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ORIGINAL ARTICLE

Antimicrobial and Antimycobacterial Activities of Methyl Caffeate Isolated from *Solanum torvum* Swartz. Fruit

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Abstract *Solanum torvum* Swartz. (Solanaceae) fruit is traditionally used for the treatment of bacterial and fungal infections. The methanolic extract was subjected to activity guided fractionation by column chromatography over silica gel. The structure of the compound was elucidated using physical and spectroscopic data. The antimicrobial activity was screened using five Gram-positive bacteria, six Gram-negative bacteria, seven clinical isolates and four fungi. Antimycobacterial activity was screened against two *Mycobacterium* strains. The zone of inhibition by methyl caffeate ranged from 0 to 22 mm. The lowest minimum inhibitory concentration (MIC) values of methyl caffeate were: 50 μg/ml against *P. vulgaris*, 25 μg/ml against *K. pneumoniae* (ESBL-3971), 8 μg/ml against *M. tuberculosis* ($H^{37}Rv$) and 8 μg/ml against *M.* tuberculosis (Rif^{R}). Methyl caffeate showed moderate antimicrobial and prominent antimycobacterial activities. Methyl caffeate can be evaluated further for drug development.

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Introduction

In recent years there has been an increasing interest in the use of natural substances against infectious diseases [1]. The development of antibiotic resistance and undesirable side effects due to excessive use of synthetic drugs has necessitated the search for new plant products to treat bacterial and fungal infections [2]. Herbs contain copious amount of chemicals that could be exploited to prevent microbial infections [3]. The emergence of antibiotic-resistant mycobacterial strains underscores the need for novel effective drugs against resistant mycobacteria as first-line antituberculosis medications. According to World Health Organisation (WHO), between 1980 and 2005, 90 million cases of TB worldwide were reported.

Solanum torvum Swartz (Solanaceae) is a shrub widely distributed in South India, Malaysia, China, Philippines, Thailand, West Indies and Tropical America. Antimicrobial activities of the leaf and fruit of this plant have been reported [4, 5]. The fruits of *S. torvum* are edible and traditionally used for the treatment of abscesses, jigger wounds, skin infections and athlete's foot [6]. Pharmacological studies revealed antiviral [7], immunosecretory [8], antioxidant [9], analgesic, anti-inflammatory [10] and antiulcerogenic *activities* [11]. Chemical constituents reported from this fruit include triacontane derivatives [12, 13], chlorogenone and neochlorogenone [14], isoflavonoid sulfate and steroidal glycosides [7, 15], 22-*β*-*O*-spirostanol oligoglycosides [16], 26-*β*-*O*-glucosidase [17]; methyl caffeate isolated from *S. torvum* showed α-glucosidase inhibition activity [18]. Methyl caffeate also showed

oxidative stress inhibiting activity [19], anti-platelet activity [20], antiproliferative activity in cervix adenocarcinoma [21] and anticancer activity in lung and leukmia cell lines [22]. Hence the present study was carried out to isolate methyl caffeate from *S. torvum* fruits and to assess its antimicrobial and antimycobacterial activities. Methyl caffeate showed good antimycobacterial activity. Hence it can be probed further for drug development.

Materials and Methods

Plant Material

Fresh unripe fruits of the *Solanum torvum* were collected during June 2010 from medicinal farm Koyambedu, Chennai, India. The species was identified by the taxonomist at Entomology Research Institute, Loyola College, Chennai, India. A voucher specimen (No. ERI/ETHPH/ST/120) has been deposited at the herbarium of the institute.

Extraction and Isolation

The collected fruits were shade dried at room temperature and powdered in a hammer mill. One kilogram of the powder was extracted with methanol twice at room temperature for 48 h. The combined extract was evaporated to dryness at 40 °C under reduced pressure. Dark green residue was obtained (50 g). The total methanol extract was chromotographed over silica gel (Merck 60–120 mesh) packed with chloroform. The column was successively eluted with chloroform, chloroform–ethyl acetate mixtures with increasing amounts of ethyl acetate and finally with ethyl acetate:methanol (1:1). Based on thin layer chromatography (TLC) profiles similar fractions were combined to give eleven fractions. When the fractions were bioassayed, fraction 4 (Yield 4.5 g) showed maximum antimicrobial activity. Fraction 4 was further purified using column chromatography and eluted with chloroform:ethyl acetate (4:1); it yielded light yellow crystals of methyl caffeate (70 mg) (m.p.: 156–162).

