Anti tubercular minimum inhibitory concentration (MIC) and chemical characterization of ethnobotanical mixture used in the treatment of tuberculosis

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Abstract

Introduction: Tuberculosis **(**TB) is a national health priority and with failing current treatment regimens, uses of ethnobotanical therapies are being more vigorously explored. Treatment of TB using oral and inhalational administration (*hawan*) of a mixture of herbs (*hawan samagri*, HS) has been practiced in India since ages. In vitro antitubercular effect of extract of HS against H37Ra strain of *Mycobacterium tuberculosis* (MTB) was recently reported in an ICMR funded research project.[1] Hence this study aims to document scientific data on antitubercular effect of this HS extract against clinical MTB strains.

Materials and Methods: Minimum inhibitory concentration (MIC) against clinical MTB strains of two different ethnobotanical mixtures (HS_1 and HS_2) used traditionally in India for the indigenous treatment of TB was compared. 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). Resazurin Microtiter Assay (REMA) was done to study antitubercular MIC.

Result and Discussion: Crude essential oil (EO) extract of HS showed significant antitubercular effect in MIC range 50 - 400 µg/ml. HS_1 showed lower MIC values compared to HS_2. GCMS analysis of EO showed an array of bioactive compounds known to have antitubercular and other beneficial effects.

Conclusion: Scientific validation of ethnobotanical use of HS extract for antitubercular activity was obtained in this work. HS crude EO seems to be a promising natural product with potential for new drug development in the treatment of TB.

Keywords: Ethnobotanical, Antitubercular activity, Essential oil, Multi drug resistant (MDR), Resazurin microtiter assay (REMA), Gas Chromatography and mass spectroscopy (GCMS), Phytochemical, Terpenoids, Phenolics, *Hawan samagri* (HS).

Introduction

Tuberculosis (TB) is the top infectious disease killer in the world. *Mycobacterium tuberculosis* (MTB*)* is the etiological agent of TB. The World Health Organization (WHO) has estimated that 10 million people develop active TB in year 2017 and the situation is complicated by an increase of MTB strains resistant to antitubercular drugs: Multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB. India accounted for 33% of global TB deaths among HIV-negative people, and for 26% of the combined total of TB deaths in HIV-negative and HIV-positive people.[2] A major problem for the control of TB is the requirement of drug regimens for six to nine months. These lengthy regimens lead to non-compliance with therapy, relapse and development of drug resistance. In order to shorten the duration of therapy, novel drugs that are active against MTB, which act through mechanisms different from those employed in the existing first line and secondary anti-TB regimes are urgently needed.[3] Hence there is much regained interest in use of herbs and *other* alternative therapies for the treatment of TB. Phytochemical aspects of most medicinal plants have been known and used since time immemorial.[4-6] Ethnobotanical advantages conferred by these plant based products have surpassed the chemical counterparts owing to their lesser side effect and more potent therapeutic effect. Development of effective anti TB drug is a global health priority. It is in this scenario that ancient traditional therapies for TB seem to be worth exploring. *Hawan* is an ancient fumigation method devised and used by sages (scientists of ancient India), that involves the use of clarified cow's butter and a mixture of odoriferous and medicinal plant parts (known as Hawan Samagr*i*, HS) for oblation onto consecrated fire of specific medicinal wood.[7] Inhalation of medicinal fumes emanating from this is proved to be effective in treatment of TB.[8] The extract of this same HS mixture is also used as oral medicine in such patients as advised by *Charak*, the famous physician of ancient India, in his treatise *Charak Samhita*.[9] However due to lack of scientific validation, the use of these has not been attempted in modern medicine. Recently few studies have tried to prove the depolluting and antibacterial effect of *hawan.*[10-12] Also, In-vitro antitubercular effect of *hawan* and HS extract was reported by Rastogi et al. against H37Ra strain of MTB.[12] However, In-vitro antitubercular effect of HS extract against clinical MTB strain has not been documented in literature *impeding* its possible use in TB treatment. This study attempts to fill the above knowledge gap in an effort to explore possibility of effective ethnobotanical alternative therapy for TB.

