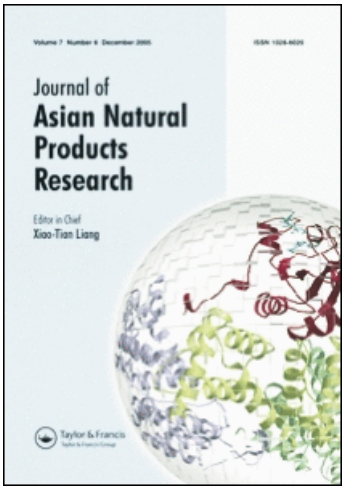

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Chemical constituents from the leaves of *Boehmeria rugulosa* with antidiabetic and antimicrobial activities

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Three new flavonoid glycosides, named chalcone-6′-hydroxy-2′,3,4-trimethoxy-4′-*O*-β-D-glucopyranoside (**1**), isoflavone-3′,4′,5,6-tetrahydroxy-7-*O*-{β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranoside} (**2**), and isoflavone-3′,4′,5,6-tetrahydroxy-7-*O*-{β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranoside} (**3**), were isolated from the leaves of *Boehmeria rugulosa*, together with five known compounds, β-sitosterol, quercetin, 3,4-dimethoxy-ω-(2′-piperidyl)-acetophenone (**4**), boehmeriasin A (**5**), and quercetin-7-*O*-β-D-glucopyranoside. The structures of the isolated compounds were determined by means of chemical and spectral data including 2D NMR experiments. The ethanolic extract of leaves showed significant hypoglycemic activity on alloxan-induced diabetic mice. Glibenclamide, an oral hypoglycemic agent (5 mg/kg, p.o.), was used as a positive control. The ethanolic extract of the plant as well as the isolated compounds **1**–**3** (25 μg/ml) showed potent antimicrobial activity against two bacterial species (*Staphylococcus aureus* and *Streptococcus mutans*) and three fungus pathogens (*Microsporum gypseum, Microsporum canis*, and *Trichophyton rubrum*). The activities of the isolated compounds **1**–**3** have been compared with positive controls, novobiocin, and erythromycin (15 μg/ml).

Keywords: *Boehmeria rugulosa*; Urticaceae; alloxan; isoflavone; *Staphylococcus aureus*; *Microsporum canis*

1. Introduction

Boehmeria rugulosa (BR) Wedd. (Urticaceae) is an evergreen tree, distributed in submontane to montane Himalaya, Himachal to Bhutan, and Myanmar. The paste obtained from the stem bark has been used as a remedy for fractured bone in the Indian traditional system of medicine [1]. Previous studies on the plants of this genus led to the isolation of different compounds, such as alkaloids [2–7], terpenoids and their glycosides [8–10], carbohydrates [11], lignans [12], and flavones [13]. The aqueous extract of plant leaves has been used by some ethnic groups of Uttarakhand, India (Central Himalaya), in the treatment of diabetes mellitus. However, no scientific or traditional data are

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available regarding the hypoglycemic effect of BR. The compounds having antimicrobial activity may occur in stems, roots, leaves, bark, flowers, or fruits of the plant. Plant-derived polyphenols such as flavones, isoflavone, chalcone, etc. have demonstrated antimicrobial activities [14,15]. In view of the fact that plant leaves contain a number of polyphenols (compounds **1**–**3**) along with quercetin and its monoglucoside in significant concentrations, consequently, the ethanolic leaves extract of the plant was subjected to antimicrobial activity. The antimicrobial activities of compounds **1**–**3** (25 μg/ml) have also been evaluated in comparison with positive control, novobiocin (15 μg/ml), and erythromycin (15 μg/ml), and showed significant inhibition of microbial growth. In the present paper, we report the isolation and characterization of chemical constituents along with antidiabetic and antimicrobial activities of the ethanolic extract. The evaluation of phytochemistry, antidiabetic, and antimicrobial activities of the BR leaves has been reported for the first time. To the best of our knowledge, compounds **1**–**3** are new reports and hitherto unreported in the literature.