Bacterial Strains

The following Gram positive, Gram negative bacteria and clinical isolates were used for the experiment. Gram positive bacteria: *Staphylococcus aureus* MTCC 96, *Enterobacter aerogenes* MTCC 111, *Micrococcus luteus*, *Staphylococcus epidermidis* MTCC 3615, Gram negative bacteria: *Shigella flexneri* MTCC 1457, *Proteus vulgaris* MTCC 1771, *Klebsiella pneumoniae* MTCC 109, *Salmonella typhimurium* MTCC 1251, *Salmonella paratyphi-B*, *Pseudomonas aeruginosa* MTCC 741 and *Bacillus subtilis* MTCC 441; the clinical isolates : *Escherichia coli* (ESBL-3984, Extended Spectrum Beta Lactamase), *Escherichia coli* (ESBL-3904), *Klebsiella pneumoniae* (ESBL-3971), *Klebsiella pneumoniae* (ESBL-75799), *Klebsiella pneumoniae* (ESBL-3894), *Klebsiella pneumoniae* (ESBL-3967) and *Staphylococcus aureus* (MRSA-methicillin resistant, clinical pathogen). The reference cultures were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India-160 036.

Fungal Strains

Candida albicans MTCC 227, *Malassesia pachydermatis*, *Trichophyton mentagrophytes* 66/01 and *Aspergillus flavus* were used for antifungal assays. All the cultures were obtained from the Department of Microbiology, Christian Medical College, Vellore, Tamil Nadu, India.

Preparation of Inoculum

Bacterial inoculums were prepared by growing cells in Mueller–Hinton broth (MHB) (Himedia) for 24 h at 37 °C. The filamentous fungi were grown on sabouraud dextrose agar (SDA) slants at 28 °C for 10 days and the spores were collected using sterile doubled distilled water and homogenized. Yeast was grown on sabouraud dextrose broth (SDB) at 28 °C for 48 h.

Antimicrobial Activity

Antibacterial and antifungal activities were carried out using disc-diffusion method [23]. Petri plates were prepared with 20 ml of sterile Mueller–Hinton agar (MHA) (Hi-media, Mumbai). The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min and a specific amount (25 µl from the 20 mg/ml) of compound was added to each disc. The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Negative control was prepared using respective solvents. Streptomycin (10 µg/disc) was used as positive control. The plates were incubated for 24 h at 37 °C for bacteria and for 48 h at 28 °C for fungi. Zones of inhibition were recorded in millimetres and the experiment was repeated twice.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration studies of the isolated compound were performed according to the standard reference method for bacteria [24], for filamentous fungi [25] and yeasts [26]. The concentrations (500, 250, 100, 50, 25, 12.5 6.25, 3.125 and 1.56 µg/ml) of the compound were dissolved in DMSO (2 %) and used. They were added to

each medium in 96 well plates. An inoculum of 100 µl from each well was inoculated. The antifungal agent fluconazole for fungi and antibacterial agent streptomycin for bacteria were included in the assays as positive controls. For fungi, the plates were incubated for 48–72 h at 28 °C and for bacteria the plates were incubated for 24 h at 37 °C. The MIC for fungi was defined as the lowest extract concentration, showing no visible fungal growth after incubation time. 5 µl of tested broth was placed on the sterile MHA plates for bacteria and incubated at respective temperature. The MIC for bacteria was determined as the lowest concentration of the compound inhibiting the visual growth of the test cultures on the agar plate.

Antimycobacterial Assay

The anti-TB activity of methyl caffeate was evaluated against standard sensitive strain *Mycobacterium tuberculosis* $H_{37}Rv$ and an rifampicin isolate *M. tuberculosis* Rif^r. The MIC was determined using broth micro-dilution assay [27–29]. The experiment was performed in sterile Middlebrook 7H9 broth supplemented with10 % ADC (BD Biosciences, USA). The above-mentioned test bacteria were grown to mid-log phase (10–12 days) at 37 °C with shaking in the test media (Middlebrook 7H9 broth supplemented with 10 % ADC). Stock solution (1 mg/ml) of methyl caffeate was prepared in DMSO and 6.4 µl volume of these stock solutions were added to the wells of a 96 well U bottom microtitre plates (Tarson, Mumbai, India) and nine twofold serial dilutions of the compound were prepared in 100 µl of test media. The turbidity of the cultures was adjusted to be equivalent to one McFarland turbidity standard ($\sim 1 \times 10^7$CFU/ml), which was further diluted 1:10 in test media and a 100 µl volume of this diluted inoculums was added to each well of the plate, resulting in a final inoculums of 5×10^5 CFU/ml. The final concentrations of the methyl caffeate after the addition of inoculums ranged from 0.12 to 32 µg/ml. Rifampicin in the concentration range from 0.12 to 32 µg/ml was used as control drug in the experiment. Periphery wells of the plate were filled with sterile distilled water to prevent evaporation of media in the wells. The plates were incubated at 37 °C under 5 % CO_2 for 3 weeks. Inhibition of growth was determined both by visual examination and with a spectrophotometer at an OD_{600} (Multiskan spectrum; Thermo Scientific, USA). The lowest concentration of the compound showing no turbidity was recorded as MIC.

