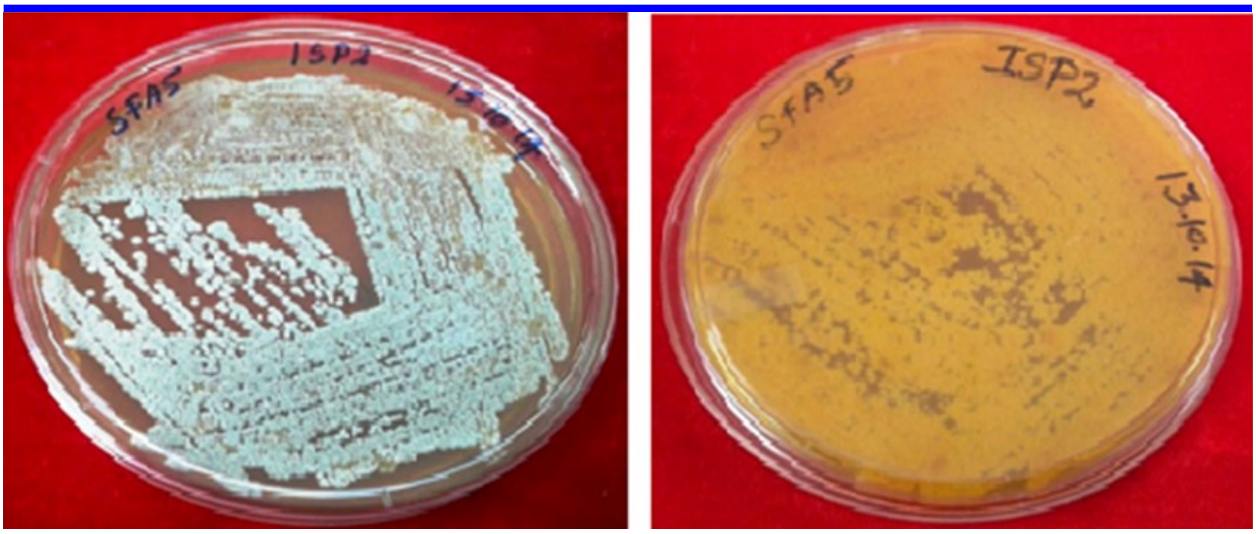


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# HPTLC fingerprint profile of antibacterial compound produced from forest soil *Streptomyces SFA5*

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## Abstract

The present study investigates the presence of antimicrobial compounds from potent isolate *Streptomyces* sp. SFA5 from the Sabarimala forest ecosystem, Western Ghats, Kerala, India. Preliminary screening revealed *Streptomyces* sp. SFA5 isolate shows significant inhibition against the bacterial and fungal pathogens. The antibacterial compound from isolate SFA5 was produced by submerged fermentation using yeast extract malt extract broth and extracted using different solvents. Among the different solvent extracts tested, the ethyl acetate extract of SFA5 shows maximum zone of inhibition against *Staphylococcus aureus* (23 mm) and *Bacillus cereus* (24 mm). A simple, sensitive and accurate HPTLC method has been performed for the quantitative estimation of bioactive compounds from ethyl acetate extract of *Streptomyces* sp. SFA5. The optimized solvent system for mobile phase in HPTLC was toluene: ethyl acetate: methanol: acetic acid (5:3:1:0.5, v/v/v/v). Results of HPTLC finger printing of ethyl acetate extract of the strain SFA5 at UV 254 and 366 with corresponding $R_f$ values substantiate the presence of bioactive compounds.

## Introduction

Microbial natural products continue to represent as important route for the discovery of novel chemicals for the development of new therapeutic agents (Skoko et al., 2005). Among the microbial producers, action-bacteria holds a prominent position as targets in screening program due to their diversity and account for the production of most of discovered bioactive secondary metabolites, primarily antibiotics, immuno-suppressive agents, enzymes and enzyme inhibitors (Thakur et al., 2007). Due to uniqueness, large geographic variation, different soil types and their contents of forest, it is quite like that there is a vast distribution of antibiotic producing *Actinobacteria* in forest environment (Aravamuthan et al., 2010).

