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TLC-MS Bioautography-Based Identification of Free-Radical Scavenging, α-Amylase, and α-Glucosidase Inhibitor Compounds of Antidiabetic Tablet BGR-34

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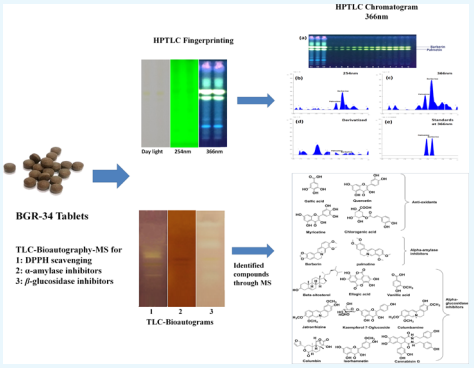
ABSTRACT: BGR-34 is a polyherbal formulation frequently used to combat diabetes around the globe especially in Asian countries. It provides an attractive treatment option to prediabetics, diabetics, and in metabolic disorders by controlling the altered blood glucose level. The lack of phytopharmacological studies on BGR-34 prompted as to reveal the compounds responsible for the antidiabetic and free-radical scavenging activity of BGR-34. An attempt was made to assess *in vitro* α-amylase and α-glucosidase enzyme inhibition of BGR-34 along with its free-radical scavenging potential via DPPH scavenging activity. Further, HPTLC profiling and quantitative analysis of berberine and palmatine in BGR-34 were carried out. Thereafter, the TLC-bioautographic-MS analysis was performed to identify the compounds responsible for antidiabetic and antioxidant activities in BGR-34. The results had shown a significant and dose-dependent inhibition potential of BGR-34 against *in vitro* α-amylase and α-glucosidase enzymatic reactions along with significant inhibition in DPPH free-radical scavenging activity. The HPTLC profiling and quantitative validation studies showed the presence of berberine and palmatine 44.926 ± 0.2907 and 10.507 ± 0.154 μg/g, respectively. The TLC-MS bioautography revealed a total of four DPPH-active, two α-amylase-active, and nine α-glucosidase-active compounds in BGR-34. It was observed from the study that BGR-34 possesses verities of bioactive compounds, which are reasonable not only for its antidiabetic effect but also for its antioxidant activity.



1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disease characterized by lacking in production and or utilization of insulin. Hyperglycemia or increased blood sugar leads to serious damage to many of the body systems, especially the nerves and blood vessels.[1] Type-2 diabetes mellitus (T2DM) characterized due to metabolic disorders in the endocrine system where insufficient insulin secretion and its resistance are the basic pathologic characteristics.[2] Although α-amylase and α-glucosidase enzymes play a crucial role as digestive enzymes in hyperglycemia, the α-amylase enzyme is responsible in breaking down alpha bonds of alpha-linked polysaccharides, whereas α-glucosidase acknowledged to control postprandial hyperglycemia. Along with, a simultaneous therapy as antioxidants, α-amylase, and α-glucosidase inhibitors provides enough potential to present the onsets of diabetes and give a reversal therapy to a diabetic patient.[3]

A statistical probable view of World Health Organization (WHO) considered that diabetes will be the seventh-leading cause of death by 2030 and reported about 1.5 million death caused by diabetes in 2012 and which was found more abundant in female than male.[4] As per the 9th edition of the International Diabetes Federation (IDF 2019) report, diabetes is the fastest-growing health emergencies of the 21st century, globally. In 2019, IDF is estimated that 463 million people suffered from diabetes and the number can be triggered to 578 million by 2030 and 700 million by 2045.[5]

The modern system of medicine deals with many classes of oral hypoglycemic drugs, namely, sulfonylureas, biguanides, α-glucosidase inhibitors, thiazolidinediones, and nonsulfonylureas secretagogues. Despite the significant progress of these medicines, the results of the treatment of diabetic patients are still far from perfect. Due to the several limitations with the use of synthetic antidiabetic drugs, such as drug resistance, adverse effects, and even toxicity, it prompts the healthcare researchers

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for the development of newer and alternative antidiabetic drugs from natural sources.[6]

In the last few decades, there has been an exponential growth in the field of herbal medicines, which are gaining popularity both in developed and developing countries because of their natural origin, easy availability and lesser side effects. The analysis of herbal medicine deals with the long history, especially in the assessment of the plant's quality and safety. Nevertheless, numerous researches have been focused on medicinal plant analysis, particularly in the development of chromatographic and spectroscopic methods. The techniques are hyphenated with biotools to understand the verities of phytoconstituents of medicinal plants for the target-based screening of activity. However, the advancement in technologies provides us with all the steps toward more formalized analytical and biological investigations.[7]

Thin-layer chromatography (TLC) is one of the most used, less expensive, and sophisticated separation techniques and its advancement with high-performance thin-layer chromatography (HPTLC) based on the full capabilities of TLC made us far easier in the field of the natural product and phytochemical analysis. The direct coupling of the compound library or active constituents separated on the TLC plate with visualizable enzyme reactions can be used for activity screening, and the assay based on direct screening of activity-oriented constituents is known as a TLC-bioautography assay. This technique provides quick access to information concerning both the localization of the bioactive compounds present in complex plant matrices and defines their biological activities. Such TLC-based bioautography through enzymatic reaction provides us the initial stage to develop activity-based screening of phytochemical constituents from herbal medicine. The bioautographic analysis coupled with mass spectroscopy (MS) techniques is purposed to a rapid, high-throughput, and target-directed isolation and identification of active molecules based on their enzymatic activity from fractions or multicompound mixtures.[8]

BGR-34 is an ayurvedic polyherbal antidiabetic formulation claimed to be jointly developed by the Council of Scientific & Industrial Research, India (CSIR), National Botanical Research Institute (NBRI), and Central Institute for Medicinal and Aromatic Plant (CIMAP). The formulation contains varieties of herbal ingredients like the stem of Daruharidar (*Berberis aristata*) and Giloy (*Tinospora cordifolia*), the heartwood of Vijaysar (*Pterocarpus marsupium*), Gudmar leaves (*Gymnema sylvestre*), Manjeet root (*Rubia cordifolia*), and Methika seeds (*Trigonella foenumgraecum*). AIMIL Pharmaceuticals (India) Ltd. owns the license for the manufacturing and marketing of BGR-34 around the globe. Further, the clinical studies on BGR-34 confirms that it postulates several mechanisms that are clinically relevant and statistically significant in the reduction of the three key measures of glucose control such as fasting plasma glucose (FPG), improved postprandial glucose (PPBG), and hemoglobin A1c (HbA1c). The trial is registered to the clinical trial registry of India (CTRI registration number: CTRI/2016/11/007476).[9]

However, until now, many of the preclinical and clinical studies have been conducted on BGR-34, but still, there is a lack of research data that could reveal the bioactive compounds responsible for the antidiabetic therapeutic potential in BGR-34. Therefore, the present study is aimed to determine α-amylase, α-glucosidase, and free-radical scavenging-bioactive compounds that potentiate antidiabetic and antioxidants activity of BGR-34 through TLC-bioautographic coupled with MS.