Materials and Methods

Composition of Herbal Mixture (Hawan Samagri, HS)

In this study two types of ethnobotanical mixtures HS_1 and HS_2 were used. HS1 consists of- *Acacia arabica* (*Babool*) bark; *Achyranthes aspera* L. (*Chirchita*) seed; *Acorus calamus* L. (*Vacha*) root; Albizia *lebbeck* (L.) *Benth* (*Sirish*) bark; *Allium sativum* L. (*Lehsun*, Garlic) tuber; *Areca catechu* L.(*Supari*, Arecanut) fruit; *Argemone Mexicana* (*Satyanaashi*) all parts; *Azadirachta indica* A. *Juss.* (*Neem*) leaves; *Berberis aristata* (*Daruhaldi*) wood;

Brassiaca campestris L. (*Sarsoan,* Yellow mustard) seed; *Butea monosperma* (Lam.) *Taub*. (*Dhaak*) seed; *Calotropis procera* (*Aak*) all parts; *Cassia tora* L.(*Chakvad*) seed; *Centratherum anthelminticum* (L.) *Kuntze* (*Kalizeeri,* Black cumin) seed; *Cinnamomum camphora* (*Kapur,* Camphor); *Commiphora mukul* (*Guggal*) latex; *Crocus sativus* L.(*Kesar,* Baby saffron) Stigma; *Curcuma longa* L. (*Haldi,* Turmeric) tube; *Cyperus scariosus* (*Nagarmotha*) root; *Datura metel* (*Dhatur*) leaves; *Elletaria cardamomum Maton*. (*Elaichi,* Green cardamom) seed; *Embelia ribes Burm. f.* (*Vayviding*) fruit; *Moringa oleifera Lam*. (*Sahijan,* Drumstick) bark; *Myristica fragrans Houtt.* (*Jaifal,* Nutmeg) fruit; *Ocimum basilicum* L.(*Van Tulsi*) leaves & aerial parts; *Ocimum sanctum* L. (*Tulsi,* Basil) leaves; *Pinus roxburghii Sarg*.(*Cheed*) wood; *Piper longum* L.(*Peepal*) fruit; *Plumbago zeylanica* L. (*Cheeta*) root; *Pongamia pinnata* (L.) *Pierre* (*Dithori*) seed; *Sisymbrium irio* (*Khoobkala*) seed; *Solanum surattense* (*Kanthkari*) all parts; *Styrasx benzoin* Dry (*Lobaan*) latex; *Surya robus* (*Raal*) latex; *Swertia chirayta* (*Chirayata*) all parts; *Tinospora cordifolia Miers* (*Giloy*) creepers; *Trachyspermum ammi* (*Ajwain*) seed; *Vitex negundo* L. (*Sambalu*) leaves and *Zingiber officinale Roscoe* (*Soonth,* zinger) rhizome. This was indigenously prepared. These herbs are recommended in ancient Indian literature, for use in various fumigation procedures, especially *hawan* and also as oral medicine in the treatment of TB patients.[8,9,13-15] HS_2 was obtained from "Rishi Udhyan" Arya Samaj, Ajmer, where *hawan* is being performed daily since ages using the same mixture.

Characterization of MTB Strains

A total of 20 pure isolates of MTB grown on Lowenstein-Jensen (LJ) medium from sputum samples of patients attending Intermediate Reference Laboratory (IRL), State Tuberculosis Training and Diagnosis Centre (STDC), Ajmer, Rajasthan were tested for their sensitivity to the HS extract. All processing was done as per standard guidelines.[16,17] MTB H37Ra ATCC 25177 was used as standard reference strain. Characterization of isolated strains was done by Line probe assay (LPA).[18,19]

Extraction and Chemical Characterization of EO from HS

The extraction of oil from HS_1 and HS_2 mixtures was done by hydro distillation technique. Water constituents in the oil collected after hydro distillation was removed by using anhydrous sodium sulphate.[20] Obtained oil was stored at -4°C in a dark colored bottle.[20,21] Castor oil was used as an oil control. Chemical analysis of all EO was done by Gas Chromatography and Mass Spectroscopy (GC-MS) (Thermo Scientific DSQ II) at National Botanical Research Institute (NBRI), Lucknow, using injection volume 1 µl and run time of 60 minutes.