High performance thin layer chromatography (HPTLC) is one of the modern sophisticated techniques that can be used for quick and easy determination of quality, authenticity and purity of the natural extracts, crude drugs and market formulations (Mamatha et al., 2011; Miyadoh et al., 1993). HPTLC provides valuable tool for reliable identification through chromatographic fingerprints that can be visualized and stored as electronic images.

The present study reports the HPTLC fingerprint profile and qualitative analysis of bioactive compounds produced by soil *Streptomyces* sp. *SFA5* isolated from the Sabarimala forest ecosystem, Western Ghats, India.

## Materials and Methods

### *Description of Streptomyces species SFA5*

Streptomyces species (SFA5) was isolated from Sabari-

mala Forest Ecosystem, Western Ghats, Kerala, India (Latitude: 09° 25′ 59″ N; Longitude: 77° 04′ 59″ E) using starch casein agar medium supplemented with filter sterilized nystatin (20 µg/mL) and nalidixic acid (100 µg/mL) by serial dilution plating method. Viability of strain SFA5 was maintained on ISP2 agar slants at 4°C (Shekar et al., 2011). Strain SFA5 produced soluble yellow pigment on ISP2 agar medium.

### *Production and extraction of bioactive compounds*

For the preparation of inoculum, spores of *Streptomyces* sp. SFA5 were inoculated into 100 mL yeast extract malt extract (YEME) broth in 500 mL conical flask at 28°C in rotary shaker with 90 rpm for 7 days (Saravanan et al., 2012). After incubation the fermented broth which contains the bioactive compounds was centrifuged at 10,000 rpm for 30 min at 4°C. The cell free supernatant was tested for antimicrobial activity by agar well diffusion method (Chaudhary et al., 2013). To extract the antimicrobial compounds, cell free supernatant was extracted by liquid-liquid extraction method (Shekar et al., 2012) using equal volume of (1:1 v/v) different solvents such as ethyl acetate, methanol, chloroform, dichloromethane, and *n*-hexane and allowed to dry. The concentrated crude extract was impregnated in sterile 5 mm filter disc at 100 µg concentration placed over the test organism. Test organisms used for screening include standard pathogens such as *Staphylococcus aureus* NCIM 2079, *Bacillus cereus* NCIM 2106, *Escherichia coli* NCIM 2256, *Pseudomonas aeruginosa* NCIM 5031, *Candida albicans* ATCC 90028, *Aspergillus fumigatus* NCIM 6645 and *Cryptococcus neoformans* ATCC 66031 and clinical pathogens such as *S. aureus, enteropathogenic E. coli, P. aeruginasa, K. pneumoniae* and *C. albicans*. Zone of inhibition was measured after 24 hours of incubation at 37°C (Video clip).

### *HPTLC profiling of ethyl acetate extract*

The crude ethyl acetate extract of *Streptomyces* sp. SFA5 which showed good activity against test pathogens was subjected to HPTLC analysis. The ethyl acetate extract was dissolved in 200 µL of ethyl acetate from which one micro liter of crude extract was loaded as 6 mm band length in the 3 x 10 cm silica gel 60F254 coated TLC plate using hamilton syringe and camag linomat 5 instrument.

### *Spot development*

The crude extract loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phase and the plate was developed in the mobile phase up to 90 mm and it was then dried by hot air to evaporate solvents from the plate. Using UV light torch, the developed spots were marked, the distance travelled by each spot in baseline and relative $R_f$ values were calculated (Baskar et al., 2010).

$$R_f \text{ value} = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by the solvent}}$$

### *Photo documentation*

The TLC plate was kept in photo documentation chamber and the images were taken at white light, UV 254 nm and UV 366 nm. The developed TLC plates were sprayed with anisaldehyde sulfuric acid reagent and dried at 100°C in hot air oven. After derivatization, the TLC plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at 366 nm. The peak table and peak densitogram were also noted.