2. Results and discussion

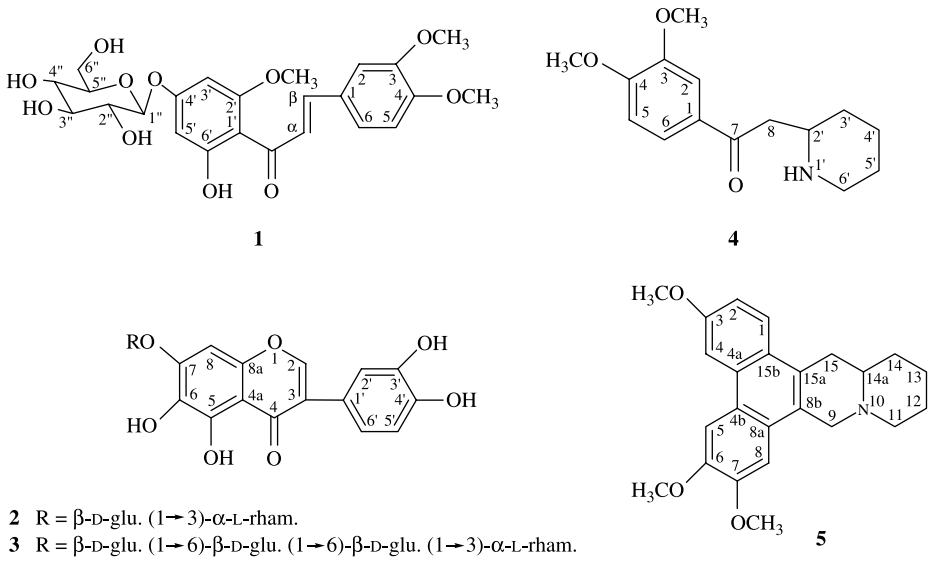
Compound **1** was isolated as yellowish crystals and the molecular formula was deduced as $C_{24}H_{28}O_{11}$ by molecular ion peak at *m/z* 492.1632 in HR-FAB-MS. The UV absorption maxima at 236sh, 272, 325sh, 383 nm in MeOH were indicative of a chalcone [16,17]. The IR absorption band at 1630 cm^{-1} showed the presence of an α,β-unsaturated carbonyl group. In the ^{1}H NMR spectrum, three doublets at δ 6.75 ($J = 1.4$ Hz, H-5), 7.63 ($J = 2.5$ Hz, H-6), and 7.26 ($J = 2.5$ Hz, H-2) were suggestive of a 3,4-disubstituted chalcone. Slightly higher field value of H-2 than that of H-6 indicated the methoxy substitution at C-3 and C-4 [18]. The absence of a signal at δ 8.0–8.5 (characteristic for H-2′) indicated substitution at 2′ position [18]. A sharp band at 3410 cm^{-1} in the IR spectrum and a downfield singlet at δ 12.0 in the ^{1}H NMR spectrum were indicative of an aromatic hydroxy function. The addition of $AlCl_3$ and $NaOAc–H_3BO_3$ in compound **1** with MeOH caused bathochromic shift of 98 and 96 nm, respectively, indicating that the hydroxy group was located at C-6′ [18,19]. The presence of the methoxy groups at C-3 and C-4 positions was confirmed by the NOESY spectrum, which showed a strong correlation between two methoxy groups. Positive reaction with Molisch's reagent suggested the presence of a sugar moiety. The doublet at δ 5.23 ($J = 8.5$ Hz, H-1″) in the ^{1}H NMR spectrum was suggestive of a β-configuration in glucose. Two downfield *meta*-coupled ($J = 2.5$ Hz) signals at δ 6.96 (H-3′) and 6.62 (H-5′) indicated that the sugar moiety was attached at C-4′. Permethylate of compound **1** on hydrolysis furnished 4′-hydroxy-2′,3,4,6′-tetramethoxychalcone (aglycone) and 2,3,4,6-tetra-*O*-methyl-D-glucose (R_f, co-PC, co-IR). The glycosidic linkage was further confirmed by the NOESY spectrum, which showed correlations of H-1″ (δ 5.23) with H-3′ (δ 6.96) and H-5′ (δ 6.62). On acid hydrolysis, it gave aglycone (mp 131–133°C) and a sugar identified as D-glucose (co-PC).

Compound **3** was recognized as isoflavone by UV absorption maxima at 269, 296sh, 348 nm, and a characteristic proton singlet at δ 8.46 (H-2) in the ^{1}H NMR spectrum [20]. The IR absorption bands at 3431 and 1632 cm^{-1} were corroborated for hydroxy and carbonyl groups, respectively. On the addition of NaOMe with MeOH showed bathochromic shifts of 26 and 20 nm in UV absorption bands I and II with reduced intensity, indicating the substitution at C-3′ and C-4′, whereas the bathochromic shift of 72 nm with $AlCl_3–HCl$ indicated the hydroxy substitution at C-5 [18].

