



Evaluation of In-Vitro Anti-Mycobacterial Activity and Isolation of Active Constituents from *Crocus sativus* L. (Iridaceae)

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ABSTRACT: Emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* against present day antitubercular agents is a major challenge to tuberculosis management programme. So, there is a need for novel drug discovery that may leads for development of alternative antitubercular agents. *Crocus sativus* is an important plant of Iridaceae family, known for its aroma; colour and medicinal properties used as traditional medicine to treat several disorders. The main constituents of the plant mostly contain antimycobacterial agents which were screened for antimycobacterial activity against non-pathogenic *Mycobacterium semegmatis* by disk diffusion method. The active extracts and their semipurified fractions were then tested against pathogenic *M.tuberculosis* H37RV, MDR and some clinical isolates by absolute concentration and proportion methods on LJ (Lewisten Jensen) media. The active extracts were subjected to bio-autoassay on TLC (Thin Layer Chromatography) plates followed by silica column chromatography for isolation of potential drug leads. Hexane extract of *C.sativus* (HECS) and methanol extract of *C.sativus* (MECS) showed a promising activity against *M.semegmatis*. HECS and its semi purified fractions F11 and F15 (4%v/v) showed 74%, 68% and 66% inhibition while as MECS and its semi purified fraction F5 showed 72% and 68% inhibition respectively against MDR strain of *M.tuberculosis*.

Key words: Antimycobacterial agents, Tuberculosis, *Crocus sativus*

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INTRODUCTION

Tuberculosis (TB) being a highly infectious disease (Agarwal, 2004) with nine million cases of active TB and 1.3 million deaths occurring every year (WHO, 2010), shares major TB burden with 55% and 30% of the total reported cases in Asian and African countries respectively (WHO, 2009). The aim to reduce the global mortality and to eliminate the disease (Raviglione and Uplekar, 2006) several drugs like Rifampicin, Isoniazid, Streptomycin and Ethambutamol were introduced in TB control. Indiscriminate use of these drugs has led to widespread multiple drug resistance MDR and extra drug resistance XDR development in *M. tuberculosis* (WHO, 2008; Iseman, 1994; Crofton and Mitchison, 1948) against first and second line anti-tuberculosis drugs (Singh, 2007). New antimicrobial drugs from plants in the research and development (R&D) pipeline have become increasingly more receptive to the potential use of antimicrobials and other drugs (Akinpelu and Onakoya, 2006). A number of medicinal plants have been screened for antimycobacterial activity from various parts of the world (Soejarto *et al.*, 2012; Gupta *et al.*, 2011; Renu *et al.*, 2010; Okunade *et al.*, 2004; Copp, 2003; Newton *et al.*, 2002 & Cantrell *et al.*, 2001.) Indian sub-continent being rich in traditional

knowledge of ayurved, few studies has been carried out on antimycobacterial activity of medicinal plants.

Crocus sativus L., commonly known as saffron, belongs to family Iridaceae, known for its aroma, colour and medicinal properties and is regarded as the most costly spice in the world (Mohammad *et al.*, 2011). Each plant grows to 20–30 cm (8–12 in) and bears up to four flowers, each with three vivid crimson stigmas. Chemical analyses of plant extracts have revealed that the main constituents of the plant to be carotenoids, glycosides, monoterpenes, aldehydes, picrocrocin and anthocyanin's, flavonoids, vitamins (especially riboflavin and thiamine), amino acids, proteins, starch, mineral matter, and gums; other chemical compounds have also been reported in saffron (Fernandez, 2006). The plant has been used in folk medicine as an ant catarrhal, eupeptic, expectorant and emmenagogue (Rios *et al.*, 1996), antitumor effects (Nair *et al.*, 1991; Salomi *et al.*, 1991; Tarantilis *et al.*, 1994), counteract atherosclerosis (Gainer and Jones, 1975), hepatic damage (Wang *et al.*, 1991), antispasmodic (Zargari, 1990), anticonvulsant (Hosseinzadeh and Khosravan 2002), antidepressant (Hosseinzadeh *et al.*, 2004), ant nociceptive and anti-inflammatory (Hosseinzadeh and Younesi, 2002),

antioxidant (Hosseinzadeh *et al.*, 2009), acetyl cholinesterase inhibiting (Geromichalos *et al.*, 2012), antitussive (Hosseinzadeh and Ghenaati, 2006), reducing withdrawal syndrome (Hosseinzadeh and Jahanian, 2010), improving male erectile dysfunction (Hosseinzadeh and Ziaee, 2008), enhancing spatial cognitive abilities after chronic cerebral hypo perfusion (Hosseinzadeh *et al.*, 2012), hypotensive (Imenshahidi *et al.*, 2010) and ant solar (Golmohammadzadeh *et al.*, 2010) properties. Chemical analysis reveals presence of more than 150 chemicals present in saffron stigmas (Bathaie and Mousavi, 2010) among which, all these pharmacological effects have been related to saffron main chemical compounds such as crocin, picrocrocins and safranal which are responsible for saffron exclusive colour, taste and odor, respectively (Melnik *et al.*, 2010).

