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Table of Contents: Volume 8 Number 18 10 May, 2014

ARTICLES

Research Articles

Detection of tamarixetin and kaempferide in different tissues by high-performance liquid chromatography in tamarixetin and kaempferide treated rats 664
Fu-quan Xu, Yuan-yuan Feng and Bin-lun Yan

Development of phytochemical fingerprint of an Indian medicinal plant Chitrak (Plumbago zeylanica L) using high performance thin layer chromatography (HPTLC) 669
Ramasamy Rajesh, Nanjundan Jaivel and Ponnusamy Marimuthu

Antibacterial activity of two triterpenes from stem bark of Protorhus longifolia 686
Rebamang A. Mosa, Mandlakayise L. Nhleko, Thandeka V. Dladla and Andy R. Opoku

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Journal of Medicinal Plant Research

Full Length Research Paper

Detection of tamarixetin and kaempferide in different tissues by high-performance liquid chromatography in tamarixetin and kaempferide treated rats

Fu-quan Xu[1]*, Yuan-yuan Feng[2] and Bin-lun Yan[1]

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Tamarixetin and kaempferide were considered as the major active constituents of *Tamarix chinensis*, and had several known bioactivities. An effective and inexpensive high-performance liquid chromatographic method was established and validated for the determination of tamarixetin and kaempferide in rat tissues following a single oral administration. Tissue distribution showed the highest level of tamarixetin was observed in lung, then in heart; and the highest concentration of kaempferide was determined in heart.

Key words: Liquid chromatography, tissues distribution, tamarixetin, kaempferide.

INTRODUCTION

The twig of *Tamarix chinensis* was used for the treatment of coughs with dyspnea and wind chill cold in Chinese folk (National Pharmacopoeia Commission, 2005). Tamarixetin and kaempferide were considered as the major active constituents of *T. chinensis* (Zhang et al., 1991). Tamarixetin had been found to have antibacterial (Sultanova et al., 2001), free radical scavenging (Fazilatun et al., 2005; Nessa et al., 2004), hepatic protective (Yannai et al., 1998) and antioxidant activity (von Moltke et al., 2004). Kaempferide was reported had the peroxynitrite free radical scavenging (Calgarotto et al., 2007), antitrypanosomal and antileishmanial (Tasdemir et al., 2006), antioxidant activities (Burda and Oleszek, 2001). Among variety of bioactive flavonoids, which were in took by daily dietary (Androutsopoulos et al., 2010), tamarixetin and kaempferide were widely found in many plants (Lai et al., 2007). With the growing significance of a potential beneficial role of tamarixetin and kaempferide in human health, there is a demand for analyzing them simultaneously. thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) had been applied to quantification of tamarixetin or kaempferide from various herbal medicines and foods (Liang et al., 2009; Lai et al., 2007; Wang et al., 2008; Paulke et al., 2006), but no simultaneous determination reported in crude plant's extract and biological fluids, let alone in tissues. It is well known that tissue distribution can help predict a variety of events related to the efficacy and toxicity of herbal preparations. Therefore, it is necessary for an intensive investigation on tissue distribution of tamarixetin and kaempferide for a good understanding of the mechanism of action and facilitating further research and development of *T. chinensis*. In the present paper,

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we developed and validated a rapid and sensitive HPLC method to determine tamarixetin and kaempferide in rat tissues. The method was successfully applied to tissue distribution study after oral administration of 600 mg/kg the total flavonoides from *T. chinensis* to healthy rats.

MATERIALS AND METHODS

Chemical, reagents and animals

Tamarixetin and kaempferide were isolated from thin-film transistor (TFT) their structures were identified on the basis of spectral data (Galeotti et al., 2008; Blasa et al., 2011) and the purity was >98.5%. Quercetin used as internal standard (IS) and its purity was >99.0%. HPLC-grade methanol was purchased from Honeywell International (Burdick and Jackson, Muskegon, MI, USA). Analytical and C_{18} cartridge columns were purchased from Waters (Waters, Milford, MA, USA). Pure tamarixetin and kaempferide solutions were prepared in methanol to furnish working solutions at concentrations 800, 400, 160, 80, 16, 8, 3.2, 1.6 µg mL^{-1} and 1400, 700, 280, 140, 28, 14, 5.6, 2.8 µg mL^{-1}, respectively. Their working solutions were added to different tissue homogenates of untreated rat and obtained the standard calibration sample in the concentration range of 0.32 to 160 µg mL^{-1} and 0.28 to 280 µg mL^{-1}. Quercetin solution of concentration 275 µg mL^{-1} was prepared in methanol. All the solutions were stored at -20ºC and were brought to room temperature before use. Wistar rats (male, 170 to 190 g) were purchased from Qingdao Institute of Drug Control (Qingdao, P. R. China), and kept in environmentally controlled breeding room (relative huminidity: 65%, temperature: 23 ± 2ºC, 12 h light-12 h dark cycle) for three days before the test, fed with food and water *ad arbitrium*. All animal studies were performed according to the requirement of the National Act on the Use of Experimental Animal (China).

Instrumentation

LC analysis was performed on Waters 2695 high performance liquid chromatography system equipped with diode array detector (2996) and Empower software (Waters, Milford, MA, USA). A Waters Sunfire^{TM} C_{18} reversed-phase column (5 µm particles, 250 mm × 4.6 mm) was used for separation and quantification.

Chromatographic conditions

The mobile phase for tissue samples was a gradient prepared from methanol (component A) and 0.15% aqueous formic acid solution (component B, pH: 2.8) and the flow rate was 1.0 mL min^{-1}. The initial mobile phase composition condition was A to B 10:90 (v/v). This was changed linearly to A to B 35:65 (v/v) at 15 min and held at this composition until 25 min, the composition was changed linearly to A to B 60:40 (v/v) at 40 min. Chromatograms were monitored at 254 nm and the temperature of column was kept at 35ºC.

Sample preparation

0.2 g tissues (heart, liver, spleen, lung, kidney, prostate and brain) were shredded in ice-bath, and then homogenized in 2 mL ice-cold 1% phosphoric acid normal saline solution. The homogenate was added 10 µL IS and vortex-mixed for 60 s. The supernatant, prepared by centrifugation at 6000 rpm (10 min), was extracted by C_{18} cartridge and eluted with water (0.6 mL) and methanol (0.6 mL) successively. The methanol fraction was evaporated to dryness under a stream of nitrogen at 40ºC. The residue was redissolved in150 µL of methanol, and then stored 30 min at 4ºC. A 10 µL aliquot was injected into the high performance liquid chromatography system after centrifugation at 12000 rpm for 10 min.

Method validation

Calibration curves were constructed by plotting peak area versus concentrations in the standard samples. The limit of detection (LOD) and limit of quantitation (LOQ) were determined to evaluate the sensitivity, defined as the concentration that produced a signal-to-noise ratio of 3:1 and 10:1, respectively. Intra- and inter-day precision, assessed by relative standard deviation (RSD) and mean concentration, were measured by performing replicate analysis (n = 5) for each concentration within one day and three continual days. The recoveries from tissue samples were calculated by comparing peak areas extracted from tissue samples with those of the same quantities added to the mobile phase. The freeze-thaw stability was tested after frozen at -20ºC for 24 h and completely thawed at room temperature. The long-time stability and short-time stability was assessed within 30 days and 24 h storage periods. All samples were tested at three concentration levels and repeated at least three times in method validation section.

Tissues distribution study

Tissues of 25 rats were removed at 10, 30, 60, 90 and 120 min after dosing 600 mg kg^{-1} TFT, washed with physiological saline solution and blotted dry twice, finally weighed and stored at -20ºC until disposal (within 24 h). Blank tissues were collected from rats free of TFT and processed as tissue samples.

RESULTS AND DISCUSSION

Extraction recovery

In different tissues, the range of two analytes was from 86.6 to 99.2% and from 87.1 to 101% and not less than 89.7% for IS. The results guaranteed the established method was suitable for tissue distribution study of tamarixetin and kaempferide.

Selectivity

The interference in tissue homogenate made it difficult to determine analytes exactly, after treaded in this study, the endogenous components were not appeared at the retention time of analytes in tissue analysis (Figure 1). It was concluded that acceptable selectivity was obtained by using the developed method.

Precision and accuracy

The measured mean concentration was very close to the added concentration (>91.3%) and the RSD of tamarixetin and kaempferide was less than 5.03% and 4.12%, respectively, which indicated that the developed method had good precision and accuracy for tissue

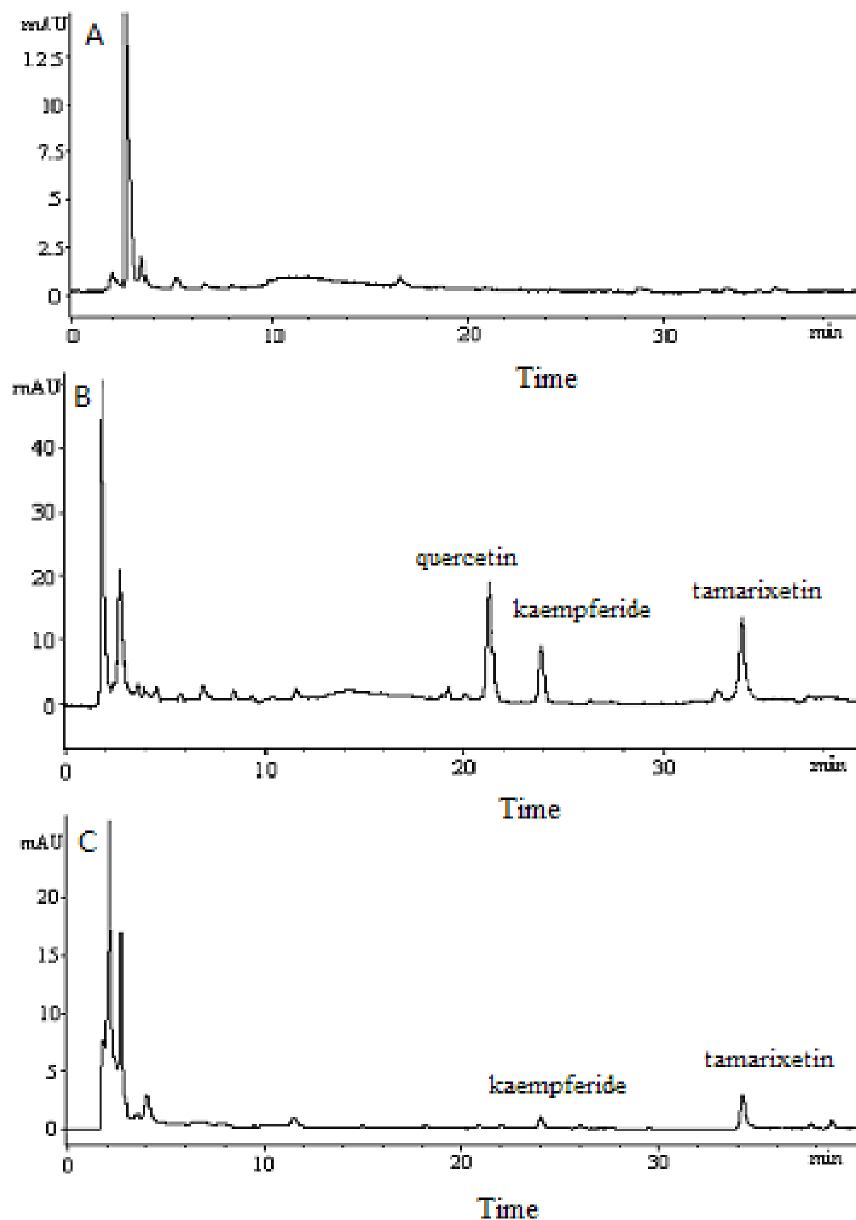

Figure 1. Chromatographic profiles of tissue samples: blank heart; **A**, blank heart spiked with tamarixetin, kaempferide and quercetin; **B**, heart obtained at 30 min; **C**.