Positive Molisch's test was suggestive of a sugar moiety. Methylation of **3**, by the Hakomori method [21], gave permethylated product, which on acid hydrolysis furnished 7-hydroxy-3′,4′,5,6-tetramethoxyisoflavone characterized by spectral analysis. The hydroxy group at C-7 in the hydrolyzed product showed that the sugar moiety was linked to this position. The linkage of sugar at C-7 was also confirmed by a downfield singlet at δ 7.39 (H-8) in the ^{1}H NMR spectrum and by the NOESY spectrum, which showed correlation of H-8 with H-1″. The methylated sugar residues were identified in the hydrolysate as 2,3,4-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, and 2,3,4-tri-*O*-methyl-L-rhamnose. Three anomeric carbon signals at δ 104.0, 103.8, and 102.2 in the ^{13}C NMR spectrum and proton signals at δ 5.13 ($J = 6.9$ Hz, H-1″), 5.06 ($J = 7.2$ Hz, H-1‴), and 4.78 ($J = 7.6$ Hz, H-1⁗) in the ^{1}H NMR spectrum indicated the presence of three glucose moieties with a β-D configuration because H-1 proton of β-linked sugar has a diaxial coupling with H-2. A broad anomeric signal at δ 4.47 (H-1⁗′) indicated an α-L configuration of a rhamnose because of the equatorial–equatorial coupling between H-1 and H-2 of the sugar. The upfield 3H doublet at δ 0.98 ($J = 6.0$ Hz) in the ^{1}H NMR spectrum and at δ 17.8 in the ^{13}C NMR spectrum indicated the existence of the methyl protons of the rhamnosyl group. In order to determine exact linkages in the sugar moieties of compound **3**, it was partially hydrolyzed to afford PH (partially hydrolyzed product) 1, PH2, PH3, and PH4. Acid hydrolysis of PH1, PH2, and PH3 gave D-glucose. PH1, PH2, and PH3 permethylates on acid hydrolysis gave 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucose; 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucose; and 2,3,4,6-tetra-*O*-methyl-D-glucose, respectively. The identities of these sugars were further confirmed by direct comparison with authentic samples. The linkages at glycosidic points in **3** were found to be D-glucose-β and L-rhamnose-α by the ^{13}C NMR spectrum [22]. The signals of C-3 and C-5 of the glucosyl group at δ 74.8 and 76.1, 72.5 and 76.9, and 78.9 and 74.4, respectively, showed β-linked glucose, whereas C-3 signal of the rhamnosyl group at δ 70.1 indicated α-linked rhamnose [22]. The glycosylation shift of **3** in the ^{13}C NMR spectrum clearly indicated that an inner glucose was substituted at position 6 (δ 67.29, downfield shift of ~5 ppm) with a glucose substituted at position 6 (δ 68.43, downfield ~6 ppm) with another glucose. This glucose molecule is substituted at position 3 (δ 78.90, ~5 ppm) with rhamnose [23]. Thus, compound **3** was characterized as isoflavone-3′,4′,5,6,-tetrahydroxy-7-*O*-{β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranoside}.

Compound **2** was isolated as a yellow crystalline solid and gave almost identical data to those of compound **3** except for the number of sugars. On acid hydrolysis, compound **2** afforded aglycone similar to that of compound **3** (3′,4′,5,6,7-pentahydroxyisoflavone), D-glucose, and L-rhamnose (1:1). The configuration and the linkage of glycosidic points of sugars in **2** were confirmed by a similar method as described for compound **3**. Hence, compound **2** was characterized as isoflavone-3′,4′,5,6-tetrahydroxy-7-*O*-{β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranoside}. Based on the evidences, the structures of the isolated compounds are shown in Figure 1.

The known compounds, β-sitosterol, quercetin, and its monoglucoside, were identified by comparison with their literature values [24,25] and direct comparison with authentic samples. The spectroscopic data for compounds **4** and **5** were found similar to those of previously published data for 3,4-dimethoxy-ω-(2′-piperidyl)-



1 **4** **5**

2 R = β-D-glu. (1→3)-α-L-rham.
3 R = β-D-glu. (1→6)-β-D-glu. (1→6)-β-D-glu. (1→3)-α-L-rham.

Figure 1. Chemical structures of compounds **1**–**5**.

acetophenone (**4**) [2,5] and boehmeriasin A (**5**) [4], respectively.

The hypoglycemic effect of the different doses of the ethanolic extract of BR leaves on alloxan-induced diabetic mice is given in Figure 2. The oral doses of BR at 100, 200, and 500 mg/kg p.o., produced significant hypoglycemic activity. The oral dose at 100, 200, and 500 mg/kg reduced the blood glucose level in test mice by 19, 37, and 52%, respectively, whereas the normal control and diabetic control groups showed the reduction in blood glucose by 21 and 27%, respectively. The oral dose in high concentration (500 mg/kg) showed significant results in comparison with glibenclamide (an oral hypoglycemic agent), which reduced the blood glucose level by 55%.