Determination of Antitubercular MIC of EO

21-28 days old pure cultures of MTB on LJ slants were used for preparing inoculums as per recommended guidelines of RNTCP manual for drug susceptibility testing[16,19] and Resazurin Microtiter Assay (REMA) was performed as described by Palomino et al.[22-24] All procedures were done as per procedure manual of Colorimetric redox indicator (CRI)[22] with following minor modifications - Instead of microbroth dilution, macrobroth dilution of EO was prepared in 7H9 broth containing $3x10^7$cfu/ml of MTB. A macro broth dilution of EO ranging from 400 µg/ml to 3.125 µg/ml was prepared. Final concentration of 0.5µg/ml of rifampicin was used as drug control. All tubes having different concentrations of oil and same number of MTB were then incubated at 37°C. After six weeks incubation, 200 µl of each dilution was transferred to wells of REMA plate and 30 µl of resazurin (0.02%) was added to all the wells. The plates were sealed and incubated overnight for color development. A change in color in wells from blue to pink was taken as an indicator of growth of the isolate at that concentration of the drug.[22] The well with lowest concentration of EO showing no colour change was taken as the MIC for that strain. All tests were done in triplicate to ensure reproducibility.

Result and Discussion

Percent inhibition of MTB strains by HS_1 was 100% at 400 µg/ml, 50% at 200 µg/ml, 30% at 100 µg/ml and 5% at 50 µg/ml. Whereas, percent inhibition shown by HS_2 was 100% at 400 µg/ml, 40% at 200 µg/ml and 5% at 100 µg/ml. Below this concentration i.e., from 25 µg/ml to 3.125 µg/ml no inhibition was observed (Table 1). HS crude EO extract showed antitubercular effect on clinical strains of MTB with MIC ranging 50µg/ml to 400 µg/ml. HS_1 (indigenously prepared) showed lower MIC and hence more effective antitubercular effect than HS_2. Control (Castor) oil showed no inhibition on any of the strains. Reference strain H37Ra tested sensitive to RIF (control drug) at concentration 0.5 µg/ml. One of the clinical isolate showed multiple drug resistance (MDR), against which HS_1 and HS_2 both showed inhibition with 200 µg/ml and 400 µg/ml respectively (Table 2). It indicates that EO extract of HS has potential to inhibit MDR MTB also, more so by HS_1. All the remaining (18) isolates were RIF and INZ sensitive and were found to be inhibited by EO extracts of HS_1 and HS_2 at different concentrations (Table 2). Since MIC of HS extract against clinical MTB has not been reported in literature, this being a novel work as undertaken in this study, MIC value results for comparison are not available. However, in 2015 Rastogi et al. reported HS_1 EO MIC value of 31.25 µg/ml against H37Ra MTB reference strain.[11] The MIC of 200 µg/ml reported against the same strain in this study is probably due to testing against $3x10^7$ cfu/ml of MTB, as recommended for testing both first line and second line drug sensitivity, in contrast to $0.5x10^6$ cfu/ml in the work of V. Rastogi et al. A study conducted by Andrade-Ochoa Sergio et al., reported anti tubercular effect of five different EO extracts of individual herbs viz., Cumin, Clove, Cinnamon, Anis and Laurel against MTB strain H37Ra, with MIC values ranging 6.25 µg/ml to 25 µg/ml.[25] This is quite lower concentration range, though not comparable to results of this study. Also lower inoculum of $1.5x10^7$ cfu/ml were used

and EO was extracted from individual herbs. Hence, ideally our study results are not comparable to theirs. HS_1 showed percent yield of 0.5% whereas HS_2 showed percent yield of 0.4%.

GCMS analysis of hydrodistilled crude EO of HS revealed an array of bioactive chemical compounds corroborating with the observed antitubercular activity viz. Longifolene (HS_1),[26] Alpha cadinal (HS_1),[27] Tridecanone (HS_2),[28] Myristicin (HS_1,HS_2),[29] Dodecanoic acid (HS_2),[30] Cembrene (HS_2).[31] It is well known fact that terpenoids such as monoterpenes, sesquiterpenes and related alcohol/ phenols have antimycobacterial activity.[31,32] All these are found to be present in both HS_1 and HS_2 (Table 3), again proving the chemical basis of their antitubercular effect. Fig. 1 & 2 show the compound peaks of GCMS chromatogram of HS_1 and HS_2 respectively. GCMS analysis of castor oil used as oil control in this study is known to contain only long chain fatty acids (Fig. 3)[33] explaining the absence of antitubercular effect observed.