## Results

*Streptomyces sp SFA5* was showed good growth with ash white color aerial mycelium, brown color substrate mycelium and diffusible yellow pigment on YEME agar medium (Figure 1). The cell free supernatant of *Streptomyces sp.SFA5* exhibited good activity over the

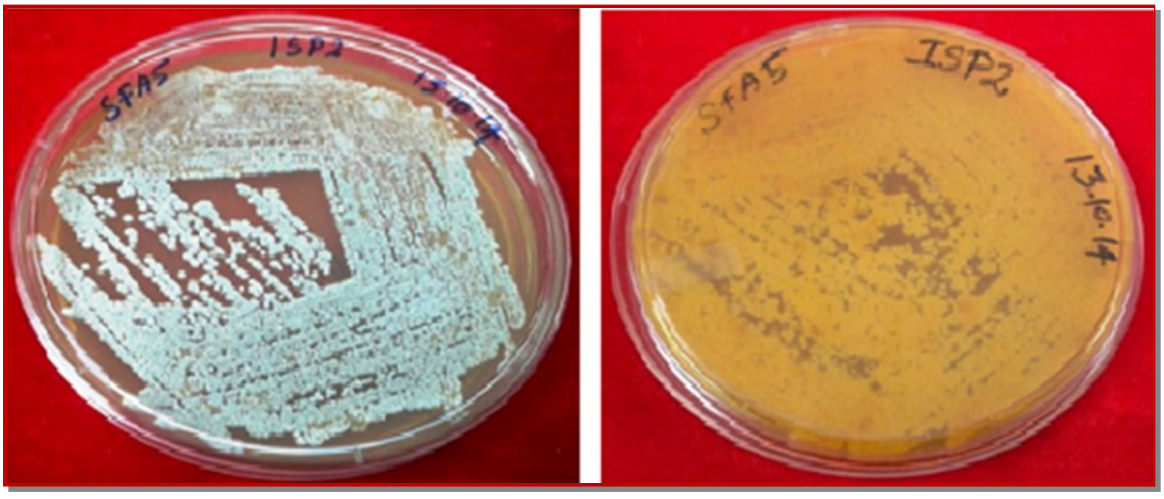


Figure 1: Morphological pattern of *Streptomyces* sp SFA5

standard as well as clinical pathogens in YEME broth (Radhakrishnan et al., 2013).

In total, the supernatant showed good activity over Gram positive bacteria and fungi than Gram negative bacteria. The supernatant of *Streptomyces sp. SFA5* inhibited standard bacterial pathogen such as *Staphylococcus aureus* NCIM 2079 (22 mm) and *Bacillus cereus* NCIM 2106 *(23mm)* as well clinical pathogen such as *Staphylococcus aureus* (19 mm) with a maximum zone of inhibition (Table I). Further cell free supernatant of *Streptomyces sp.* SFA5 inhibited the standard fungal pathogen *Candida albicans* ATCC 90028 (18 mm) and clinical fungal pathogen *Candida albicans* (22 mm) with maximum zone of inhibition. Five different solvent tested for extraction, ethyl acetate extract of *Streptomyces sp. SFA5* grown on YEME broth showed maximum zone of inhibition on antimicrobial activity, while other solvent extract such as dichloromethane, chloroform and *n*-hexane extract showed no zone of inhibition (Table II).

The HPTLC profiling of ethyl acetate extract of *Streptomyces* sp. *SFA5* shows presence of antimicrobial compounds in the chromatograph as well as in UV after derivatization (Febina et al., 2013). The analysis was carried at volume of 10 µL *Streptomyces* sp. *SFA5* extract at UV 254 nm and UV 366 nm (Figure 2). Yellow brown color spots were observed after derivatization confirmed the presence of antimicrobial compound and optimized solvent system for mobile phase involves toluene: ethyl acetate: methanol: acetic acid (5:3:1:0.5, v/v/v/v).