Calibration curves and sensitivity

Calibration curves were shown as follows: heart (kaempferide: $Y = 0.00336X + 0.0411$, $r = 0.994$, 0.28-280 μg mL^{-1}, tamarixetin: $Y = 0.00352X + 0.0341$, $r = 0.993$, 0.32-160 μg mL^{-1}), liver (kaempferide: $Y = 0.00343X + 0.0423$, $r = 0.996$, 0.28-280 μg mL^{-1}, tamarixetin: $Y = 0.00347X + 0.0402$, $r = 0.996$, 0.32-160 μg mL^{-1}), spleen (kaempferide: $Y = 0.00341X + 0.0435$, $r = 0.995$, 0.28-280 μg mL^{-1}, tamarixetin: $Y = 0.00354X + 0.0369$, $r = 0.996$, 0.32-160 μg mL^{-1}), lung (kaempferide: $Y = 0.00332X + 0.0431$, $r = 0.993$, 0.28-280 μg mL^{-1}, tamarixetin: $Y = 0.00338X + 0.0472$, $r = 0.991$, 0.32-160 μg mL^{-1}), kidney (kaempferide: $Y = 0.00329X + 0.0443$, $r = 0.994$, 0.28-280 μg mL^{-1}, tamarixetin: $Y = 0.00342X + 0.0360$, $r = 0.995$, 0.32-160 μg mL^{-1}), prostate (kaempferide: $Y = 0.00334X + 0.0433$, $r = 0.990$, 0.28-280 μg mL^{-1}, tamarixetin: $Y = 0.00346X + 0.0373$, $r = 0.994$, 0.32-160 μg mL^{-1}), brain (kaempferide: $Y = 0.00343X + 0.0414$, $r = 0.995$, 0.28-280 μg mL^{-1}, tamarixetin: $Y = 0.00337X + 0.0318$, $r = 0.993$, 0.32-160 μg mL^{-1}) . Y was peak area ratio and X represented the concentration (μg mL^{-1}) in the linear regression equation. The LOD and LOQ of this method were 0.06 and 0.16 μg mL^{-1}, which was the foundation of HPLC method for tissue distribution study.

Table 1. The results of tamarixetin and kaempferide in tissues (μg mL^{-1}) (n = 5).

Compound	Liver	Heart	Spleen	Lung	Kidney	Prostate	Brain
Tamarixetin (at 10 min)	0.57	1.45	0.32	1.34	0.56	0.15	0.28
Kaempferide (at 10 min)	0.32	0.76	0.24	0.85	0.15	0.16	0.21
Tamarixetin (at 30 min)	1.76	2.14	0.37	2.31	1.21	0.31	0.45
Kaempferide (at 30 min)	0.84	1.08	0.26	1.07	0.28	0.22	0.14
Tamarixetin (at 60 min)	0.24	0.37	0.45	1.13	1.45	0.23	0.72
Kaempferide (at 60 min)	0.14	0.21	0.18	0.60	0.72	0.17	0.34

Stability

The analytes stability was evaluated by long-time, short-time and freeze-thaw stability experiment. The recoveries of freeze-thaw stability after three cycles were 88.7 to 92.1% (tamarixetin) and 86.3 to 93.5% (kaempferide). The values of short-time stability were no less than 86.4% (tamarixetin) and 87.3% (kaempferide), and the long-time stability results were more than 89.6% (tamarixetin) and 92.1% (kaempferide). These results indicated that all the biological samples were stable after three freeze (-20ºC)-thaw cycles, for 24 h at room temperature and for 30 days at -20ºC, with a reduction of less than15%.

Tissues distribution

Concentrations of tamarixetin and kaempferide were determined in various tissues of rat such as heart, liver, lung, spleen, kidney, prostate and brain, respectively. At 30 min after oral administration of TFT to rats, high level of two analytes was observed in heart, liver and lung, and at 60 min the high tissues were spleen, kidney and brain. Tamarixetin and kaempferide were few in all collected tissues after 90 min and were undetectable in most tissues after 120 min; there was no long-term accumulation after administration. Table 1 showed the concentrations of the tamarixetin and kaempferide in rat tissues at 10, 30 and 60 min after oral dose of TFT. This study showed that analytes was mainly distributed in abundant blood-supply tissues such as lung, liver and heart, which implied that the distribution of analytes maybe depended on the blood flow. The high distribution in lung confirmed that the twig of *T. chinensis* could treat cough.

Conclusion

The assay procedure presented in this report provides a simple, rapid and sensitive procedure for the determination of tamarixetin and kaempferide in tissues after oral administration to rats. The achieved tissue distribution results may be useful for further study of TFT, and be good for the development of *T. chinensis* to modern medicine. This was the first study of tamarixetin and kaempferide on tissue distribution after oral administration of TFT to rats.

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Conflict of Interests

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

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Full Length Research Paper

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

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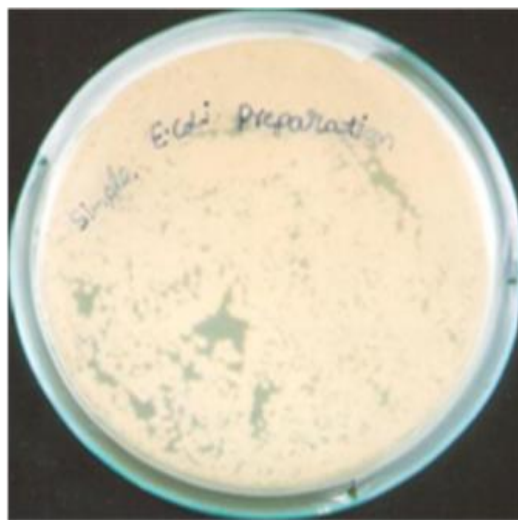
Table 3. Characterization of HPTLC profile of different extracts at 366 nm.

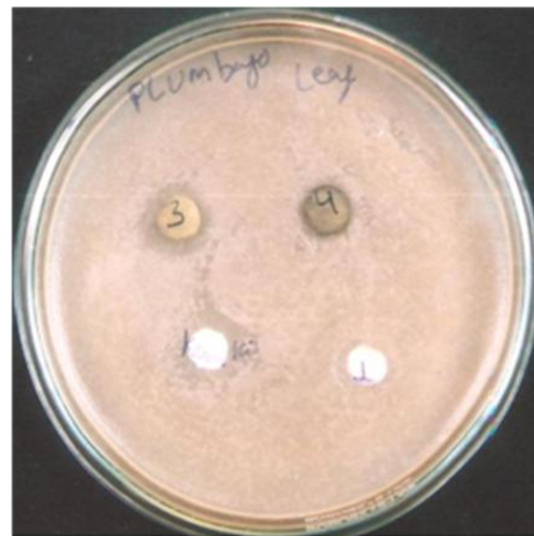
Track	Peak	Rf	Area (AU)	Area (%)
1 Ethanol-leaf extract (purified)	1	0.01	2565.0	31.00
	2	0.14	249.5	3.02
	3	0.22	927.1	11.21
	4	0.24	445.4	5.38
	5	0.35	1408.9	17.03
	6	0.37	603.3	7.29
	7	0.41	882.7	10.67
	8	0.55	845.3	10.22
	9	0.96	346.1	4.18
2 Methanol-leaf	1	0.01	5853.0	92.00
	2	0.18	509.1	8.00
3 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



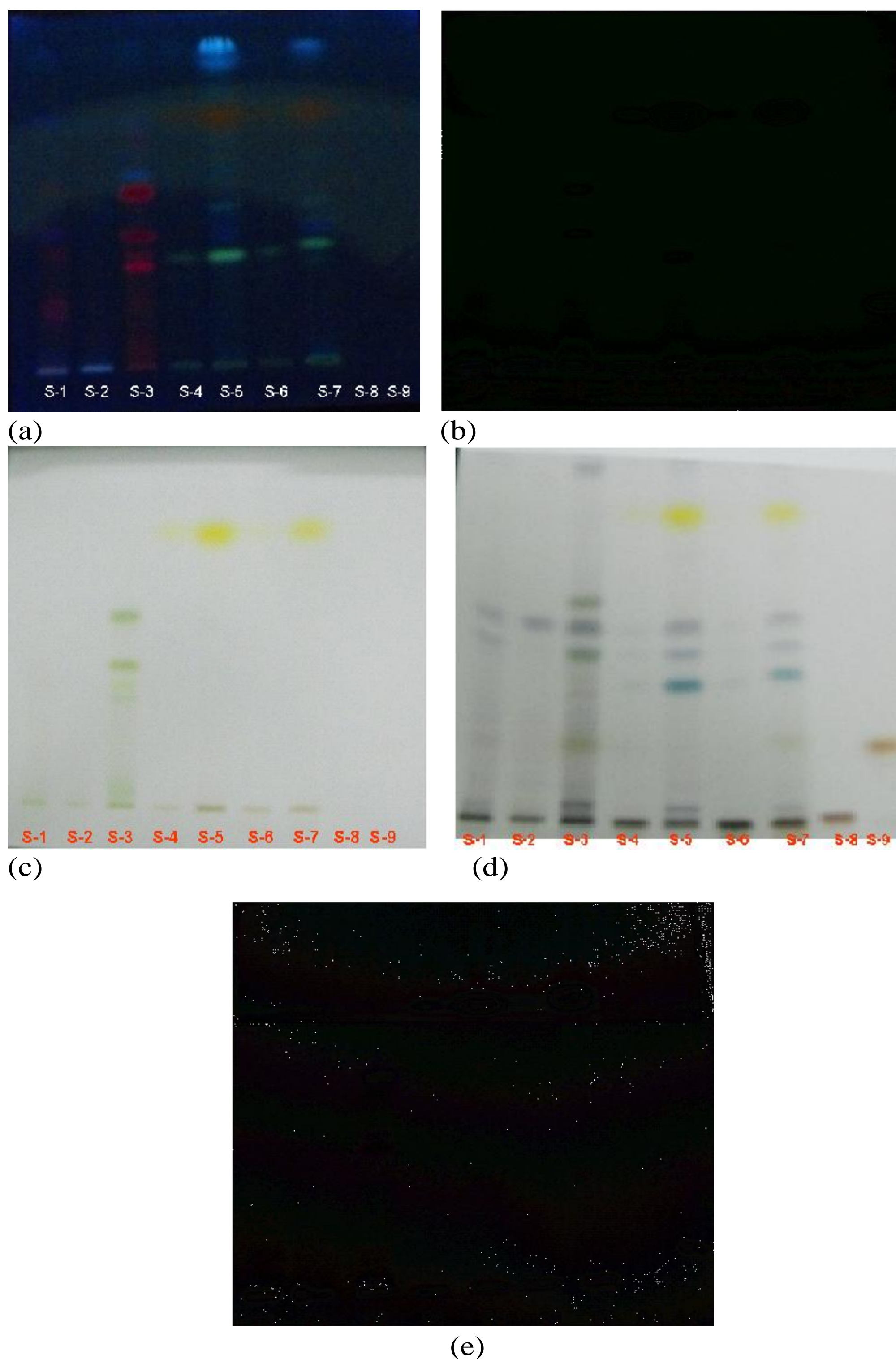
(a)



(b)

(c)

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.