All forms of diabetes mellitus are due to a decrease in the circulating concentration of insulin (insulin deficiency) and a decrease in the response of peripheral tissues to insulin (insulin resistance). These abnormalities lead to alterations in the metabolism of carbohydrates, lipids, ketones, and amino acids; the fundamental characteristic of the disorder is hyperglycemia. Alloxan produced a significant increase in the blood glucose level by damaging pancreatic β-cells, resulting in a decrease in endogenous insulin secretion, which decreases the utilization of glucose by the tissues and thus is called an effective diabetes-induction agent [26]. In the present case, the anti-hyperglycemic activity was probably shown by the polar constituents of the plant (i.e. flavonoid glycosides). Previously isolated compound **5** has been reported as cytotoxic [4] but in the present case, no toxicity was observed in experimental mice, probably due to the low concentration of the compound when compared with other constituents; in a crude extract, in fact, the synergistic effect also played an important role in bioactivity. Moreover, a crude extract without toxic constituents can show the toxicity in animals whereas conceal the toxicity even containing toxic substances. The biphasic effect of the BR extract has been observed with maximum hypoglycemic effect at 24th hour. The extract can be used for further biological studies as no lethality and other behavioral

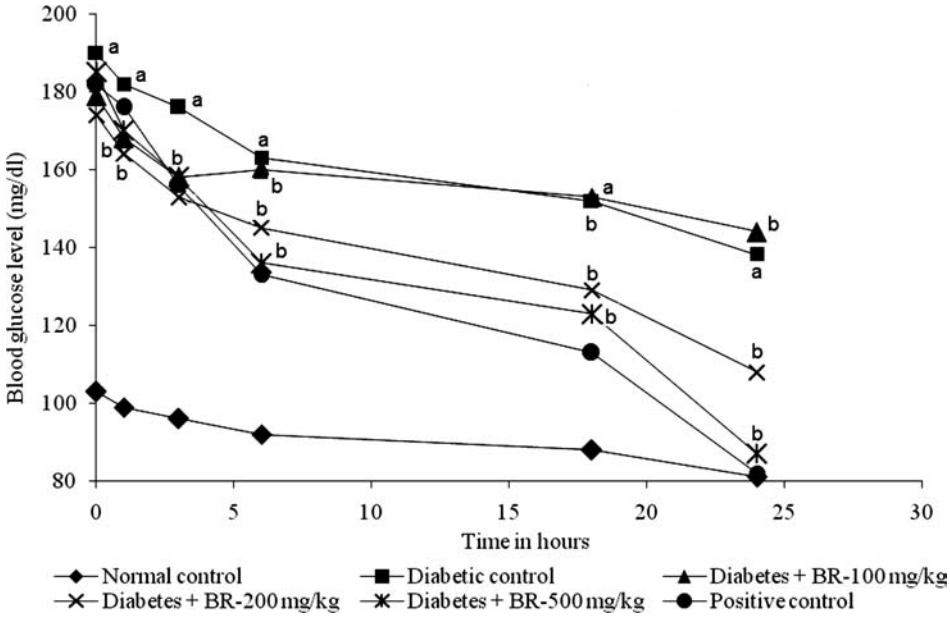


Figure 2. Effect of oral administration of different doses of BR extract on the blood glucose level. Values are mean ± SEM for six animals. a = $p < 0.05$ vs. normal control, b = $p < 0.05$ vs. diabetic control; BR, *Boehmeria rugulosa*; positive control, glibenclamide.

changes were observed up to 1000 mg/kg p.o., for 21 days in the experimental mice. Anti-hyperglycemic studies with the crude extract and isolated compounds are underway for the evaluation of other parameters such as lipids, triglycerides, total cholesterol, HDL cholesterol, etc. and further elucidation of their mechanism of action in the experimental animals.

The ethanolic extract from the leaves showed significant activity against some micro-organisms *in vitro* (Table 1). It showed poor activity against *Klebsiella pneumoniae* and *Escherichia coli*. A potent activity was showed against *Staphylococcus aureus* and its 10 hospital stains. Crude plant extracts are generally a mixture of active and non-active compounds. With more refined and solitary compounds, minimum inhibitory concentrations (MICs) of less than 100 μg/ml may be suggestive of good antimicrobial activity. Since crude mixtures will have higher MICs, an MIC of less than 100 μg/ml is interpreted in this study as showing strong antimicrobial potential whereas 25 and 50 μg/ml as showing most strong antimicrobial potential. Compounds **1**–**3** (25 μg/ml) showed significant activity against *S. aureus*, *Streptococcus mutans*, *Microsporum canis*, and *Trichophyton rubrum* in comparison with standard antimicrobial drugs (Table 2). The micro-organisms, *E. coli*, *S. epidermidis*, *K. pneumoniae, Aspergillus niger*, *A. fumigates*, and *Penicillium citrinum*, unaffected by the ethanolic extract were also found unaffected by isolated compounds **1**–**3**. This fact concluded that the antimicrobial activity shown by the crude extract was conceivably due to the polyphenolic compounds (**1**–**3**) present in the extract. Since the isolated compounds were found to have more significant antimicrobial activities, these can be a source of novel antimicrobial drugs for pharmaceutical industries.

Table 1. IZD (cm) and MICs (μg/ml) of the ethanolic extract of BR leaves against different bacteria and fungus pathogens.

