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# Development of phytochemical fingerprint of an Indian medicinal plant Chitrak (*Plumbago zeylanica* L) using high performance thin layer chromatography (HPTLC)

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**Fresh, shade-dried and powdered samples of leaf, stem and root of Chitrak (*Plumbago zeylanica* L) were subjected to fractional distillation in a Soxhlet apparatus using four organic solvents. Chloroform, acetone and ethanol extracts from root of *Plumbago* showed higher zone of inhibition of 22.66±1.52, 21.5±1.29 and 16.5±1.29 mm and minimum inhibitory concentration (MIC) of 1.0, 10.0 and 10.0 µg/ml, respectively against *Escherichia coli*. Root and leaf extracts by ethanol, chloroform and acetone showed higher antibacterial activity against *E. coli* as compared to standard Kanamycin (MIC-100 µg/ml). The high performance thin layer chromatography (HPTLC) fingerprints were used for the quantitation of two bioactive markers: gibberellic acid and quinol R in the plant powder of different organs. Maximum content of gibberellic acid was found in acetone extract of the root (59.74%, $R_f$ 0.79) followed by methanol root extract (53.01%). HPTLC provides a chromatographic fingerprint of phytochemicals and is suitable for confirming the identity and purity of medicinal plant raw materials.**

**Key words:** *Plumbago zeylanica*, antimicrobial property, bioactive compound, kanamycin, *Escherichia coli*, high performance thin layer chromatography (HPTLC), phytochemical fingerprint.

## INTRODUCTION

Medicinal plants are a vital source of medication in developing countries. Of course, these are used by all sections of people either directly as folk remedies or in different indigenous system of medicines or indirectly in the pharmaceutical preparations of modern medicines. Besides, the threats in drug resistant pathogenic microbial strains can be prevented by the un-tapped medicinal principles from plants (Davis, 1994). Although, hundreds of plant species have been tested for antimicrobial properties, the vast majority have not yet been adequately evaluated. In every developing country, it is necessary that the documentation of medicinal plants be treated as a matter of extreme urgency. Chitraka (*Plumbago zeylanica* L., Family: Plumbaginaceae) is a well known herb in traditional Indian medicine known to have effective medicinal properties like antirheumatic, carminative,

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anthelmintic, anti-inflammatory and promotes appetite, helps digestion and used for dyspepsia, piles, and skin diseases (Chopra et al., 1992). Biological activities of crude extract and active constituents of this plant reported so far include antimicrobial, antimutagenic, antitumor and radio modifying properties (Krishnaswami et al., 1980; Devi et al., 1994). Since different parts of this plant like roots, leaves and stems have medicinal values with different clinical indications, it is important to establish quality of the plant raw material for its constituent plant part composition. The active chemical constituents in a plant material serve as a characteristic fingerprint for that plant and help to develop analytical techniques to ascertain the quantity of the active constituents in botanically derived products. Chromatographic techniques can be used to document phytochemical fingerprints and quantitate chemical markers to identify morphological variations in the herbal raw material. High performance thin layer chromatography (HPTLC) is a sophisticated and automated form of TLC which can be used to purify the bioactive compounds qualitatively and quantitatively. It has better analytical precision and accuracy where both sample and standard are processed simultaneously (Sutar et al., 2002). Two important chemical compounds like quinol, otherwise known as hydroquinone (benzene-1,4-diol), an aromatic organic compound which is used as a topical application in human medicine and gibberellic acid, a plant growth hormone produced in different plant parts found to have antimicrobial property (Morshed et al., 2005) were taken as standards. The present investigation, in this context was undertaken to screen the aforementioned medicinal plant grown widely in Orissa for antimicrobial activity and purification, quantification of antimicrobial compounds by HPTLC, and developing HPTLC fingerprint. The active compound isolated from these extracts is compared with kanamycin (commercial antibacterial compound) for its antibacterial activity against *Escherichia coli.*

## MATERIALS AND METHODS

### Sample preparation

The different plant parts: roots, leaves and stems of the medicinal plant, *Plumbago* were collected from the Golden Jubilee Herbal Garden, Department of Horticulture, College of Agriculture, Orissa University of Agriculture and Technology, Bhubaneswar. The pure isolates of bacterial strain *E. coli* was collected from the Department of Bacteriology, OVC, OUAT, Bhubaneswar. The different plant parts like leaves, stems, and roots were collected and washed in tap water, followed by distilled water and air dried under shade for one week. The dried plant material was ground to powder of small particle size of 2 mm by the grinder.

### Active compound extraction

Specified amounts of powdered leaf, stem and root of *Plumbago* were taken separately and extracted with 250 ml of acetone, ethanol, chloroform and methanol separately in Soxhlet apparatus for 2 to 6 h, following modified procedures as developed by Soxhlet in 1879. The plant extracts were collected and excess solvents were evaporated through rotary vacuum evaporator and the crude residues were collected. The percentage of crude extract in the initial sample was calculated. The dried extracts were weighed and dissolved in 2 ml of the respective solvents and stored at 4°C. The different solvent extracts were purified by silica column chromatography. The crude extracts were subjected to silica gel 60 to 120 mesh column chromatography and eluted with the corresponding solvents. These elutants were evaporated to dryness with the rotary evaporator and the dried samples were weighed on dry weight basis, dissolved in respective solvents and assayed for their antimicrobial activity.

### Antibacterial assay

Antibacterial activity of the plant extracts and reference standard kanamycin was tested by the Kirbey-Bauer disc diffusion technique against *E. coli*. Twenty milliliters of sterile medium was poured in a sterile Petri plate and was allowed to solidify. Then, 100 µl of overnight grown bacterial culture was spread on this plate using L-rod rotator. A sterile filter paper disc impregnated with a plant extract (30 µl from a stock of 10 mg/ml) was placed on agar plate and the plate was incubated overnight at 37°C. The bioactive compound diffused from the disc into the medium and inhibited the growth of the bacteria. The area of no growth (Zone of inhibition) was determined. A positive control with kanamycin and a negative control with the respective solvent were maintained.

### Determination of minimum inhibitory concentration (MIC) of bioactive compound

Minimum inhibitory concentration was determined based on the microbial growth after overnight incubation against different concentrations of plant extract used in MIC method. Six sets, each set containing 4 test tubes were maintained. Sets 1 and 2 were taken for plant extract (100 mg/ml of solvent); Sets 3 and 4 was for negative control with solvent; and Sets 5 and 6 was for positive control with kanamycin (100 mg/ml of water). Besides, two test tubes as cells control were maintained. Then, 900 µl of diluted bacterial stock ($10^{-6}$) was added to each test tube. Plant extracts measuring 100 µl from a stock of 100 mg/ml was added to tube 1 of set 1 and was thoroughly mixed which gave $10^{-1}$ dilution. This process was followed up to tube 4 so as to get $10^{-4}$ dilution. The set 2 was just replicated like set 1. In a similar fashion, 100 µl of solvent and 100 µl of kanamycin were added to the first tube in sets 3 and 4 and sets 5 and 6, respectively and serial dilution was made. All the tubes were incubated overnight at 37°C. The bacterial growth was observed on the plate (cell control) and in the tubes. The result was tabulated as ‘G’ for growth indicating compound as not effective and ‘N’ for no growth indicating compound inhibiting growth.

### HPTLC fingerprint of bioactive compounds

Chromatography was performed on a pre-activated silica gel HPTLC plate (10 × 10 cm, 0.2 mm layer thickness). Sample stocks were prepared in their respective solvents. Samples of 5.0 µl and known concentrations of standards were applied to the plate as 6 mm wide bands using the Camag 100 µl sample syringe with an automated Camag TLC applicator, positioned 10 mm from the bottom and 10 mm from side of the plate. The space between two bands was 10 mm. The application parameters were identical for all the analyses performed. The HPTLC plates were developed in a Camag twin trough glass tank (20 mm × 10 cm) which was pre-saturated with the mobile phase for 30 min. The length of each run

**Table 1.** Organic-solvent extraction in *Plumbago*.

| S/N | Solvent-plant part combination | Dry powder (g) | Extract (g) | Extraction (%) |
|---|---|---|---|---|
| 1 | S1P1 | 1.45 | 0.5 | 34.48 |
| 2 | S1P2 | 10 | 0.66 | 6.60 |
| 3 | S1P3 | 8 | 0.12 | 1.50 |
| 4 | S2P1 | 2 | 0.3 | 15.00 |
| 5 | S2P2 | 10 | 0.45 | 4.50 |
| 6 | S2P3 | 8 | 0.13 | 1.63 |
| 7 | S3P1 | 3.24 | 0.5 | 15.43 |
| 8 | S3P2 | 10 | 1.09 | 10.90 |
| 9 | S3P3 | 8 | 0.30 | 3.75 |
| 10 | S4P1 | 3.24 | 0.4 | 12.35 |
| 11 | S4P2 | 10 | 0.23 | 2.30 |
| 12 | S4P3 | 8 | 0.12 | 1.50 |

S1: Ethanol, S2: Chloroform, S3: Methanol, S4: Acetone and P1: Leaf, P2: Root, P3: Stem.

was 9 cm. The TLC runs were performed under laboratory conditions of 25±5°C and 50% relative humidity. After development, plates were derivatized in p-anisoldehyde and ferric chloride and heated at 110°C for 15 min. The TLC spots corresponding to sample and standard were quantified at 366 nm using a Camag TLC scanner with Camag Wincats software and a tungsten source.