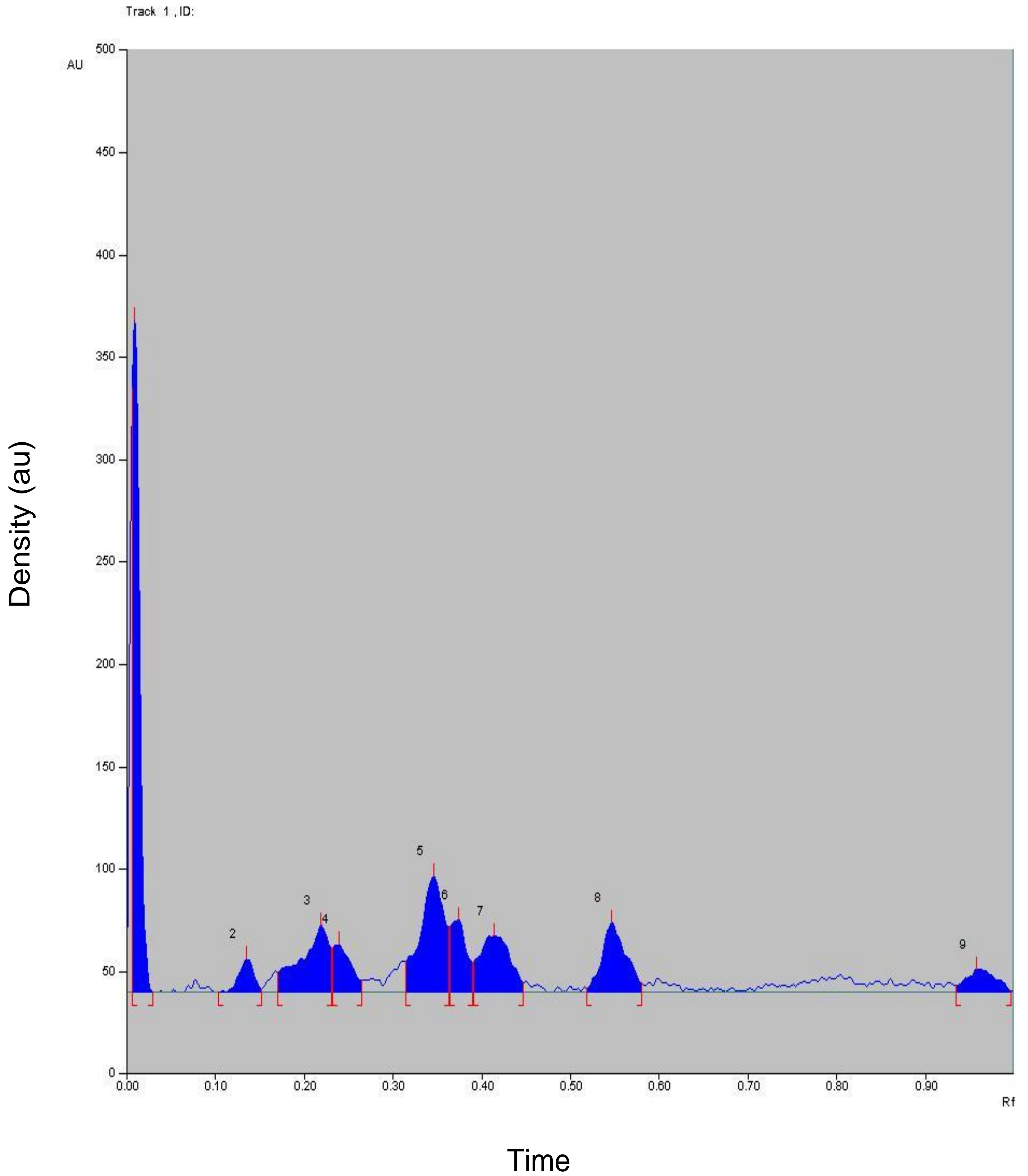


Figure 3a. Scan (366nm) of HPTLC layer showing separation of phytochemicals in ethanol extract of Leaf.

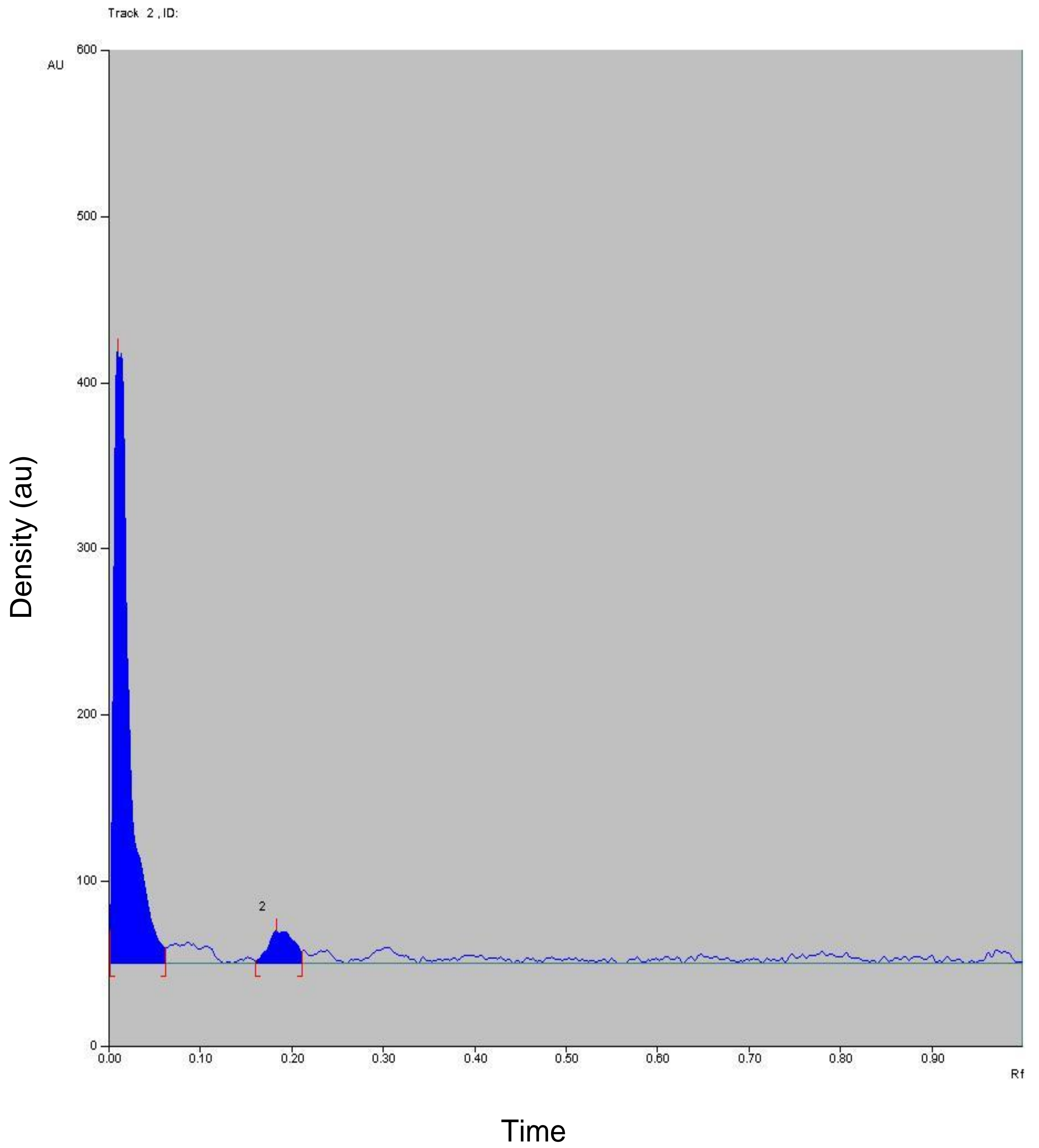


Figure 3b. Scan (366 nm) of HPTLC layer showing separation of phytochemicals in methanol extract of leaf.

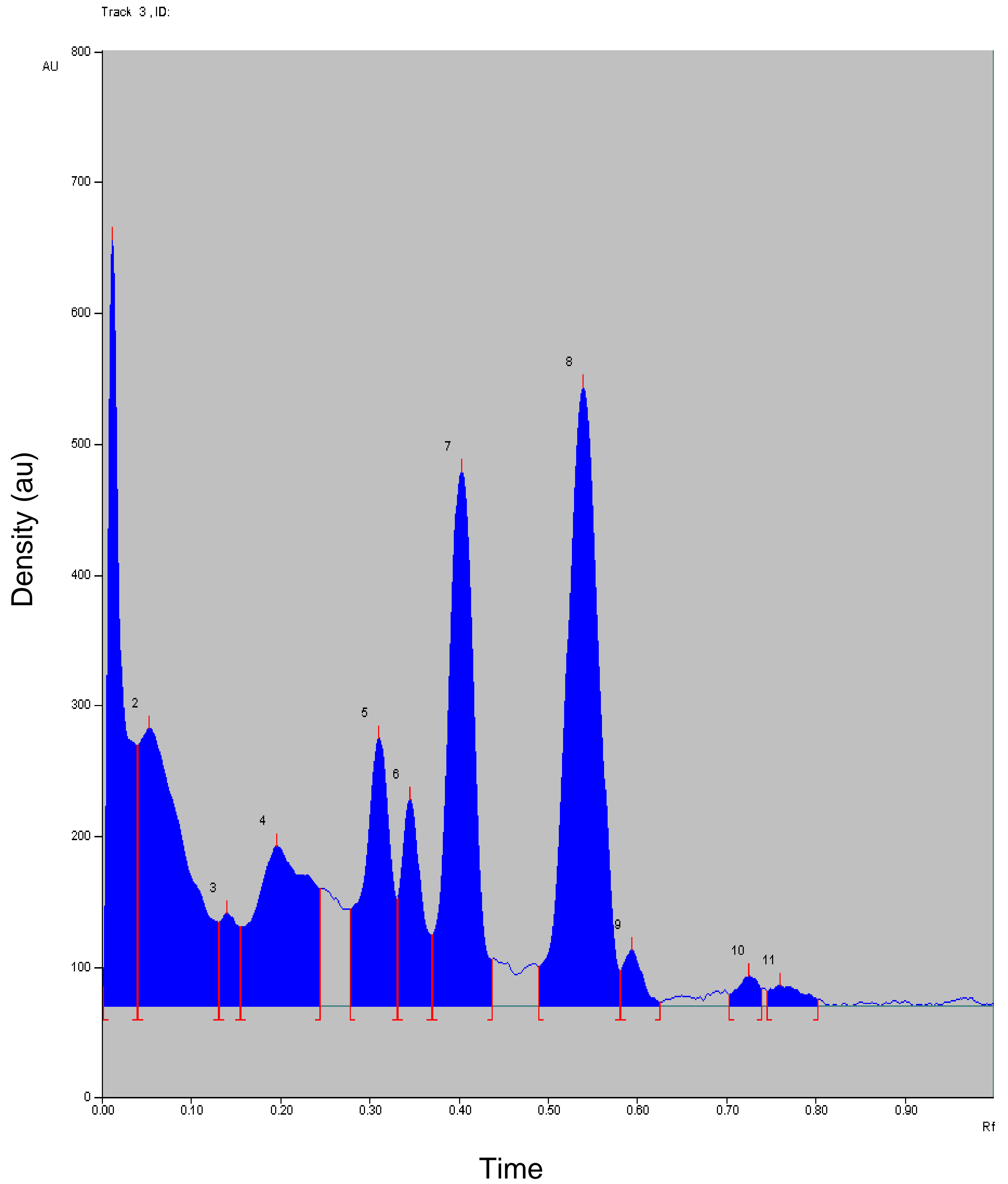


Figure 3c. Scan (366 nm) of HPTLC layer showing separation of phytochemicals in acetone extract of leaf.