2. RESULTS AND DISCUSSION

The extractive values of different batches of the samples showed more significant difference, and the percentage yield of the methanolic extract was obtained as 8.14 ± 0.07 for three batches of BGR-34.

2.1. Total Phenol and Flavonoid Contents. An average of total phenolic contents of different batches of BGR-34 was found as 124.5 ± 0.34 mg equivalent to GAE/gm extract, whereas the total flavonoid content was found as 112.12 ± 0.36 mg equivalent to rutin/gm extract, which does not showed statistically significant differences ($p < 0.001$) in different three batches of BGR-34. The phenolic and flavonoid content were found higher than previously reported in a single ingredient of BGR-34.[10−14]

Further, the result suggests that BGR-34 is enriched with polyphenols and plays a key role in the management of T2DM by regulating PPBG, HbA1c, and FPG. The bioactive phytoconstituents regulate the functional ability of α-glycosidase, DPP-4, and enzymes linked to the antioxidant defense system and suppress oxidative stress induced by increased free radicals in T2DM.[9]

Polyphenols are the most abundant antioxidants in medicinal plants as well as in the daily uses of the human diet. A study cited by Aryaeian et al. on T2DM cited that phenol and flavonoid exert antioxidant and antidiabetic activity through enduring the role of the antioxidant defense system, pancreatic β-cell, and cAMP to function properly. Besides that, these also regulate phosphodiesterase (PDEs) activity, which is responsible to suppress β-cell and cAMP function.[15]

Polyphenols are one of the key factors that play a crucial role in the management of diabetes by regulating postprandial blood glucose levels and protection against the deleterious effects of hyperglycemia-induced oxidative stress. Further, the author claims that the antioxidant effect of polyphenols enhances insulin sensitivity and catalytic activity in glucose phosphorylation, which are considered as core alternatives in the treatment of diabetes mellitus. Polyphenol-rich medicinal herbs/foods inhibit insulin resistance and pancreatic β-cell damage induced by oxidative stress (ROS/RSN) resulting in prevention from the progression of T2DM. Similarly, in our findings, BGR-34 possesses a high content of phenol and flavonoid, which acts as a factor to exert an antioxidant and antidiabetic effect.[16]

2.2. DPPH Free-Radical Scavenging Activity. The varieties of phytochemicals present in BGR-34 are contributed to exert antioxidant activity. The free-radical scavenging activity of different batches of BGR-34 methanolic extract on DPPH radicals has shown significant and dose-dependent inhibitory potential with average inhibition of 64.18 ± 2.60% at the higher concentration of 1000 μg/mL with an IC_{50} value of 677.69 ± 2.29 μg/mL. Ascorbic acid was used as a positive control, which showed an average inhibition of 99.35 ± 1.97% at the concentration of 1000 μg/mL with an IC_{50} value of 80.07 ± 2.15 μg/mL. BGR-34 was found to possess statistically significant antioxidant properties with p-value summary** and p-value (one-tailed) 0.0043.

The DPPH assay is the most used method for the assessment of the antioxidant capacity of natural products through spectrophotometric techniques based on the quench-

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ing of stable colored radicals. BGR-34 is rich in secondary metabolites and includes phenol and flavonoid that have antioxidant activities owing to their redox characteristics. Dietary phenols and flavonoids have enough ability to scavenge with free radicals induced by oxidative stress. These polyphenols that strengthen the efficacy of the antioxidant enzyme defense system have several types of enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT). These enzymes are known for their pivotal role in suppression of the excessive ROS by breaking the autoxidative chain reaction, quenching the trace elements, and preventing the overall cellular injury. Apart from this, maintaining a normal level of glutathione is important for diabetic patients. Diabetes induces an alteration in glutathione peroxidase and glutathione reductase activity, and where diabetic nephropathy is often linked to low renal glutathione levels.[16,17] The antioxidant activities of a herbal drug and dietary supplements depend on a large heterogeneous group of polyphenols and are naturally occurring antioxidants that play a crucial role in oxidative suppression and cure various ailments including diabetes (T2DM).[18]

2.3. α-Amylase Inhibitory Potential. The α-amylase is a calcium-containing metalloenzyme that breaks down the α-1,4 linkages of polysaccharide to monosaccharide in the oral cavity. The imbalanced cellular carbohydrate and lipid metabolism can cause an increase in the postprandial blood glucose level, which eventually leads to the onset and progression of T2DM.[19] Therefore, it is important to find natural alternatives to drugs that can inhibit α-amylase. Amylase-inhibitory potential of different batches of BGR-34 had shown significant dose-dependent inhibition with an average inhibition of 88.31% ± 2.53 at the concentration of 1000 μg/mL and an IC_{50} value of 45.65 ± 1.855 μg/mL, whereas acarbose, which was used as a positive control, showed a 56.5 ± 0.27% inhibition at the concentration of 1000 μg/mL and an IC_{50} value of 775.27 ± 3.44 μg/mL. The measurement was taken in triplicate for three different batches, and average reading is presented. BGR-34 was found to possess the significant α-amylase inhibitory property with *p*-value summary*** and *p*-value (one-tailed) 0.0006. The average percentage of amylase inhibition of different samples of BGR-34 at different concentrations (μg/mL) is shown in Table 1. Hence, the study supports that α-amylase inhibition potential of the methanolic extract of BGR-34 found more efficient and significant than acarbose.