	Antibacterial activities														
	Sa	Ec	Sm	Se	Kp	SH3	SH4	SH5	SH7	SH8	SH10	SH11	SH15	SH23	SH25
IZD	15	–	12	–	–	14	14	12	12	9	15	10	11	12	11
MIC	100	–	100	–	–	100	100	50	50	100	100	25	100	100	100

	Antifungal activity								
	An		Af		Pc		Tr	Mg	Mc
IZD	–		–		–		6	11	8
MIC	–		–		–		100	100	100

Notes: –, no inhibition zone; Sa, *S. aureus*; Ec, *E. coli*; Sm, *S. mutans*; Se, *Staphylococcus epidermidis*; Kp, *K. pneumoniae*; SH, hospital stains of *S. aureus*; An, *A. niger*; Af, *Aspergillus fumigatus*; Pc, *P. citrinum*; Tr, *T. rubrum*; Mg, *M. gypseum*; Mc, *M canis*.

3. Experimental

3.1 General experimental procedures

Melting points were recorded on a Perfit melting point apparatus and are uncorrected. UV spectra were measured on a Perkin-Elmer Lambda-25 spectrometer in MeOH. IR spectra were recorded on a Perkin-Elmer, Spectrum RX I FT-IR spectrometer (KBr disks). NMR spectra were obtained on a JEOL NMR spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C, DMSO-d_6 as a solvent, TMS as an internal standard). Mass spectra were recorded on a Finnigan MAT spectrometer (San Jose, CA, USA; xcalibur ver-2 software). Column chromatography was performed on silica gel (Merck, 60–120 mesh, 15 × 100 cm; Mumbai, India). TLC was carried out on silica gel (Merck, 10–40 μ) precoated plates and the spots were visualized by spraying with 7% H_2SO_4. Alloxan was obtained from Central Drug House (P) Ltd (Daryaganj, New Delhi, India), novobiocin from Merck Ltd (Mumbai, Maharashtra, India), erythromycin from Alembic Ltd (Baroda, Gujarat, India), and glibenclamide from Ind-Swift Ltd (Parwanoo, Gujarat, India).

3.2 Plant material

Fresh leaves (10 kg) of BR were collected from Kartik swami temple, Chamoli, during the month of July and identified at Taxonomy Laboratory, Department of Botany, H.N.B. Garhwal University, Srinagar. A voucher specimen (GUH-17599) has been deposited in the departmental herbarium.

3.3 Extraction and isolation

The powdered leaves were extracted with 95% ethanol (5000 ml, 4–5 times) on a heating mantle. After the removal of the solvent under reduced pressure, the residue (500 g) was fractionated repeatedly with EtOAc to yield soluble (216 g) and insoluble (284 g) portions. These portions

Table 2. Antimicrobial activities of compounds **1**–**3** (25 μg/ml) against test micro-organisms comparing with reference compounds.

S. no.	Test micro-organisms	**1** ± SD	**2** ± SD	**3** ± SD	Novobiocin ± SD	Erythromycin ± SD
1	*Staphylococcus aureus*	21 ± 2	18 ± 2	20 ± 2	21 ± 2	NT
2	*Streptococcus mutans*	25 ± 4	13 ± 4	17 ± 4	22 ± 2	NT
3	*Microsporum gypseum*	06 ± 1	11 ± 3	11 ± 2	NT	15 ± 2
4	*Microsporum canis*	21 ± 4	15 ± 4	18 ± 3	NT	18 ± 3
5	*Trichophyton rubrum*	23 ± 3	17 ± 2	17 ± 2	NT	20 ± 3

Notes: SD, standard deviation; NT, not tested.

were chromatographed followed by pre-adsorption onto silica gel and then added on the top of silica gel (500 g) packed column. The EtOAc-soluble portion with C_6H_6– $CHCl_3$ = 9:1, 17:3, 4:1, 17:5, 7:3, 17:7, 3:2, 17:9, and 1:1 as eluents afforded four known compounds named β-sitosterol [24], quercetin [25], 3,4-dimethoxy-ω-(2′-piperidyl)-acetophenone (**4**) [2,5], and boehmeriasin A (**5**) [4]. The EtOAc-insoluble portion was chromatographed with $CHCl_3$–MeOH = 23:2, 9:1, 22:3, 43:7, 21:4, 41:9, and 4:1 as eluents. Fractions were collected every 100 ml and combined according to the TLC analysis. The elution with $CHCl_3$–MeOH (22:3) afforded quarcetin-7-*O*-β-D-glucopyranoside [25], $CHCl_3$–MeOH (43:7) afforded chalcone-6′-hydroxy-2′,3,4-trimethoxy-4′-*O*-β-D-glucopyranoside (**1**), $CHCl_3$–MeOH (21:4) afforded isoflavone-3′,4′,5,6-tetrahydroxy-7-*O*-{β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranoside} (**2**), and $CHCl_3$–MeOH (4:1) gave isoflavone 3′,4′,5,6-tetrahydroxy-7-*O*-{β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranoside} (**3**) .