Although study of mechanism of action (m.o.a.) was not attempted in this study, it is a known fact that the lipophilic character of their hydrocarbon skeleton and the hydrophilic character of their functional group are of main importance in the antimicrobial action of EO components.[31,34] The antimycobacterial activity of terpenes is said to be due to perturbation of the plasma membrane lipid fraction of the microorganism, resulting in alteration of membrane permeability and leakage of intracellular materials.[34]

Tuberculosis is mostly asymptomatic and is aggravated when impairment of immunity arises due to conditions like malnutrition, diabetes, malignancy, and AIDS.[2] Interestingly alpha turmerone was seen to be the most abundant (18.78%) compound in GCMS peaks of HS1 EO and absent in HS2. This compound is known to have potent antioxidant, anti inflammatory, antimicrobial and neural stem cell proliferative property[35] which shall contribute to enhancement of immunity. EO analysis showed presence of beta-caryophyllene known to have anti-inflammatory, analgesic action.[36,37] Recent research has demonstrated that the bicyclic sesquiterpene beta-caryophyllene has the ability to bind to CB2 cannabinoid receptors. What this means is that along with its cellular and skin-health supporting properties, due to its activation of CB2 receptors, beta-caryophyllene may also support healthy nervous and immune function, and have soothing and relaxing (analgesic, anti-inflammatory), properties on the body.[37] The therapeutic attributes of Myristicin identified in both HS1 and HS2 includes hepatoprotective, antibacterial, anti-inflammatory, antioxidant, cytotoxicity and anti-proliferation of cancerous cells, psychoactive and anti-cholinergic effects. Beta–elemene is muscle relaxing, antiinflammatory and is able to pass the blood–brain barrier.[38] It has demonstrated in vitro, in vivo and clinical antitumoral effects.[38] Isolongifoline, Alphaterpinol, Methyl eugenol, Alpha Curcumene, etc., constitute a complex mixture of volatile compounds including terpene hydrocarbons and their oxygenated derivatives (alcohols, esters, aldehydes, ketones, phenols, and ethers) having multi-system health benefits.[5,7,32,38,39] Thus, clinically observed antitubercular effect of the HS extract seems to be a sum total of its anti-oxidant, anti-inflammatory, analgesic, antimicrobial, stem cell proliferative, immunomodulatory, neuroprotective, anxiolytic effects, etc. Together they bring about the desired cure evidenced in traditional medical practice. It is very likely that each of the constituents of the HS EO show activity against MTB as well.

Thus documentation of the chemical fingerprint of EO extract of traditionally used HS and documentation of in-vitro anti-tubercular activity enabled scientific validation of the ethnobotanical use of these for TB treatment. Antitubercular screening for the phytochemicals from indigenously used plant extract represents a starting point for newer potent antimycobacterial drug discovery,[39] that might prove to be a panacea in the treatment of TB. However, the exact m.o.a. needs to be further elucidated by in-vivo animal studies and safety/toxicity studies, which were beyond the scope of the present work, but nevertheless warranted in future.

Table 1: MIC of various EO against MTB

Concentration µg/ml(ppm)*	% Inhibition of MTB strains		
	HS_1	**HS_2**	**Control castor oil**
25- 3.125	No inhibition	No inhibition	No inhibition
50	5% (n=1)	No inhibition	No inhibition
100	30% (n=6)	5% (n=1)	No inhibition
200	50% (n=10)	40% (n=8)	No inhibition
400	100% (n=20)	100% (n=20)	No inhibition

*****ppm-parts per million

Table 2: In vitro anti tubercular effect of different essential oil extracts on MTB strains

