

## Research Article

# HPTLC Fingerprint Profile and Identification of Antidiabetic and Antioxidant Leads from *Bauhinia rufescens* L

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Diabetes is one of the world's major health problems, and many reports have supported the role of oxidative stress in the pathogenesis of both type 1 and type 2 diabetes. The present study aims to evaluate the antidiabetic and antioxidant activity of *Bauhinia rufescens*, a plant used in Sudanese folkloric medicine for the treatment of diabetes. It was also aimed to identify isolates and characterize the bioactive antidiabetic and antioxidant compounds using bioactivity-guided fractionation followed by high-performance thin-layer chromatography (HPTLC) autography, liquid chromatography-mass spectrometric analysis, and nuclear magnetic resonance (NMR). Two potential compounds were successfully isolated and identified which may provide new leads for more potent analogues in drug discovery.

## 1. Introduction

In recent years, the interest in folk medicine from different cultures has increased significantly in industrialized countries due to the fact that many prescription drugs worldwide have originated from the tropical flora [1].

Diabetes mellitus is a group of disorders with multiple etiologies resulting from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyperglycemia (very high blood glucose levels) with disturbances in carbohydrate, fat, and protein metabolism. [2] Treatment of diabetes mellitus is considered as the main global problem, and successful treatment has yet to be discovered. Traditional medicines might provide useful sources for the development of new drugs for treatment of diabetes mellitus and its complications [3]. Medicinal plants, mostly with antioxidant activity, are the major source of drugs for the treatment of oxidative stress-induced diabetic complications since they have no or only few side effects [4]. Several plant extracts with hypoglycemic properties and protective activities act either through their positive impact

on glucose homeostasis in diabetic patients or by lowering blood pressure and improving the renal and cardiovascular functions [5, 6]. The most common herbal active ingredients used in treating diabetes are flavonoids, tannins, phenolic, and alkaloids that improve the performance of pancreatic tissues by increasing the insulin secretion or decreasing the intestinal absorption of glucose [7]. The existence of these compounds implies the importance of evaluating the antidiabetic properties of these plants [8].

Plants belonging to the genus *Bauhinia* have been reported as having potent antioxidant, antidiabetic, anti-inflammatory [9, 10, 11], and other biological properties. [12, 13]. The leaves and stem bark of these plants are used in different phytopreparations to lower blood glucose levels, and they are also frequently used in folk medicine to treat infectious diseases, and several experimental studies have confirmed their antimicrobial potential [14, 15, 16, 17, 18].

The plant *Bauhinia rufescens* Lam common name Kharoub (family: Fabaceae) has been used traditionally in Sudan, Nigeria, and Senegal for various pharmacotherapeutic purposes. However, till date, there are no reports on

scientific validation of its traditional claim neither its bioactivity. Hence, it was thought worthwhile to determine its antioxidant and antidiabetic potential and isolate and characterize the bioactive compounds.

## 2. Materials and Methods

**2.1. Chemicals and Reagents.** All chemicals and reagents were of analytical grade quality. Methanol, chloroform, petroleum ether, acetonitrile, toluene, ethylacetate, and DMSO were obtained from S.D. Fine Chemical Limited, Bombay, India. Formic acid from MRS Scientific, UK, DPPH from Sigma-Aldrich Company, USA, and Silica gel  $G_{60} F_{254}$  from S.D. Fine Chemical Limited, Bombay, India, were obtained

**2.2. Equipment.** Chromatographic analysis was carried out using Waters ACQUITY UPLC<sup>(TM)</sup> system (Waters Corp., MA, USA). Silica gel 60 F254 precoated HPTLC plates,  $10 \times 10$  cm, were used (Merck, Germany). Camag Linomat-V applicator was also used (CAMAG, Switzerland). <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were carried out on the Bruker AM 500 spectrometer (Germany) operating at 700 MHz (<sup>1</sup>H-NMR) and 125 MHz (<sup>13</sup>C-NMR) in a spectroscopic grade DMSO-d<sub>6</sub>.

**2.3. Chromatographic Conditions.** Separation was performed on a UPLC system equipped with a binary solvent delivery system, an auto-sampler, a column manager, and a tunable MS detector (Waters, Manchester, UK). The system was operated under the Empower software (Waters, USA). Data acquisition has been done in positive modes. Chromatography was performed using acetonitrile (A) and 0.5% v/v formic acid in water (B) as the mobile phases on the monolithic capillary silica-based C<sub>18</sub> column (ACQUITY UPLC<sup>(R)</sup> BEH C<sub>18</sub> 1.7 μm, 2.1 × 100 mm), with the pre-column split ratio 1 : 5 and a flow rate 10 μL/min at ambient temperature.

Different solvent systems were investigated in order to obtain suitable elution in TLC plates. Toluene : ethyl acetate : formic acid (5 : 4 : 1, v/v/v) was finally selected as the specific solvent.

**2.4. Plant Material.** The plant was collected from Northern Kordofan and authenticated at the herbarium of the Medicinal and Aromatic Plants Research Institute (MAPRI), and a voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Khartoum.

**2.5. Preparation of Plant Extract.** Air-dried leaves powder (1 kg) was exhaustively extracted with 500 ml petroleum ether using a soxhlet apparatus. The extract was then concentrated under reduced pressure using a rotary evaporator. The remaining marc was dried and extracted using 500 ml chloroform and then by 500 ml methanol. Both chloroform

and methanol extracts were concentrated under reduced pressure using a rotary evaporator.

**2.6. Fractionation of the Active Extract.** The methanol extract (active extract) was fractionated with ethyl acetate, chloroform, and n-butanol (250 ml for each) sequentially after suspending it in water.

### 2.7. Antioxidant Assay

**2.7.1. DPPH Radical Scavenging Assay:** The DPPH radical scavenging was determined according to Brand et al., a standard method with some modification [19]. In a 96-well plate, the test extracts were dissolved in DMSO and allowed to react with 300 μM ethanolic solution of 2, 2-di (4-tert-octylphenyl)-1-picryl-hydrazyl stable free radical (DPPH) for half an hour at 37°C. After incubation, the absorbance was measured at 517 nm against a blank of DPPH in DMSO using a multiplate reader spectrophotometer. Propyl gallate was used as a standard drug at a concentration of 0.5 mM. All tests were run in triplicate, and percentage radical scavenging activity (%RSA) by samples was determined in comparison with a DMSO-treated control group using the following formula:

$$\% \text{RSA} = \left[ \frac{(A_c - A_t)}{A_c} \right] \times 100, \quad (1)$$

where RSA is the radical scavenging activity percent,  $A_c$  is the absorbance of the control, and  $A_t$  is the absorbance of the tested extract at 517 nm.

The obtained data were statistically analyzed and presented as mean ± standard deviation of the mean.

**2.7.2. Bioautography Method.** The extracts were run on the TLC silica gel  $G_{60} F_{254}$  plate using the specific solvent system. Plates were then air-dried, and visualization was conducted by spraying the plate with DPPH (0.001 g in 95% methanol/ethanol).