3.3.1 Chalcone-6′-hydroxy-2′,3,4-trimethoxy-4′-O-β-D-glucopyranoside (*1*)

Yellowish crystals (MeOH–$CHCl_3$, 190 mg), $[\alpha]_D^{20}$ −54 (c = 0.5, MeOH); UV λ_{max}^{MeOH} (nm): 236sh, 272, 325sh, 383 (MeOH), 250sh, 312, 354, 481 (MeOH+$AlCl_3$) and 274, 335, 458, 479 (MeOH+NaOAc–H_3BO_3); IR ν_{max}^{KBr}: 3410, 2965, 1630, 1480 cm^{-1}; ^1H NMR (ppm): δ 7.26 (d, J = 2.5 Hz, H-2), 6.75 (d, J = 7.5 Hz, H-5), 7.63 (d, J = 7.5 Hz, H-6), 6.15 (d, J = 17.0 Hz, H-α), 7.37 (d, J = 17.0 H-β), 6.96 (d, J = 2.5 Hz, H-3′), 6.62 (d, J = 2.5 Hz, H-5′), 5.23 (d, J = 8.5 Hz, H-1″), 3.93–3.11 (m, H-2″-H-6″), 3.32 (s, OMe-3), 3.67 (s, OMe-4), 3.89 (s, OMe-2′), 12.01 (s, OH-6′); ^{13}C NMR (ppm): δ 125.1 (C-1), 114.7 (C-2), 157.3 (C-3), 159.2 (C-4), 115.6 (C-5), 114.1 (C-6), 129.9 (C-α), 134.4 (C-β), 183.5 (C=O), 120.5 (C-1′), 162.4 (C-2′), 98.7 (C-3′), 158.8 (C-4′), 99.1 (C-5′), 148.1 (C-6′), 105.8 (C-1″), 73.4 (C-2″), 77.9 (C-3″), 75.8 (C-4″), 76.5 (C-5″), 62.4 (C-6″), 56.3 (OMe-3), 55.2 (OMe-4), 58.6 (OMe-2′); LC-EI-MS: 494 $[M+2H]^+$, 492 $[M]^+$, 332 $[M+2H-162(glu)]^+$, 237 $[M-162-3OCH_3]^+$163 $[M+3H-162-3OCH_3-77]^+$; Elemental analysis: found C, 58.62%, H, 5.70%, O, 35.64%; calcd for $C_{24}H_{28}O_{11}$: C, 58.53%, H, 5.73%, O, 35.70%.

*3.3.1.1 Acid hydrolysis of **1**.* Compound **1** (10 mg) was refluxed with 10% aqueous HCl for 5 h, afforded aglycone (mp 131–133°C), and D-glucose identified by PC (*n*-BuOH–H_2O–AcOH, 4:1:5, R_f 0.18) with an authentic sample.

*3.3.1.2 Methanolysis of **1**.* Compound **1** (10 mg) was refluxed with NaH (25 mg) and MeI (2 ml) in DMSO (10 ml) for 3 h; after acid hydrolysis (7% HCl, 7 h reflux)

furnished 4′-hydroxy-2′,3,4,6′-tetramethoxychalcone (aglycone) and 2,3,4,6-tetra-*O*-methyl-D-glucose (R_f, co-PC, co-IR).

*3.3.2 Isoflavone-3′,4′,5,6-tetrahydroxy-7-O-{β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranoside} (**2**)*

A yellow crystalline solid (MeOH, 115 mg); mp 243–246°C; $[\alpha]_D^{20}$ + 54 (c = 0.5, MeOH); UV λ_{max} (nm): 269, 288, 343sh (MeOH) 289, 363 (MeOH+NaOMe); 331, 356 (MeOH+$AlCl_3$–HCl); IR ν_{max}^{KBr}: 3394, 2913, 1642, 1471 cm^{-1}; ^{1}H NMR (ppm): δ 8.21 (s, H-2), 7.53 (s, H-8), 6.48 (d, J = 8.0 Hz, H-2′), 6.76 (d, J = 6.5 Hz, H-5′), 6.96 (d, J = 8.0 Hz, H-6′), 5.38 (d, J = 7.5 Hz, H-1″), 3.16–3.86 (m, H-2″-H-6″; H-2‴-H-5‴), 4.92 (s, br, H-1‴), 1.03 (d, J = 6.0 Hz, H-6‴), 12.61 (s, OH-5), 10.84 (s, OH-6), 10.12 (s, OH-3′), 9.66 (s, OH-4′); ^{13}C NMR (ppm): 157.5 (C-2), 117.1 (C-3), 177.5 (C-4), 108.2 (C-4a), 149.2 (C-5), 153.0 (C-6), 167.1 (C-7), 101.2 (C-8), 162.8 (C-8a), 121.7 (C-1′), 115.6 (C-2′), 158.2 (C-3′), 156.6 (C-4′), 116.4 (C-5′), 114.5 (C-6′); LC-EI-MS: 611 $[M+H]^+$, 610 $[M]^+$, 465 $[M+H-146]^+$, 303 (100%) $[M+H-146-162)]^+$; Elemental analysis: C, 53.13%, H, 4.91%, O, 41.96%; calcd for $C_{27}H_{30}O_{16}$: C, 53.12%, H, 4.95%, O, 41.93%.