Several previous studies demonstrate that amylase inhibitors, particularly acarbose (pseudotetrasaccharide), have been used in the treatment of diabetes where it regulates the modulatory effects of PPG and HbA1c in hyperglycemia.[20] The α-amylase inhibitors block or slows down the absorption of starch into the gastrointestinal tract (GIT) mainly by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose and other simple sugars.[21] Diabetes-induced oxidative stress occurs as a result of slow progress in the treatment of diabetes due to the formation of free radicals generated through glucose oxidation and the subsequent oxidative degradation of glycated proteins in the body. Therefore, the phenol, flavonoid, and alkaloid compounds are frequently recommended avoiding such complications.[22]

Table 1. α-Amylase and α-Glucosidase Inhibition of BGR-34 Tablet (*n* = 9)

	activity (% inhibition)	
concentration (μg/mL)	α-amylase	α-glucosidase
1000	88.31 ± 2.53	84.05 ± 2.33
500	85.27 ± 2.77	74.61 ± 2.42
250	82.85 ± 1.95	61.60 ± 2.02
125	75.75 ± 1.97	40.83 ± 1.68
62.5	61.38 ± 2.05	15.96 ± 1.48
31.25	27.85 ± 1.78	12.30 ± 0.85
15.62	8.81 ± 1.65	5.86 ± 0.33
7.81	1.19 ± 0.12	1.52 ± 0.40

2.4. α-Glucosidase Inhibitory Potential. The α-glucosidase enzyme can significantly increase the postprandial blood glucose in type-2 diabetes patients. Therefore, α-glucosidase inhibitors regulate postprandial hyperglycemia by delaying carbohydrate absorption.[23] BGR-34 is the most credible natural and alternative therapy that has an immense potential against the α-glucosidase activity. The present study was conducted to evaluate the inhibitory potential of BGR-34 against α-glucosidase activity. The results show a significant dose-dependent inhibitory potential with an average inhibition of 84.05 ± 2.33% at the concentration of 1000 μg/mL and an IC_{50} of value 136.35 ± 2.35 μg/mL, whereas acarbose showed 46.5 ± 0.53% inhibition at the concentration of 1000 μg/mL. The mean values of three different batches (n = 9) are presented. BGR-34 was found to possess the significant α-glucosidase inhibitory property with *P*-value summary*** and *P*-value (one-tailed) <0.0001. The average percentage of α-glucosidase inhibition of different samples of BGR-34 at different concentrations (μg/mL) is shown in Table 1. Hence, the study suggests that α-glucosidase inhibition potential of BGR-34 is more efficient and significant than acarbose.

In a recent study conducted on healthy subjects, it demonstrates that the treatment effect of natural α-glucosidase inhibitors (acarbose) is not only based on delayed digestion of complex carbohydrates in the gut but also on the metabolic effects of colonic starch fermentation.[24] Hence, α-glucosidase inhibitors may be an effective therapy in the treatment of type-2 diabetes as it specifically targets postprandial hyperglycemia. The main advantage of α-glucosidase inhibitors is the absence of any hypoglycemic events or other life-threatening events on overdoses.[25]

2.5. HPTLC Fingerprinting. Herbal products contain several verities of secondary metabolites with large chemical diversity, and it is very difficult to target a particular constituent concerning their biological activity. In these cases, TLC fingerprint profiling of herbal products is very useful and widely used for qualitative and quantitative evaluation. The compounds separated on the TLC plate at different R_f values represent the number of chemical constituents present in the mixture/extract.[26] Moreover, in HPTLC fingerprinting of BGR-34, the plate was developed in the defined solvent system *n*-butanol:water:glacial acetic acid (6:3:1, v/v/v) and scanned at 254 and 366 nm before derivatization and at 540 nm post derivatization. HPTLC chromatographic analysis of different batches of samples showed 9, 7, and 11 prominent spots at different values at 254, 366, and 540 nm, respectively. The chromatographic profiling revealed that several major and minor constituents present in the methanolic extract of BGR-34. The constituent intensity was identified according to their AUC value/peak intensity (Figure 1, Table 2).

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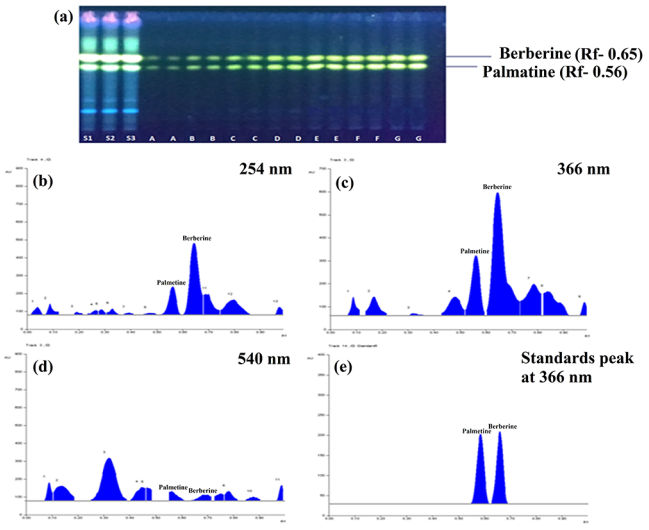

Figure 1. HPTLC plate view at 366 nm and different chromatograms of BGR-34 at different wavelengths. Panel (a) represents track numbers S1−S3 of different three batches of BGR-34, and track numbers A−G are the mixed standard of berberine and palmatine in duplicates at 366 nm. Panels (b)−(d) show chromatograms of the sample at 254, 366, and 540 nm, respectively, whereas panel (e) shows chromatogram of mixed standard at 366 nm.

2.6. Quantitative Estimation of Berberine and Palmatine. For the quantitative estimation of berberine and Palmatine, a presample-applied HPTLC plate was developed in a development chamber containing *n*-butanol:water:glacial acetic acid (6:3:1, v/v/v) as a solvent system and densitometry scanning was performed at 366 nm. The developed method for berberine and palmatine was linear, ranging from 100 to 4000 ng for both with regression coefficient (r^2) 0.999 and 0.998, respectively. The limit of detections (LODs) of the developed method for berberine and palmatine were 12.0 and 12.7 ng/spot, whereas the limit of quantifications (LOQs) were 36.3 and 38.4 ng/spot, respectively. The percentage accuracy values of the developed method for berberine and palmatine were 99.71−100.13% and 99.36−100.6%, respectively. The average quantity of berberine and palmatine in all three batches was found to be 44.926 ± 0.2907 and 10.507 ± 0.154 μg/g, respectively. The HPTLC plate and their chromatogram are shown in Figure 1.