MATERIAL AND METHODS

Collection of Plant material and extraction: Fresh aerial parts of *C. sativus* were collected from local hills of Pampered Kashmir in August-September. The plant materials were identified with the help of experts in Department of Botany, University of Kashmir and shade dried before use. The dried plant material was grinded separately and 100 g of plant material was sequentially extracted by Hexane, Chloroform, Ethyl acetate, Methanol and Water (Scheme-1). Organic solvent extracts were filtered twice with Whatman's filter paper to remove any residual material and dried in rotary evaporator under vacuum. The water extracts were filtered with Whatman's filter paper, filtrate was centrifuged at 5000 rpm for 10 minutes at room temperature to remove any residual material and the supernatant was lyophilized.

Phytochemical screening:

The bioactive extracts were tested for various phytochemicals such as alkaloids, terpenoids, reducing sugars, saponins, tannins, Phlobatannins etc. (Adetuyi *et al.*, 2001; Sofowora, 1982).

Antimicrobial testing:

Preliminary screening of various solvent extracts was carried out on nonpathogenic fast growing mycobacterial strain, *M. smegmatis* (MC2-155). The active extracts were tested on *M. tuberculosis* standard strain H37RV, clinical isolates CL-1 (+3 strain) and CL-2 (+2 strain) and MDR strain.

Preliminary screening on *M. smegmatis* by disk diffusion method (Bauer *et al.*, 1996): Stock solutions (200mg/ml) of hexane, chloroform, ethyl acetate and methanol extracts of selected plant were prepared in Dimethyl-Sulphoxide (DMSO), whereas water extracts were dissolved in water. All the extracts were stored at 4°C prior to use. Petri plates with Middlebrook 7H11 agar were seeded with 100µl of *M. smegmatis* culture at a concentration of 1.5×10^6 cells/ml (adjusted to the 0.5 McFarland turbidity standards). Sterile filter paper discs (6

mm in diameter) impregnated with 2mg/10µl/disc of plant extracts were placed on the petri plates. Sterile discs (6mm diameter) of Rifampicin (20µg/disc) were used as positive control and a disc soaked in 10µl of DMSO was solvent control. The inoculated plates were incubated and the results were recorded by measuring the zones of growth inhibition (Inhibition zone diameter) after 72 hrs. All experiments were carried out in triplicate.

Minimum inhibitory concentration (MIC) by broth dilution method: Bio-active plant extracts were dissolved in DMSO and stock solutions (2mg/ml) in Middlebrook 7H9 broth was prepared. Ten serial dilutions of the extracts ranging from 2000 µg/ml to 3.9µg/ml concentrations were prepared and the tubes were inoculated with 100 µl of *M. smegmatis* (1×10^6 cell/ml). Rifampicin (64 µg/ml) was used as a standard drug for comparison and DMSO (10 µl/disc) was used as negative control. The tubes were incubated aerobically at 37°C for 24 hrs 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 tested for color change and the concentration at which a decrease in red color (reduction of INT to Formosan by bacteria) was apparent compared to the next higher concentration was taken as MIC value. All experiments were carried out in triplicate.

Bio-auto assay guided purification of active plant extracts:

Methanol extract of *C. sativus* (MECS) and Hexane extract of *C. sativus* (HECS) possessing good activity in disc diffusion assay with promising MIC were subjected to Bio-auto assay on silica TLC plates (Merck). The TLC plates were dipped in Middlebrook 7H 11 agar petri plates, seeded with *M. smegmatis* and disc diffusion assay was performed. After 72hrs of incubation, plates were sprayed with INT to record zones of growth inhibition.

Isolation of bioactive compounds from MECS and HECS by column chromatography:

MECS and HECS were fractionated on open 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, compared with TLC bio-auto assay and pooled to 5 fractions (F1-5) whereas for HECS the column was eluted with hexane/ethyl acetate gradient (10:0-0:10). The fractions were analyzed by TLC, compared with TLC bio-auto assay and pooled to give 20 fractions (F1-F20). All the fractions were tested for antimicrobial activity on *M. smegmatis* by disc diffusion assay (Fig. 1 & 2).

