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Anti-mycobacterial screening of five Indian medicinal plants and partial purification of active extracts of *Cassia sophera* and *Urtica dioica*

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ABSTRACT

Objective: To find out the anti-mycobacterial potential of *Cassia sophera* (*C. sophera*), *Urtica dioica* (*U. dioica*), *Momordica dioica*, *Tribulus terrestris* and *Coccinia indica* plants against multi-drug resistant (MDR) strain of *Mycobacterium tuberculosis* (*M. tuberculosis*). **Methods:** Plant materials were extracted successively with solvents of increasing polarity. Solvent extracts were screened for anti-mycobacterial activity against fast growing, non-pathogenic mycobacterium strain, *Mycobacterium semegmatis*, by disk diffusion method. The active extracts were tested against MDR and clinical isolates of *M. tuberculosis* by absolute concentration and proportion methods. The active extracts were subjected to bio-autoassay on TLC followed by silica column chromatography for isolation of potential drug leads. **Results:** Hexane extract of *U. dioica* (HEUD) and methanol extract of *C. sophera* (MECS) produced inhibition zone of 20 mm in disc diffusion assay and MIC of 250 and 125 μg/mL respectively in broth dilution assay against *Mycobacterium semegmatis*. Semipurified fraction F2 from MECS produced 86% inhibition against clinical isolate and 60% inhibition against MDR strain of *M. tuberculosis*. F18 from HEUD produced 81% inhibition against clinical isolate and 60% inhibition against MDR strain of *M. tuberculosis*. Phytochemical analysis indicated that anti-mycobacterial activity of MECS may be due to presence of alkaloids or flavonoids and that of HEUD due to terpenoids. **Conclusions:** *C. sophera* and *U. dioica* plant extracts exhibited promising anti-mycobacterial activity against MDR strain of *M. tuberculosis*. This is the first report of anti-mycobacterial activity form *C. sophera*. This study showed possibility of purifying novel anti-mycobacterial compound(s) from *C. sophera* and *U. dioica*.

1. Introduction

Tuberculosis (TB) is a highly infectious disease currently infecting around 1/3 of the world's population[1]. As per the WHO estimate, there are 9 million cases of active TB with 1.3 million reported deaths every year at present[2]. Asian and African countries shares major TB burden with 55% and 30% of the total reported cases respectively[3]. Currently used anti-mycobacterial drugs, namely, rifampicin, isoniazid, streptomycin and ethambutol, were introduced in TB control programmes almost three decades ago. Indiscriminate use of these drugs has led to the development of multi-drug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (*M. tuberculosis*), making the treatment difficult and increasing TB burden[4-7]. The emergence of MDR and XDR strains and increase in TB burden necessitated the urgent need for new anti-mycobacterial drugs.

Medicinal plants have been used worldwide by traditional medicinal practitioner for the treatment of various diseases. Approximately 60% of world's population still relies on medicinal plants for their primary healthcare. The advantage of plant based drug discovery is that the phytochemicals provide novel drug leads with novel mechanism of action. Anti-mycobacterial phytochemicals may be helpful in identification of new molecular targets in MDR and XDR strains of mycobacterium opening new vistas for anti-tubercular drug discovery. A number of medicinal plants have been screened for anti-mycobacterial activity in past few years[8-12]. Some plants have shown promising

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results[13], but very few active molecules have been purified. In the Indian subcontinent which is rich in traditional knowledge of "Ayurveda", studies have been carried out on anti-mycobacterial activity of medicinal plants[14-15]. Present study was carried out to evaluate *invitro* anti-mycobacterial activity of *Cassia sophera* (*C. sophera*), *Urtica dioica* (*U. dioica*), *Momordica dioica* (*M. dioica*), *Tribulus terrestris* (*T. terrestris*) and *Coccinia indica* (*C. indica*) plants. *C. sophera* Linn. (Caesalpiniaceae) is used for treatment of psoriasis, cough, arthritis, diabetes and convulsions[16]. Its extracts are used against ringworm[17] and also possess hepatoprotective activity[18]. *M. dioica* Roxb. (Cucurbitaceae) possess anti-allergic[19], analgesic and anti-inflammatory[20] and anti-diabetic activity[21]. *T. terrestris* Linn. (Zygophyllaceae) is used as tonic, aphrodisiac, analgesic, astringent, stomachic, antihypertensive, diuretic and lithontriptic and also for the treatment of urinary tract infections[22]. *U. dioica* Linn. (Urticaceae) possess antioxidant, antimicrobial, antiulcer and analgesic activities[23].