KNSTDC ID No.	Antitubercular drug sensitivity by LPA		In vitro anti tubercular effect			
			Rifampicin (Drug control) 0.5 µg/ml	Mean M IC of EO (µg/ml)		
	RIF	INZ		HS_1	HS_2	Castor oil
Control	S	S	S	200	400	No inhibition
6163	R	R	R	400	400	No inhibition
6165	S	S	S	100	200	No inhibition
6167	S	S	S	200	400	No inhibition
6168	S	S	S	100	200	No inhibition
6169	S	S	S	200	200	No inhibition
6174	S	S	S	50	100	No inhibition
6175	S	S	S	200	400	No inhibition
6176	S	S	S	400	400	No inhibition
6177	S	S	S	200	200	No inhibition
6181	S	S	S	200	400	No inhibition
6182	S	S	S	100	400	No inhibition
6184	S	S	S	200	400	No inhibition
6185	S	S	S	200	400	No inhibition
6187	S	S	S	100	200	No inhibition
6189	S	S	S	100	200	No inhibition
6190	S	S	S	200	200	No inhibition
6191	S	S	S	100	200	No inhibition
6192	S	S	S	200	400	No inhibition
6193	S	S	S	200	400	No inhibition

Table No 3: Comparative properties of control (castor oil) and test oils HS_1 and HS_2 EO

S. No.	Property	Castor Oil	HS_1 EO	HS_2 EO
1	Solubility	DMSO	Ether, Ethanol, DMSO, Benene, Di ethyl ether	Ether, Ethanol, DMSO, Benene, Di ethyl ether
2	Density	0.95 gm/ml	0.9 gm/ml	0.8 gm/ml
3	Refractive index	1.47	1.49	1.46
4	Water resistant	Yes	Yes	Yes
5	Freezing	No	No	Yes
6	Percentage yield (%)	NA	0.50%	0.40%
7	Colour	Colourless	Pale yellow	Light brown
8	Odour	Odourless	Aromatic	Aromatic
9	Physical state at room temperature	Liquid	Liquid	Liquid
10	GCMS Characterization with percent abundance	Oleic acid(C18), Palmitic acid (C16), Stearic acid (C17), Undecylenic acid (C11), Methyl ricinoleate (C19), Behenic acid (C21), Tridecylenic acid (C11), nonadecyclic acid (C19)	Camphor (23.9%), Thyme camphor (6.94%), Myristicin (2.39%), Longifolene (1.49%), Alpha Terpineol (1.80%), Alpha Copaene (1.08%), Beta-elemene (2.08%), Caryophyllene (5.87%), Muuroline (4.64%), Alpha-Himachaline (1.72%), Alpha-Curcumene (1.39%), Methyl eugenol (4.09%), Caryophyllene oxide (2.93%), Humulane (4.99%), Elemicine (3.11%), Alpha- Cadinal (1.39%), Turmerone (1.00%), Ar-Tumerone (18.78%), Asarone (0.59%), Verticiol (0.63%)	Camphor (6.43%), Myristicin (1.11%), Cyclo isolongifolene (12.03%), 2 Nonanone (1.81%), 2-Undicanone (9.18%), Decanoic Acid (7.36%), Beta –selinene (0.71%), Tridecanone (0.45%), Dodecanoic Acid (31.39%), Epicedrol 1.68%, Patchoulol (1.82%), Germacrone (3.82%), Methoxy cinnamate (0.76%), Cembrene (0.30%), Dehydrotestosterone (0.45%)