Statistical Analysis

Antimicrobial activity of methyl caffeate was statistically analyzed by one way Analysis of Variance. Significant differences between the zones of inhibition (mm) were determined using Duncan multiple range test at $p = 0.05$ with the help of SPSS 11.5 version software package.

Results and Discussion

The methanol extract of *S. torvum* fruit was previously shown to have in vitro antimicrobial activity against *A. pyogenes*, *B. subtilis*, *P. aeruginosa*, *S. aureus*, *S. pyogenes*, *A. niger* and *C. albicans* [6]. In the present study methanol extract of *S. torvum* unripe fruit was fractionated on a silica gel column by eluting with solvents of increasing polarity and the fractions were monitored by TLC. Similar fractions were combined. The active principle was identified as methyl caffeate. The structure was elucidated using spectroscopic methods. IR: V KBr/max = 3484 (hydroxyl), 2955, 2840, 1682 (ester carbonyl), 1601, 1529, 1442 (aromatic), 1367, 1277 (ester C–O–C), 1181, 1105, 974, 850, 812, 773 cm^{-1}. ^{1}H NMR (δ, DMSO-d_6, 400 MHz): 6.77 (1H, d, $J = 8.0$ H_2, H-5) 7.00 (1H, dd, $J = 8.0$ and 2.0 H_2, H-6), 7.06 (1H, d, $J = 2.0$ H_2, H-2), 6.27(1H, d, $J = 16.0$ H_2, H-8), 7.49 (1H, d, $J = 16.0$ H_2, H-7), 3.69(3H, S, –COOMe). ^{13}C NMR (δ, DMSO-d_6, 100 MHz): 125.50 (C-1), 115.69 (C-2), 145.58(C-3), 148.42(C-4), 114.73 (C-5), 121.41(C-6), 145.16(C-7), 115.07(C-8), 167.08 (–COOMe), 51.20 (–COOMe). EI-MS (*m/z*) for $C_{10}H_{10}O_4$: 194 (M^+), 163 $[M\text{-}OMe]^+$ 134 $[M\text{-}60]^+$. The identity of the compound was confirmed by comparison of the physical and spectroscopic data (IR, ^{1}HNMR, ^{13}CNMR and MS) with those reported in the literature [30] (Fig. 1).

Methyl caffeate exhibited (Table 1) activity against bacteria using disc diffusion method. The MIC values of methyl caffeate against bacteria and fungi are given in Tables 2 and 3. Methyl caffeate showed only moderate effect when compared to control drugs of fluconazole for fungi and streptomycin for bacteria. *P. aeruginosa* has emerged as one of the most problematic Gram-negative pathogen, with an alarmingly high antibiotic resistance rate [31, 32]. Even with the most effective antibiotics against this pathogen, namely carbapenems (imipenem and meropenem), the resistance

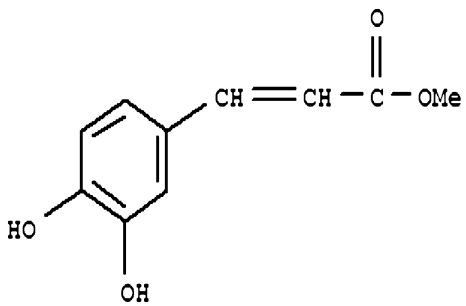

Fig. 1 Isolated compound methyl caffeate from *S. torvum* fruit

Table 1 Antimicrobial activity of methyl caffeate isolated from *S. torvum* (2 mg/ml) using disc-diffusion method (zone of inhibition in mm)

Organism	Methyl caffeate	Streptomycin (25 µg/disc)
Gram positive		
Staphylococcus aureus	20 ± 0.71^{f}	14 ± 0.71^{a}
Enterobacter aerogenes	0^{a}	$22 \pm 0.71^{c,d}$
Micrococcus luteus	9 ± 0.45^{b}	$24 \pm 0.90^{d,e}$
Shigella flexneri	0^{a}	$24 \pm 0.90^{d,e}$
Staphylococcus epidermidis	0^{a}	14 ± 0.71^{a}
Gram negative		
Proteus vulgaris	22 ± 0.71^{g}	30 ± 0.71^{g}
Salmonella typhimurium	12 ± 0.71^{c}	$24 \pm 0.90^{d,e}$
Salmonella paratyphi-B	16 ± 0.45^{d}	18 ± 0.71^{b}
Pseudomonas aeruginosa	20 ± 0.71^{f}	30 ± 0.54^{g}
Bacillus subtilis	19 ± 0.71^{e}	$22 \pm 0.84^{c,d}$
Klebsiella pneumoniae	9 ± 0.45^{b}	20 ± 0.71^{b}
Fungi		Fluconazole
Candida albicans	20 ± 0.71^{f}	$28 \pm 0.71^{f,g}$
Aspergillus flavus	22 ± 0.81^{g}	$29 \pm 0.71^{f,g}$