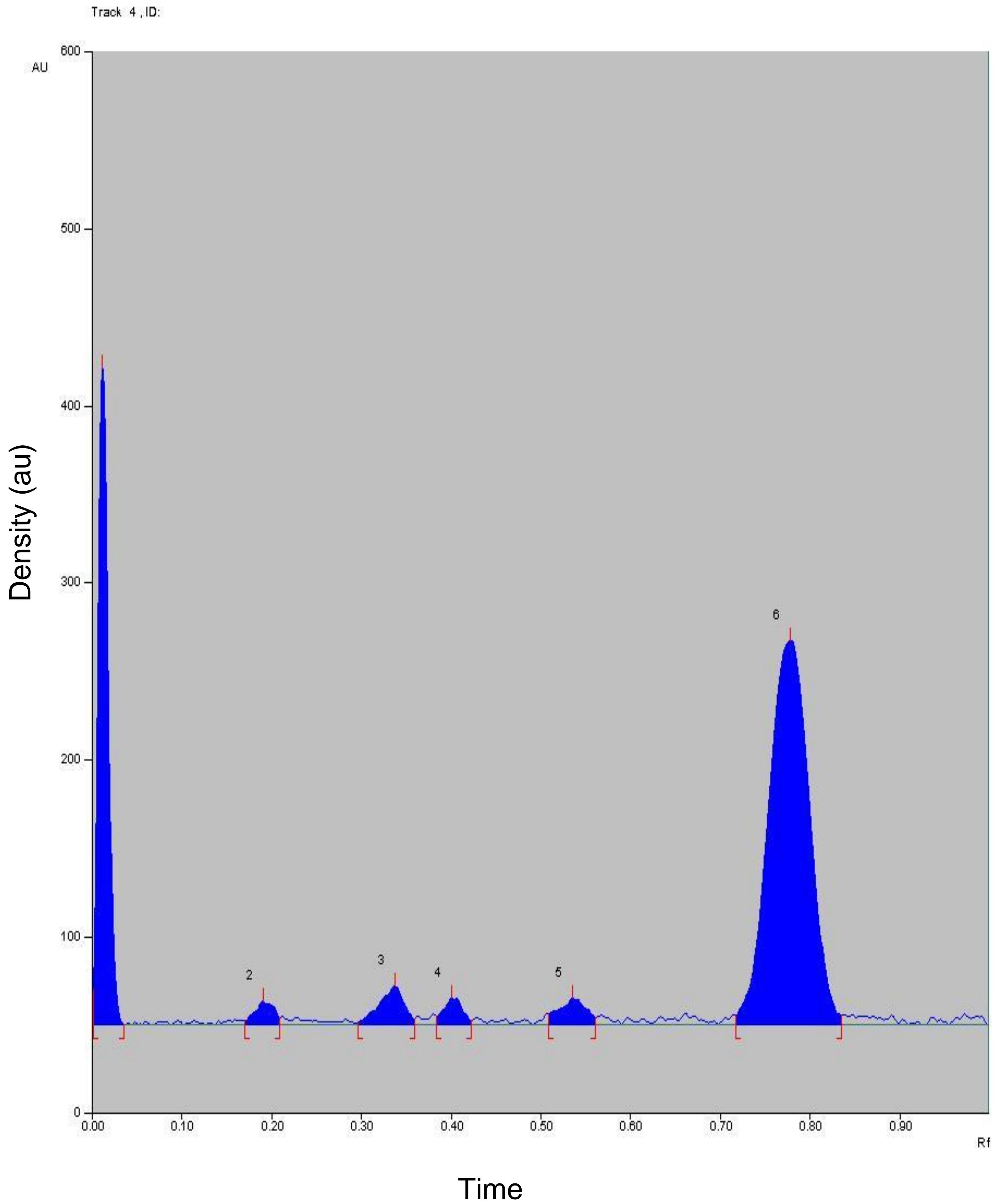


Figure 3d. Scan (366 nm) of HPTLC layer showing separation of phytochemicals in ethanol extract of Root.

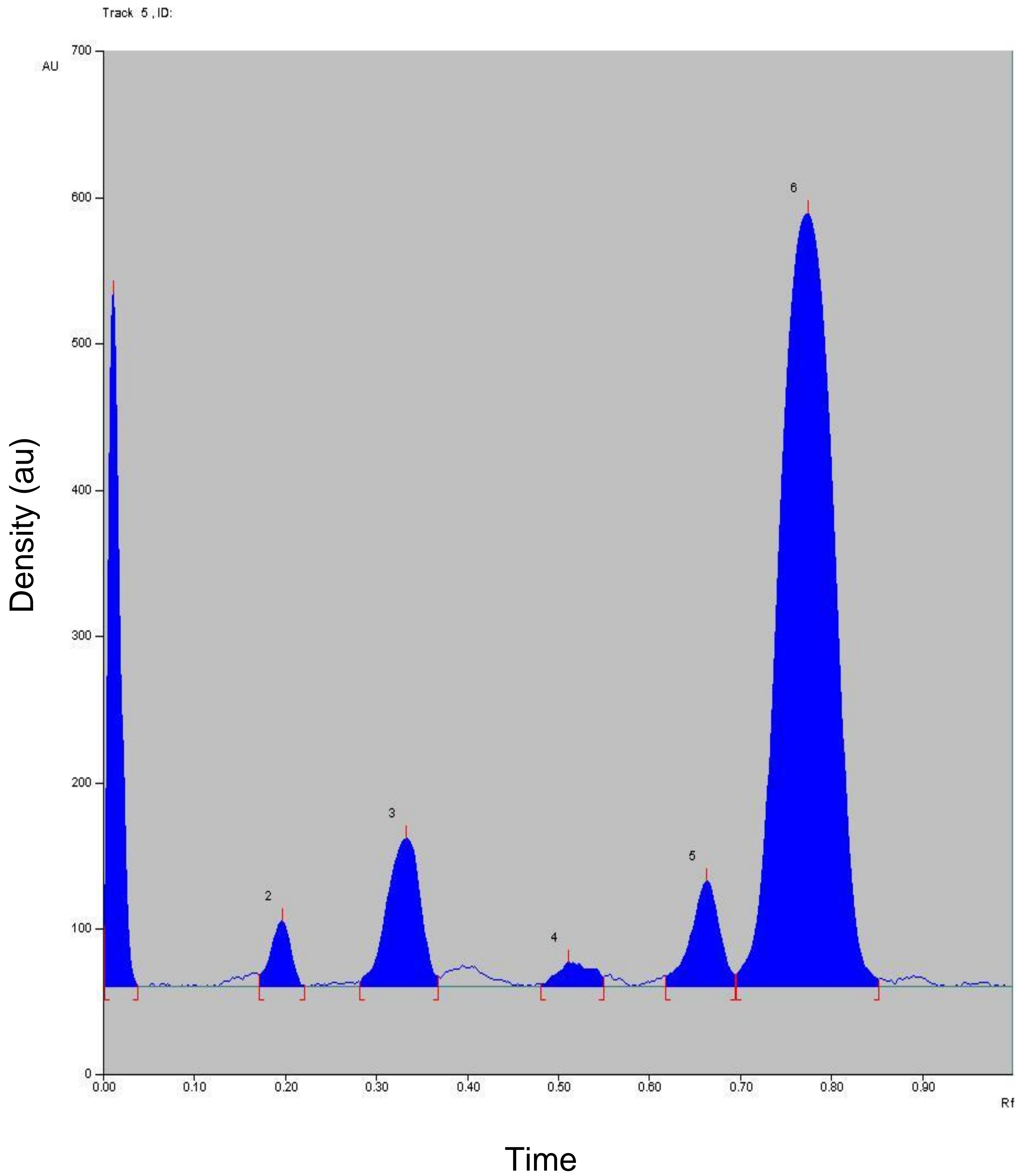


Figure 3e. Scan (366 nm) of HPTLC layer showing separation of phytochemicals in chloroform extract of root.

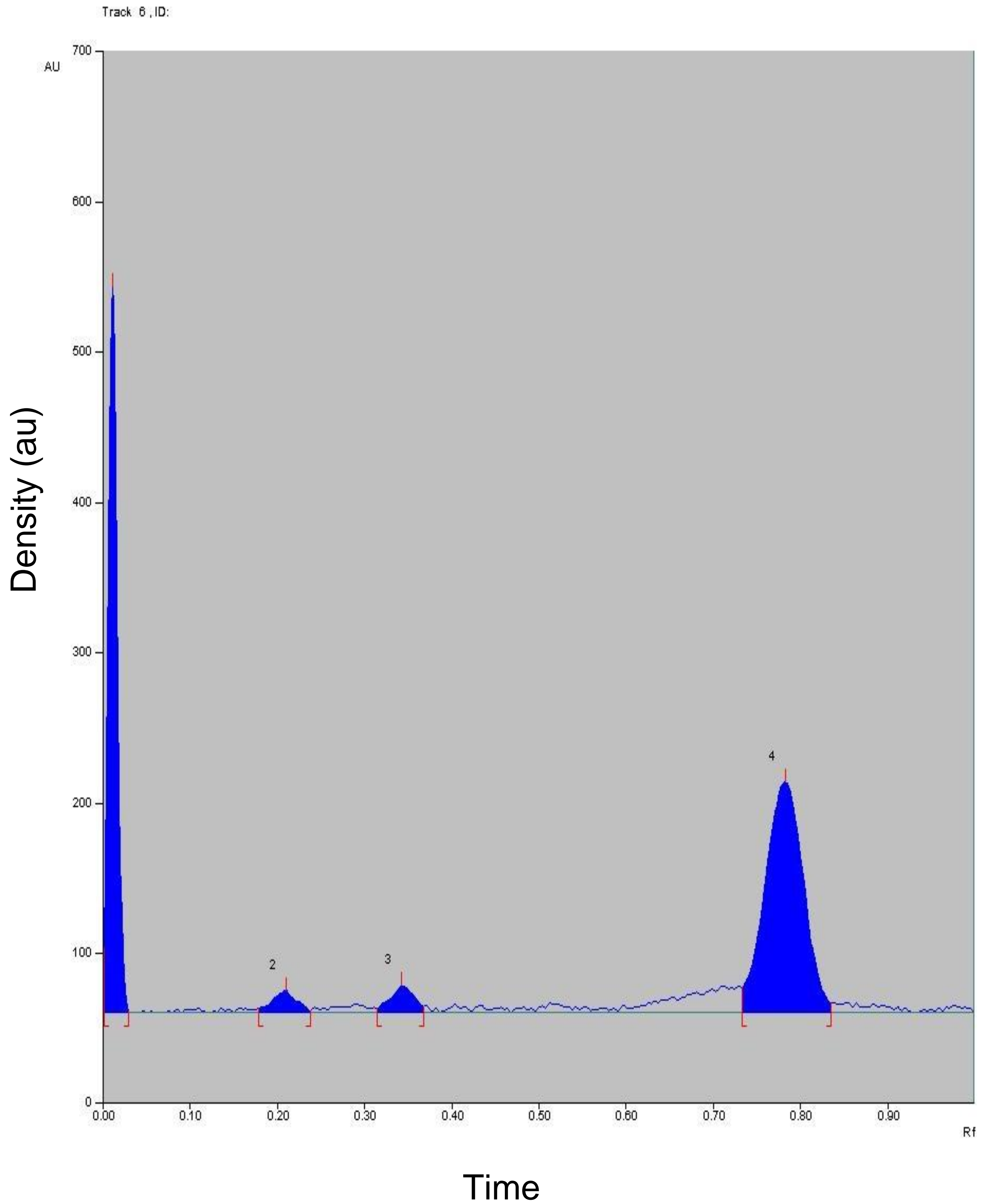


Figure 3f. Scan (366 nm) of HPTLC layer showing separation of phytochemicals in methanol extract of root.