Screening of medicinal plant for anti-mycobacterial activity involves large number of solvent extracts. Screening large number of extracts on slow growing, pathogenic *M. tuberculosis* strain is a major problem. In this study, various solvent extracts of five selected medicinal plants were screened against fast growing, non-pathogenic *Mycobacterium semegmatis* (*M. semegmatis*). The extracts possessing promising activity were tested against standard, MDR and clinical isolates of *M. tuberculosis*. The most active extracts were further fractionated and tested against *M. tuberculosis*.

2. Materials and methods

2.1. Strains, culture media and chemicals

Preliminary screening of various solvent extracts was carried out on non-pathogenic fast growing mycobacterial strain, *M. semegmatis* (MC2-155). The active extracts were tested on *M. tuberculosis* standard strain H37Rv (ATCC-35838), MDR strains, and clinical isolates CL-1 (+3) and CL-2 (+2). Anti-mycobacterial activity of extracts against these strains was observed in Department of Microbiology, Maharani Laxmi Bai Medical College, Jhansi, India. Mycobacterial culture media [Middle brook 7H11 agar, Middle brook 7H9 broth, and Lowenstein-Jensen (L-J) medium], media supplements and standard antibiotics were obtained from Himedia (Mumbai, India). Other chemicals used in the study were of high purity and purchased commercially.

2.2. Collection of plant materials and extraction

Fresh aerial parts of *T. terrerstris* and fruits of *C. indica* and *M. dioica* were collected from local hills of Bundelkhand University during May-August, 2010. Dried seeds of *C. sophera* were obtained from drug manufacturing unit of Sree Baidyanath Ayurved Bhavan Ltd (Jhansi, India). The plant materials were identified with the help of experts in Department of Botany, Bundelkhand University. The voucher specimens with no. BU/BMS/VS/2010/01 (*C. indica*), BU/BMS/VS/2010/02 (*T. terristeris*), BU/BMS/VS/2010/03 (*M. dioca*) and BU/BMS/VS/2010/04 (*C. sophera*) were preserved in Department of Biomedical Sciences, Bundelkhand University. Leaves of *U. dioica* were collected from Anantnag district of the Kashmir Valley, India in October, 2009 and authenticated by the Centre of Biodiversity & Taxonomy, Department of Botany, University of Kashmir and a voucher specimen (28100 KASH) was deposited. The plant materials were dried in shade, ground to obtain coarse powder and stored at -40 °C in airtight containers until use.

Plant materials (100 g) were sequentially extracted for 12 h with hexane, chloroform, ethyl acetate and methanol in soxhlet apparatus. The residues after methanol extraction was soaked in distilled water at 60 °C for 12 h with constant stirring. The solvent extracts were filtered twice with Whatman's filter paper and centrifuged at 3 000 r/min for 15 min to remove any residual material. The organic solvents extracts were dried in a rotary evaporator (Heidolph, Germany) under vacuum and water extracts were lyophilized in freeze dryer (Martin Christ, Germany). The extracts were stored in glass vials at -40 °C until use.