Values in each column followed by the same numerical were not significantly different by Duncan test at $p \leq 0.05$. Streptomycin—standard antibacterial agent; fluconazole—standard antifungal agent. No activity—a and b, c, d, e, f, g increasing the mode of activity from lower to higher

rate was found to be 15–20.4 % amongst 152 *P. aeruginosa* strains [32]. Our study showed that methyl caffeate was active against *P. aeruginosa.* This activity might be due to its ability to complex with cell wall [33] to inhibit microbial growth [34]. Methyl caffeate is a phenolic compound and antimicrobial capacity of phenolic compounds is well known [35]. Table 4 shows the MIC values of methyl caffeate against *M. tuberculosis* using micro-broth dilution assays. Methyl caffeate exhibited potent anti-TB activity against both the tested isolates (8 µg/ml). The MIC values showed good activity against both *M. tuberculosis* ($H^{37}Rv$) and *M. tuberculosis* (Rif^{R}) at 8 µg/ml. The methods described here could be useful in determining the mycobactericidal activity of natural products because these assays require smaller volumes and can be performed faster than other methods described by Rastogi et al. [36] and Friis-Moller et al. [37]. *Thesium chinense* Turcz. has been used as a Korean traditional medicine to treat tuberculosis and methyl caffeate was isolated from it [38]. The in vitro antibacterial and antimycobacterial activities may support the use of *S. torvum* species in traditional medicine to treat microbial infections. The bioactivity guided fractionation of the methanol extract of the unripe fruit led to the isolation of methyl caffeate as the major active principle. Our experiment suggests that *S. torvum*, an important medicinal herb in Indian medicine, may be a potent candidate for clinical trial for tuberculosis.

Table 2 Minimum inhibitory concentration (µg/ml) of methyl caffeate isolated from *S. torvum* against tested bacteria

Organism	Methyl caffeate	Streptomycin
Gram positive		
Staphylococcus aureus	250	6.25
Micrococcus luteus	100	6.25
Gram negative		
Proteus vulgaris	50	6.25
Salmonella paratyphi-B	100	25
Salmonella typhimurium	500	30
Pseudomonas aeruginosa	100	25
Bacillus subtilis	250	25
Klebsiella pneumoniae	100	6.25
Clinical isolates		
Escherichia coli (ESBL-3984)	250	25
Escherichia coli (ESBL-3904)	500	25
Klebsiella pneumoniae (ESBL-3971)	25	6.25
Klebsiella pneumoniae (ESBL-75799)	100	25
Klebsiella pneumoniae (ESBL-3894)	500	6.25
Klebsiella pneumoniae (ESBL-3967)	250	25
Staphylococcus aureus (MRSA)	500	6.25

Streptomycin (standard antibacterial agent); methyl caffeate (isolated compound)

Table 3 Minimum inhibitory concentration (µg/ml) of methyl caffeate isolated from *S. torvum* against tested fungi

Organism	Methyl caffeate	Fluconazole
Candida albicans	250	>100
Malassesia pachydermatis	500	12.5
Trichophyton mentagrophytes	100	25
Aspergillus flavus	100	25

Fluconazole (standard antifungal agent); methyl caffeate (isolated compound)

Table 4 Minimum inhibitory concentration values for inhibition by methyl caffeate against *M. tuberculosis* culture using absolute concentration method

Strains	Lab code	Methyl caffeate MIC (µg/ml)	Rifampicin (µg/ml)
M. tuberculosis $H^{37}Rv$ (HR-Sen) (ATCC 27294)	$H_{37}Rv$	8	0.12
M. tuberculosis Rif^{R}	Rif^{R}	8	32

Conclusion

Methyl caffeate isolated from the methanolic extract of the fruits of *S. torvum* showed moderate antibacterial and antifungal activities against the tested strains when compared to

control drugs. Methyl caffeate showed a potent in vitro anti-TB activity against both the tested isolates of *M. tuberculosis* using micro-broth dilution assays at the dose level of 8 μg/ml. The results showed that methyl caffeate could be probed further in drug discovery programme related to tuberculosis.

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