### 2.8. Antidiabetic Activity

**2.8.1. In Vitro Assay Using TLC Bioautographic Method (α-Amylase Inhibitors).** The in vitro assay of antidiabetic activity of plant extracts was determined according to Washim Khan et al., a standard method [20]. The ethyl acetate fraction (active fraction) was spotted on the TLC plate and developed using toluene : ethyl acetate : formic acid (5 : 4 : 1 v/v/v) as the mobile phase. After solvent migration, the TLC plate was dried and dipped into a previously prepared enzyme solution (alpha-amylase (200 unit) which was dissolved in a buffer solution (pH 7.15) and kept at 4°C). Incubation in a humid desiccator was firstly performed for 30 min at room temperature for the primary reaction between the enzyme and enzyme inhibitors, followed by another 15 min for enzyme substrate reaction and then air-dried and finally dipped in an iodine solution for the visualization.

**2.8.2. In Vivo Assay of Plant Extracts.** The in vivo assay of antidiabetic activity of plant extracts was also determined according to Washim Khan et al. [20]. Diabetes was induced in the rats by the administration of streptozotocin (in citrate buffer PH 5.5) intraperitoneally at a dose of 60 mg/kg. Three days later, the blood glucose level was measured. Animals with a blood glucose level around 300 mg/100 ml of blood were used. To test the effect of extracts in normal and streptozotocin diabetic rats, the animals were fasted for 12 hours and then were administered the extracts in doses of 1 g/kg intraperitoneally. Two hours later, the animals were anaesthetized with ether and blood was collected via a cardiac puncture. Blood glucose level was measured using the Reflotron instrument and the provided glucose kits (Roche Diagnostics).

### 2.9. Qualitative Analysis of the Active Fraction

**2.9.1. LC-MS Analysis.** LC-MS analysis was performed with the same methods followed by Washim Khan et al. [21]. Sample solutions (5 mg/mL) were filtered through a 0.2  $\mu$ M PTFE membrane filter and injected into the UPLC-MS chromatograph. Separation was achieved by stepwise gradients from 5% B to 100% B for 20 min. The flow rate of the nebulizer gas was set to 500 L/h, for cone gas it was set to 50 L/h, and the source temperature was fixed to 100  $^{\circ}$ C. The capillary voltages and cone voltage were set to 3.0 and 40 KV, respectively. For collision, argon was employed at a pressure of  $5.3 \times 10^{-5}$  torr. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the Mass Lynx V 4.1 software incorporated in the instrument. Data obtained from UPLC-MS were processed by Mass Lynx V4.1 (Waters, USA) and further used for metabolomics analysis of different samples. Separated metabolites present in different samples were tentatively identified based on their m/z ration and on the literature.

**2.9.2. High-Performance Thin-Layer Chromatography (HPTLC) Analysis.** HPTLC analysis was performed using Washim Khan et al. methods [20], where 5  $\mu$ l of the active fraction solution was separately applied to a 6 mm wide band on the HPTLC plates using a Camag Linomat-V automated TLC applicator with the nitrogen flow providing a delivery speed of 150 nL/s from the syringe. Plates were developed in a Camag Twin through a glass tank presaturated with the selected solvent system for 40 min. The plate was developed horizontally in a Camag horizontal developing chamber (10  $\times$  10 cm) at room temperature. The plate was scanned at different wavelengths such as 254 nm, 354 nm, and 454 nm. After heating the plate at 100  $^{\circ}$ C for 5 min, derivatization of the chromatogram was done with 5% anisaldehyde sulphuric acid in methanol. The scanning was carried out at 565 nm with a Camag TLC scanner III using the Wincats1.2.3 software.

**2.10. Isolation and Structural Elucidation of Active Compounds.** Isolation of some active antidiabetic and

antioxidant principles was achieved by preparative thin-layer chromatography (PTLC), and structure determination of the isolated compound was achieved by NMR. The chemical shift values are expressed in  $\delta$  (ppm) units using (TMS) as an internal standard, and the coupling constants ( $J$ ) are expressed in Hertz (Hz). Standard pulse sequences were used for generating COSY, HMQC, and HMBC spectra (2D experiments).

## 3. Results and Discussion

Although insulin therapy and oral hypoglycemic agents are the first line of treatment for diabetes mellitus, they have some side effects and fail to significantly alter the course of diabetic complications [22]; therefore, medicinal plants had been claimed to provide another source of diabetes treatment. Many reports have supported the role of oxidative stress in the pathogenesis of both type 1 and type 2 diabetes. Bioactivity-guided fractionation led to isolation and identification of different compounds with antidiabetic and antioxidant activity, and most of them are phenolic compounds, especially flavonoids. The antioxidant property of flavonoids was the first mechanism of action studied, in particular with regard to their protective effect against cardiovascular diseases [23]. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals that are probably involved in several diseases [24]. Mechanism of antioxidant action can include suppressing reactive oxygen species formation, either by chelating trace elements involved in free radical production, scavenging reactive species, and upregulating or protecting the antioxidant natural defenses [25]. Antioxidant activity of flavonoids has beneficial effect in treatment of diabetes, and especially many reports have supported the role of oxidative stress in the pathogenesis of both type 1 and type 2 diabetes. In the present study, the antioxidant and antidiabetic activities of our selected plant were performed for the first time.

**3.1. DPPH Radical Scavenging Assay of Plant Extracts.** The radical scavenging ability measured by the DPPH assay showed that extracts exhibited extremely large variation in their capacity to inhibit DPPH. As summarized in Table 1, the highest effect of free radical neutralization was obtained by the methanolic extract. It showed strong antioxidant activity (89%), almost similar in effect to the standard propyl gallate (92 %).

**3.2. Antidiabetic Assay of Plant Extracts.** The three different extracts of *Bauhinia rufescens*, petroleum ether, chloroform, and methanol extracts, were administered in a dose of 1 g/kg intraperitoneally into normal fasted rats and at the same above dose to streptozotocin-treated rats. The methanol extract clearly decreased the blood glucose level in normal fasted rats and streptozotocin-treated rats by  $27 \pm 3.1\%$  and  $16.9 \pm 1.7\%$ , respectively, after two hours following administration, while the chloroform and petroleum ether extracts did not show significant effect on the glucose level (Table 2).

TABLE 1: Antioxidant activity *B. rufescens* extracts.

Sample code	%RSA $\pm$ SD (DPPH)
Methanol extract	89 $\pm$ 0.01
Pet. ether extract	12 $\pm$ 0.01
Chloroform extract	13 $\pm$ 0.03
Propyl gallate	92 $\pm$ 0.01

TABLE 2: In vivo antidiabetic assay of *Bauhinia rufescens* extracts.