2.3. Anti-mycobacterial activity against M. semegmatis

2.3.1. Preliminary screening by disk diffusion method

Stock solutions (200 mg/mL) of hexane, chloroform, ethyl acetate and methanol extracts were prepared in dimethyl sulphoxide (DMSO), whereas water extracts were dissolved in water. Petri plates with Middle brook 7H11 agar were seeded with 100 μL of *M. semegmatis* culture at a concentration of 1.5×10^6 cells/mL (OD adjusted to the 0.5 McFarland's turbidity standards). Sterile filter paper discs (6 mm in diameter) impregnated with 2 mg/disc of plant extracts were placed on the Petri plates. Rifampicin (20 μg/disc) was used as positive and a disc soaked in 10 μL of DMSO served as solvent control. The inoculated plates were incubated for 72 h and the results were recorded by measuring diameter of growth inhibition zone[24]. All experiments were carried out in triplicate.

2.3.2. Minimum inhibitory concentration (MIC) by broth dilution method

MIC was determined for plant extracts exhibiting growth inhibition zone of more than 10 mm in disc diffusion assay. The extracts were dissolved in DMSO and stock solution (2 mg/mL) was prepared in Middle brook 7H9 broth. The extracts were successively diluted to half (10 times) with 7H9 broth to obtain 10 concentrations, ranging from 2 000 to 3.9 μg/mL. The tubes were inoculated with 100 μl of *M. semegmatis* ($1x10^6$ cells/mL, OD adjusted to the 0.5 McFarland's turbidity standards). Rifampicin (64 μg/mL) was used as a standard drug for comparison and DSMO (10 μL/mL) served as solvent control. The tubes were incubated aerobically at 37 °C for 72 h followed by addition of 50 μL of 0.2 mg/mL 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) solution. The tubes were observed for color change and the concentration at which a decrease in red color (reduction of INT to formazan by bacteria) was apparent compared to the next higher concentration was taken as MIC value[25]. All experiments were carried out in triplicate.

2.4. Phytochemical screening

The bioactive extracts were tested for alkaloids, saponins, flavonoids, tannins, terpenoids, anthraquinones, phlobatannins and cardiacglycosides[26-28].

2.5. M. semegmatis activity guided purification of methanol extract of C. sophera (MECS) and hexane extract of U. dioica (HEUD)

2.5.1. Thin layer chromatography (TLC) bio-autoassay

MECS and HEUD possessing good activity in disc diffusion assay with promising MIC were subjected to bio-autoassay on silica TLC plates (Merck, Kiesel 60 F254, 0.2 mm thickness). MECS and HEUD were resolved on TLC plates with standardized mobile phase. The TLC plates were dried in air and dipped in Middle brook 7H11 agar Petri plates and seeded with *M. semegmatis* as per procedure followed for disc diffusion assay. After 72 h of incubation, plates were sprayed with INT to record zones of growth inhibition to indentify active band on TLC.

2.5.2. Column chromatography

MECS and HEUD were fractionated on silica gel (Merck, 60-120 mesh) column chromatography. For MECS, the column was eluted with chloroform/methanol gradient (10:0-0:10). The fractions were analyzed by TLC and pooled to 10 fractions (F1, F2, F3..., F10). For HEUD, the column was eluted with hexane/ethyl acetate gradient (10:0-0:10). The fractions were analyzed by TLC and pooled to give 20 fractions (F1, F2, F3..., F20). All the fractions were tested for anti-mycobacterial activity on *M. semegmatis* by disc diffusion assay and compared with active bands of TLC bio-auto-assay for identification of anti-mycobacterial active subfractions.

2.6. Anti-mycobacterial activity against M. tuberculosis

MECS, HEUD and their active subfractions were tested for anti-mycobacterial activity on standard, MDR and clinical isolates of *M. tuberculosis* by the proportion and absolute concentration methods.

2.6.1. Preparation of mycobacterial suspension

Approximately 4 mg of *M. tuberculosis* culture [visualized as 2/3 loopful, 3 mm internal diameter, 0.558 mm (24 SWG) thick wire loop] was added in 0.3 mL of sterile distilled water in a Bijou bottle and vortexed for 30 s to prepare a uniform suspension. Sterile distilled water (3.7 mL) was added to obtain 1 mg/mL suspension (S1). The suspension was kept on the bench for 15-20 min to allow the coarser particles to settle down.