The *Streptomyces* sp. SFA5 shows nearly 14 peaks with good Rf and area % values for the peak 7 (0.4 and 29%) and peak 8 (0.5 and 18%) for finger print profile ethyl acetate extract indicates firmly the presence of antimicrobial compound at UV 254 nm and UV 366 nm (Figure 3). The *Streptomyces* sp. *SFA5* shows nearly 6 peaks with good Rf and area percent values for the peak 2 (0.2 and 28%) and peak 3 (0.3 and 51%) for finger print profile of ethyl acetate extract indicates stoutly the

**Table I**

**Antimicrobial activity of *Streptomyces* sp. SFA5 by agar well diffusion method**

| Test organism | Zone of inhibition (mm in diameter) |
|---|---|
| | YEME broth |
| Standard pathogen | |
| *Staphylococcus aureus* NCIM 2079 | 22 |
| *Bacillus cereus* NCIM 2106 | 23 |
| *Escherichia coli* NCIM 2256 | - |
| *Pseudomonas aeruginosa* NCIM 5031 | - |
| *Candida albicans* ATCC 90028, | 18 |
| *Aspergillus fumigatus* NCIM 6645 | - |
| *Cryptococcus neoformans* ATCC 66031 | - |
| Clinical pathogens | |
| *S. aureus* | 19 |
| Enteropathogenic *E. Coli* | - |
| *P. aeruginasa* | - |
| *K. pneumonia* | - |
| *C. albicans* | 22 |

**Table II**

**Effect of different solvents on the extraction of bioactive compounds from *Streptomyces* sp SFA5**

| Test organism | Solvent extracts | | | | |
|---|---|---|---|---|---|
| | Ethyl acetate extract | Methanol extract | Dichloromethane extract | Chloroform extract | n-Hexane extract |
| *S. aureus* NCIM 2079 | 23 | 19 | - | - | - |
| *B. cereus* NCIM 2106 | 24 | 20 | - | - | - |
| *E. coli* NCIM 2256 | - | - | - | - | - |
| *P. aeruginosa* NCIM 5031 | - | - | - | - | - |

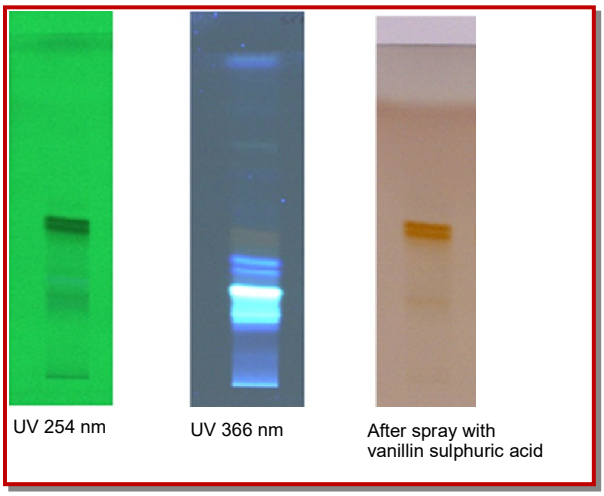


Figure 2: HPTLC profiling showing the presence of antimicrobial compound at UV 254 and UV 366 after derivatization

presence of antimicrobial compound at UV 366 nm.

Figure 4 shows the profiling results of SFA5 ethyl acetate extract at UV 254 nm.

## Discusion

*Streptomyces species* SFA5 from forest ecosystem exhibited good activity against Gram positive bacteria and fungi in agar well diffusion. Most of the metabolites reported from *Actinomycetes* are found to be active against Gram positive bacteria when compared to Gram negative bacteria (Berdy, 2005; Radhakrishnan et al., 2010). The antimicrobial activity of ethyl acetate extract confirmed the extracellular nature of bioactive compounds. Most of the bioactive metabolites from actinomycetes are extracellular in nature (Radhakrishnan et al., 2011). HPTLC is a reliable chromatographic method for analysing several samples of divergent nature and