Treatment (1 g/kg)	Normal rats		Streptozotocin-treated rats	
	Blood glucose level mg/100 ml			
	Before	After	Before	After
<i>B. rufescens</i> pet. ether	98.3 $\pm$ 3.9	101.5 $\pm$ 3.7	289.4 $\pm$ 8.9	292.5 $\pm$ 5.9
<i>B. rufescens</i> chloroform extract	93.0 $\pm$ 4.6	99.3 $\pm$ 1.7	277.6 $\pm$ 4.3	290.5 $\pm$ 1.9
<i>B. rufescens</i> methanol extract	98.3 $\pm$ 2.9	71.7 $\pm$ 3.0	289.4 $\pm$ 7.9	240.5 $\pm$ 5.3

\* $P < 0.05$ .

**3.3. Fractionation of Active Methanol Extract and Activity Evaluation.** The obtained results revealed that the methanol extract has the best antioxidant and antidiabetic activities compared to the other extracts. Accordingly, the methanol extract was fractionated by a separatory funnel using different solvents. The fractions were then subjected to different tests to quantify their activities, isolate, and identify the active compounds.

The antioxidant activity of the four fractions was assayed using the DPPH radical scavenging assay. Table 3 summarizes the obtained results which revealed that the ethyl acetate fraction has potent antioxidant activity (90%).

TLC bioautographic method was then performed to detect the antioxidant compounds and the  $\alpha$ -amylase enzyme inhibitors which reflect the antidiabetic activity.

The active ethyl acetate fraction was spotted on the TLC plate and developed using the selected solvent. By spraying the plate with DPPH (0.001 g in 95% methanol/ethanol), a bright yellow or cream spot was shown against a purple background, which indicates the presence of compounds with antioxidant activity (Figure 1).

Furthermore, to investigate the antidiabetic activity, the TLC plate was spotted with the active fraction, eluted, and dipped in an iodine solution.  $\alpha$ -Amylase inhibitors were visible on the TLC plate by the appearance of a yellowish-greyish spot on a dark brown background (Figure 2).

**3.4. Isolation and Structure Determination of the Active Compounds (1) and (2).** From antidiabetic and antioxidant test results (Figures 1 and 2), it is clear that compounds 1 and 2 possess antidiabetic activity beside their activity against oxidative stress. For the isolation of these active compounds, the ethyl acetate fraction was subjected to preparative thin-layer chromatography (PTLC) using toluene : ethyl acetate : formic acid (5 : 4 : 1, v/v/v) as the mobile phase.

The structures of the isolated compounds were determined by  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra.  $^1\text{H}$ -NMR spectrum of compound 1 (Table 4) indicated the presence of two singlet aromatic protons, and each proton integrated for one proton at  $\delta$  6.44 and  $\delta$  6.77 and were assigned to H-6 and H-8,

TABLE 3: Antioxidant activity of different methanolic fractions.

Sample code	%RSA $\pm$ SD (DPPH)
Aqueous F	80 $\pm$ 0.01
Ethyl acetate F	90 $\pm$ 0.01
Butanol F	79 $\pm$ 0.01
Chloroform F	85 $\pm$ 0.01
Propyl gallate	92 $\pm$ 0.01

F: fraction.

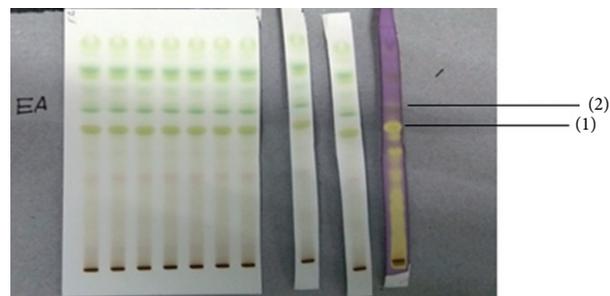


FIGURE 1: Bioautographic assay showing the inhibition of  $\alpha$ -amylase by some compounds developed on a silica gel G60 F254 plate which was eluted with toluene : ethyl acetate : formic acid (5 : 4 : 1, v/v/v) as the mobile phase.

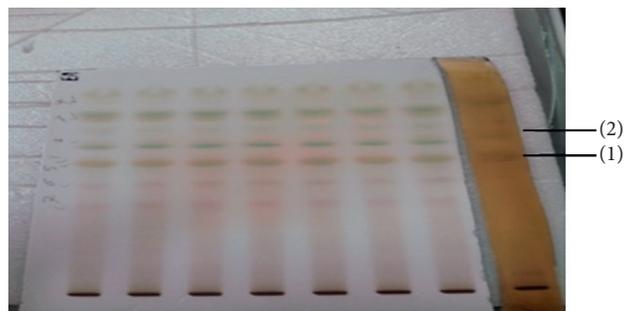


FIGURE 2: Bioautographic assay showing the inhibition of  $\alpha$ -amylase by some compounds developed on a silica gel G60 F254 plate which was eluted with toluene : ethyl acetate : formic acid (5 : 4 : 1, v/v/v) as the mobile phase.

TABLE 4:  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR assignments of compound 2 (700 MHz, DMSO) in comparison with reported data for rutin (quercetin-3-O-rutinoside) (100 MHz,  $\text{CD}_3\text{OD}$ ) [27].

Position	$\delta\text{H}$ compound (2)	$\delta\text{H}$ rutin	$\delta\text{C}$ compound (2)	$\delta\text{C}$ rutin
2		—	158.08	158.22
3		—	134.70	134.52
4		—	178.21	178.39
5		—	162.88	162.48
6	6.44	6.10 s	99.70	99.72
7		—	166.0	166.58
8	6.77	6.28 s	94.84	94.90
9		—	159.06	158.91
10		—	105.99	105.32
1'		—	120.73	122.77
2'		7.64 s	119.34	117.37
3'		—	144.00	144.32
4'		—	150.00	150.23
5'	6.90 d	6.85 d	115.67	115.46
6'	7.77 d	7.63 d	120.51	122.47
1''	5.22–5.27	4.96 d	103.69	103.63
2''	5.22–5.27	4.96 d	76.00	74.64
3''	5.22–5.27	4.96 d	70.65	—
4''	3.11–3.97 m	3.20–3.90 m	70.13	—
5''	3.11–3.97 m	3.20–3.90 m	70.01	—
1'''	4.69	4.50 d	101.53	101.92
2'''	4.69	4.50 d	71.79	71.32
3'''	4.69	4.50 d		
4'''	3.11–3.97 m	3.20–3.90 m		
5'''	3.11–3.97 m	3.20–3.90 m		
6'''	3.11–3.97 m	3.20–3.90 m		

$\delta$  = chemical shift in ppm,  $s$  = singlet,  $d$  = doublet, and  $m$  = multiplet.