2.6.2. Absolute concentration method

Standardized inoculums of *M. tuberculosis* (4 μL of 1 mg/mL bacterial suspension) was cultured on drug-free L-J media and media containing rifampicin (32, 64 and 128 μg/mL), isoniazid (0.2, 1.0, 5.0 μg/mL), ethambutol (2, 4 and 6 μg/mL), streptomycin (8, 16 and 32 μg/mL), MECS, F2 of MECS, HEUD and F18 of HEUD (62.5, 125.0, 250.0, 500.0, 1 000.0 μg/mL). L-J media slopes were incubated aerobically at 37 °C for 4 weeks. The lowest concentration of the plant extracts which inhibited the growth of *M. tuberculosis* (less than 20 colonies) was recorded as MIC[29].

2.6.3. Proportion method

Ten fold serial dilution suspensions S2, S3 and S4 were prepared from S1 in distilled water and inoculated [1 loopful, 3 mm external diameter, 0.558 mm (24 SWG) thin wire loop] on L-J slopes. The plant extracts were incorporated in the L-J media at concentration of 2% (v/v) and 4% (v/v) (2 mg and 4 mg of plant extracts were dissolved in minimum amount of DMSO and added to 100 mL of culture medium). Rifampicin (40 μg/mL), isoniazid (0.2 μg/mL), ethambutol (2 μg/mL) and streptomycin (8 μg/mL) were used as positive controls in the assay. The cultures were incubated for 4 weeks and visible colonies were counted[29].

3. Results

3.1. Anti-mycobacterial activity against M. semegmatis

The plant extracts showed low to good anti-mycobacterial activity in the disc diffusion assay. The HEUD and MECS were the most active extracts producing zone of inhibition with a diameter of 20 mm. The aqueous extracts of all plants except that of *C. sophera* showed low activity (Table 1).

The MIC of the most active extract of each plant (HEUD, MECS, and methanol extracts of *M. dioica*, *C. indica* and *T. terrestris*) was determined by broth dilution assay. The HEUD and MECS were the most active extracts showing MIC value of 250 and 125 μg/mL, respectively. The methanol extracts of *M. dioica*, *C. indica* and *T. terrestris* showed MIC values of 500, 1 000 and 500 μg/mL, respectively. The most active extracts MECS and HEUD were subjected to TLC bio-autoassay and fractionated by column chromatography to isolate active molecule(s).

3.2. Phytochemical analysis

The most active extract of each plant was tested for various

Table 1
Inhibition zone diameters (mm) by various plant extracts against *M. semegmatis* in disc diffusion assay.

Plant	Solvent extract					DMSO	Rifampicin
	Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous		
C. sophera	9	7	9	20	12	7	29
M. dioica	6	9	12	13	7	6	29
C. indica	10	8	9	12	9	7	28
T. terrestris	9	9	12	13	8	6	28
U. dioica	20	15	10	10	7	6	28

phytochemicals. Alkaloids, saponins, terpenoids, tannins and flavonoids were predominantly present in the active extracts (Table 2). Alkaloids and saponins were present in all active extracts except the HEUD. Phlobatannins were absent in all the extracts. Cardiac glycosides were present only in the MECS. The MECS contained alkaloids, saponins, flavonoids, tannins, anthraquinons and cardiac glycosides. The HEUD contained tannins, terpenoids and anthraquinons.

3.3. Isolation of bioactive compounds by TLC bio-autoassay and column chromatography

Active zones of the HEUD and MECS on TLC were identified bio-autoassay (Results not shown). TLC bio-autoassay helped to identify the active fractions of HEUD and MECS obtained from column chromatography.

Ten pooled fractions (F1-F10) obtained on silica column and eluted with chloroform/methanol gradient were subjected to anti-mycobacterial disc diffusion assay on *M. semegmatis*. F1-F3 were active. The F2 obtained at 98:2 chloroform:methanol was the most active and it produced 27 mm zone of inhibition in disc diffusion assay. TLC of F2 was compared with TLC bio-autoassay of MECS for identification of active bands.