Track 1, ID: SFA5 Ethyl acetate extract **A**

| Peak | Start Position | Start Height | Max Position | Max Height | Max % | End Position | End Height | Area | Area % | Assigned substance |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.01 Rf | 0.0 AU | 0.02 Rf | 211.8 AU | 11.00 % | 0.06 Rf | 17.6 AU | 3044.6 AU | 6.43 % | unknown * |
| 2 | 0.17 Rf | 6.1 AU | 0.23 Rf | 140.3 AU | 7.29 % | 0.24 Rf | 36.1 AU | 3823.6 AU | 8.08 % | unknown * |
| 3 | 0.24 Rf | 137.2 AU | 0.25 Rf | 154.5 AU | 8.03 % | 0.28 Rf | 88.9 AU | 4046.1 AU | 8.55 % | unknown * |
| 4 | 0.28 Rf | 89.4 AU | 0.30 Rf | 111.7 AU | 5.81 % | 0.31 Rf | 69.3 AU | 2668.3 AU | 5.64 % | unknown * |
| 5 | 0.31 Rf | 69.5 AU | 0.32 Rf | 78.4 AU | 4.07 % | 0.34 Rf | 48.7 AU | 1656.7 AU | 3.50 % | unknown * |
| 6 | 0.35 Rf | 48.8 AU | 0.37 Rf | 64.0 AU | 3.33 % | 0.38 Rf | 62.7 AU | 1537.8 AU | 3.25 % | unknown * |
| 7 | 0.38 Rf | 63.1 AU | 0.45 Rf | 448.8 AU | 23.32 % | 0.46 Rf | 20.2 AU | 13717.8 AU | 28.98 % | unknown * |
| 8 | 0.46 Rf | 422.9 AU | 0.47 Rf | 465.5 AU | 24.19 % | 0.50 Rf | 20.4 AU | 8371.6 AU | 17.69 % | unknown * |
| 9 | 0.50 Rf | 20.6 AU | 0.51 Rf | 39.8 AU | 2.07 % | 0.56 Rf | 17.1 AU | 1367.4 AU | 2.89 % | unknown * |
| 10 | 0.58 Rf | 14.3 AU | 0.61 Rf | 26.9 AU | 1.40 % | 0.63 Rf | 17.0 AU | 924.8 AU | 1.95 % | unknown * |
| 11 | 0.64 Rf | 22.8 AU | 0.66 Rf | 27.3 AU | 1.42 % | 0.68 Rf | 24.7 AU | 719.2 AU | 1.52 % | unknown * |
| 12 | 0.69 Rf | 25.6 AU | 0.72 Rf | 41.5 AU | 2.16 % | 0.75 Rf | 31.3 AU | 1587.8 AU | 3.35 % | unknown * |
| 13 | 0.76 Rf | 31.0 AU | 0.79 Rf | 35.8 AU | 1.86 % | 0.84 Rf | 0.8 AU | 1436.7 AU | 3.04 % | unknown * |
| 14 | 0.93 Rf | 0.1 AU | 0.98 Rf | 78.2 AU | 4.06 % | 1.00 Rf | 1.4 AU | 2427.3 AU | 5.13 % | unknown * |

Track 1, ID: SFA5 Ethyl acetate extract **B**

| Peak | Start Position | Start Height | Max Position | Max Height | Max % | End Position | End Height | Area | Area % | Assigned substance |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.01 Rf | 11.4 AU | 0.02 Rf | 85.7 AU | 7.34 % | 0.14 Rf | 1.4 AU | 2252.6 AU | 7.74 % | unknown * |
| 2 | 0.16 Rf | 1.0 AU | 0.23 Rf | 236.4 AU | 20.25 % | 0.26 Rf | 94.8 AU | 8030.2 AU | 27.59 % | unknown * |
| 3 | 0.26 Rf | 95.5 AU | 0.30 Rf | 644.9 AU | 55.25 % | 0.33 Rf | 34.1 AU | 14605.0 AU | 50.17 % | unknown * |
| 4 | 0.33 Rf | 34.1 AU | 0.36 Rf | 84.7 AU | 7.25 % | 0.37 Rf | 40.7 AU | 1887.1 AU | 6.48 % | unknown * |
| 5 | 0.37 Rf | 42.2 AU | 0.39 Rf | 98.0 AU | 8.40 % | 0.45 Rf | 0.0 AU | 1886.2 AU | 6.48 % | unknown * |
| 6 | 0.94 Rf | 0.1 AU | 0.98 Rf | 17.5 AU | 1.50 % | 1.00 Rf | 1.1 AU | 448.0 AU | 1.54 % | unknown * |

Figure 3: Rf values of ethyl acetate extract of SFA5 at UV 254 nm (A) and 366 nm (B)