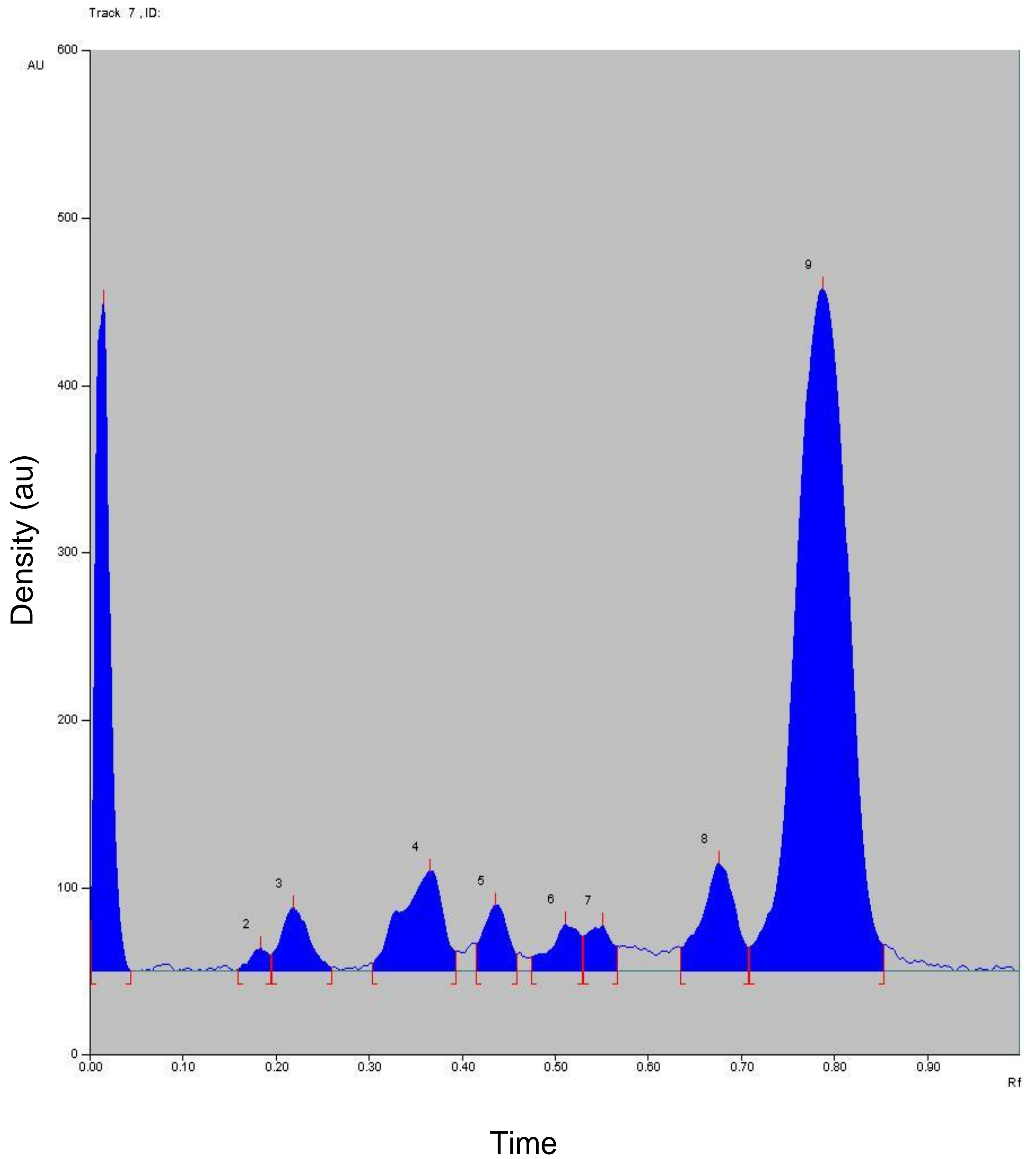


Figure 3g. Scan (366 nm) of HPTLC layer showing separation of phytochemicals in acetone extract of root.

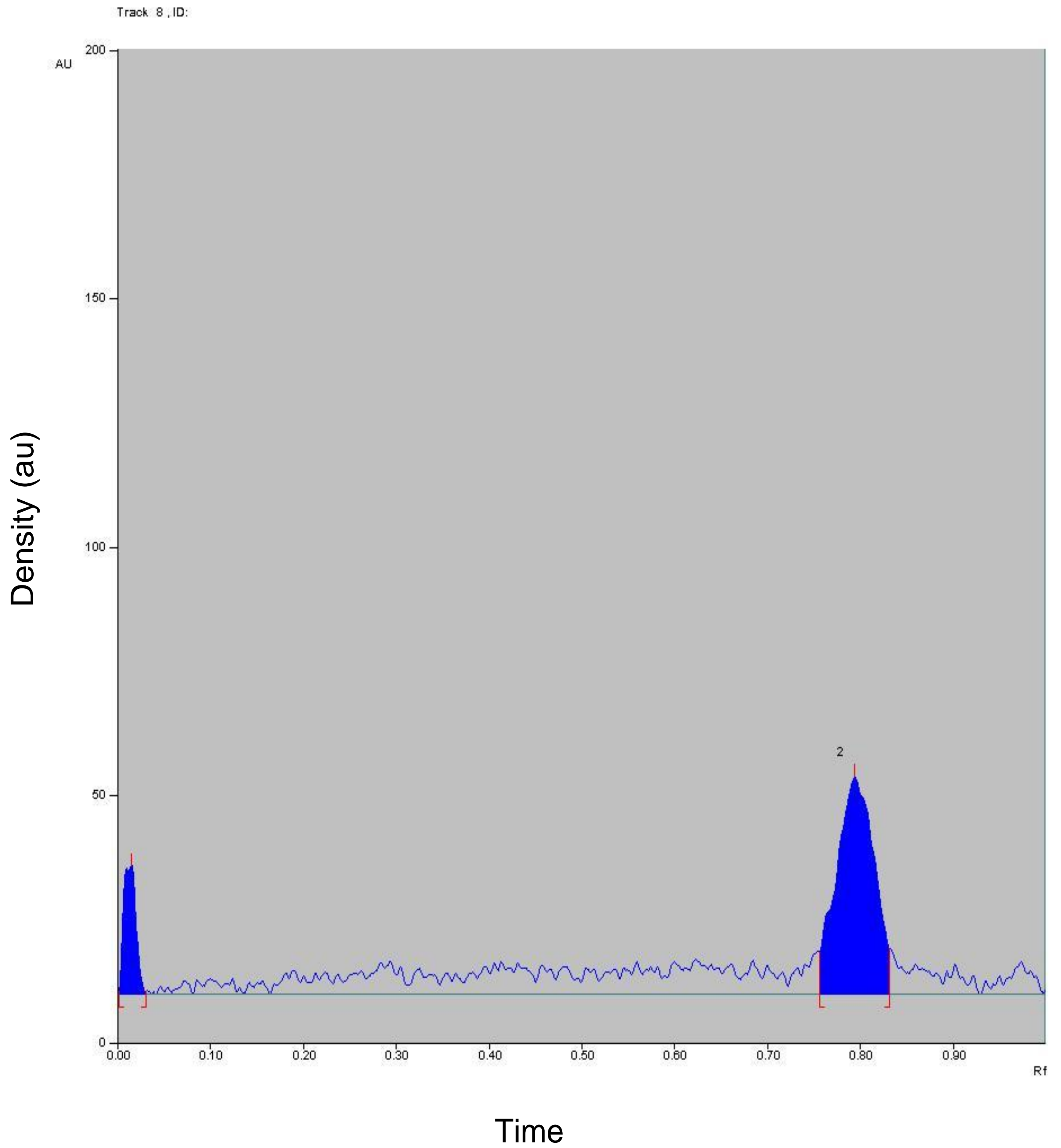


Figure 3h. Scan (366 nm) of HPTLC layer showing separation of phytochemicals in standard gibberillic acid.

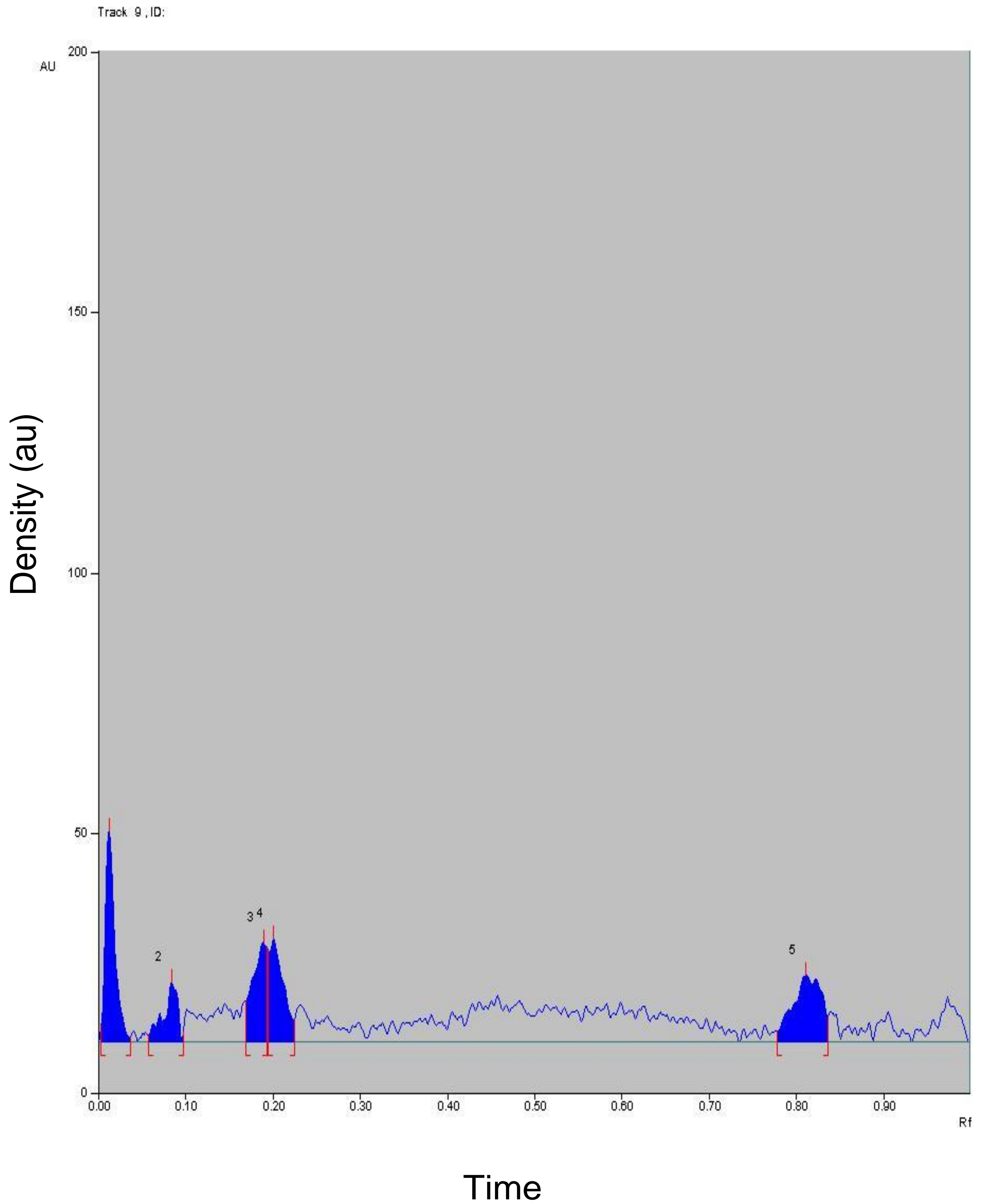


Figure 3i. Scan (366 nm) of HPTLC layer showing separation of phytochemicals in standard Quinol-R.