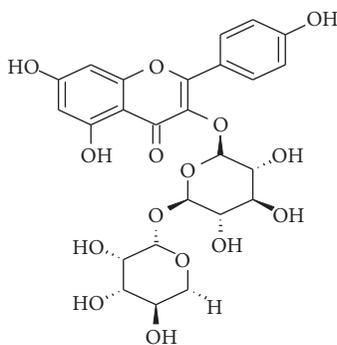


FIGURE 3: Chemical structure of rutin (quercetin-3-O-rutinoside).

respectively. Other aromatic proton areas were assigned for ring-B protons, and two doublet signals at  $\delta$  7.77 and  $\delta$  6.90 were assigned to H-6' and H-5', respectively.  $^1\text{H}$ -NMR spectrum also indicated the presence of a group of signals ranging between  $\delta$  5.2 and  $-5.27$  attributed to H-1'', H-2'', and H-3'' in rhamnose sugar. Furthermore, a multiplet protons signal appeared at  $\delta$  3.11–3.97 which assigned to the remaining rhamnose sugar, H-4'' and H-5''. The glucose sugar protons, H-1''', H-2''', H-3''', H-4''', H-5''', and H-6''', appear at  $\delta$  ranging between 3.11 and 4.69.

$^{13}\text{C}$ -NMR spectrum (Table 4) indicated the presence of ten quaternary carbons including one carbonyl group at  $\delta$  178.21 assigned to C-4. The downfield shift of C-4 indicated

the presence of a hydroxyl group at C-5 [26]. Four oxygenated aromatic carbons at  $\delta$  166.0, 162.88, 150.00, and 144.00 were assigned to C-7, C-5, C-4', and C-3', respectively. The other five remaining quaternary carbons at  $\delta$  159.06, 158.08, 134.70, 120.73, and 105.99 were assigned to C-9, C-2, C-3, C-1', and C-10, respectively.  $^{13}\text{C}$ -NMR spectrum experiment also indicated the presence of a group of signals ranging between  $\delta$  70.01 and 71.79 which were assigned for sugar protons.  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra of compound 1 were in full agreement with that reported for rutin (structure shown in Figure 3) [27].

$^1\text{H}$ -NMR spectrum of compound 2 (Table 5) showed that the spectrum is different from the previous identified

TABLE 5:  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR assignments of compound 1 (500 MHz, DMSO) in comparison with reported data for kaempferol-3-O-rutinoside (100 MHz, CD $_3$ OD) [27].

Position	$\delta\text{H}$ compound (2)	$\delta\text{H}$ kaempferol-3-O-rutinoside	$\delta\text{C}$ compound (2)	$\delta\text{C}$ kaempferol-3-O-rutinoside
2		—	158.08	155.98
3		—	134.70	134.58
4		—	178.21	177.23
5		—	162.88	162.04
6	6.44 d	6.22 d	99.70	98.88
7		—	166.0	163.31
8	6.77 d	6.33 d	94.84	93.98
9		—	159.06	156.82
10		—	105.99	104.79
1'		—	120.73	121.39
2'	8.01	8.19 d	130.96	130.76
3'	6.90 d	6.92 d	112.50	113.40
4'	—	—	160.41	160.92
5'	6.90 d	6.92	115.67	114.87
6'	7.77 d	8.19 d	131.00	131.03
1''	5.22–5.27	5.02 d	103.69	102.11
2''	5.22–5.27	5.02 d	76.00	74.83
3''	5.22–5.27	5.02 d	70.65	75.48
4''	3.11–3.97 m	3.15–3.90 m	70.13	69.23
5''	3.11–3.97 m	3.15–3.90 m	70.01	77.65
6''	3.11–3.97 m	3.15–3.90 m		67.08
1'''	4.69	4.45 d	101.53	100.10
2'''	4.69	4.45 d	71.79	
3'''	4.69	4.45 d		
4'''	3.11–3.97 m	3.15–3.90 m		
5'''	3.11–3.97 m	3.15–3.90 m		

$\delta$ =chemical shift in ppm,  $d$ =doublet, and  $m$ =multiplet.

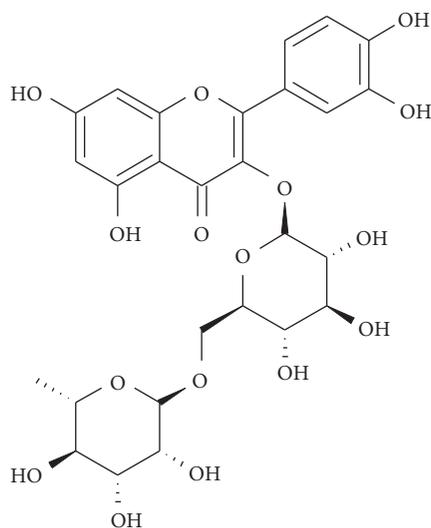


FIGURE 4: Chemical structure of kaempferol-3-O-rutinoside.

compound, in presence of two signals at  $\delta$  8.01 and  $\delta$  6.90 attributing to H-2' and H-3', respectively.

$^{13}\text{C}$ -NMR spectrum (Table 5) showed the presence of one oxygenated aromatic carbon at  $\delta$ 160.41 in ring-B

carbons. Two equivalent signals at  $\delta$ 130.96 and 131.00 were attributed to C-2' and C-3', respectively. The presence of two equivalent signals indicated clearly the presence of only one hydroxyl group in ring-B. Therefore, compound 2 was

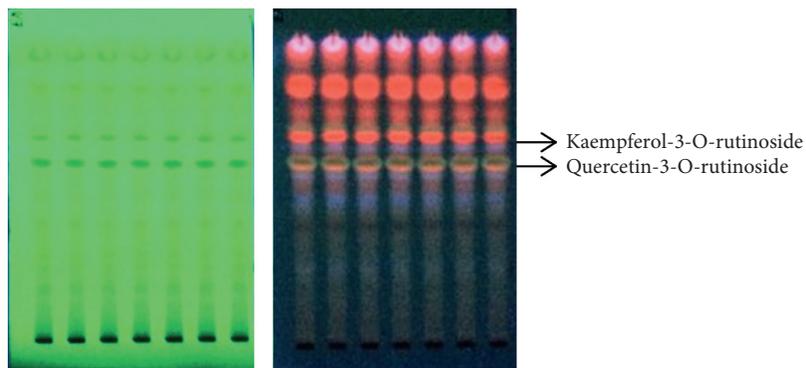


FIGURE 5: Thin-layer chromatogram of the ethyl acetate fraction as shown by a TLC scanner (254 and 366 nm), developed in toluene : ethyl acetate : formic acid (5 : 4 : 1, v/v/v).