*3.3.3 Isoflavone-3′,4′,5,6-tetrahydroxy-7-O-{β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranoside} (**3**)*

A yellow amorphous solid (MeOH, 173 mg), $[\alpha]_D^{20}$ + 78 (c = 0.3, MeOH); UV λ_{max}^{MeOH} (nm): 269, 296sh, 348sh (MeOH); 295, 368 (MeOH+NaOMe); 341, 358 (MeOH+$AlCl_3$–HCl); IR ν_{max}^{KBr}: 3431, 2896, 1632, 1475 cm^{-1}; ^{1}H NMR (ppm): δ 8.46 (s, H-2), 7.39 (s, H-8), 6.37 (d, J = 8.0 Hz, H-2′), 6.68 (d, J = 6.5 Hz, H-5′), 6.94 (d, J = 8.0 Hz, H-6′), 5.13 (d, J = 6.9 Hz, H-1″), 3.07–3.81 (m, H-2″–H-6″; H-2‴–H-6‴; H-2⁗–H-6⁗ and H-2⁗′–H-5⁗′), 5.06 (d, J = 7.2 Hz, H-1‴), 4.78 (d, J = 7.6 Hz, H-1⁗), 4.47 (s, br, H-1⁗′), 0.98 (d, J = 6.0 Hz, H-6⁗′), 12.56 (s, OH-5), 10.93 (s, OH-6), 9.81 (s, OH-3′), 9.17 (s, OH-4′); ^{13}C NMR (ppm): 158.5 (C-2), 116.9 (C-3), 177.5 (C-4), 106.7 (C-4a), 154.8 (C-5), 151.1 (C-6), 164.1 (C-7), 98.7 (C-8), 160.3 (C-8a), 121.7 (C-1′), 115.3 (C-2′), 156.7 (C-3′), 156.4 (C-4′), 116.3 (C-5′), 114.1 (C-6′), 104.0 (C-1″), 68.3–77.5 (C-2″–C5″; C-2‴–C-5‴; C-2⁗–C-5⁗, and C-2⁗′–C-5⁗′), 65.3 (C-6″), 103.8 (C-1‴), 62.1 (C-6‴), 102.2 (C-1⁗), 66.4 (C-6⁗), 98.9 (C-1⁗′), 17.8 (C-6⁗′); DEPT-135°: CH (25), CH_2 (3), and CH_3 (1); LC-EI-MS: 935 $[M+H]^+$, 934 $[M]^+$, 789 $[M+H-146(rham)]^+$, 627 $[M+H-146+162(glu)]^+$, 464 $[M+H-146+2\times162]^+$ 303 (100%) $[M+H-146+3\times162]^+$; Elemental analysis: C, 50.15%, H, 5.31%, O, 44.54%; calcd for $C_{39}H_{50}O_{26}$: C, 50.11%, H, 5.39%, O, 44.50%.

*3.3.3.1 Acid hydrolysis of **3**.* Compound **3** (20 mg) was hydrolyzed with 2 N HCl–MeOH (1:1), afforded an aglycone with mp 163°C and $[\alpha]_D^{20}$ + 46, and identified as 3′,4′,5,6,7-pentahydroxyisoflavone. The neutralized (Ag_2CO_3) aqueous hydrolysate showed the presence of D-glucose and L-rhamnose in a 3:1 ratio (photo colorimeter; PC, *n*-BuOH–AcOH–H_2O 4:1:5, R_f 0.17 and 0.35, respectively).

*3.3.3.2 Partial hydrolysis of **3**.* Twenty milligrams of compound **3** were heated with 2 N HCl–BuOH (1:1, 10 ml) at 70°C for 5 h. The BuOH layer was washed with water, evaporated to dryness, and subjected to preparative TLC to afford isoflavone 3′,4′,5,6-tetrahydroxy-7-*O*-glucoside, isoflavone 3′,4′,5,6-tetrahydroxy-7-*O*-glucosyl-(1 → 6)-glucoside, and isoflavone 3′,4′,5,6-tetrahydroxy-7-*O*-glucosyl-(1 → 6)-glucosyl-(1 → 6)-glucoside,

identified by further hydrolysis of these products. The sugar moieties were confirmed by co-PC with authentic samples.