Antimicrobial activity of active fractions of MECS and HECS on *M. tuberculosis*:

MECS, HECS and their active sub-fractions were tested for antimicrobial activity on *M. tuberculosis* by the Absolute Concentration and Proportion methods. The

Absolute Concentration Method or minimum inhibitory concentration (MIC):

Standardized inoculums of *M.tuberculosis* (4µl of 4mg/ml bacterial suspension) was grown on drug-free LJ media and media containing Rifampicin (32,64,128 µg/ml), Isoniazid (0.2,1,5 µg/ml), Ethambutol (2,4,6 µg/ml) Streptomycin (8,16,32 µg/ml), MECS, F5 of MECS, HECS and F11 and F15 of HECS (62.5, 125, 250, 500, 1000 µg/ml). Resistance was expressed in terms of the lowest concentration (MIC) of the drug inhibiting growth showing development of ≥ 20 colonies (Kent and Kubica, 1985).

Proportion method:

A suspension of *M.tuberculosis*, by scrapping approximately 4 mg moist weight of samples (visualized as 2/3 loopful of 3mm internal diameter 24SWG (Thick) wire loop) into 0.3 ml of sterile distilled water in a Bijou Bottle was prepared and vortexed for 30 seconds to produce a uniform suspension. 3.7 ml of sterile distilled water was then added to the suspension to make it approximately 1mg/ml (S1) and the suspension was kept on the bench for 15-20 minutes to allow the coarser particles to settle down. From this suspension a 10-fold dilution was made by carefully adding 0.2 ml to 1.8 ml sterile distilled water (S2, 10-1) and two further serial dilutions (S3 & S4) were prepared in the similar manner and inoculated one loopful (3 mm external diameter, 24 SWG Thin) on drug free and drug containing LJ slopes. The L.J media with plant extracts was prepared by same procedure as for drug free L.J medium and the plant extracts were incorporated in the medium at concentration of 2 per cent v/v and 4 per cent v/v (2 ml and 4 ml of fresh plant extract was dissolved into 100 ml of culture medium. For positive controls the concentration of drugs was Rifampicin 40 µg/ml, Isoniazid 0.2µg/ml, Ethambutol 2 µg/ml, Streptomycin 8µg/ml (Kent and Kubica, 1985). Determination of colony forming units (CFU) on Lowenstein-Jensen (L-J) media: The ten-fold dilution of standard 1 mg/ml *M. tuberculosis* suspension (Canetti *et al.*, 1969) were streaked on L-J medium for determining CFU in the presence and absence of plant extracts. A suspension of *M. tuberculosis* 1 mg/ml is equivalent to Mac-Farland standard-1 (Kent and Kubica, 1985). One loopful (6 µl) of this suspension was streaked on the L-J slants using 3 mm external diameter loop.

RESULTS

Antimycobacterial susceptibility testing of extracts against *M.semegmatis*:

The selected plant extracts showed moderate to good antimycobacterial activity in disc diffusion assay. Results showed HECS and MECS were the most active extracts producing zone of inhibition of 22 and 20 mm. respectively. Aqueous extract of plants showed least activity (Table 1). The extracts showing inhibition zone

diameter (IZD) greater than 10 were subjected to MIC determination. HECS and MECS were the most active extracts showing MIC value of 250 and 125µg/ml respectively.

Phytochemical analysis:

The active extracts showing inhibition zone diameter greater than 10 mm were tested for phytochemicals. The results of the phytochemical analysis (Table 2) prominently indicate the presence of saponins, steroids, tannins, terpenoids and anthraquinones. Phlobatannins were found to be absent in all the extracts. Saponins were found to be present in several extracts. Phytochemical analysis of active extracts revealed that alkaloids were found to be present in all active extracts except HECS. The polar solvent extracts showed moderate to good activity as compared to non-polar solvents. Alkaloids, saponins, terpenoids, tannins and flavonoids were predominantly present in active extracts. MECS contains alkaloids, saponins, flavonoids, tannins, terpenoids, anthraquinones and cardiac glycosides. HECS contains terpenoids, tannins and anthraquinones, alkaloids, saponins, tannins and anthraquinones. **Isolation of bioactive fractions from MECS and HECS by column chromatography:**

MECS was fractionated on open silica gel (Merck, 60-120 mesh) column chromatography. The column was eluted with chloroform/methanol gradient to obtain 5 fractions out of which F₅ obtained at 98:2 (Chloroform: MeOH) was most active and produced 27mm zone of inhibition in disc diffusion assay.

Similarly HECS was fractionated on open silica gel (Merck, 60-120 mesh) column chromatography. The column was eluted with hexane/ethyl acetate gradient to obtain 20 fractions out of which F₁₁ and F₁₅ obtained at 77:23 and 71:29 (hexane: ethyl acetate) were most active and produced 23mm and 24 mm zone of inhibition in disc diffusion assay respectively.