## RESULTS AND DISCUSSION

### Isolation of phytochemical by organic solvent extraction

The content of different phytochemicals present in leaf, stem and roots soluble in different organic solvents was quantified after their extraction through Soxhlet apparatus and as shown in Table 1. The plant part: leaf has the highest percentage of phytochemical extractable in all four different solvents in *Plumbago* with the extracting efficiency, the highest in ethanol (34.48%), followed by methanol (15.43%), chloroform (15.0%) and acetone (12.35%). The root contains least amount of organic solvent-extractable phytochemicals as compared to leaf and stem.

### Antimicrobial activity of crude extract

The antimicrobial activity of crude extract was determined through disc-diffusion. Different crude extracts of *Plumbago* were assayed for antimicrobial activity by loading 10 µg/sterile disc. Antimicrobial activity from crude fractions of the solvent extracts against *E. coli* was determined as shown in form of zone of inhibition (Figure 1) and the diameter of zone is as shown in Table 2. The root extracts of all four organic solvents (Ethanol, Chloroform, Methanol and Acetone) from *Plumbago* were found to have antibacterial activity against Gram negative bacteria: *E. coli*, whereas the leaf extracts by ethanol, methanol and acetone after purification showed antibacterial activity. The highest activity was found in both chloroform and acetone extracts from root of the *Plumbago*. Among various combinations, chloroform, acetone and ethanol extracts from root of *Plumbago* were found to have higher zone of inhibition of 22.66±1.52, 21.5±1.29 and 16.5±1.29 mm, respectively against the tested organism, *E. coli*. The inhibition zone size of *E. coli* against antibiotic kanamycin disc at 10 µg was only 11.5±0.70 mm, but all the crude and purified extracts of root and leaf in *Plumbago* showed higher activity as compared to kanamycin. Therefore, the susceptibility of *E. coli* to all the extracts was more pronounced when compared with the antibiotic kanamycin. Beg and Ahmad (2000) tested the alcoholic extract of *Plumbago zeylanica* (root) against multidrug-resistant clinical isolates of bacteria (*Salmonella paratyphi, Staphylococcus aureus, E. coli, Shigella dysenteriae*). The extract exhibited strong antibacterial activity against all test bacteria irrespective of their antibiotic resistance behavior. This finding was well correlated to the present report by their efficient antimicrobial effect on *E. coli* by both chloroform and acetone extracts.

### MIC value of purified compounds

MIC value of the crude and purified extracts from different plant parts with different organic solvents ranges from 1.0 to 100 µg/ml for the Gram negative bacteria, *E. coli* (Table 2). The commercial antibiotic kanamycin which was used as a positive control showed the MIC value of 100 µg/ml. In this context, the root extracts of all organic solvents taken were found to be more active in comparison to the commercial antibiotic (Table 2). From this study, it is clear that the active compound isolated from root and leaf extracts of *Plumbago* are more potent in controlling *E. coli*. These active compounds are best

**Table 2.** Antimicrobial activity and MIC of crude and purified extracts of *Plumbago* against *E. coli*.

| S/N | Solvent and plant part combination | Zone of inhibition (mm) | MIC value (µg/ml) |
|---|---|---|---|
| 1 | Ethanol leaf extract (Purified) | 11.5±0.70 | 100 |
| 2 | Methanol leaf extract (Purified) | 12.5±0.70 | 10 |
| 3 | Acetone leaf extract (Purified) | 12±1.41 | 100 |
| 4 | Ethanol root extract (Crude) | 16.5±1.29 | 10 |
| 5 | Chloroform root extract (Crude) | 22.66±1.52 | 1.0 |
| 6 | Methanol root extract (Crude) | 11.5±0.70 | 100 |
| 7 | Acetone root extract (Crude) | 21.5±1.29 | 10 |
| 8 | Negative control with all solvents | - | - |
| 9 | Kanamycin | 11.5±0.70 | 100 |

alternative for the antibiotics to which resistance has been developed. Since these compounds showed similar kind of antimicrobial activity towards the tested organism, it leads to a conclusion that the plant extracts have the similar properties. The structural characterization of these molecules promote the utilization of this active principle as a lead in case of drug discovery from plant based formulations to control the emerging infectious drug resistant pathogenic microorganisms.

## HPTLC fingerprint of bioactive compounds

The different plant parts of the same medicinal plant have different therapeutic applications as known from Ayurved. Therefore, it is important to distinctively identify the plant part constituent of the plant powder which can easily be done with chromatographic techniques by documenting phytochemical fingerprints and quantitating chemical markers. In this study, HPTLC fingerprint patterns have been evolved for powders of different plant parts of *Plumbago* along with two standards, namely, quinol R and gibberellic acid known for their antimicrobial properties.

The composition of the mobile phase for TLC was optimized using different solvents of varying polarity and good resolution was achieved using Toluene: Ethyl acetate: Formic acid, 8.5: 1.0:0.5 as mobile phase. In a full scan experiment, chromatogram at wavelength of 366 nm (Figure 2a) and 254 nm (Figure 2b) showed more components information and better separation than at visible range (white light) (Figure 2c). Visualization of the components of different plant extracts after development of HPTLC was carried out by staining with p-anisoldehyde (Figure 2d) and ferric chloride (Figure 2e), because most organic compounds are colorless. The different spots of individual tracks have completely been resolved as could be seen from the scan showing various peaks in seven plant extracts (Figure 3a to g), two standards (Figure 3h and i) and global peak data for all 9 tracks are as shown in Figure 3j for comparison.

The presence of quinol and gibberellic acid was detected using silica gel HPTLC pre-coated plates with the mobile phase Toluene:Ethyl acetate:Formic acid, 8.5:1.0:0.5 (v/v). Chromatograms showing variations in phytochemical profiles of Chitrak were developed using HPTLC technique and were used for evaluating morphological variations. The HPTLC fingerprints were used for the quantitation of two bioactive markers gibberellic acid and quinol R in the plant powder of different organs. Among different tracks indicating individual sample (plant extract) in a chromatogram, track 3 containing leaf extract of acetone was resolved into maximum 11 components (spots) (Figure 3c), whereas root extract of methanol was resolved into 4 spots only (Figure 3f).

The $R_f$ value of 0.79 for gibberellic acid with maximum area of 83.54% was taken for determining its content in the samples; similarly, an $R_f$ value of 0.81 was taken for quinol. Thus, maximum content of gibberellic acid was found to be present in acetone extract of root part of *Plumbago* (59.74%, $R_f$ 0.79), followed by methanol root extract (53.01%) as shown in Table 3.

## Conclusion

The visual observation and direct recording of the entire chromatogram including all sample components and the ability to repeat detection and quantification steps under different conditions make the HPTLC method more suitable for rapid analysis of a large number of samples. HPTLC as reported in this study provides a chromatographic fingerprint of phytochemicals and is suitable for confirming the identity and purity of medicinal plant raw materials.

## ACKNOWLEDGEMENTS

The authors thank the Department of Biotechnology, Government of India for funding the PG teaching and research programme in Agricultural Biotechnology.