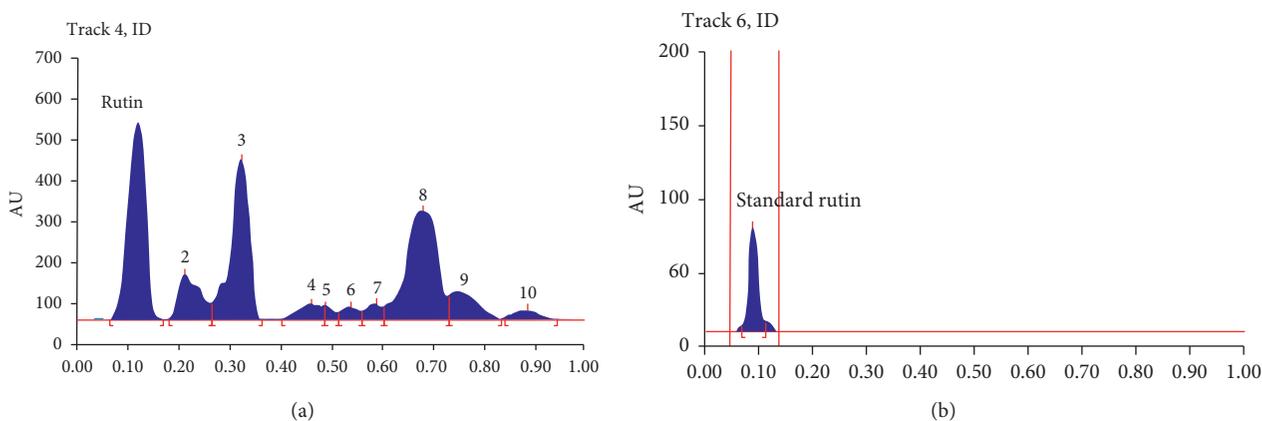


FIGURE 6: HPTLC analysis of the ethyl acetate fraction of *Bauhinia rufescens* on a silica gel plate, developed in toluene : ethyl acetate : formic acid (5 : 4 : 1, v/v/v), showing number of compounds and rutin as the standard.

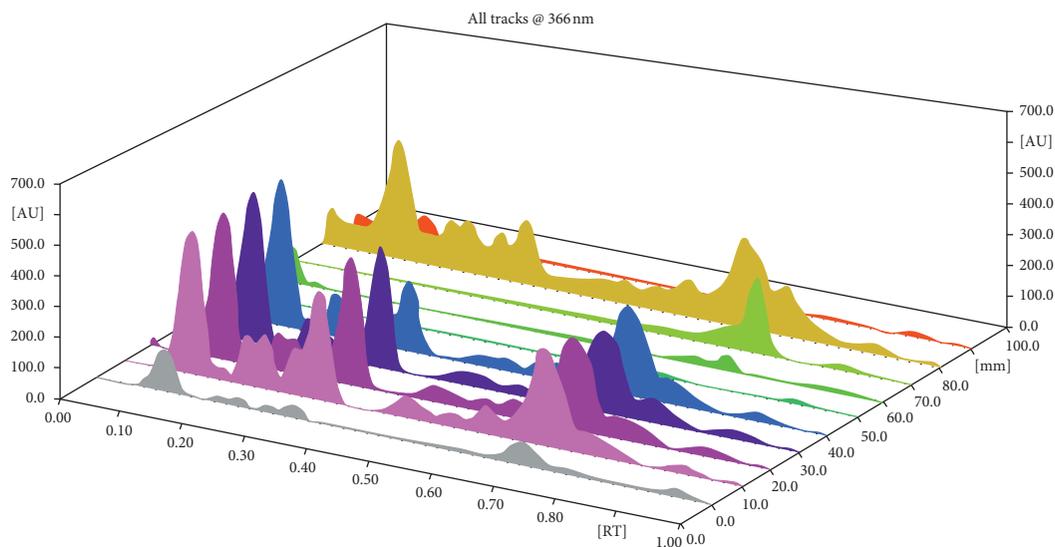


FIGURE 7: 3D graph of the HPTLC chromatogram.

TABLE 6: Predicted identified compounds using LC-MS.

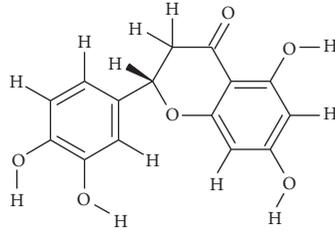
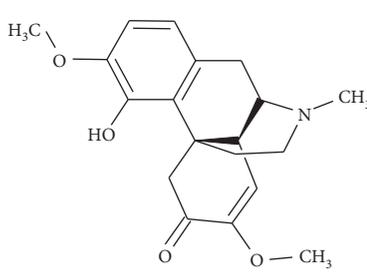
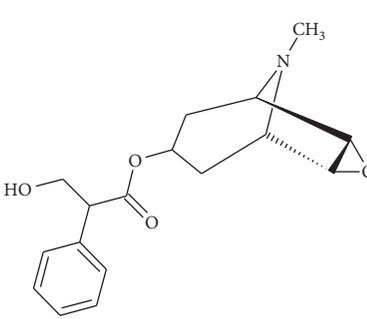
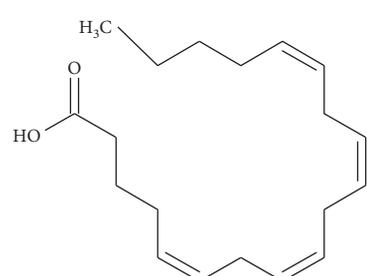
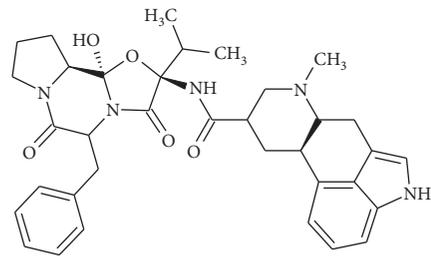
Sl. No.	m/z	Compound name	Structure	Mass bank no.
1	288.1740	Eriodictyol		PR020015
2	329.2419	Sinomenine		TY000053
3	302.9715	Scopolamine		KO009233
4	303.9746	Arachidonic acid		BML80765
5	611.0244	Dihydroergocristine		BML81110

TABLE 6: Continued.

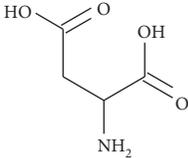
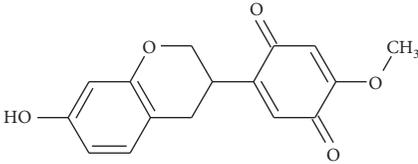
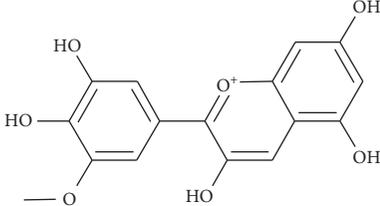
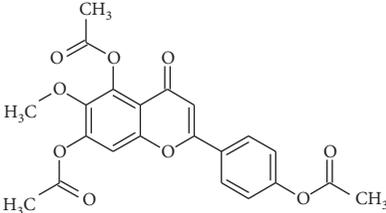
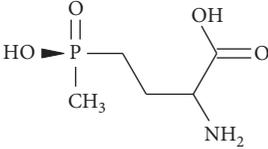
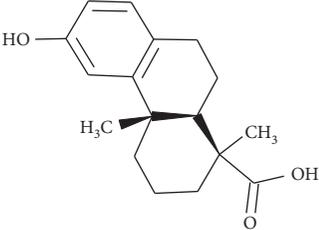
Sl. No.	m/z	Compound name	Structure	Mass bank no.
6	133.0590	Aspartic acid		BML80790
7	286.9800	Clausequinone		FIO00282
8	316.9833	Petunidin		PR100450
9	426.2412	Hispidulin acetate		TY000235
10	181.0699	Phosphinothricin		WA002712
11	274.2022	Podocarpic acid		BML81985

TABLE 6: Continued.