2.7. TLC-Bioautography Assay. The TLC-bioautography assay was performed to screen antioxidant, α-amylase- and α-glucosidase-active compounds present in BGR-34. After MS analysis, the TLC DPPH-bioautography-active spots spectral data revealed four free-radical scavenging compounds as gallic acid (m/z = 170.12), quercetin (m/z = 302.04), chlorogenic acid (m/z = 354.09), and myricetin (m/z = 318.03) with R_f values of 0.77, 0.74, 0.46, and 0.26, respectively (Figure 2, Table 3).

Although the current shreds of evidence strongly support gallic acid, quercetin, chromogenic acid, and myricetin to have excellent antioxidant potential evaluated through several experimental models, many authors suggested that bioactive compounds from herbal products have been used as a nutritional supplement to combat different kinds of diseases, including diabetes, cardiac disorders, and cancer.[27−30] The *in vitro* study conducted by Viapiana and Wesolowski concluded that dietary intake of natural products containing phenolic and flavonoid such as gallic acid, quercetin, chlorogenic acid, and myricetin has recently received great attention due to the epidemiological evidence that correlates a regular intake of these products protects against several diseases.[31]

Furthermore, in α-amylase TLC bioautography, the MS analysis of the scraped bioactive bands from the controlled TLC revealed two prominent bioactive compounds as berberine (m/z = 336.12) and palmatine (m/z = 352.15) with R_f values of 0.65 and 0.56, respectively (Figure 2, Table 3).

Berberine and palmatine are the well-known natural isoquinoline alkaloidal compounds present in the roots, rhizomes, stem, and bark of *Berberis aristata* and *Tinospora cordifolia* of BGR-34. The bioautographic result reveals that berberine and palmatine are the major constituents of BGR-34, which shows inhibitory potential against α-amylase. An extensive search in electronic databases revealed their action to improve insulin resistance and show a reversal effect against

Table 2. Comparative TLC Profiling of BGR-34 at Different Wavelengths

	wavelength								
	254 nm			366 nm			540 nm		
R_f	batch 1	batch 2	batch 3	batch 1	batch 2	batch 3	batch 1	batch 2	batch 3
0.09	+	+	+	+	+	+	+	+	+
0.19	+	+	−	+	+	+	−	−	−
0.27	+	+	+	−	−	−	−	−	−
0.29	+	+	+	−	−	−	−	−	−
0.33	+	+	+	−	−	−	+	+	+
0.45	−	−	−	−	−	−	+	+	+
0.48	−	−	−	+	+	+	+	+	+
0.56	+	+	+	+	+	+	+	+	+
0.64	+	+	+	+	+	+	+	+	+
0.69	+	+	+	−	−	−	+	+	+
0.75	−	−	−	−	−	−	+	+	+
0.80	+	+	+	+	+	+	+	+	+
0.84	−	−	−	+	+	+	−	−	−
0.88	−	−	−	−	−	−	+	+	+
0.98	+	+	+	+	+	+	+	+	+
total number of metabolites	10	10	09	08	08	07	11	11	11

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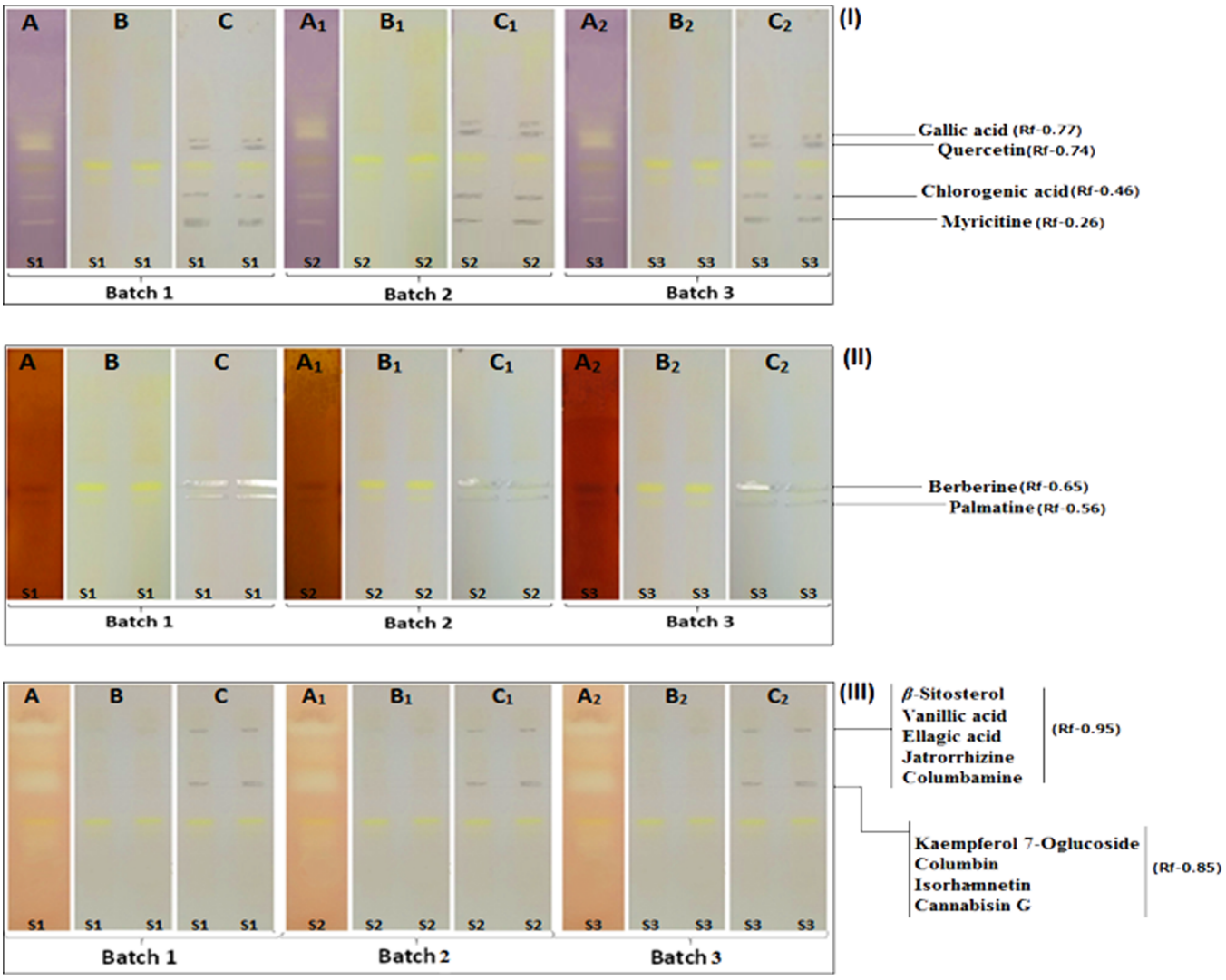