**Table 3.** Characterization of HPTLC profile of different extracts at 366 nm.

| Track | Peak | Rf | Area (AU) | Area (%) |
|---|---|---|---|---|
| 1 Ethanol-leaf extract (purified) | 1 | 0.01 | 2565.0 | 31.00 |
| | 2 | 0.14 | 249.5 | 3.02 |
| | 3 | 0.22 | 927.1 | 11.21 |
| | 4 | 0.24 | 445.4 | 5.38 |
| | 5 | 0.35 | 1408.9 | 17.03 |
| | 6 | 0.37 | 603.3 | 7.29 |
| | 7 | 0.41 | 882.7 | 10.67 |
| | 8 | 0.55 | 845.3 | 10.22 |
| | 9 | 0.96 | 346.1 | 4.18 |
| 2 Methanol-leaf | 1 | 0.01 | 5853.0 | 92.00 |
| | 2 | 0.18 | 509.1 | 8.00 |
| 3 Acetone-leaf | 1 | 0.01 | 8900.9 | 13.84 |
| | 2 | 0.05 | 10166.1 | 15.81 |
| | 3 | 0.14 | 1257.4 | 1.96 |
| | 4 | 0.20 | 6855.0 | 10.66 |
| | 5 | 0.31 | 5431.6 | 8.45 |
| | 6 | 0.34 | 3268.8 | 5.08 |
| | 7 | 0.40 | 10851.3 | 16.87 |
| | 8 | 0.54 | 15705.5 | 24.42 |
| | 9 | 0.59 | 834.6 | 1.30 |
| | 10 | 0.72 | 501.1 | 0.78 |
| | 11 | 0.76 | 543.1 | 0.84 |
| 4 Ethanol-root (crude) | 1 | 0.01 | 3958.4 | 26.93 |
| | 2 | 0.19 | 264.0 | 1.80 |
| | 3 | 0.34 | 564.5 | 3.84 |
| | 4 | 0.40 | 297.4 | 2.02 |
| | 5 | 0.54 | 421.4 | 2.87 |
| | 6 | 0.78 | 9193.5 | 62.54 |
| 5 Chloroform-root (crude) | 1 | 0.01 | 5555.5 | 13.48 |
| | 2 | 0.20 | 892.9 | 2.17 |
| | 3 | 0.33 | 3409.5 | 8.27 |
| | 4 | 0.51 | 594.2 | 1.44 |
| | 5 | 0.66 | 2087.7 | 5.07 |
| | 6 | 0.77 | 28666.6 | 69.57 |
| 6 Methanol-root (crude) | 1 | 0.01 | 4776.7 | 40.05 |
| | 2 | 0.21 | 371.5 | 3.11 |
| | 3 | 0.34 | 455.8 | 3.82 |
| | 4 | 0.78 | 6322.8 | 53.01 |
| 7 Acetone-root (crude) | 1 | 0.01 | 5656.3 | 16.37 |
| | 2 | 0.18 | 235.0 | 0.68 |
| | 3 | 0.22 | 1031.6 | 2.99 |
| | 4 | 0.36 | 2471.2 | 7.15 |
| | 5 | 0.44 | 941.2 | 2.72 |
| | 6 | 0.51 | 785.0 | 2.27 |
| | 7 | 0.55 | 668.8 | 1.94 |

**Table 3.** Contd.

| | | | | |
|---|---|---|---|---|
| | 8 | 0.68 | 2118.1 | 6.13 |
| | 9 | 0.79 | 20638.5 | 59.74 |
| 8 Gibberellic acid | 1 | 0.01 | 326.5 | 16.46 |
| | 2 | 0.79 | 1657.7 | 83.54 |
| 9 Quinol R | 1 | 0.01 | 413.4 | 26.20 |
| | 2 | 0.08 | 172.5 | 10.93 |
| | 3 | 0.19 | 277.5 | 17.58 |
| | 4 | 0.20 | 321.7 | 20.38 |
| | 5 | 0.81 | 392.9 | 24.90 |

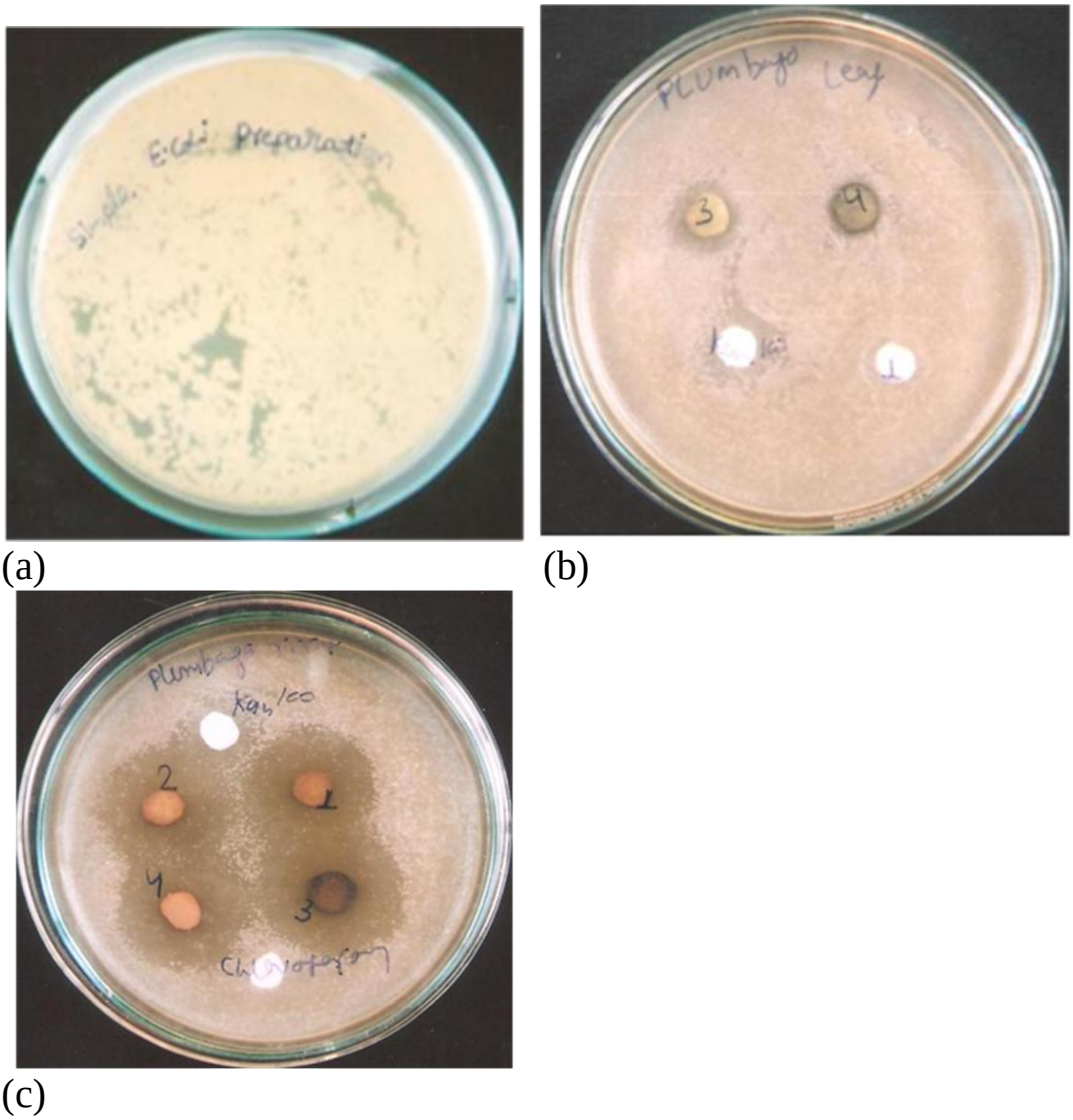
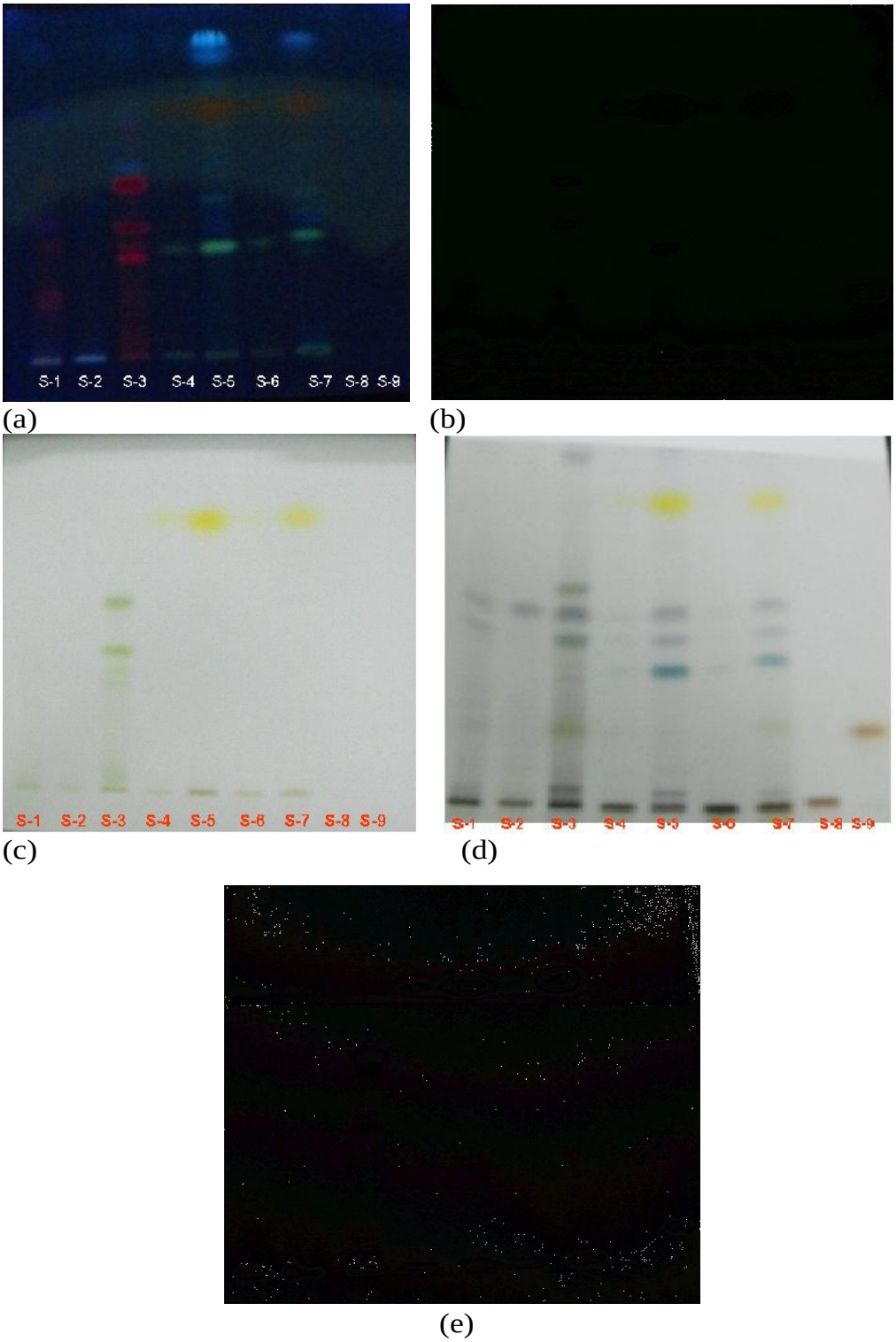