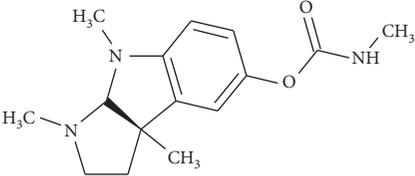
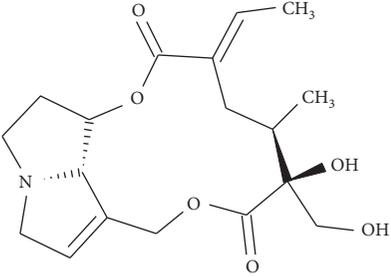
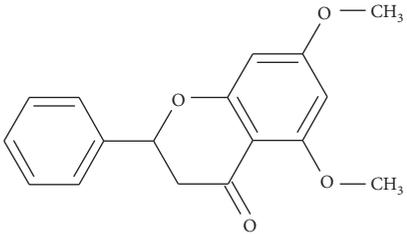
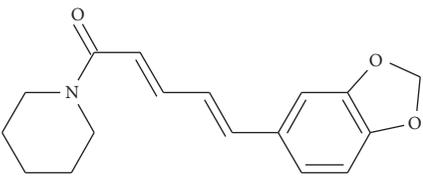
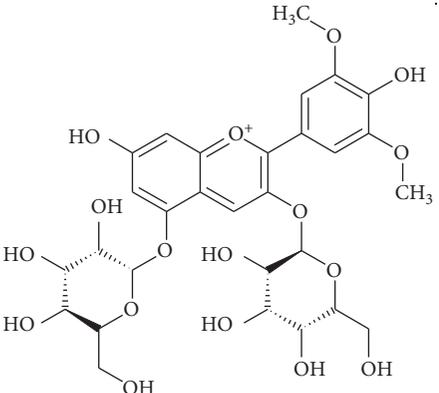
Sl. No.	m/z	Compound name	Structure	Mass bank no.
12	275.2051	Eserine (physiostigmine)		KO008958
13	351.1658	Retrorsine		BML82060
14	284.2592	5,7-Dimethoxyflavanone		BML01475
15	285.2819	Piperine		MT000119
16	655.1729	Malvin		PR020064

TABLE 6: Continued.

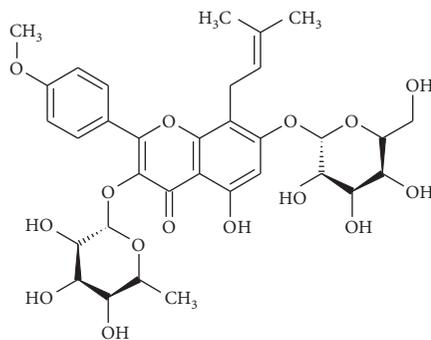
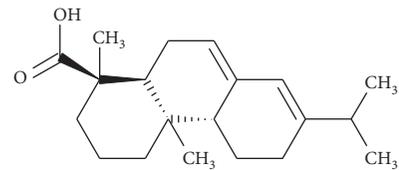
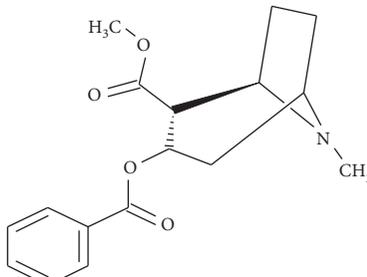
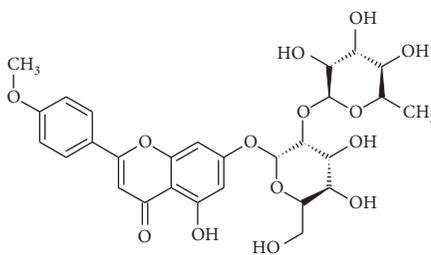
Sl. No.	m/z	Compound name	Structure	Mass bank no.
17	677.2285	Icariin		TY000207
18	302.2279	Abietic acid		BML80660
19	303.2309	Cocaine		EA281708
20	593.1495	Fortunellin		TY000229

TABLE 6: Continued.

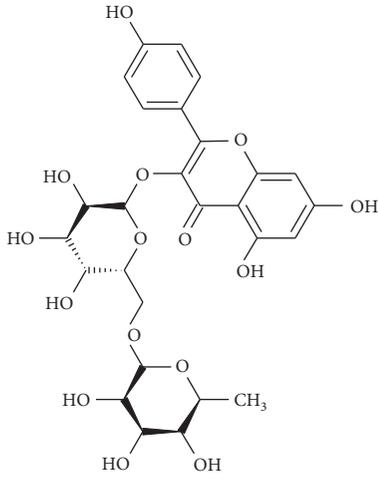
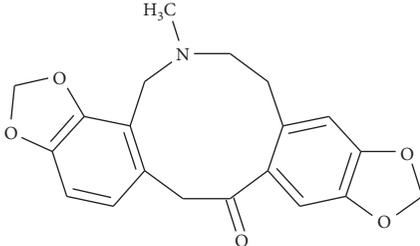
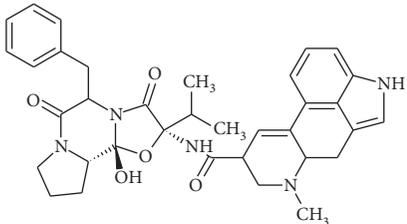
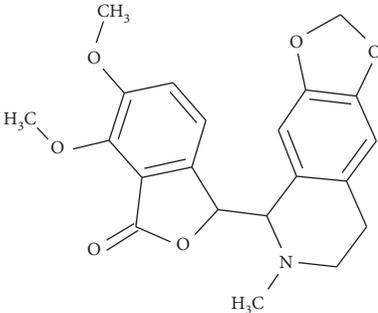
Sl. No.	m/z	Compound name	Structure	Mass bank no.
21	594.1522	Kaempferol-3-O-rutinoside		PR020056
22	353.1820	Protopine		KO009201
23	609.1419	Ergocristine		BML81190
24	383.2260	Hydrastine		BML81430

TABLE 6: Continued.