Twenty pooled fractions (F1-F20) obtained on silica column and eluted with hexane/ethyl acetate gradient were subjected to anti-mycobacterial disc diffusion assay on *M. semegmatis*. F15-F18 were active. F18 obtained at 77:23 hexane:ethyl acetate was the most active and produced 24 mm zone of inhibition in disc diffusion assay. TLC of F18 was compared with TLC bio-autoassay of HEUD for identification of active bands.

3.4. Anti-mycobacterial activity against M. tuberculosis

3.4.1. MIC

MIC, mean CFU value, inhibition rate by HEUD, MECS and their semipurified fractions were evaluated against H37Rv, CL-1 and CL-2 (Table 3).

3.4.2. Mean CFU and inhibition rate

MECS, HEUD and their semipurified fractions showed significant *M. tuberculosis* growth inhibition on L-J media slopes. Semipurified fraction F2 from MECS produced 86% inhibition, whereas F18 from HEUD produced 81% inhibition at 4% (v/v) concentration in CL-1 culture. F2 and F18 produced 60% inhibition against MDR and 68% and 79% inhibition against standard strain, respectively. The result showed that *M. tuberculosis* growth inhibition by HEUD, MECS and their purified subfractions was dose dependant with 4% (v/v) concentration producing more effect as compared to 2% concentration (Table 3).

Table 2
Phytochemical analysis.

Plant	Extract	Alkaloids	Saponins	Flavonoids	Tannins	Terpenoids	Anthraquinones	Phlobatannins	Cardiac glycosides
C. sophera	Methanol	+	+	+	+	-	+	-	+
M. dioica	Ethylacetate	+	+	+	+	-	-	-	-
C. indica	Methanol	+	+	+	+	+	+	-	-
T. terrestris	Methanol	+	+	-	+	-	-	-	-
U. dioica	Hexane	-	-	-	+	+	+	-	-

Table 3
Anti-mycobacterial assay using plant extracts against *M. tuberculosis*.

Extract/fraction	Strain	MIC[a] (μg/mL)	Mean CFU on media			Inhibition rate (%)	
			Control	2% (v/v) plant extract	4% (v/v) plant extract	2% (v/v) plant extract	4% (v/v) plant extract
Methanol extract of *C. sophera* (MECS)	H37RV	500	47	27	13	42	72
	MDR	500	27	14	10	49	62
	CL-1	250	71	25	15	65	79
	CL-2	125	66	24	12	63	81
Hexane extract of *U. dioica* (HEUD)	H37RV	250	47	22	12	53	74
	MDR	500	28	16	15	43	46
	CL-1	250	72	21	17	71	76
	CL-2	250	65	24	20	63	69
F2 fraction of MECS	H37RV	250	47	26	15	45	68
	MDR	250	25	12	10	52	60
	CL-1	125	72	21	10	70	86
	CL-2	125	65	27	11	58	83
F18 fraction of HEUD	H37RV	250	47	19	10	60	79
	MDR	250	30	14	12	54	60
	CL-1	125	72	19	14	74	81
	CL-2	125	65	19	10	71	78

[a]MIC was determined by absolute concentration method.

4. Discussion

WHO declared TB as global health emergency because of the increase in HIV co-infection and the appearance of MDR and XDR strains[30]. No new anti-mycobacterial drug has been introduced in past 30 years, hence, there is an urgent need to develop novel, safe, effective and affordable drug for treating resistant forms of TB. There has been a renewed interest in phytochemicals as source of novel therapeutics in the past decade, hence, plants have been investigated for various pharmacological effects including anti-mycobacterial activity. A number of plants have been reported to possess anti-mycobacterial activity[11,31]. Phytochemicals may become the base for new drug development by providing a pharmacophore which could be used for the development of new drug with novel mechanism of action. Recently anti-mycobacterial activity of *Adhatoda vasica*, *Acalypha indica*, *Aloe vera*, *Allium cepa* and *Allium sativum* have been reported against MDR strains of *M. tuberculosis*[14].