Figure 2. TLC-bioautography. Panel (I) represents DPPH scavenged treated TLC bioautogram of three different batches of BGR-34 where (I-A,A_1,A_2) is showing yellow spots against the dark purple color background, (I-B,B_1,B_2) represents the normal view (control TLC) of plates before DPPH scavenging, and (I-C,C_1,C_2) represents scrapped DPPH active spots from TLC plates. Panel (II) represents α-amylase-treated TLC bioautogram of three different batches of BGR-34 where (II-A,A_1,A_2) is showing dark blue/violet spots at the brown color background, (II-B,B_1,B_2) represents the normal view (control TLC) of plates before amylase treatment, and (II-C,C_1,C_2) represents scrapped α-amylase active spots from TLC plates. Panel (III) represents α-glucosidase-treated TLC bioautogram of three different batches of BGR-34 where (III-A,A_1,A_2) is showing white/ceramic spots against the purple color background, (III-B,B_1,B_2) represents the normal view (control TLC) of plates before α-glucosidase treatment, and (III-C,C_1,C_2) represents scrapped α-glucosidase active spots from TLC plates.

hyperglycemia. The study, cited by Cicero, reported that berberine and palmatine exert their antidiabetic potential by affecting glucose metabolism, increasing insulin secretion, stimulating glycolysis, suppressing adipogenesis, inhibiting mitochondrial function, activating the 5′ adenosine monophosphate-activated protein kinase (AMPK) pathway, and increasing glucokinase activity. The previous studies support that berberine and palmatine not only inhibit α-amylase activity in hyperglycemia but also increase the glucose uptake by muscles, which leads to a reversal effect on hyperglycemia. These isoquinoline alkaloids also protect the injury of hearts that occurs due to oxidative and inflammatory stress. The herbal formulation containing berberine and palmatine is considered as safe due to the low incidence of adverse reactions, such as gastrointestinal discomfort, and transient increases in the plasma bilirubin level.[32−34]

Moreover, in the case of α-glucosidase TLC-bioautography, two wide white active zones appeared on TLC-bioautogram. The corresponding spot from the control plate of both active zones were scrapped, and MS analysis was performed. Data obtained from MS revealed five compounds at an R_f value of 0.95 including β-sitosterol (m/z = 414.70), vanillic acid (m/z = 168.04), ellagic acid (m/z = 302.19), jatrorrhizine (m/z = 338.38), and columbamine (m/z = 338.38) while four componds at a 0.85 R_f value identified as kaempferol 7-O-glucoside (m/z = 448.38), columbin (m/z = 358.39), isorhamnetin (m/z = 316.26), and cannabisin G (624.69) (Figure 2 and Table 3).

The inhibition of α-amylase and α-glucosidase is a clinical strategy for the management of T2DM, and herbal medicines have been reported to credibly alleviate hyperglycemia.[35] Previous studies performed on β-sitosterol support it as established α-glucosidase inhibitors or antidiabetic compounds

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Table 3. Isolated Bioactive Compounds from BGR-34 Tablet through TLC-Bioautography-MSm

TLC R_f value	name of compounds	exact mass	tentative mass	molecular formula	mass ID	activities
0.77	gallic acid	170.12	170.19	$C_6H_2(OH)_3COOH$	PubChem CID: 370	antioxidant activity
0.74	quercetin	302.04	302.10	$C_{15}H_{10}O_7$	MBID-BS003389	
0.46	chlorogenic acid	354.09	354.98	$C_{16}H_{18}O_9$	MBID-PB005541	
0.26	myricitine	318.03	317.16	$C_{15}H_{10}O_8$	MBID BS003379	
0.65	berberine	336.12	336.14	$C_{20}H_{18}NO_4+$	MBID TY000105	amylase activity
0.56	palmatine	352.15	352.33	$C_{21}H_{24}NO_4+$	MBID TY000051	
0.95	β-sitosterol	414.70	413.46	$C_{29}H_{50}O$	NISTR: 83−46-5	glucosidase activity
	vanillic acid	168.04	167.22	$C_8H_8O_4$	MBID BS003123	
	ellagic acid	302.19	301.39	$C_{14}H_6O_8$	PCID: 5281855	
	jatrorrhizine	338.38	338.58	$C_{20}H_{20}NO_4$	PCID 72323	
	columbamine	338.38	338.58	$C_{20}H_{20}NO_4$	PCID 72310	
0.85	kaempferol 7-O-glucoside	448.38	448.18	$C_{21}H_{20}O_{11}$	PCID 10095180	
	columbin	358.39	359.18	$C_{20}H_{22}O_6$	40	
	isorhamnetin	316.26	317.26	$C_{16}H_{12}O_7$	PCID 5281654	
	cannabisin G	624.69	624.99	$C_{36}H_{36}N_2O_8$	PCID 10438919	

present in varieties of herbs and herbal formulations.[36] Phenolic compounds such as vanillic acid and ellagic acid and kaempferol 7-O-glucoside were also detected as α-glucosidase inhibitors and also considered as a strong candidate for antidiabetic and antioxidant activity. These compounds lead to strong credentials of α-glucosidase inhibitors and a natural approach to treat diabetes.[37−39] Although Columbine, columbamine, and jatrorrhizine are the key markers of *Tinospora cordifolia* that possess hypoglycemic potential by lowering the elevated blood glucose level, which is confirmed by several experimental models.[40−42] Ishaq et al. reported that flavonoid such as isorhamnetin ameliorates the hyperglycemic effect through its α-glucosidase inhibitory potential and antioxidative stress potential also.[43] According to the report of Subramoniam et al., cannabisin G, a naturally occurring lignanamide, has an immense effect as an α-glucosidase inhibitor.[44] The reported antioxidants and antidiabetic compounds with their chemical structure are summarized in Table 4 and Figure 3.