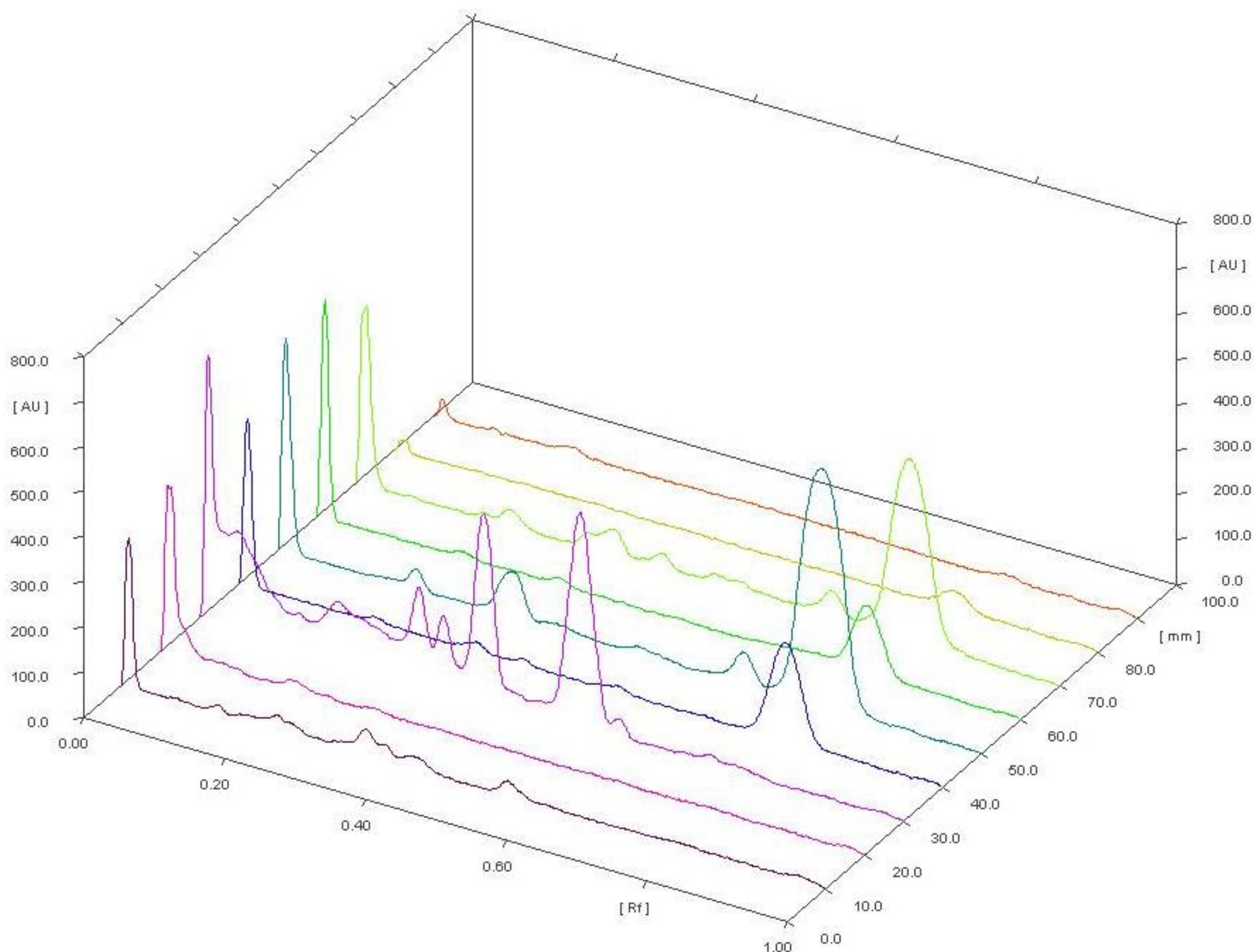


Figure 3j. Global peak data of different extracts of Plumbago. All tracks are at 366 nm.

Conflict of Interests

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

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Full Length Research Paper

Antibacterial activity of two triterpenes from stem bark of *Protorhus longifolia*

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Antibiotic resistance of pathogenic strains has hugely contributed to the wide spread of new and re-emerging infectious diseases. There is thus the need for the discovery of new (non-penicillin based) antibiotics. The triterpenes [3β-hydroxylanosta-9,24-dien-21-oic acid (1) and methyl-3β-hydroxylanosta-9,24-dienoate (2)] isolated from stem bark of *Protorhus longifolia* (Benrh.) Engl. were evaluated for their antibacterial activity against a panel of selected general and antibiotic resistant Gram positive and Gram negative bacteria. The antibacterial activity of the compounds was determined using disc diffusion method. The compounds exhibited antibacterial activity against most of the tested bacteria with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranging from 0.16 to 5.00 mg/ml and 0.63 to 5.00 mg/ml, respectively. The triterpenes did not exhibit any bacterial DNA damaging effects, but apparently affected the microbial cell membrane integrity. The triterpenes could be a potentially effective antimicrobial agent to combat infectious diseases.

Key words: Triterpenes, antibacterial activity, antibiotic resistance, DNA damage.

INTRODUCTION

Pathogenic microorganisms are the major cause of a wide range of infectious diseases, with high rate of mortality in humans resulting from bacterial infections (Tajbakhsh et al., 2011). Despite great progress made in the discovery of antibiotics, the development of bacterial resistance to some of the current antibiotics is a serious global challenge (Hoffmann et al., 2011; Chung et al., 2013). This challenge has then triggered a search for new effective antimicrobial compounds with novel mechanisms of action (Rojas et al., 2003; Zakaria et al., 2009). Plants have always been an untapped source to provide bioactive compounds as potential therapeutic agents, including antimicrobials. Thus, the wide chemical diversity of plant-derived compounds is important in the development of effective agents to combat infectious diseases resistant to conventional drugs (de León et al., 2005).

Protorhus longifolia (Benrh.) Engl. (Anacardiaceae) is a tall, ever green indigenous tree in Southern Africa. Stem bark of the plant has been traditionally used by the Zulus

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to cure various diseases such as heartwater and diarrhea in cows (Dold and Cocks, 2001). Antimicrobial (Suleiman et al., 2010) and anti-platelet aggregation (Mosa et al., 2011) activities of the crude extracts of the plant parts have been reported. Lanosteryl triterpenes isolated from the stem bark of *P. longifolia* have been reported to exhibit anti-platelet aggregation activity (Mosa et al., 2011).

Triterpenes are a versatile group of biologically active plant secondary metabolites widely distributed in the plant kingdom. They are predominantly found on plant surfaces such as leaf, stem bark and fruit waxes (Jäger et al., 2009). These compounds reportedly exhibit a wide spectrum of biological activities including anti-inflammatory (Ko et al., 2007; Mosa et al., 2011), antitumor (Gonzalez et al., 2002) and antimicrobial activity (Motlhanka et al., 2010; Kiplimo et al., 2011). The importance of plant derived triterpenes as new targets for drug development cannot be overemphasised. In this study, we evaluated antimicrobial activity of the triterpenes (3β-hydroxylanosta-9,24-dien-21-oic acid, and methyl-3β-hydroxylanosta-9,24-dienoate) isolated from stem bark of *P. longifolia.*

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all chemicals and reagents (of analytical grade) used were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

Plant collection

Fresh stem barks of *P. longifolia* were collected in March, 2012 from KwaHlabisa, KwaZulu-Natal, South Africa. The plant with the voucher specimen RA01UZ was confirmed by Mrs. N.R. Ntuli, Department of Botany, University of Zululand. The plant material was well cleaned with tap water, chopped into smaller pieces and air-dried. It was then powdered (2 mm mesh) and stored in a sterile brown bottle at 4°C till processing.

Extraction and isolation

The methods previously described by Mosa et al. (2011) were followed to extract and isolate the two triterpenes from stem bark of *P. longifolia*. Briefly, the powdered stem bark was defatted with *n*-hexane and the residue then extracted (1:5 w/v) with chloroform. Silica gel column chromatography (24 × 700 mm; Silica gel 60; 0.063 to 0.2 mm; 70 to 230 mesh ASTM; Merck, Darmstadt, Germany) was used to isolate the compounds from the chloroform extract (13 g). A hexane-ethyl acetate solvent gradient system (9:1 to 3:7) was used as the mobile phase, collecting 20 ml fractions. The collected fractions, analysed by thin layer chromatography (TLC) (silica gel 60 TLC aluminum sheets 20 cm × 20 cm, F_{254}) were combined based on their profile to yield 18 combined fractions (Fr. A-R). The ninth and fourteenth combined fractions were further separately purified with hexane and ethyl acetate to afford compounds 1 (0.72 g) and 2 (1.14 g), respectively. Stuart SMP 11 melting point apparatus (Shalom Instruments supplies, Durban, R.S.A) was used to determine melting point. Structures of the triterpenes were established and confirmed through the use of spectral techniques such as infrared (IR) (KBr, Perkin-Elmer 100 FTIR),1D and 2D nuclear magnetic resonance (NMR) techniques (^{1}H-^{1}H, ^{13}C-^{13}C, DEPT, COSY, HMQC, HMBC and NOESY) (in $CDCl_3$, Bruker 600 MHz). Chemical shifts were expressed in δ (ppm) (Appendices 1 and 2).

Compounds

Details of 3β-hydroxylanosta-9,24-dien-21-oic acid (1) have previously been given by Mosa et al. (2011) with estimated purity of more than 95%. Methyl-3β-hydroxylanosta-9,24-dien-21-oate (2) was obtained as white crystals, purity > 95% based on melting point, mp 204 to 205°C, IR (KBr) v_{max}= 3469, 1683 cm^{-1}. ^{1}H and ^{13}C NMR (Table 1), the data suggested the molecular formula $C_{31}H_{50}O_3$, MW 470.736.

Antimicrobial activity

Microorganisms

The Gram positive (*Staphylococcus aureus KZN)* and Gram negative (*Salmonella spp. KZN, Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa ATCC 19582, Proteus mirabilis* KZN) bacteria were obtained from the Department of Microbiology, University of Zululand. Antibiotic resistant strains of clinical isolates were obtained from the Lancet Pathology Laboratory (Durban, South Africa). Mueller-Hinton agar (Merck Catalogue No. 1.05435.0500) was used to maintain stock cultures and these were kept at 4°C.