In current study, *in vitro* anti-mycobacterial activity of *C. sophera*, *U. dioica*, *M. dioica*, *T. terrestris* and *C. indica* have been evaluated. There are reports of screening of large number of plant extracts against fast growing, nonpathogenic *M. semegmatis* and the active extracts have also exhibited activity against *M. tuberculosis*[26]. Out of five plants selected in this study, *C. sophera* and *U. dioica* were identified as potentially active in disc diffusion assay against *M. semegmatis*. MECS and HEUD produced 20 mm inhibition zone, whereas rifampicin produced 28 mm inhibition zone. The MIC of these extracts was 125 and 250 μg/mL respectively, which may be considered very promising for crude extracts having large number of compounds. No anti-mycobacterial activity has been reported earlier from the genus *Cassia* and this is perhaps the first report of anti-mycobacterial activity from *C. sophera*, hence, MECS was processed further for purification of active molecule(s). The semipurified fraction F2 of MECS obtained at 98:2 chloroform/methanol was the most active and it produced 27 mm zone of inhibition in disc diffusion assay on *M. semegmatis*. The MIC of was 125 μg/mL against CL-1 and CL-2 and 250 μg/mL against standard and MDR strain of *M. tuberculosis*. The findings indicated that partial purification of MECS improved the activity against the tested strains, probably due to increase in concentration of the active molecule(s). The phytopharmaology of *C. sophera* is not explored extensively having very few published reports[32]. Since this is the first report of anti-mycobacterial activity of MECS, its phytochemical analysis was carried out. MECS showed presence of alkaloids, saponins, flavonoids, tannins, anthraquinons and cardiac glycosides. Alkaloids from a number of plants have been reported to possess anti-mycobacterial activity[11]. Anti-mycobacterial activity alkaloid vasicine from *A. vasica* have already been reported[33]. Other important anti-mycobacterial alkaloids includes berberine from *Hydrastis canadensis*[34] and chabamide from *Piper chaba*[35]. Flavonoids are another important class of phytochemicals possessing anti-mycobacterial activity. Ferulenol form *Ferula communis*[36], licochalcone A from *Glycyrrhiza glabra*[37] and phaseollidin from *Erythrina gibbosa*[38] are few of the potential anti-mycobacterial flavonoids isolated form plants. Anti-mycobacterial activity of MCES may be due to presence to alkaloids or flavonoids. Antimicrobial activity of hexane extract of *U. dioica* has been reported earlier by our group. The antibacterial subfraction from hexane extract of *U. dioica* showed presence of neophytadiene (26.97%), butyl tetradecyl ester (9.53%), dibutyl phthalate (7.45%), bis (2-ethyl hexyl) maleate (8.80%) and 1,2- benzenedicarboxylic acid (9.89%) as the principal terpenes[25]. In this study, HEUD exhibited promising anti-mycobacterial activity against *M. semegmatis* and *M. tuberculosis* as compared to other solvent extracts. The phytochemical analysis of HEUD showed presence of tannins, terpenoids and anthraquinons. Plant terpenoids have already been reported to exhibit antimicrobial activity[39]. The finding suggested that terpenoids present in HEUD may be responsible for anti-mycobacterial activity. Semipurified fractions F2 of MECS and F18 from HEUD produced 86% and 81% inhibition at 4% (v/v) concentration against clinical isolate of *M. tuberculosis*. The effect of semipurified fractions was better as compared to their crude extracts, suggesting that active molecule(s) may have concentrated during the process of purification. Moreover, 4% (v/v) extract was more active as compared to 2% extract, indicating that the effect was dose dependant.

In conclusion, *C. sophera* and *U. dioica* plant extracts exhibited promising anti-mycobacterial activity against MDR strain of *M. tuberculosis*. This is the first report of anti-mycobacterial activity of *C. sophera*. This study showed possibility of purifying novel anti-mycobacterial compound(s) from *C. sophera* and *U. dioica*.

Conflict of interest statement

We declare we have no conflict of interest.

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