Hence, the entire study as per the experimental analysis and pieces of evidence-based research on BGR-34 confirms that its constituents plays many of physiological action in diabetic patients such as decreased glucose production, increase GLP-1 and& GIP, and decrease glucose absorption, increase insulin secretion, enhanced insulin sensitivity, increases glucose uptake and storage by muscle, decrease the chances of diabetic nephropathy, protection from oxidative damage, control blood glucose level, and block DPP4 enzyme activity. Herewith, TLC-bioautography coupled with MS revealed numbers of chemical constituents responsible for the antioxidant and antidiabetic activity in BGR-34. HPTLC profile, bioautographic assay, and physiological function of BGR-34 are summarized in a systematic representation and are followed in Figure 3.

3. CONCLUSIONS

BGR-34 showed the presence of a biologically relevant amount of phenolic and flavonoids. *In vitro* activities showed the excellent antidiabetic and antioxidant potential of BGR-34. Further, TLC-bioautographic coupled with MS analysis showed the numbers of antidiabetic and antioxidant bioactive compounds present in BGR-34. Hence, it is to conclude that BGR-34 has an immense potential as antidiabetic and antioxidant mediated via α-amylase, α-glucosidase, and free-radical inhibition. It controls blood glucose and reduces FBG, PPG, and HblAc.

Table 4. Reported Antioxidant and Antidiabetic Compounds Present in BGR-34

S.N	compound name	reported activity
1	gallic acid	antioxidant activity[27]
2	quercetin	antioxidant activity[28]
3	chlorogenic acid	antioxidant activity[29]
4	myricetin	antioxidant activity[30]
5	berberine	antidiabetic activity (amylase inhibitor)[32−34]
6	palmatine	
7	β-sitosterol	antidiabetic activity (glucosidase inhibitor)[36]
8	vanillic acid	antidiabetic activity (glucosidase inhibitor)[37]
9	ellagic acid	antidiabetic activity (glucosidase inhibitor)[38]
10	kaempferol 7-O-glucoside	antidiabetic activity (glucosidase inhibitor)[39]
11	columbin	antidiabetic activity (glucosidase inhibitor)[40]
12	columbamine	antidiabetic activity (glucosidase inhibitor)[41]
13	jatrorrhizine	antidiabetic activity (glucosidase inhibitor)[42]
14	isorhamnetin	antidiabetic activity (glucosidase inhibitor)[43]
15	cannabisin G	antidiabetic activity (glucosidase inhibitor)[44]

4. MATERIAL AND METHODS

HPTLC system (CAMAG, Muttenz, Switzerland), TLC Silica gel 60F_{254} (Merck KGaA, 64271 Darmstadt, Germany), and a Water's ACQUITY UPLC system (Waters Corp., MA, USA)were used for mass spectrometry analysis. C18 column (ACQUITY UPLC BEH C18 1.7 μm, 2.1 × 100 mm), Mass Lynx V4.1 (Waters, USA), and HPLC water were used. α-Amylase (CAS RN 9000-90-2), α-glucosidase (CAS number: 9001-42-7), pNPG (CAS number: 2207-68-3), fast blue (CAS number: 14263-94-6), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (CAS number:1898-66-4), and ascorbic acid (CAS number:50-81-7) were purchased from Sigma Aldrich Co., St Louis, USA. Folin Ciocalteu reagent, sodium carbonate, aluminum chloride, sodium acetate, α-amylase, and glucosidase were procured from Loba Chemie Pvt. Ltd., Mumbai, India.

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ACS Omega 2020, 5, 29688−29697