Antimycobacterial activity of semipurified fractions of MECS and HECS on *M.tuberculosis*

Absolute concentration method (MIC Method)

A significant inhibition was found on the growth and CFU of *M.tuberculosis* by presences of plant extracts. Upto 95 % inhibition was found due to presence of Plant extracts in L.J Medium that of 90% inhibition by MECS, 85% by HECS, 76% by F₅ Fraction and 86% inhibition by F₁₁ and F₁₅ Fractions of HECS with a reduction of CFU from near about 30 to 10. The activity was also found to be increased from 2% v/v to 4% v/v of plant extracts (Table 3).

Table1. Inhibition zone diameters (IZD*) and MIC of various plant extracts Against *Mycobacterium semegmatis*

S.No	Extract	IZD *(mm)	MIC (µg/ml)
1	Hexane	20	250
2	Chloroform	13	1000
3	Ethyl acetate	12	1000
4	Methanol	19	125
5	Aqueous	10	NA
6	DMSO**	9	NA

* All values in mm **DMSO= Dimethyl sulfoxide

Table2. Phytochemical analysis of solvent extracts of selected medicinal plants

S.NO	Extract	Alkaloids	Saponins	Flavonoids	Tannins	Terpenoids	Sugars
1	Methanol	+	+	+	+	+/-	-
2	Chloroform	+	+	+	-	+	-
3	Ethyl acetate	-	-	-	+	+	-
4	Hexane	+	+	-	-	+	-
5	Aqueous	-	+	-	+	+	+

Table3. Drug susceptibility of clinical specimens of *Mycobacterium tuberculosis* along with standard (H₃₇ RV ATCC No.35838), MDR strain; CL-1 (+3 strain) and CL-2 (+2 strain) by LJ Proportion Method

S. No.	Extract/Fraction Isolates	Strain	MIC (µg/ml)	LJ Proportion Method				
				Mean CFU on Media			%age Inhibition	
				Plant Extract			Plant Extract	
				Control	2%v/v	4%v/v	2%v/v	4%v/v
1.	MECS	H ₃₇ RV	500	47	27	13	42	72
		MDR	500	27	14	10	49	62
		CL-1	250	72	21	11	70	85
		CL-2	250	65	22	10	66	85
2.	HECS	H ₃₇ RV	250	47	22	12	53	74
		MDR	500	28	16	15	43	46
		CL-1	250	72	21	17	71	76
		CL-2	125	65	24	20	63	79
4.	F ₅ (MECS)	H ₃₇ RV	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
5.	F ₁₁ (HECS)	H ₃₇ RV	500	47	26	15	44	68
		MDR	500	20	12	10	40	50
		CL-1	500	72	17	11	76	85
		CL-2	250	65	24	11	63	83
6.	F ₁₅ (HECS)	H ₃₇ RV	500	47	25	16	47	66
		MDR	250	30	16	12	47	60
		CL-1	125	72	28	17	61	76
		CL-2	125	65	19	14	71	86

DISCUSSION

M. semegmatis is fast growing mycobacterium which can be very useful for preliminary screening of large number of drug candidates like the natural plant extracts (Emma *et al.*, 2010). In this study the extracts exhibiting activity against *M. semegmatis* were also active against *M. tuberculosis*, justifying use of *M. semegmatis* in initial screening. Against *M. semegmatis*, MECS and HECS were found to have MIC of 125 and 250µg/ml with

IZD of about 19 and 20 mm respectively. While as MIC of 250, 250, 125 and 125µg/ml with 74, 88 and 90% inhibition against standard, MDR and CL-1 and CL-2 strains of *M. tuberculosis* respectively. HECS was found more active against CL-2 with MIC of 125µg/ml with 76 % of inhibition. F5 produced a 125µg/ml of MIC against CL-1 by inhibiting 86% of bacterial growth while as H37RV and MDR strains were found to have inhibited by

68%. The F11 inhibited CL-2 by 83% and that of F15 inhibited CL-1 and CL-2 by 86% with MIC of 125µg/ml. The semi purified fractions (F5 of MECS; F11 and F15 of HECS) obtained by partial purification on silica column concentrated the active constituent evident from decrease in MIC and increase in percentage of inhibition. This study opens a possibility of obtaining novel compounds for the treatment of tuberculosis including MDR. There is, therefore, need to carry out some more studies in animal models of tuberculosis to reaffirm these findings. Further studies are, however, needed to reconfirm the antimycobacterial properties of these extract and identify the active component through advanced techniques, such as GC-MS.

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