**Figure 1.** (a) E. coli lawn; (b) zone of inhibition of plumbago leaf extracts of 1-Ethanol,3-methanol,4-acetone abd disc of kanamycin; (c) anti bacterial effect of plumbago root extracts of 1-Ethanol, 2-chloroform, 3-methanol, 4-acetone, 5-kanamycine, 100 ppm (positive control) and 6-chloroform (negative control).




S-1=Ethanol Plumbago Leaf Extract
S-2= Methanol Plumbago Leaf Extract
S-3= Acetone Plumbago Leaf Extract
S-4= Ethanol Plumbago Root Extract
S-5=Chloroform Plumbago Root Extract
S-6= Methanol-root extract
S-7=Acetone – root extract
S-8=Standard-1(Gibberilic Acid)
S-9= Standard-2(Quinol-R)

**Figure 2.** (a) Developed Plate in 366 nm; (b) developed Plate in 254 nm; (c) developed plate in normal Light; (d) derivatized plate in p-Anisoldehyde stain and (e) derivatized plate in $FeCl_3$ stain.

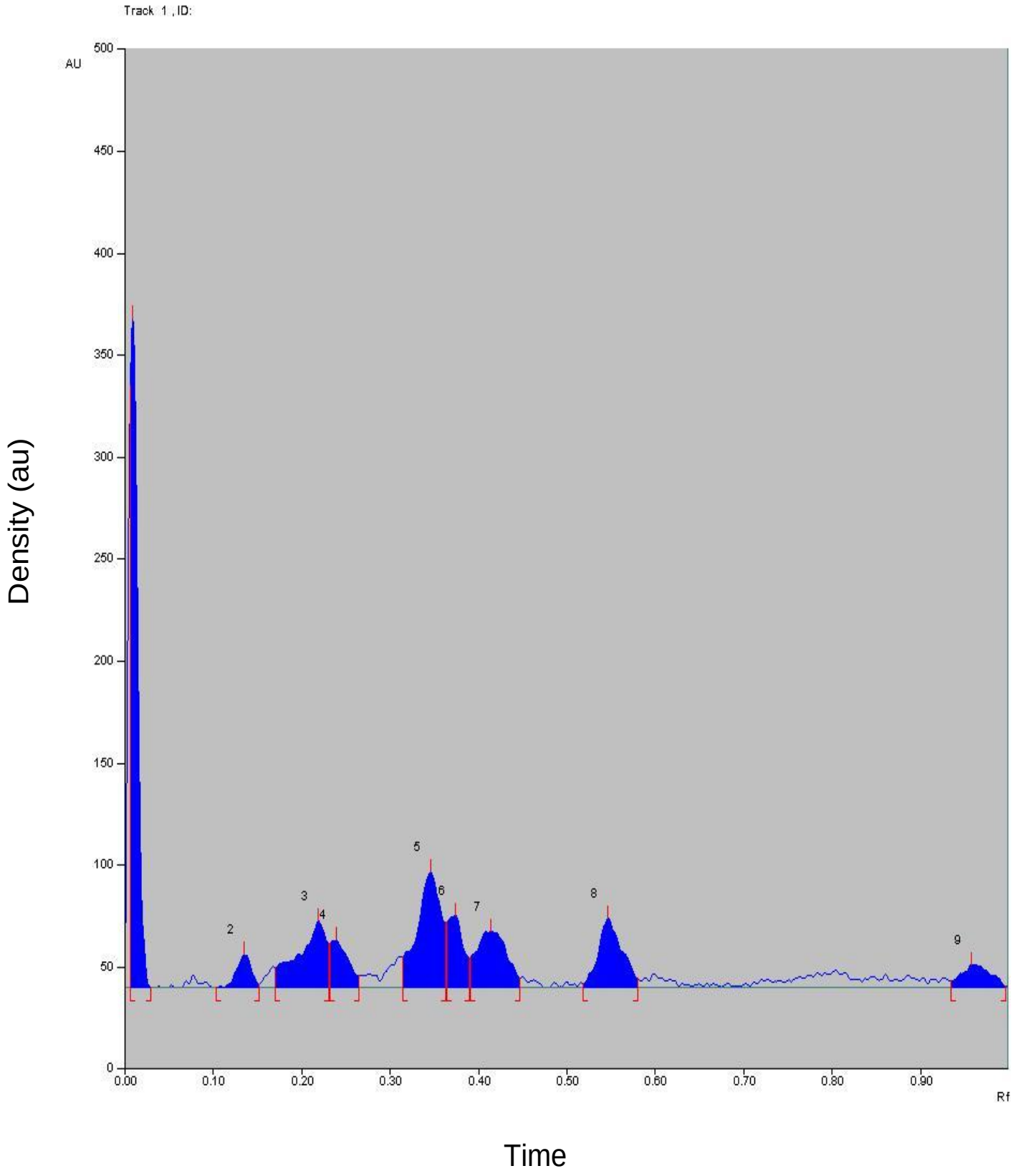



**Figure 3a.** Scan (366nm) of HPTLC layer showing separation of phytochemicals in ethanol extract of Leaf.

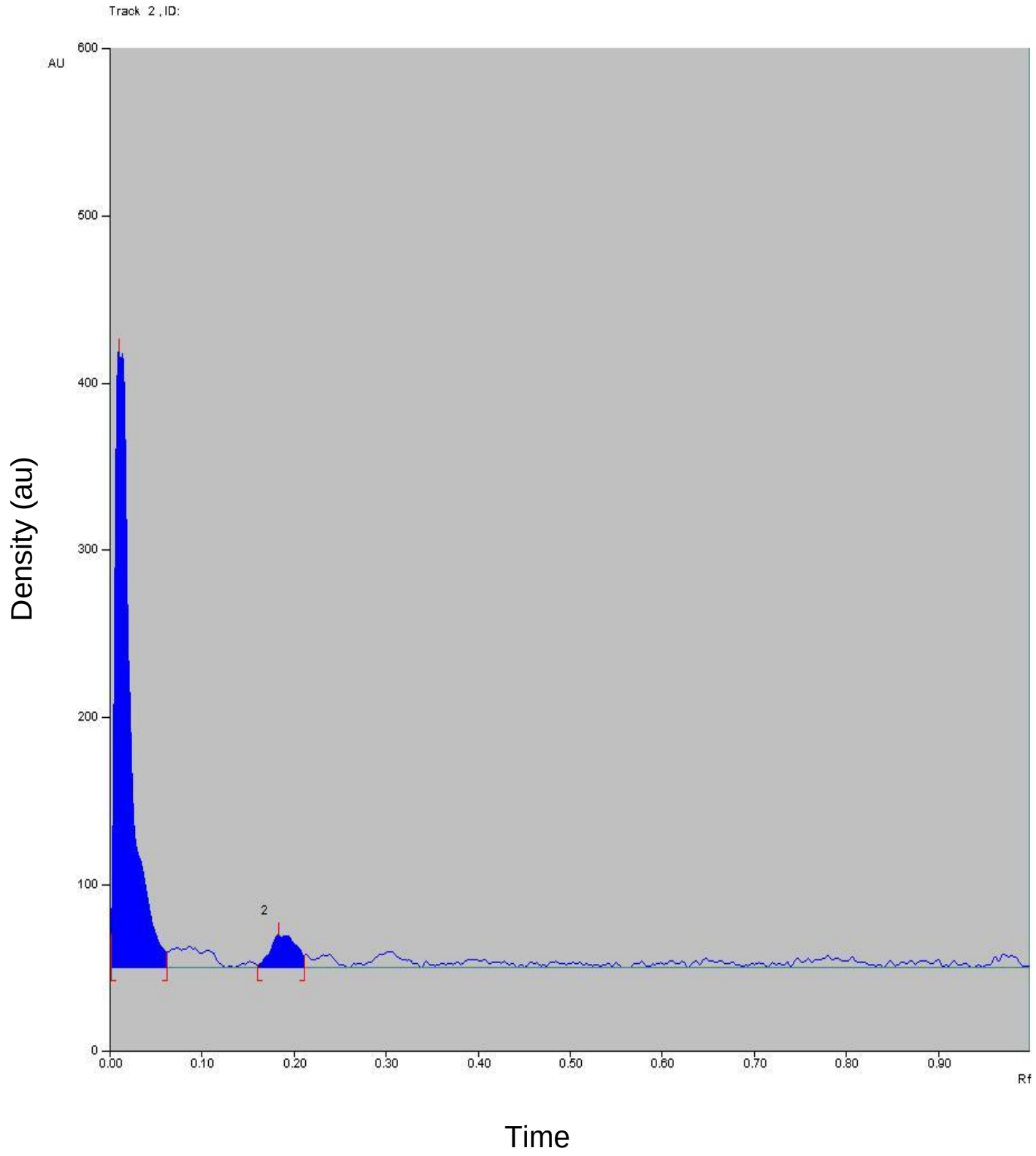