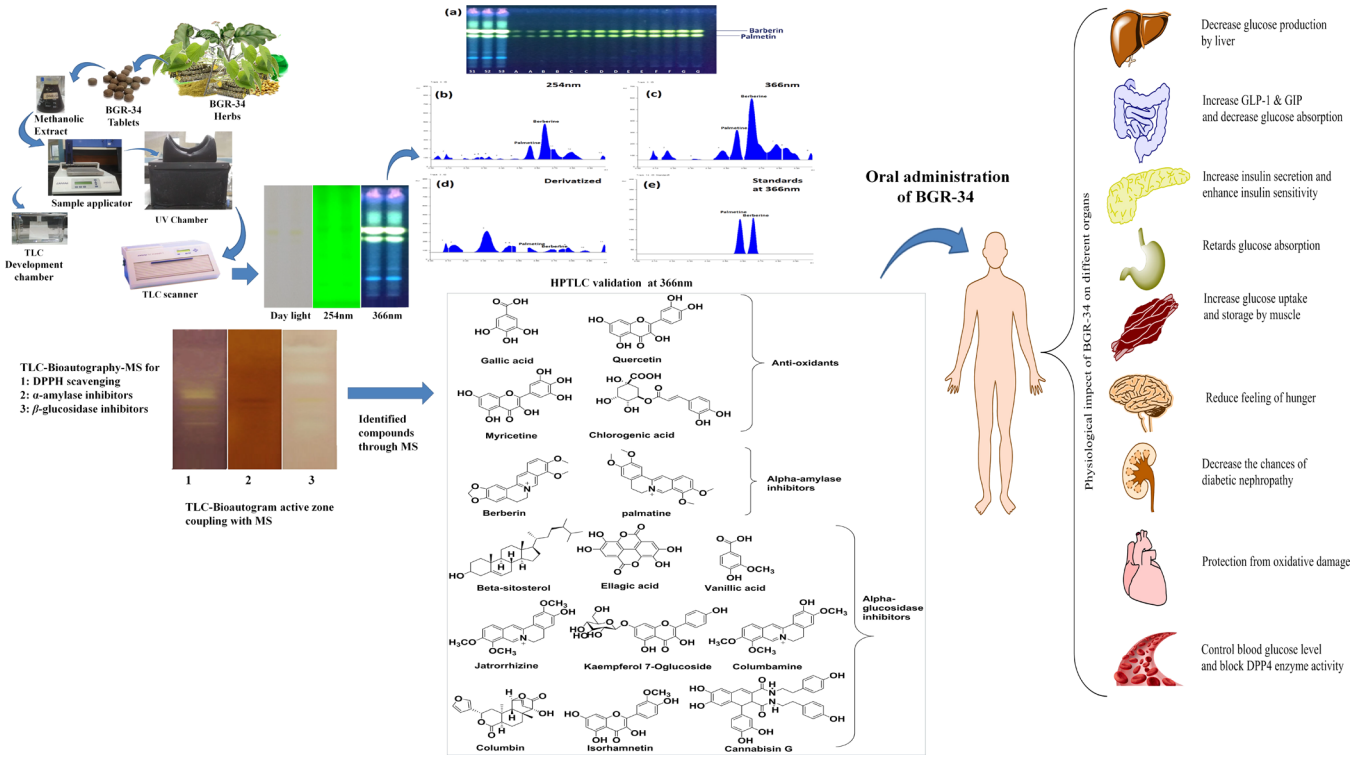

Figure 3. Systematic diagram represents phytopharmacological evaluation of BGR-34. In the study, initially, BGR-34 methanolic extract was processed to HPTLC fingerprinting at 254 nm, 366 nm before derivatization, and 540 mm post derivatization. Further, BGR-34 proceeded for the identification of free radical scavenging compounds, α-amylase, and β-glucosidase inhibitors through the TLC-bioautographic method coupled with mass spectrometry. Several numbers of phytoconstituents were identified in the methanolic extract of BGR-34 having anti-oxidant, α-amylase, and β-glucosidase inhibitory activities. The quantitative validation of BGR-34 was done using berberine and palmetin as major markers of BGR-34. As per the research DATA base of BGR-34, its ingredients, and their phytochemical constituents, it is obliged to many of physiological action in the body such as decreased glucose production by the liver, increase GLP-1 and GIP and decrease glucose absorption in the gut, increase insulin secretion and enhance insulin sensitivity, retard gastric glucose absorption, increase glucose uptake and storage by muscle, reduce the feeling of hunger, decrease the chances of diabetic nephropathy, protect from oxidative damage and control blood glucose level, and block DPP4 enzyme activity.

BGR-34 was procured as a gift sample from AIMIL pharmaceutical India Ltd. All other solvents and chemicals used were of analytical grade.

4.1. Preparation of Sample for Analysis. The gift sample of the BGR-34 was obtained from AIMIL Pharmaceutical Ltd. Thirteen tablet each (three batches) was powdered and processed for the extraction by the maceration process for 6 h at room temperature using 100 mL of methanol as a solvent. Then, the contents were filtered, and the filtrates were dried on a water bath at 50 °C temperature. After completion of the extraction, the extractive value of each sample was calculated and the residue were stored in a suitable container for further analysis at −20 °C.[45,46]

4.2. Total Phenol and Flavonoid Contents. The total phenol content of the different samples was determined by using the Folin Ciocalteu (FC) method with some modifications.[10] Briefly, 5 mg/mL stock solution of extract was prepared in methanol from each batch of BGR-34. An amount 500 μL of stock solution from each batch was mixed with 2.5 mL of FC (1:10, v/v). After mixing, 2.5 mL of sodium bicarbonate solution (7.5%) was added and allowed to stand for 30 min with intermittent shaking. The absorbance was measured at 765 nm using a spectrophotometer. The total phenolic content in BGR-34 was calculated from a calibration curve of standard gallic acid (10−1000 μg/mL), and the result was expressed as mg gallic acid equivalent/gm of extract (mg GAE/gm extract).

The total flavonoid concentration of the samples was measured by the aluminum chloride method with some modifications.[10] Briefly, 5 mg/mL stock solution of extract was prepared in methanol from each batch of BGR-34. An amount 500 μL of stock solution from each batch was mixed with 1.5 mL methanol. After 5 min, 0.1 mL aluminum chloride (10%), 0.1 mL sodium acetate (1 M), and 2.8 mL water were added. After incubation for 40 min, absorbance was measured at 415 nm using a spectrophotometer. The total flavonoid content in BGR-34 was calculated from a calibration curve of standard rutin (10−1000 μg/mL), and the result was expressed as mg rutin equivalent/gm extract (mg rutin/gm extract).

4.3. DPPH Free-Radical Scavenging Activity. The antioxidant activity of BGR-34 was determined by the described protocol with some modifications.[10] An amount 20 μL of the sample from each batch was mixed with 180 μL of DPPH solution in methanol (0.01 mM) solution in each defined well of 96-well plates. The obtained mixtures were incubated for 30 min at room temperature in a relatively dark place, and then absorbance was read using a spectrophotometer at 517 nm. Ascorbic acid was used as a positive control and considered the efficacy of BGR-34 in the proportion of ascorbic acid. The % scavenging curve was plotted against the

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ACS Omega 2020, 5, 29688−29697

extract concentration and their respective IC_{50} values. DPPH scavenging effect was calculated using the following equation

$$\text{Percentage scavenging activity} = (1\text{-}A_{\text{sample}}/A_{\text{control}}) \times 100$$

4.4. α-Amylase Inhibitory Activity. The α-amylase inhibitory activity of BGR-34 was determined by the described protocol with some modifications.[47] Briefly, 40 μL of the sample from each batch was mixed with 40 μL of amylase solution (4 units/mL in sodium phosphate buffer pH 6.7) in each defined well of 96-well plate. The obtained mixtures were incubated at 37 °C for 30 min. After incubation, 40 μL of starch solution (0.1%) was added to the mixture. After 10 min, 20 μL of hydrochloric acid (I M) was added to stop the enzyme and substrate reaction and 100 μL of iodide solution (5 mM iodine +5 mM potassium iodide in distilled water) was added and the absorbance was measured at 580 nm. Acarbose was used as the standard.

$$\text{Percentage inhibition of } \alpha\text{-amylase} = (1\text{-}A_{\text{sample}}/A_{\text{control}}) \times 100$$

4.5. α-Glucosidase Inhibitory Activity. The α-glucosidase inhibitory activity of BGR-34 was determined by the described protocol with some modifications.[47] Briefly, 120 μL of each sample was mixed with 20 μL of α-glucosidase solution (1 U/mL in 0.1 M potassium phosphate buffer, pH 6.8) in each defined well of 96-well plate. The obtained mixtures were incubated for 15 min at 37 °C. The reaction was initiated by adding 20 μL of *para*-nitrophenyl-α-D-glucopyranoside (5 mM), and the mixtures were further incubated for 15 min. The reaction was terminated by adding 80 μL of 0.2 M sodium carbonate, and then absorbance was measured at 405 nm. Acarbose was used as the standard. The percent inhibition of α-glucosidase was calculated using the equation

$$\text{Percentage inhibition of } \alpha\text{-glucosidase} = (1\text{-}A_{\text{sample}}/A_{\text{control}}) \times 100.$$