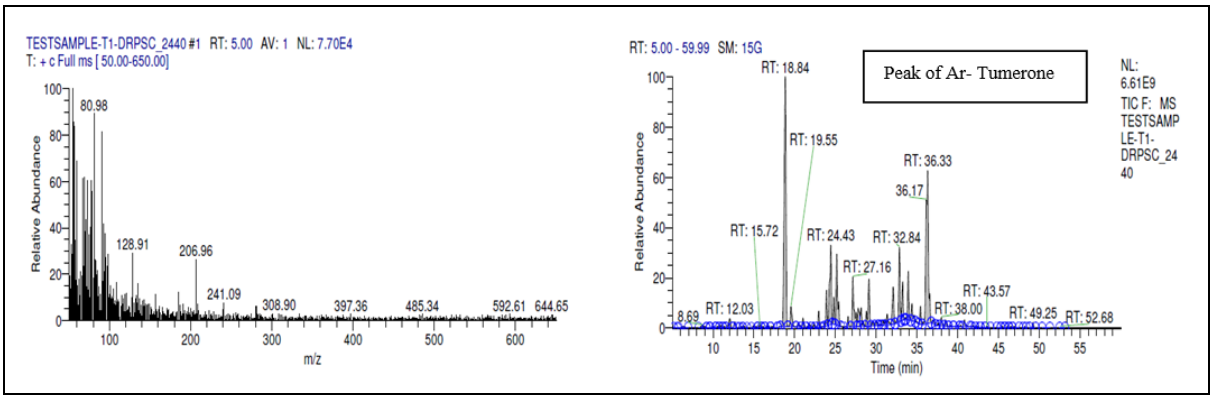


Fig. 1: Showing GC-MS peaks of HS1 EO

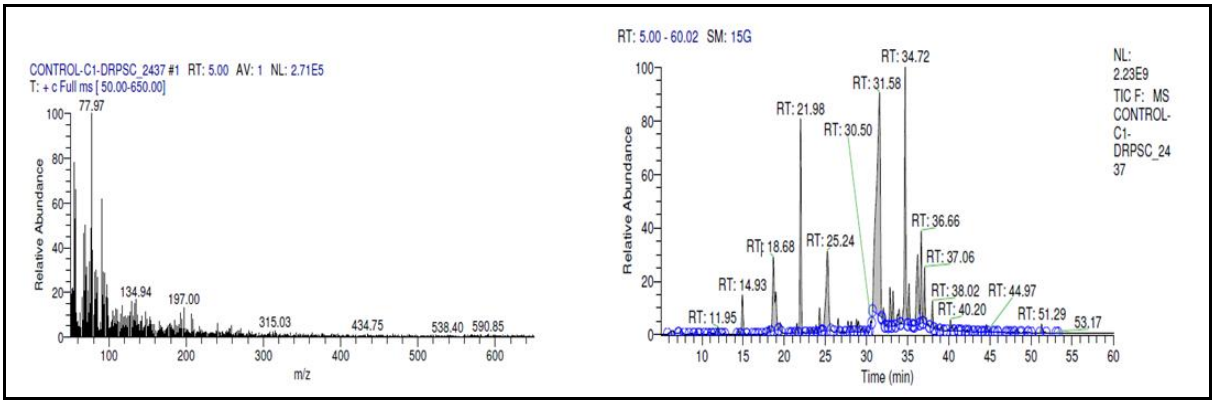
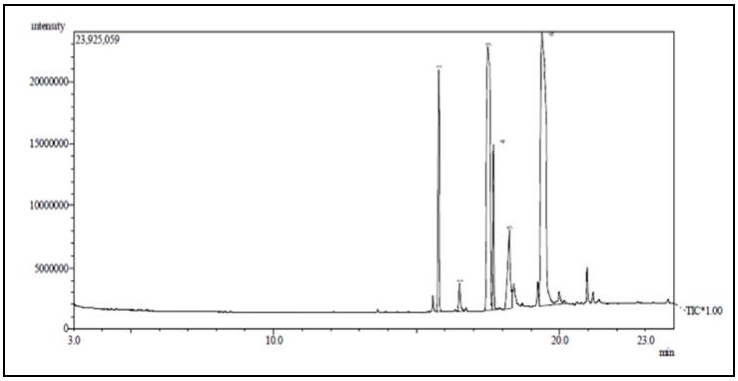


Fig. 2: Showing GC-MS peaks of HS_2 EO



Courtesy[33]: Chemistry and Material research, 7 (2), 2015

Fig. 3: Showing GC-MS total ionic chromatogram (TIC) of Castor bean oil

Conclusion

The present study found scientific validation for the ethnobotanical use of mixture of herbs, traditionally used as HS in *Hawan* fumigation and also as oral medicine for the treatment of TB. 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 HS_1 crude EO in novel TB drug discovery and management. In this perspective, we recommend bioactivity guided isolation and purification of lead compounds for anti tubercular activity. Also, efficacy and toxicity studies of the crude EO in an animal model are warranted. The pharmacobiological advantage of crude HS extract therapy as compared to purified chemical compounds also needs to be elucidated by future studies.

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