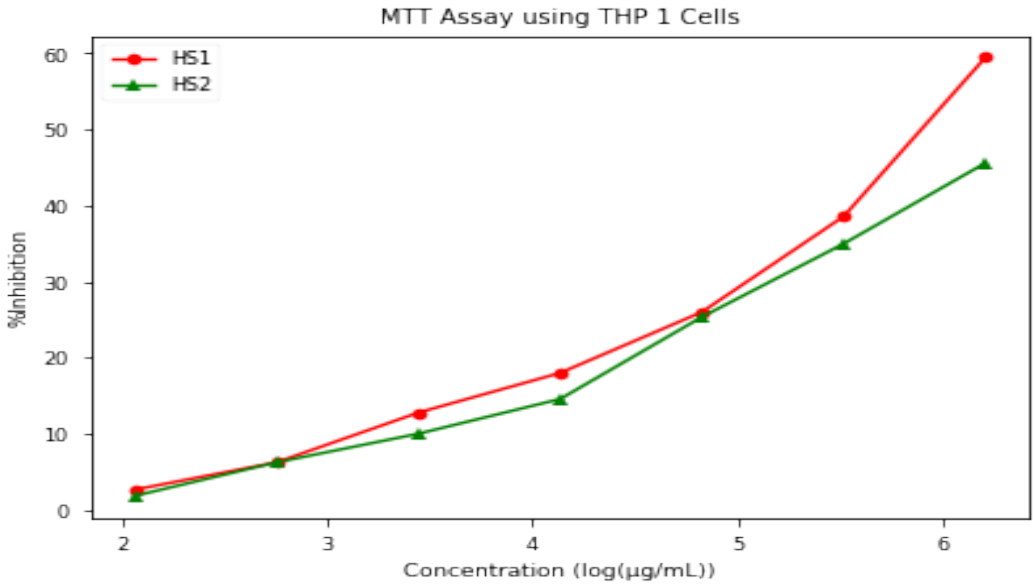

Figure 1: Sigmoid dose response curve of HS1and HS2

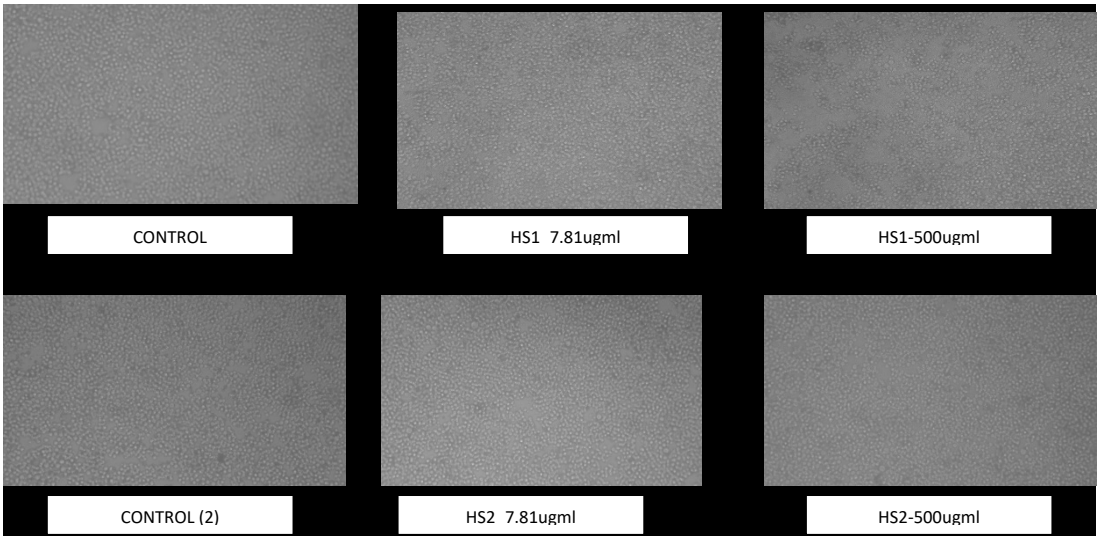

Figure 2: Phase-contrast imaging for demonstration of no cytotoxic effect of HS1 and HS2

Conclusion:

This study proves that HS1 and HS2 with potent in-vitro anti TB and anti-inflammatory effect are non-cytotoxic on human monocyte cell line, thus proving biological safety of use of these ethno botanical mixtures, traditionally used in TB treatment. This is a milestone development towards novel anti TB drug development.

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