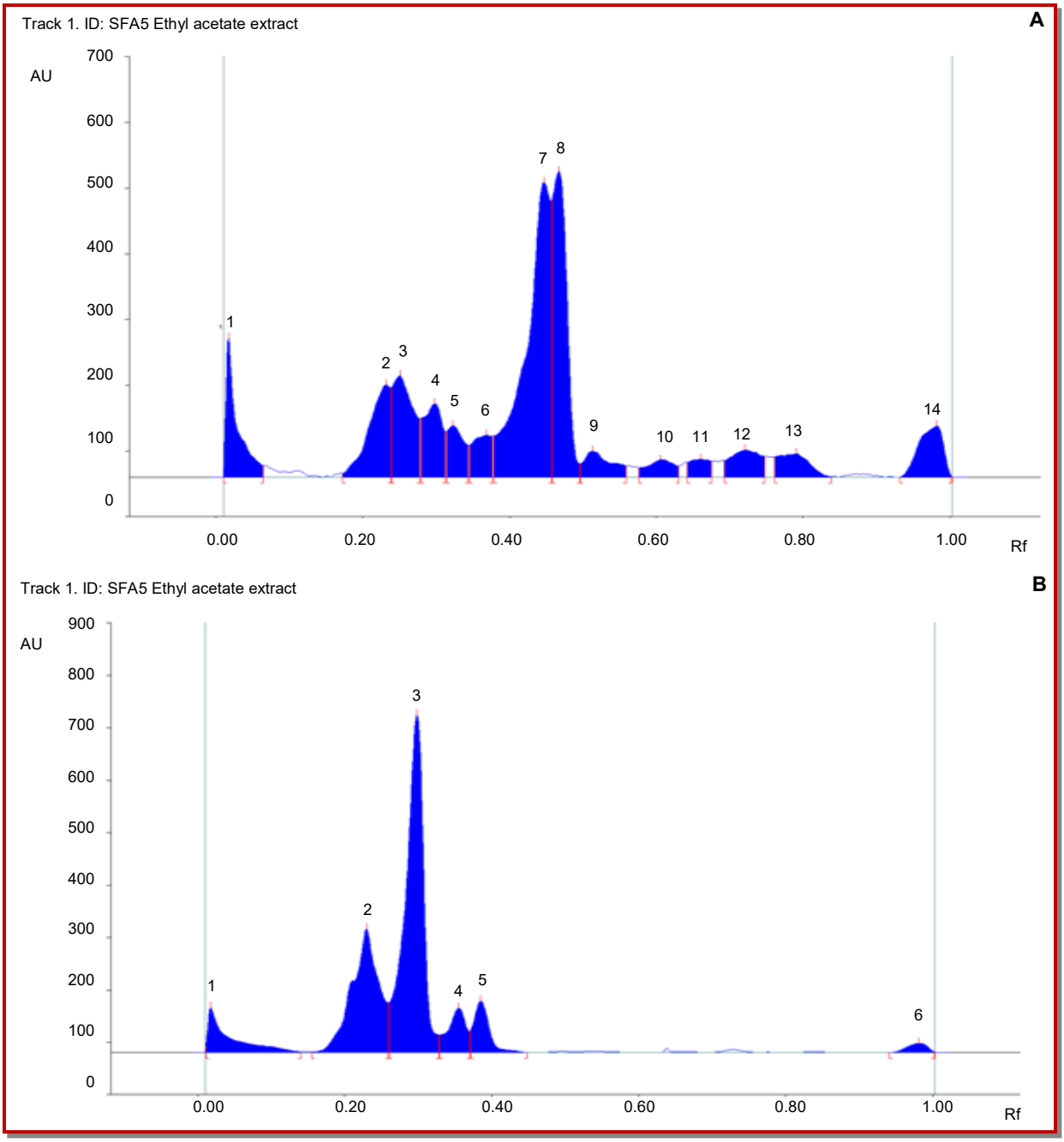


Figure 4: Finger print profile of ethyl acetate extracts of SFA5 at UV 254 nm (A) and 366 nm (B)

composition at the same time (Andola et al., 2010). HPTLC analysis of the ethyl acetate extract of SFA5 produced two yellow brown spots revealed the presence of two major antibacterial components at $R_f$ value of 0.38 and 0.46 in the chosen solvent system.

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## Conflict of Interest

Authors declare no conflict of interest

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