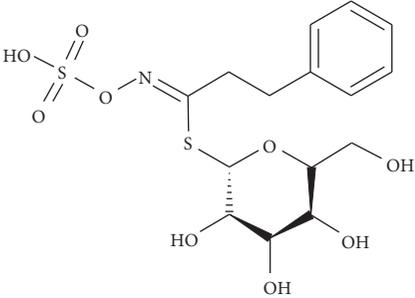
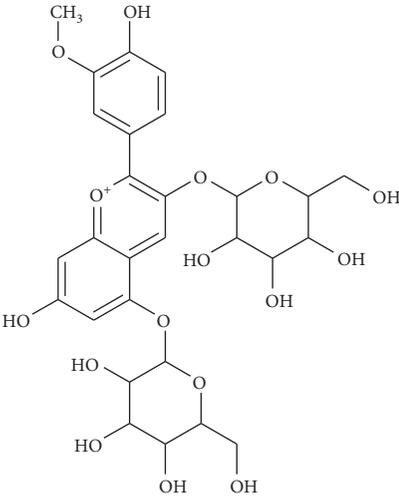
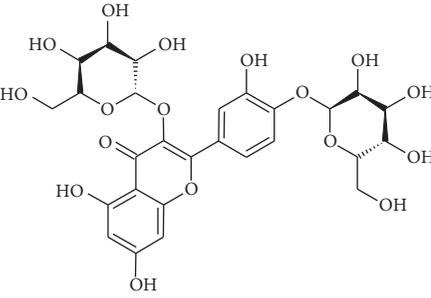
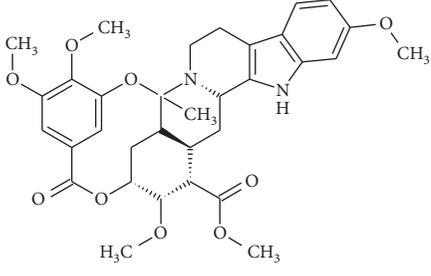
Sl. No.	m/z	Compound name	Structure	Mass bank no.
25	423.2115	Gluconasturtiin		PR100433
26	625.1345	Peonidin-3,5-O-di-beta-glucopyranoside		PR100459
27	626.1376	Quercetin-3,4'-O-di-beta-glucopyranoside		PR100456
28	609.1417	Reserpine		CE000148

TABLE 6: Continued.

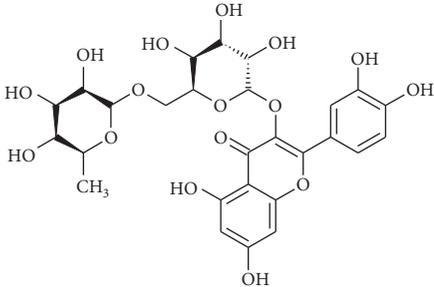
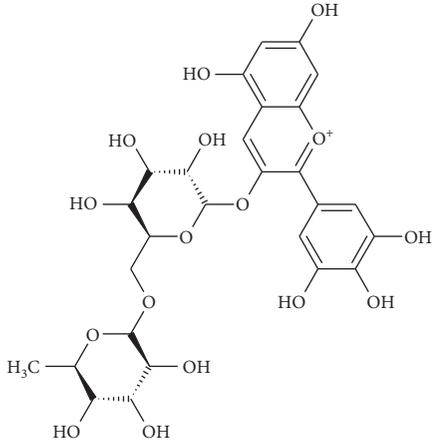
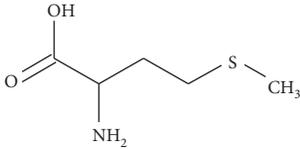
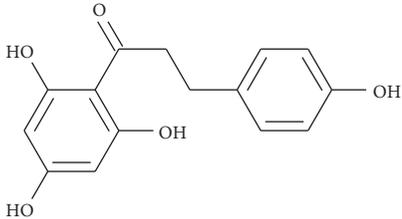
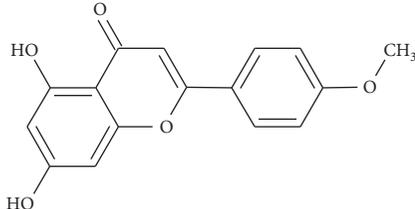
Sl. No.	m/z	Compound name	Structure	Mass bank no.
29	610.1446	Rutin		CE000136
30	611.1469	Delphinidin-3-rutinoside		CE000386
31	148.9785	Methionine		CE000452
32	274.2027	Phloretin		TY000158
33	284.2592	Acacetin		PR020028

TABLE 6: Continued.

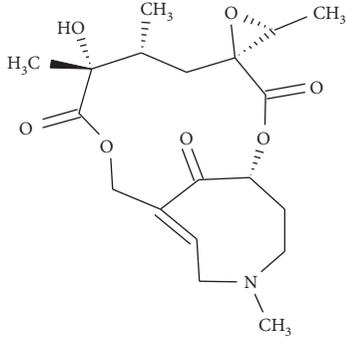
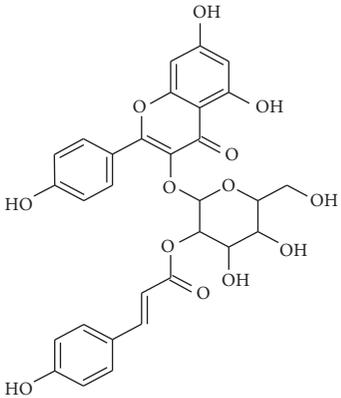
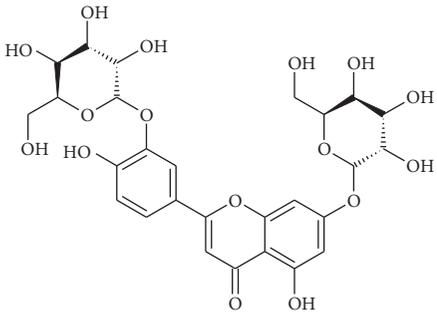
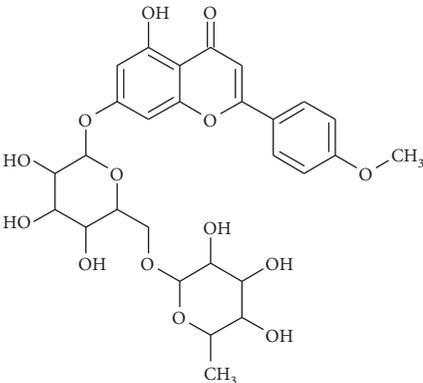
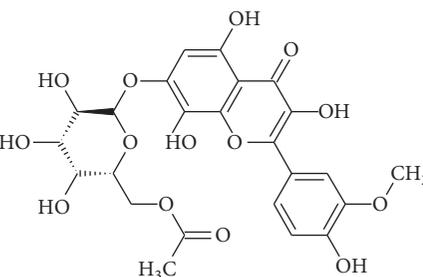
Sl. No.	m/z	Compound name	Structure	Mass bank no.
34	381.2096	Otosenine		BML81860
35	593.1494	Kaempferol-3-glucoside-2''-p-coumaroyl		PR101018
36	610.1445	Luteolin-3',7-di-O-glucoside		PR020059

TABLE 6: Continued.

Sl. No.	m/z	Compound name	Structure	Mass bank no.
37	593.1498	Acaciin		TY000123
38	681.1872	Haploside		BML81345

identified as kaempferol-3-O-rutinoside as  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  spectra were in full agreement with that reported for kaempferol-3-O-rutinoside (structure shown in Figure 4) [27].