4.6. HPTLC Profiling and Quantitative Estimation of Berberine and Palmatine. A 30 mg amounts of each extract were dissolved individually in HPLC grade methanol and then filtered using a PTFE membrane filter of 0.2 μM. Further, from the stock solution, berberine and palmatine were mixed so that the final concentration of berberine and palmatine was obtained 0.5 mg/mL. Thereafter, with the help of Camag Linomat-V (CAMAG, Switzerland), 6 μL of the sample from each of BGR-34 was applied individually with a 6 mm wide-band length to prewashed and activated Silica gel 60 F254 precoated HPTLC plates (20 × 10 cm; Merck, Germany) with the nitrogen flow providing a delivery speed of 150 nL/s. The TLC plate was developed in a presaturated TLC development chamber containing *n*-butanol:water:glacial acetic acid (6:3:1, v/v/v) as a solvent system. The plates were prepared in duplicate and developed to a distance of 7.5 cm at room temperature (25 °C). After drying the spots on the developed plates, one was visualized under visible (white), short UV (254 nm), and long UV (366 nm) light. The other plate was sprayed with anisaldehyde-sulfuric acid reagent and heated for 5 min for color reaction and visualized at 540 nm. The quantification of berberine and palmatine was carried out at 366 nm using a Camag TLC scanner III using Wincats1.2.3 software.[26]

4.7. TLC-Bioautography for Antioxidants Activity/DPPH Free-Radical Scavenging Activity. TLC-bioautographic-based determinations of DPPH free-radical scavenging active compounds were identified as per the protocol with some modifications.[48] A stock solution of DPPH (5 mM) was prepared in methanol, and the stock solution was kept at 4 °C. Further, the methanolic extract of BGR-34 6 μL was applied in duplicate to a TLC plate G_{60} F_{254} at a distance of 15 mm using Camag Linomat automatic sample applicator. The plate was developed in a TLC development chamber containing *n*-butanol:water:glacial acetic acid (6:3:1, v/v/v) as a solvent system. Thereafter, the developed TLC plate was dried and cut from mid to separate both the tracks. Further, half of plate was sprayed/dipped in methanolic solution of DPPH. The yellowish color bands appeared against the purple background, indicating that the bands are active as an antioxidant.

4.8. TLC-Bioautography for α-Amylase Activity. TLC-bioautographic-based determination of α-amylase active compounds was identified as per the protocol with some modifications.[48] Briefly, a stock solution of α-amylase (10 mg) was prepared in 20 mL of sodium acetate buffer solution and the stock solution was kept at 4 °C. Thereafter, after the sample was applied, it was developed and separated on two halves as above. The developed plate half portion was then dipped in a prepared enzyme solution and incubated for 1.5 h in a humid desiccator containing a little amount of enough water inside the chamber so that the plate should not come in contact with water but enough to maintain the humid environment inside the chamber. After the incubation period, the plate was dipped in 1% of the starch solution as substrate and further incubated for 20−30 min for enzyme and substrate reaction. Finally, the plate was dipped in Gram's iodine solution and the α-amylase activity was visible on the TLC plate by the appearance of the violet spot on a dark brown color background and then images were captured of developed bioautogram.

4.9. TLC-Bioautography for α-Glucosidase Activity. Bioautographic-based determination of α-glucosidase active compounds was identified as per the protocol with some modification.[48] Briefly, a stock solution of α-glucosidase (100 U) was prepared in 10 mL of sodium acetate buffer solution and the stock solution was kept at 4 °C. Thereafter, the sample was applied and developed in solvent system; after drying it was separated in two halves from mid as above. The developed plate was then dipped in a prepared enzyme solution and incubated for 1−2 h in a humid desiccator containing a little amount of enough water inside the chamber so that the plate should not come in contact with water but enough to maintain the humid environment inside the chamber. After the incubation period, the TLC plate was further dipped in the mixture of 2-naphthyl-α-D-glucopyranoside (2 mg/mL) and Fast-Blue B salt solution (2.5 mg/mL) in a ratio of 1: 1 as the substrate for enzyme and substrate reaction and incubated for 2 h for completion of the reaction. Glucosidase activity was visible on the TLC plate by the appearance of the white spot on a purple/violet background, and the images were captured of developed bioautogram.

4.10. TLC-Bioautography-Based Determination of Antioxidants, α-Amylase, and α-Glucosidase Compounds. To identify antioxidant and α-amylase and α-glucosidase active compounds present in the methanolic extract of BGR-34. The developed different TLC-bioautograms with active zones of DPPH, amylase, and glucosidase inhibition

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ACS Omega 2020, 5, 29688−29697

were kept simultaneously in parallel with the controlled TLC plate (second half, track), and the targeted spots were scrapped which were lying in parallel of the active zone. The scrapped spot materials with silica gel were dissolved in HPLC grade methanol through vortex and centrifuge at 4000 RPM for 10 min to separate silica gel particles. The supernatants were separated and filtered (0.2 μM PTFE membrane filter). The obtained samples were processed for mass spectrometry (MS) analysis.[48]

4.11. MS Analysis of Bioactive Metabolites Isolated from TLC. The obtained sample from the bioautographic assay, MS analysis, was coupled to identify the active metabolite based on their molecular mass as obtained from spectral data. The MS was performed on a Water's ACQUITY UPLC (TM) system (Waters Corp., MA, USA) equipped with a binary solvent delivery system, an auto-sampler, column manager, and a tunable MS detector operated through Empower software (Waters, Manchester, UK). For optimum chromatographic separation, acetonitrile (A) and water (B) were run on a monolithic capillary silica-based C18 column (ACQUITY UPLC(R) BEH C18 1.7 μm, 2.1 × 100 mm). The flow rate of the nebulizer gas was set at 500 L/h; for cone gas, it was set at 50 L/h, and the source temperature was fixed at 100 °C. The capillary and cone voltage was set at 3.0 and 40 KV, respectively. For collision, argon was employed at a pressure of 5.3×10^{-5} torr. The obtained spectral data from MS was processed by Mass Lynx V4.1 (Waters, USA) and further used for metabolomics analysis of the samples. Separated metabolites present in different samples were tentatively identified based on their m/z values from mass data sources such as Mass Bank, PubChem, Drug Bank, ChemSpider, and literature.[47,49]

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Notes

The authors declare no competing financial interest.

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