**Figure 3b.** Scan (366 nm) of HPTLC layer showing separation of phytochemicals in methanol extract of leaf.

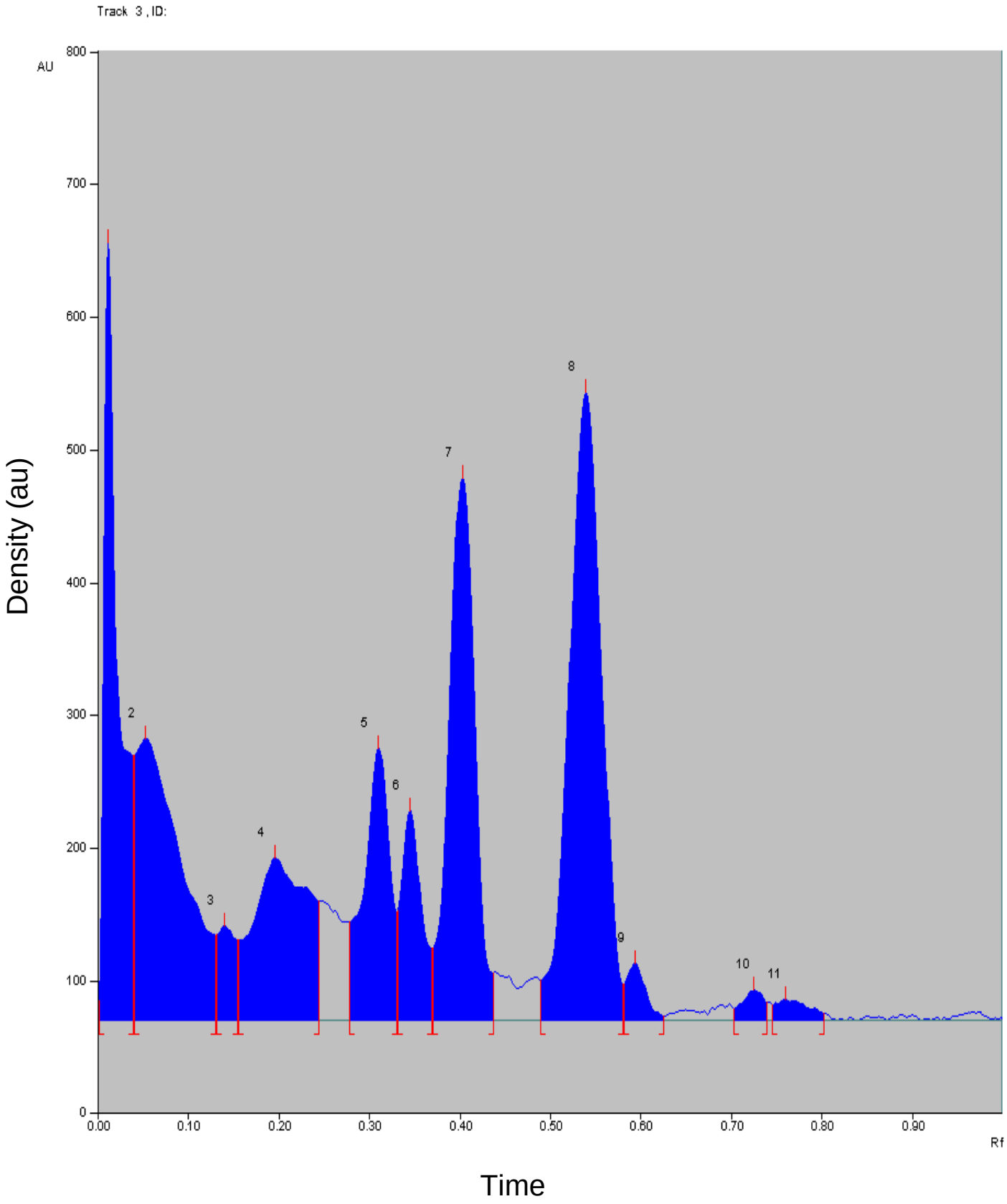



**Figure 3c.** Scan (366 nm) of HPTLC layer showing separation of phytochemicals in acetone extract of leaf.

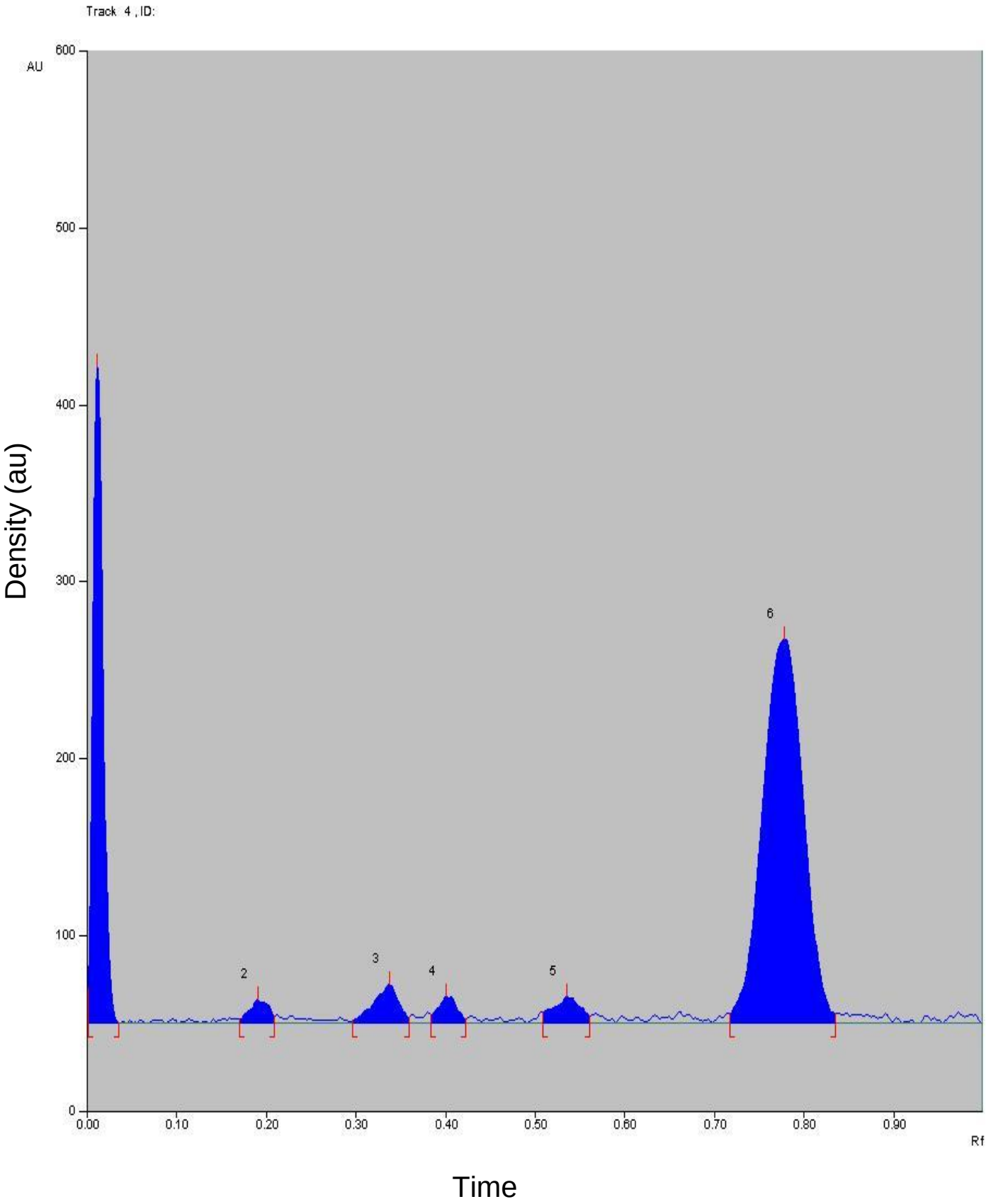



**Figure 3d.** Scan (366 nm) of HPTLC layer showing separation of phytochemicals in ethanol extract of Root.

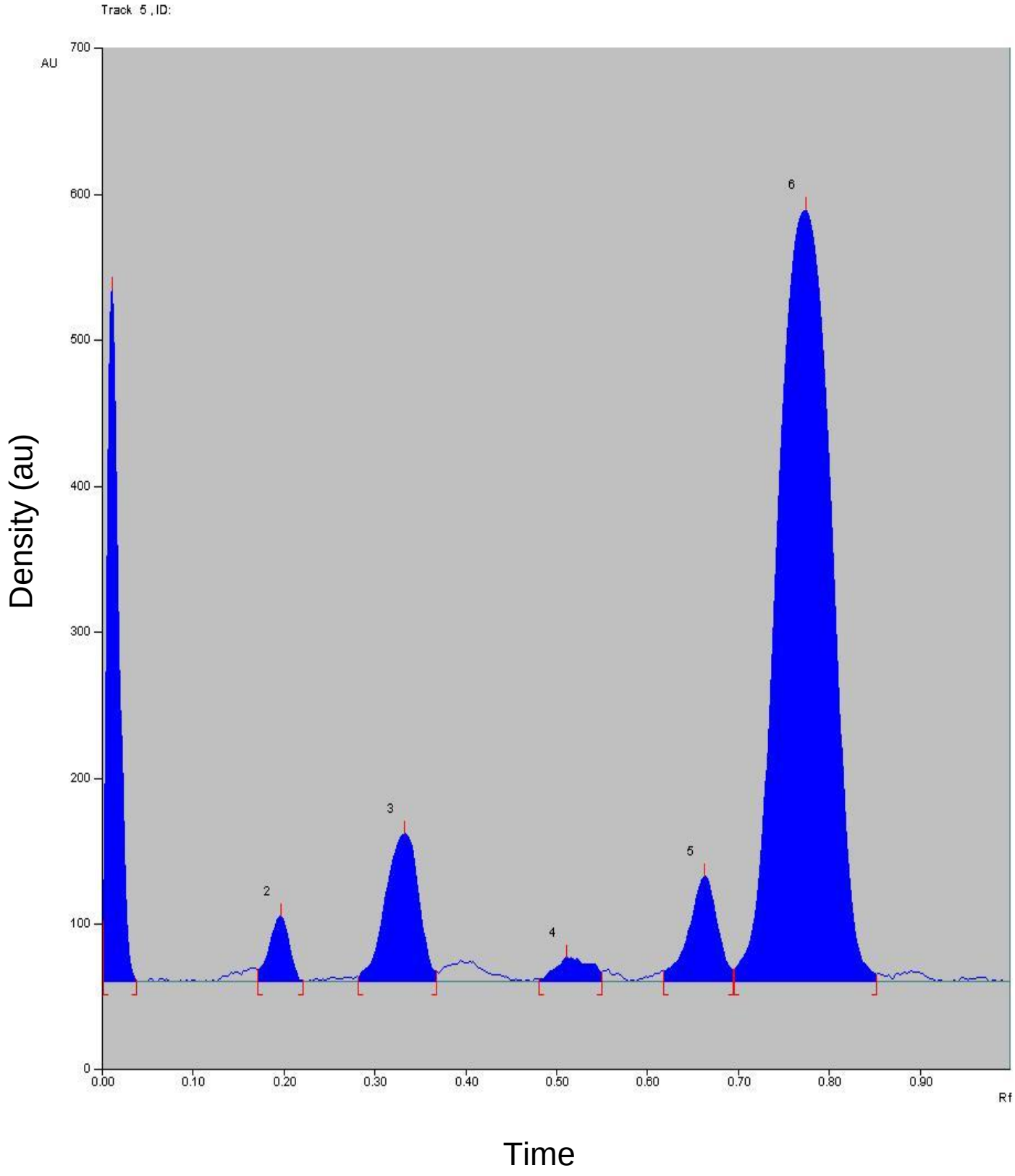