**3.5. Standardization and High-Performance Thin-Layer Chromatography (HPTLC) Fingerprint Analysis.** The active ethyl acetate fraction was developed in a high-performance thin-layer chromatographic (HPTLC) plate using toluene: ethyl acetate:formic acid (5:4:1, v/v/v) as the mobile phase (Figure 5), followed by TLC scanning at 254 and 366 nm. HPTLC chromatogram (2D and 3D chromatogram) revealed the presence of different compounds with  $R_f$

values ranging between 0.11 and 0.90 (Figures 6 and 7). The result showed the presence of flavonoid rutin which appear at  $R_f$  0.11, when compared with the standard, which can be used as a biomarker for standardization and quality control. In general, the finger printing can be useful in checking the adulterant. Therefore, it can be useful for the evaluation of different marketed herbal drugs formulated in dosage forms.

**3.6. Identification of Phytoconstituents from Ethyl Acetate Fraction by LC-MS.** The UPLC/MS analysis results in identification of more than thirteen predicted compounds, and most of them are phenolic compounds, especially flavonoids (Table 6 and Figures 8 and 9).

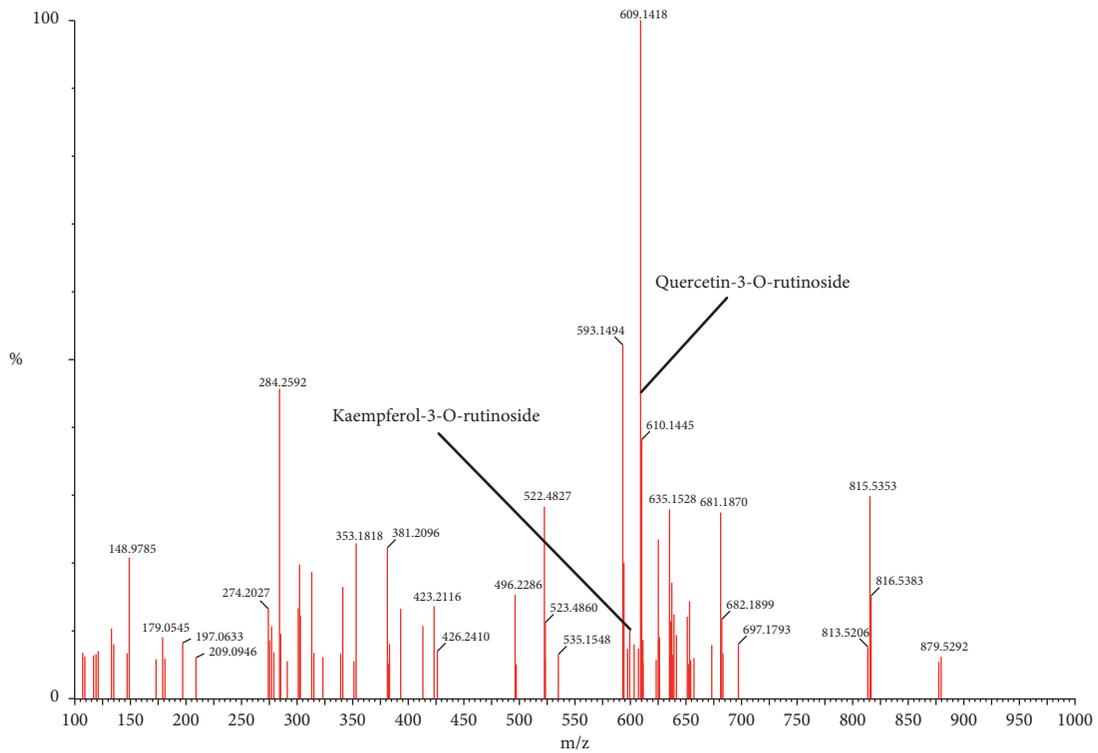


FIGURE 8: Total MS chart representing molecular ion peaks of identified compounds from the active fraction analyzed by LC/MS.

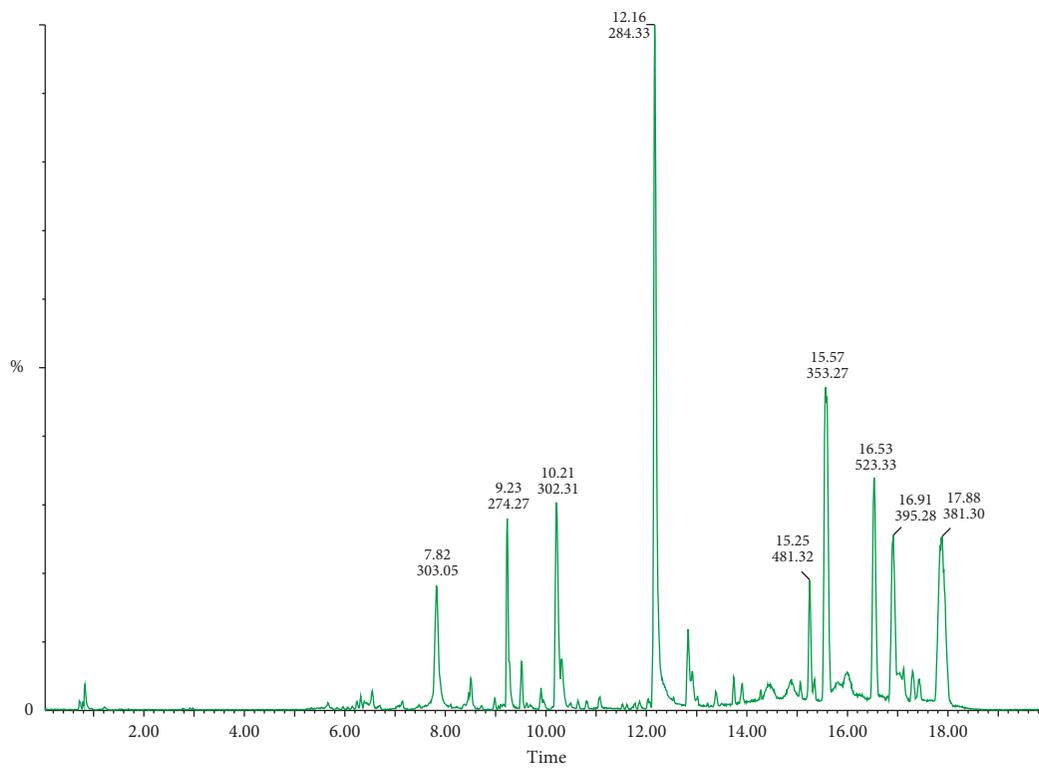


FIGURE 9: HPLC peaks of compounds identified by LC/MS.

#### 4. Conclusion

In our present study, two promising antidiabetic leads, namely, rutin and kaempferol-3-O-rutinoside, had been successfully isolated and characterized from *Bauhinia rufescens* for the first time. These results can be considered as the starting point for future studies. Toxicological studies may further be planned to develop it in the form of a new drug for management of diabetes.

#### Data Availability

The data used to support the finding of this study are available from the corresponding author upon request.

#### Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

#### Acknowledgments

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