*3.3.3.3 Permethylation of **3***. Compound **3** and other hydrolysate products (each 10 mg) were refluxed with NaH (25 mg) and MeI (2 ml) in DMSO (10 ml) for 5 h separately. The reaction mixture was extracted with $CHCl_3$ and purified by preparative TLC. Further hydrolysis of permethylated products by a similar procedure was carried out for the determination of the interglycosidic linkage.

3.3.4 7-Hydroxy-3′,4′,5,6-tetramethoxyisoflavone

^{1}H NMR (ppm): δ 8.34 (s, H-2), 7.38 (s, H-8), 6.45 (d, $J = 3.2$ Hz, H-2′), 6.67 (d, $J = 6.5$ Hz, H-5′), 6.74 (d, $J = 4.2$ Hz, H-6′), 9.91 (s, OH-7), 3.83 (s, OMe-5), 3.76 (s, OMe-6), 3.75 (s, OMe-3′), 3.24 (s, OMe-4′).

3.4 Antidiabetic evaluation

3.4.1 Preparation of doses

The oral doses of the dried ethanolic extract of BR leaves at 100, 200, and 500 mg/kg, p.o., body weight were prepared in distilled water (5% alcoholic) for the determination of the hypoglycemic effect, whereas the oral doses of 100, 200, 500, and 1000 mg/kg, p.o., were prepared for LD_{50} experiments. Glibenclamide (as a standard), 5 mg/kg p.o., was prepared with distilled water.

3.4.2 Study of the test drug and positive control on experimental animals

Swiss albino mice of either sex (35–50 g body weight) were employed for this study. These animals were deprived of food for 16 h but allowed free access to water. They were housed in the departmental animal house and exposed to normal light. Experiments were performed according to the guide for the care and use of laboratory animals, from the CPCSEA, Ministry of Environment and Forest, Govt. of India (Reg. No. 107/1999/CPCSEA). After deprived of food for 16 h, mice were divided into six groups (six animals each), (I–VI), namely normal control, diabetic control, diabetes+BR-100, diabetes+BR-200, diabetes+BR-500 mg/kg, and positive control. Induction of diabetes was performed using a modification in the method described in the literature [27]. The diabetes was produced by an injection of alloxan (60 mg/kg, dissolved in saline) in the tail vein of mice. The diabetic state was assessed by blood glucose levels, 36 h after alloxan administration, and only the mice having blood glucose more than 150 mg/dl were selected for the study. Animals that presented glucose levels lower than 150 mg/dl were rejected. The group of normal control (I) was not administered alloxan and only received distilled water (5% alcoholic). Rest of the groups (II–VI) received alloxan and after 36 h, they were treated with distilled water (diabetic control); groups III–V were treated with 100, 200, and 500 mg/kg, p.o., respectively, of the BR extract; and group VI was treated with glibenclamide (5 mg/kg, p.o. as a standard). Blood samples of normal and alloxan-induced diabetic mice were collected at 0, 1, 3, 6, 18, and 24 h during the treatment. In each case, 10 µl of the serum sample was collected and estimated for glucose by the glucose oxidase–peroxidase method [28].

3.4.3 LD_{50} experiment

The mice were administered BR, orally at doses of 100, 200, 500, and 1000 mg/kg, p.o., body weight, and observed continuously for 1 h and intermittently up to 24 h for any gross behavioral changes and death.

3.4.4 Data and statistical analysis

Results are expressed as the mean ± SEM of six independent experiments. The data

were analyzed for statistical significance by one-way ANOVA test; P values < 0.05 were considered to be significant.

3.5 Antimicrobial evaluation

The antimicrobial study of the ethanolic extract and isolated compounds **1–3** was carried out by the agar disk diffusion method [29] against five bacterial species, *S. aureus*, *S. epidermidis*, *S. mutans*, *E. coli*, and *K. pneumoniae*, and six fungus pathogens, *A. niger*, *A. fumigatus*, *P. citrinum*, *Microsporum gypseum*, *M. canis*, and *T. rubrum*, including 10 hospital stains of *S. aureus* obtained from different culture media for the determination of the inhibition zone diameter (IZD) and MIC. Nutrient broth tubes (for bacterial species, incubated at 37°C for 24 h) and Sabouraud's broth tubes (for fungus pathogens, incubated at 27°C for 5 days) were prepared for the MIC determination. The MIC was determined as the least concentration of the sample inhibiting the growth of the test organisms [30,31]. The antimicrobial activity of compounds **1–3** was performed on the basis of activity shown by crude leaves extract. The activity of a certain concentration of the isolated compounds **1–3** (25 μg/ml) was compared with standard compounds novobiocin (15 μg/ml; for bacterial species) and erythromycin (15 μg/ml; for fungus pathogen).

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