**Figure 3e.** Scan (366 nm) of HPTLC layer showing separation of phytochemicals in chloroform extract of root.

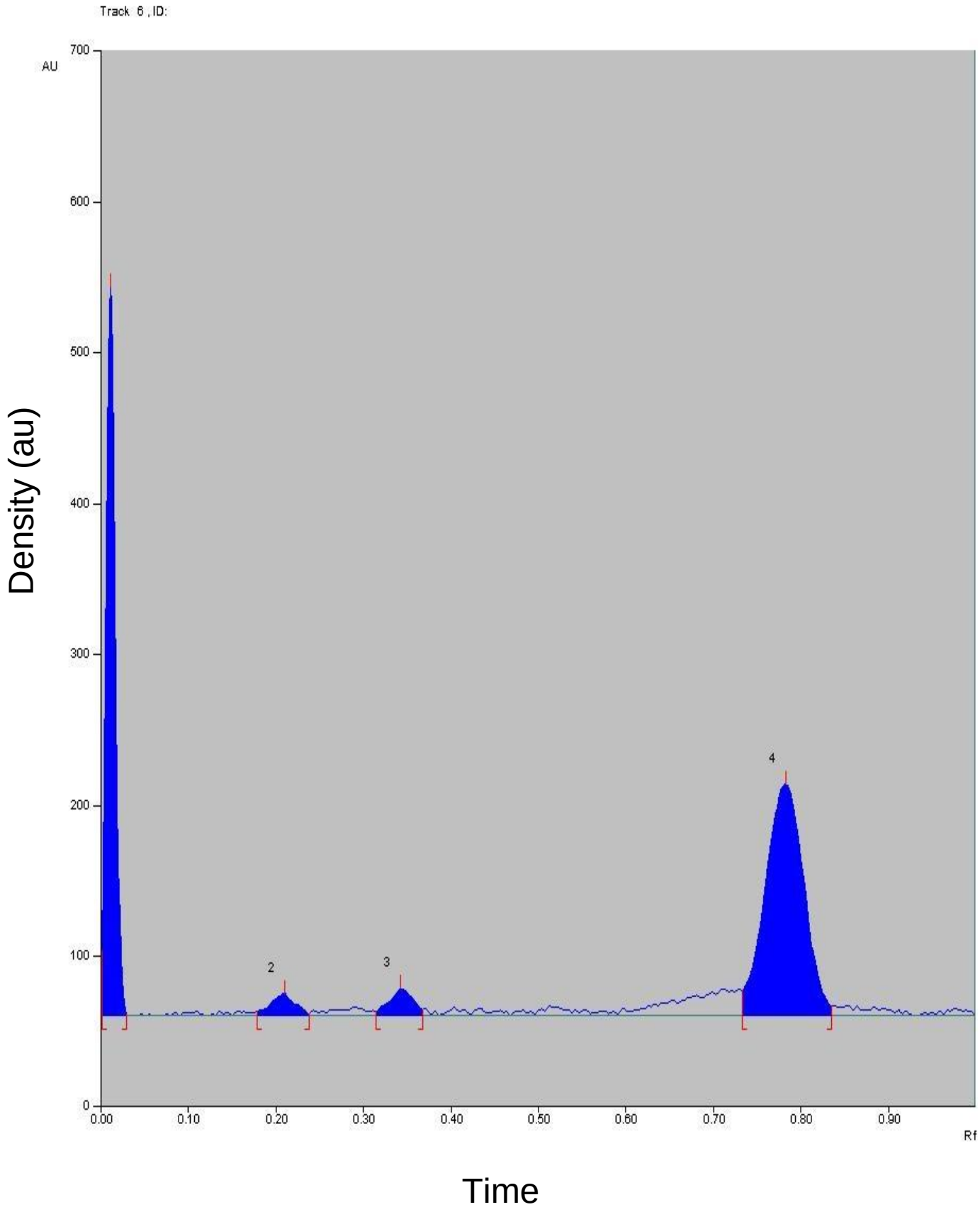



**Figure 3f.** Scan (366 nm) of HPTLC layer showing separation of phytochemicals in methanol extract of root.

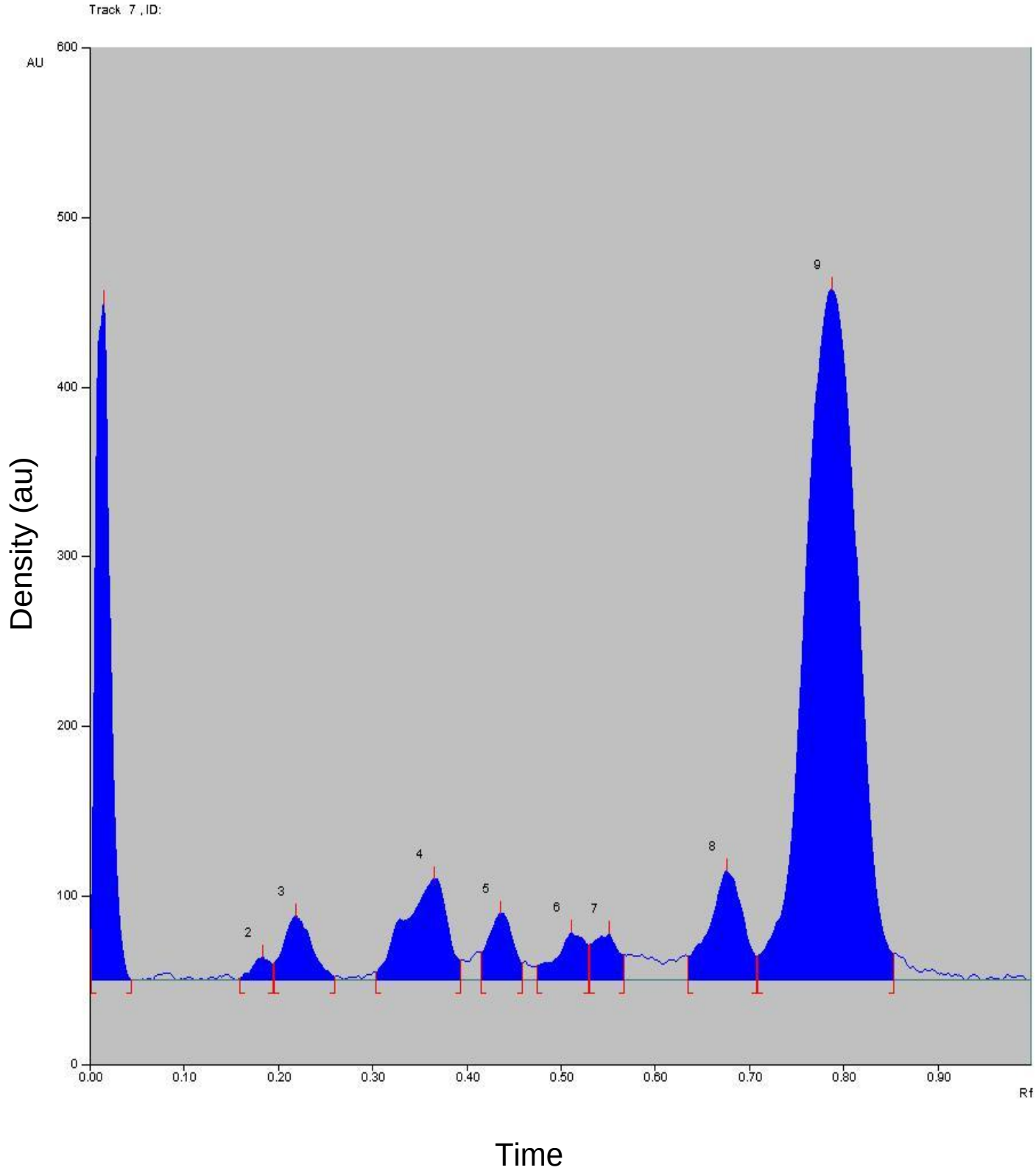



**Figure 3g.** Scan (366 nm) of HPTLC layer showing separation of phytochemicals in acetone extract of root.

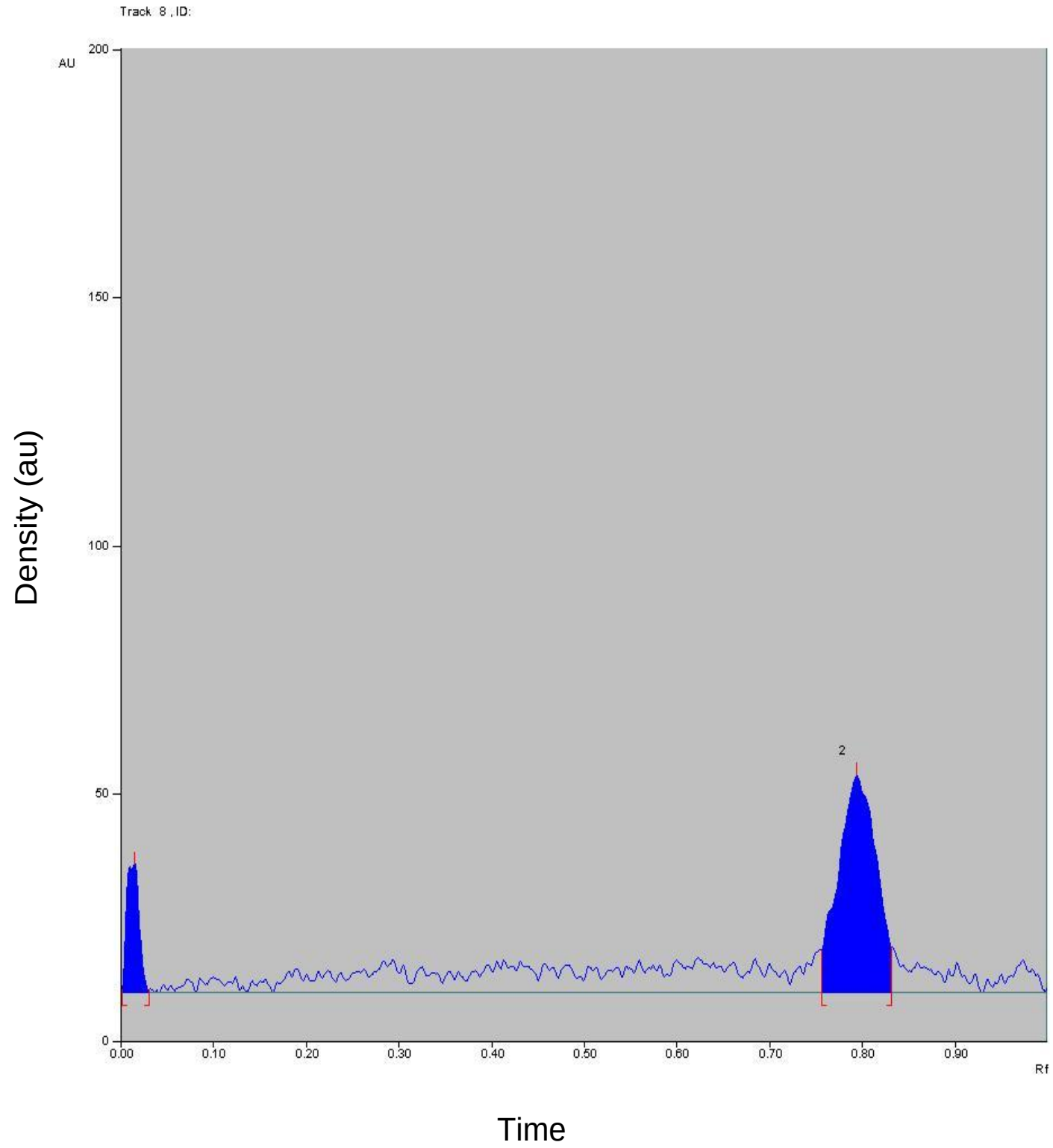