Disk diffusion method

Antimicrobial activity of the triterpenes was investigated by the agar disc diffusion method (Vlietinck et al., 1995). Overnight bacterial cultures were diluted to a final cell density (1.0 × 10^8 CFU/ml) equivalent to 0.5 McFarland standard. Sterile paper discs (6 mm diameter), impregnated with the triterpene (20 mg/ml 10% DMSO) were placed on nutrient agar which was inoculated with bacterial suspension (1.0 × 10^8 CFU/ml). Dimethyl sulphoxide (DMSO) (10%) was used as negative control while ampicillin and neomycin were used as positive controls. The inoculated plates were incubated at 37°C for 24 h. The antimicrobial activity was evaluated by measuring the zone of inhibition (mm) against the test organism.

Minimum inhibitory concentration (MIC) and Minimum bacterial concentration (MBC)

The method previously described by Eloff (1998) was adopted to determine the MIC of the triterpenes. Overnight bacterial cultures in Muller-Hinton broth were standardized to 0.5 McFarland standard and 96-well plates were used to quantitatively determine the MIC and MBC of the compounds. The tests were replicated thrice and the mean values reported. Nutrients broth (50 µl) was added to all wells of the 96-well plate and 50 µl of the triterpene (20 mg/ml, in 10% DMSO) was introduced to the wells in the first row (A) and

Table 1. ^{13}C-NMR data and significant ^{1}H-NMR data of compound 2. Chemical shifts are expressed in δ (ppm).

Position	δ_C (ppm)	Type	δ_H (ppm)
1	30.4	CH_2	-
2	23.9	CH_2	-
3	74.3	CH	4.24 (1H, s, OH)
4	37.4	C	-
5	44.4	CH	-
6	17.3	CH_2	-
7	25.8	CH_2	-
8	49.6	CH	-
9	145.9	C	-
10	34.7	C	-
11	118.3	CH	-
12	28.6	CH_2	5.13 (1H, t)
13	43.3	C	-
14	51.0	C	-
15	31.3	CH_2	-
16	27.0	CH_2	-
17	47.7	CH	-
18	13.3	CH_3	-
19	21.7	CH_3	-
20	48.4	CH	-
21	177.3	C	-
22	34.7	CH_2	-
23	25.5	CH_2	-
24	125.6	CH	-
25	136.4	C	5.22 (1H, t)
26	17.4	CH_3	1.65 (3H, s)
27	25.8	CH_3	1.67 (3H, s)
28	21.9	CH_3	1.37 (3H, s)
29	27.4	CH_3	0.92 (3H, s)
30	22.1	CH_3	1.04 (3H, s)
$-OCH_3$	59.8	CH_3	3.86 (3H, s)

mixed well. Sample mixture (50 µl) was removed from all the wells in the row A to perform a 2-fold serial dilution down the rows. The last 50 µl was discarded. Respective bacterial cultures (50 µl) were introduced into the corresponding wells. DMSO (10%) was used as negative control while ampicillin and neomycin were used as positive controls. The plates were covered and then incubated at 37°C for 24 h. Iodonitrotetrazolium chloride (INT) (20 µl, 0.2 mg/ml) was added to all of the wells and the plates were then further incubated at 37°C for 30 min. The MIC was recorded as the lowest concentration of the tested compounds at which no visible microbial growth was observed. The MBC of the triterpenes was determined by removing 10 µl of each culture medium from the wells that no bacterial growth was observed onto sterile nutrient agar plates. The plates were then incubated at 37°C for 24 h, after which they were observed for presence or absence of growth. The MBC was recorded as the minimum concentration at which no bacterial growth recurred. Ampicillin and neomycin were used as positive controls.

Lactate dehydrogenase (LDH) release assay

The effect of the triterpene on the bacterial membrane integrity was evaluated by using the cytosolic LDH release assay (Tadić et al., 2012). Fresh cultures of the most susceptible bacteria were treated and incubated (at 37°C for 24 h) with the MBC of the compound. The bacterial suspension was centrifuged at 5000 × g for 5 min. Supernatant (100 µl) was collected and mixed with 100 µl of reaction mixture (54 mM lactic acid, 0.28 mM of phenazinemethosulfate, 0.66 mM INT, 1.3 mM NAD^+). The mixture was incubated at 37°C for 10 min. DMSO (10%) was used as negative control and 3% triton X-100 was used as positive control. The pyruvate-mediated reduction of INT into highly-coloured formazan (red) was measured at 490 nm using BioTek plate reader (ELx 808 UI, Biotek Instrument Supplies). The experiment was replicated three times and the mean values reported. The amount of LDH (%) released upon cellular loss of membrane integrity was calculated using the formula:

$$\text{LDH released} = [(A_E - A_C) / (A_T - A_C)] \times 100$$

Where A_E- absorbance of test compound-treated cell culture, A_C-absorbance of control (cell medium alone) and A_T-absorbance of Triton X-100 lysed cells [representing maximal (100%) LDH released].

Bacterial DNA damage

The method described by Liu et al. (2011) was adapted to determine the effect of the triterpenes on bacterial DNA. This was determined on the bacteria that were most susceptible to the compounds. Fresh bacterial cultures were treated with the MBC of the compound. Both treated and untreated bacteria were incubated at 37°C for 24 h. Bacterial DNA was extracted and purified using ZR Fungal/bacterial DNA MiniPrep™ kit (Zymo Research, USA, Catalogue No. D6005). Briefly, bacterial samples were added directly to ZR lysis tubes and centrifuged at 10 000 × g for 10 min. The pellet was collected and resuspended in the lysis solution for 5 min. Fungal/bacterial DNA binding buffer was then added to the suspension. This was centrifuged at 10,000 × g for 10 min and supernatant was collected. The bacterial DNA was precipitated following a series of centrifugation and washing in buffers. Pure DNA was then eluted with the elution buffer. Agarose gel electrophoresis (150 v for 30 min) was used to evaluate damage of both untreated and triterpene-treated DNA. This was run along with DNA maker. The DNA was visualized using a vilberlourmate Gel documentation system.

RESULTS AND DISCUSSION

Structural elucidation and characterization

The structures of the isolated compounds (Figure 1) were established and confirmed through ^{1}H and ^{13}C NMR. The physical and spectral data of 3β-hydroxylanosta-9,24-dien-21-oic acid (1) have been previously described (Mosa et al., 2011). The IR spectrum showed absorption bands for hydroxyl (3360, 2581 cm^{-1}) and carbonyl (1702

Table 2. Zones of inhibition (mm) of the triterpenes on some sensitive and antibiotic resistant bacteria.

Bacteria	Resistance	Compd 1	Compd 2	Ampicillin	Neomycin
E. coli (ATCC 8739)	-	10±0.01	13±0.12	14±0.07	12±0.05
Salmonela spp. (KZN)	-	12±0.00	15±0.10	11±0.01	10±0.09
P. aeruginosa ATCC 19582	-	13±0.20	15±0.45	14±0.15	13±1.00
S. aureus (KZN)	-	13±0.03	12±0.01	14±0.01	13±0.01
P. mirabilis (KZN)	-	11±0.05	14±0.03	12±0.00	11±0.10
P. aeruginosa T3374	Cotrimoxazole	8.0±1.00	13±0.90	11±0.01	14±1.20
S. aureus P12724	Cipro: Levo, Clindamycin	11±1.01	13±0.60	10±0.24	11±0.50
S. aureus B10808	Oxa: Meth, Penicillin	11±0.00	16±1.14	13±0.10	9.0±0.10
S. aureus P12702	Cipro: Levo, Clindamycin	12±0.15	15±0.10	13±1.50	12±0.08
Streptococcus viridians S17141	Oxa: Meth, Oxa: Clox	10±0.50	16±1.24	8.0±0.00	11±0.25

The results are expressed as mean ± SD. Compd- Compound, Clox- Cloxacillin, Cipro- Ciprofloxacin, Levo- Levofloxacin, Meth- Methicillin, Oxa- Oxacillin.

cm^{-1}) functional groups which further confirmed the structure. The ^{1}H-NMR of the compound (2) followed the same triterpenoid pattern with a large clusters of signals of CH_3, CH_2 and CH between δ_H 2.5 and 0.8 observed in 3β-hydroxylanosta-9,24-dien-21-oic acid (Mosa et al., 2011). The ^{13}C-NMR of this compound was also similar to that of 3β-hydroxylanosta-9,24-dien-21-oic acid, with the presence of four olefinic carbon atoms between 145 to 118 ppm, and five quaternary carbon atoms confirming the lanosteryl skeletal structure. The presence of an ester carbon atom at δ_C 177.3 instead of a carboxylic carbon at δ_C 181.5 suggested that this compound is the methyl ester of 3β-hydroxylanosta-9,24-dien-21-oic acid. Table 2 presents a detailed assignment of the ^{13}C-NMR and significant ^{1}H-NMR of the triterpene. The absorption bands for hydroxyl (3469 cm^{-1}) and carbonyl (1683 cm^{-1}) functional groups observed on IR spectrum also further assisted in confirming the NMR structure.

Biological activity

Plants have always been an untapped source to provide structurally diverse bioactive compounds as potential therapeutic agents, including antimicrobials. The antibacterial activity of the triterpenes from *P. longifolia* is shown in Tables 2 to 4. The two triterpenes exhibited antibacterial activity against both Gram negative and Gram positive bacteria with the MIC and MBC values ranging from 0.16 to 5.00 mg/ml and 0.63 to 5.00 mg/ml, respectively. It is noteworthy that the triterpenes were active against the antibiotic resistant clinical isolates; the *P. aeruginosa and Staphylococcus* spp. being the most susceptible organisms. Triterpenoids from *Momordica balsamina* (Ramalhete et al., 2011), *Alisma orientale* (Jin et al., 2012) and *Carpobrotus edulis* (Martins et al., 2011) also exhibited antibacterial activity on various resistant bacterial strains including the *Staphylococcus* spp. Popova et al. (2009) also reported the antibacterial activity of lanostane triterpenes from wood-decay fungus *Fomitopsis rosea* against *S. aureus*. The ability of both compounds to exhibit strong activity even on the resistant bacterial strains indicates their potential to be developed into effective antimicrobial agents.

Antimicrobial drugs exert their therapeutic activity through various mechanisms which include inhibition of proteins, cell wall, cell membrane and nucleic acid synthesis (Riaz et al., 2011). The antimicrobial activity of terpenoids has been associated with among others, bacterial cell membrane disruption by the lipophilic compounds (Saleem et al., 2010). The evaluation of the bacterial DNA damaging effect of the triterpenes indicated that the investigated triterpenes did not exhibit any DNA damaging effect. DNA protective effect of some triterpenes has previously been reported (Ramos et al., 2010; Smina et al., 2011). The cytosolic lactate dehydrogenase (LDH) release assay was used to evaluate the effect of the compound 2 (compound 1 was not tested due sample limitation) on the bacterial cell membrane integrity. The release of the cytosolic LDH into the extracellular medium is indicative of the cell membrane damaging effect of the triterpene. It is noteworthy that despite the complex Gram negative bacteria cell wall, the highest percentage LDH release was observed in case of *P. mirabilis* (89.6%) and *E. coli* (76.3%) (Table 5). While some compounds act by damaging both membrane and DNA (Liu et al., 2011), it is apparent that the triterpenes exert their antibacterial activity by affecting the microbial cell membrane integrity rather than damaging DNA.

Conclusion

The two triterpenes (3β-hydroxylanosta-9,24-dien-21-oic acid and methyl-3β-hydroxylanosta-9,24-dien-21-oate)

Table 3. MIC and MBC in mg/ml of the triterpenes on the general bacteria.