**Figure 3h.** Scan (366 nm) of HPTLC layer showing separation of phytochemicals in standard gibberillic acid.

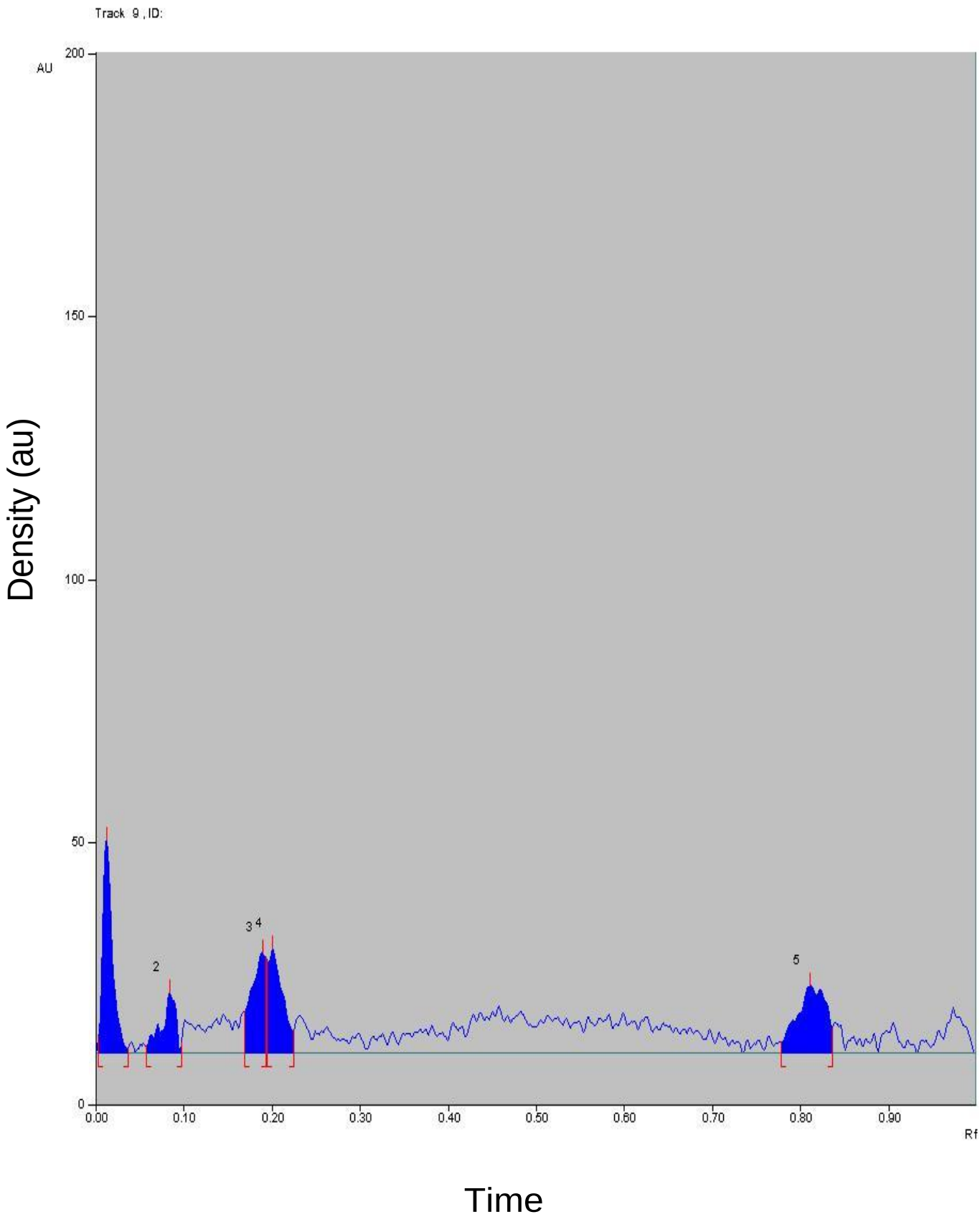



**Figure 3i.** Scan (366 nm) of HPTLC layer showing separation of phytochemicals in standard Quinol-R.

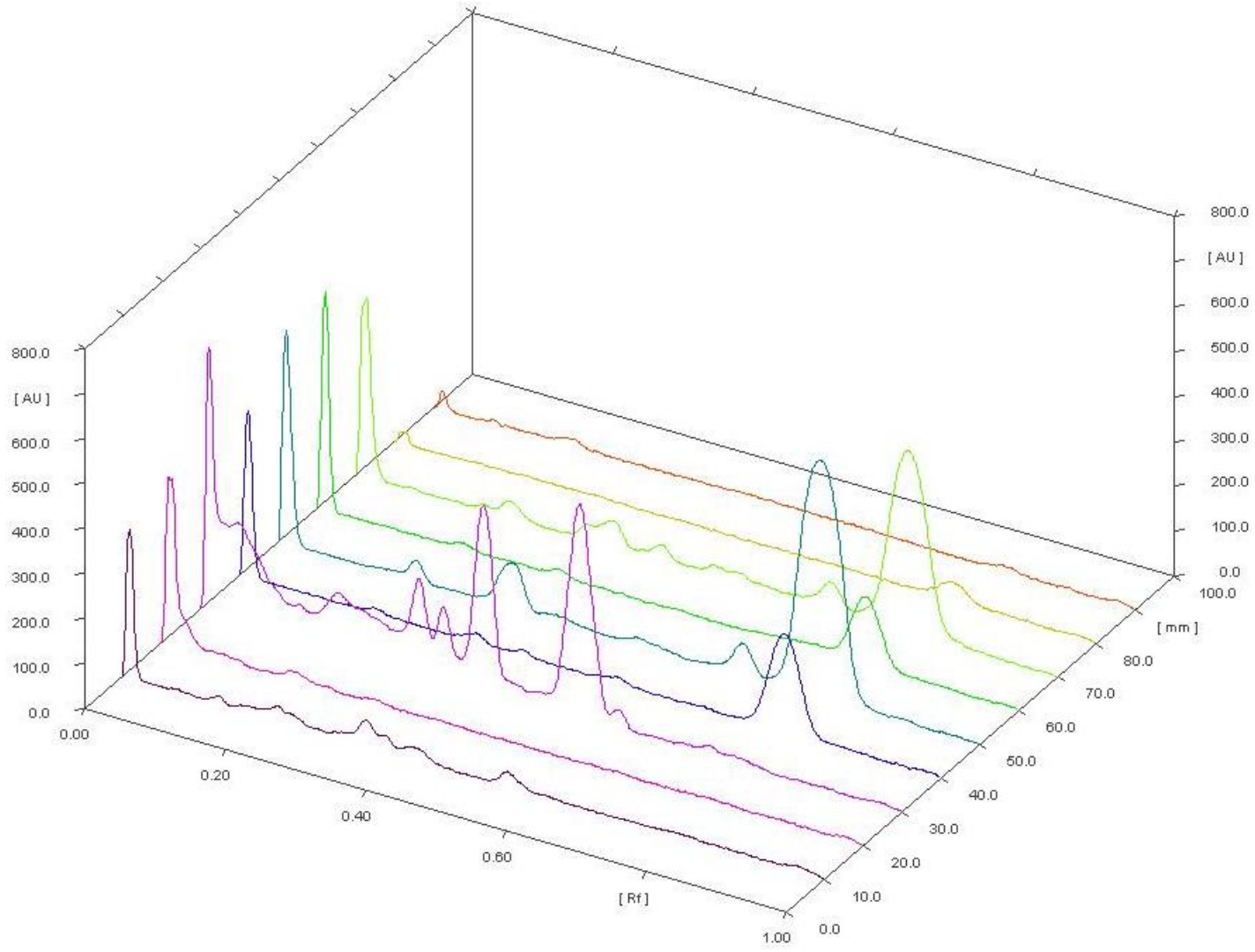



**Figure 3j.** Global peak data of different extracts of Plumbago. All tracks are at 366 nm.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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