Bacteria	Compound 1		Compound 2		Ampicillin		Neomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli (ATCC 8739)	1.25	1.25	1.25	1.25	0.16	1.25	0.63	2.50
Salmonela spp. (KZN)	0.16	1.25	1.25	2.50	0.63	0.63	0.31	1.25
P. aeruginosa ATCC	0.63	5.00	0.63	5.00	0.16	0.63	1.25	1.25
S. aureus (KZN)	0.31	2.50	0.16	1.25	0.31	1.25	0.16	0.31
P. mirabilis (KZN)	1.25	5.00	0.31	1.25	0.16	0.31	0.31	2.50

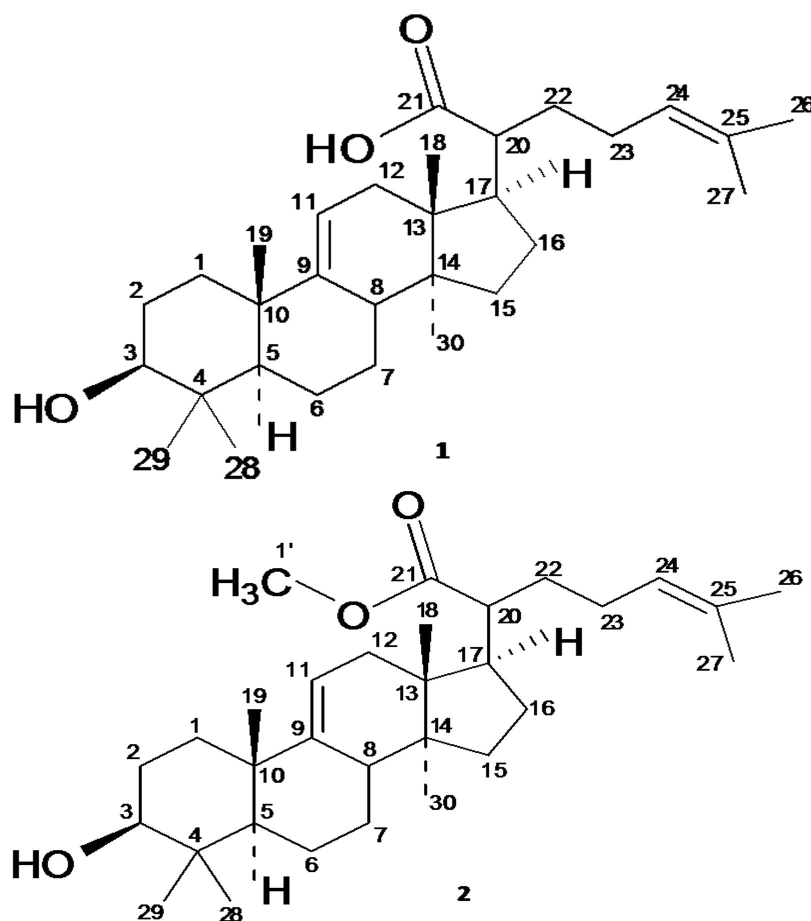

Figure 1. Chemical structures of 3β-hydroxylanosta-9,24-dien-21-oic acid (1) and methyl-3β-hydroxylanosta-9,24-dien-21-oate (2).

from *P. longifolia* possess antibacterial activity towards both Gram negative and Gram positive bacteria. The bactericidal activity of the compounds even on the antibiotic resistant strains indicates their potential as effective antimicrobial agents to combat infectious diseases resistant to conventional drugs. Further study to show the efficacy of the compounds in animal models is recommended.

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Conflict of Interest

Authors declare no conflict of interest.

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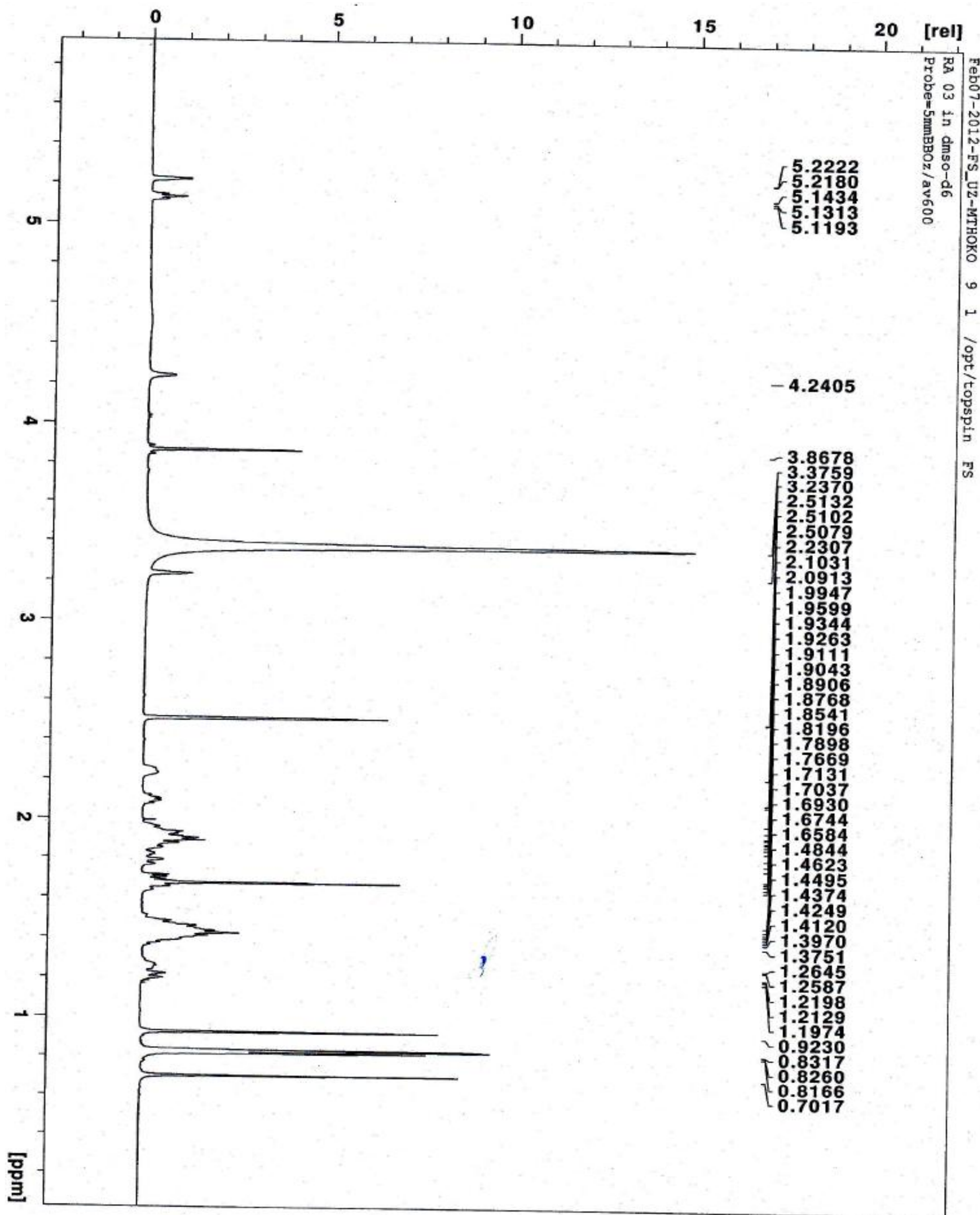
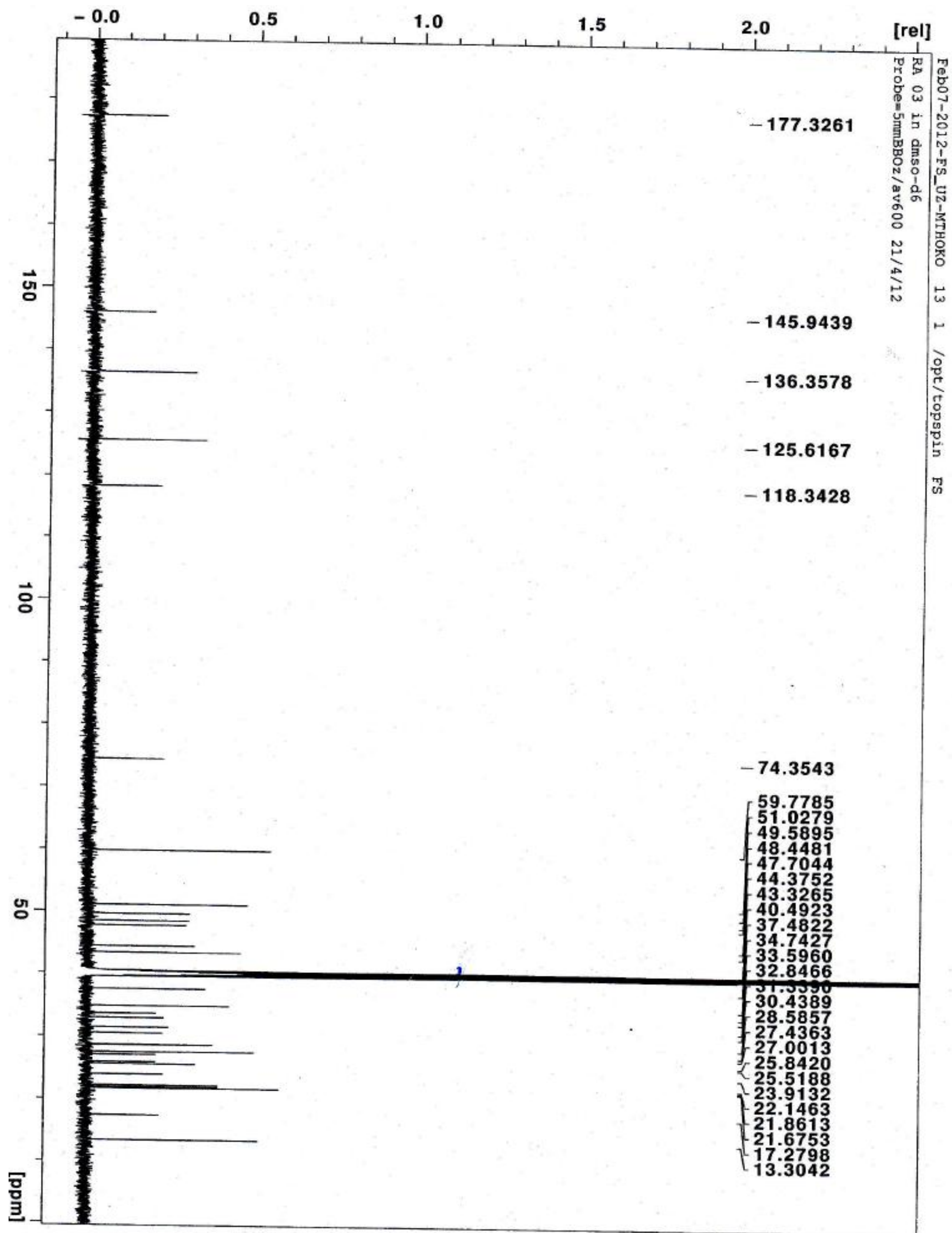


Appendix 1. ^{1}H Spectra of compound 2.



Appendix 2. ^{13}C-NMR Spectra of compound 2.

Related Journals Published by Academic Journals

- *African Journal of Pharmacy and Pharmacology*
- *Journal of Dentistry and Oral Hygiene*
- *International Journal of Nursing and Midwifery*
- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Toxicology and Environmental Health Sciences*

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