Biomedical Research 2017; 28 (22): 10026-10032 *ISSN 0970-938X*
www.biomedres.info

Urease inhibition potential and molecular docking of dihydroquercetin and dihydromyricetin isolated from *Picea smithiana* (wall) Boiss.

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Abstract

Three flavanonol namely quercetin, dihydroquercetin and dihydromyricetin were isolated from ethyl acetate fraction of *Picea smithiana* (wall) Boiss by using standard isolation schemes. The isolated flavanonol were evaluated for their enzyme inhibition potential against urease, carbonic anhydrase and Phosphodiesterases-I. Flavanonol showed significant activity against urease with IC_{50} value of 29.73 ± 1.22 µM, While quercetin and dihydroquercetin were found to be weak inhibitors of urease with IC_{50} values of 208.87 ± 2.11 and 202.87 ± 2.01 µM respectively. Thiourea was used as a standard for the inhibition of urease enzyme (IC_{50}=21 ± 0.12). On the other hand flavanonol 2 showed potent activity against phosphodieseterase-1 when compared with the standard EDTA (IC_{50}=273 ± 1.69 µM). Newly identified inhibitors of enzyme may lead for the discovery of new drug to treat urolithiasis, and cardiovascular associated disorder. In silico drug designing plays an important role in the discovery of new inhibitors against the target. Compounds 1-3 were screen for docking study. The docked conformations of compounds 1-3 and reference thiourea showed potency of compounds 1-3. From the docking statistics, it is observed that the binding affinity of compound 3 is -8.5 kcal/mol (Autodock vina docking energies), which is best than the standard thiourea (-3.4 kcal/mol).

Keywords: *Picea smithiana*, Dihydroquercetin, Dihydromyricetin, Urease, Carbonic anhydrase, EDTA, Urolithiasis, Cardiovascular associated disorder.

Accepted on October 23, 2017

Introduction

Phyto-compounds serve as a lead compound for the production of modern medicines [1]. Synthetic drugs are costly and have adverse side effects such as rendering the patients immunocompromised, apart from leading evolution of drug resistant strains of bacterial pathogens [2]. Ethno-botanical studies play their role in revealing important plant species in term of crude drug discovery [3]. This traditional knowledge of medicinal plants is important for discovery and synthesis of mainstream drugs [4]. In this regard, phytochemical, biological and pharmacological studies backed the drug industry remarkably [5]. A plethora of plants have been reported to exert health benefits yet there are many poorly evaluated species. This study deals with one of such promising specie, *Picea smithiana* (aka West Himalayan spruce) which belongs to the family Pinaceae. This tree grows at high altitude, hilly areas of different areas of Pakistan such as Dir, Kaghan, Swat, Chitral, Kashmir, Gilgit and Kurram while it can also be found in Afghanistan, India and Nepal [6]. The genus *Picea* is a repertoire of phytochemicals like glycoside, lignans, flavonoids etc. [7]. Some of the traditional uses of the plant include the relief of skin diseases, renal disorders, eye diseases, diabetes and inflammations [8].

Phosphodiesterase (PDE, EC 3.1.4.1) family is a ubiquitous enzyme group that catalyzes the hydrolysis of phosphodiester bonds. PDEs comprising at least 11 distinct enzyme families are responsible for the hydrolysis of cyclic Adenosine Monophosphate (cAMP) and cyclic Guanosine Monophosphate (cGMP). Both cAMP and, to a lesser extent, cGMP possess an important role in the regulation of inotropic mechanisms in the human myocardium. However, cAMP has numerous effects in other tissues and different phosphodiesterase iso-enzymes are found in many other tissues [9]. Drugs that inhibit the action of PDE (thus reducing the breakdown of cAMP and cGMP) exert a therapeutic action on the heart, lung, and vasculature as well as on platelet function and inflammatory mechanisms. Many of these drugs affect more than one iso-enzyme widely distributed in tissues. As a result, PDE Inhibitors (PDEI) can have a multiplicity of effects. For example; theophylline displays activity on the lung as well as cardiac and vascular effects, while amrinone affects cardiac, vascular, and platelet functions. In fact, sildenafil (Viagra®) was originally studied as a possible anti-anginal agent [9]. Up to date, many phenolic compounds such as chromones; e.g. 5-(hydroxymethyl)-7-methoxy-2-methylchromone [10], flavonoids; e.g. amentoflavone, bilobetin, sequoiaflavone, and ginkgetin [10], stilbenes; e.g. trans-4', 5-dihydroxy-3-methoxystilbene-5-O-(R-L-rhamnopyranosyl-(1,6))-β-D-glucopyranoside [11], have been reported to inhibitory potential against PDE enzyme family and, therefore, dihydromyricetin (3) appear to be a successful source for showing to ascertain new PDE1 inhibitor.

Experimental

Reagents

Snake venom phosphodiesterase I (P4631), caffeine (C0750), and bis-(p-nitrophenyl) phosphate (N3002) were purchased from Sigma-Aldrich Co. (Milan, Italy). Organic solvents such as n-hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), and n-butanol (BuOH) were obtained from Suzhou Ausun Chemical Co., Lit. (Suzhou,China).Unless otherwise stated, all the other reagents were from Searle Pakistan Ltd. (Karachi, Pakistan).

Plant material

Picea Smithiana (wall) Boiss was collected from Hilly areas of Swat, Khyber Pakhtunkhwa, Pakistan in the month of July, 2015 and it was successively identified by Dr. Lal Badshah, Department of Botany, University of Peshawar, Pakistan.

Extraction and isolation

Coarsely powdered aerial parts of *Picea Smithiana* (wall) Boiss (16 kg) were subjected to maceration at room temperature for 15 d with commercial grade methanol with periodic shaking and mixing. After 15 d it was filtered with the help of muslin cloth which separated the methanol soluble material in the form of filtrate while the remaining insoluble plant remained in the form of residue. The filtrate of plant material obtained as a result of soaking contains the solvent (methanol) and solvent soluble part of plant. So to remove the solvent it was passed through the rotary evaporator under vacuum at 40°C. As a result about 1200 grams dark green colored crude methanolic extract (crude MeOH extract) was obtained. This extract was suspended in about 600 ml distilled water to form a solution. This suspension was then separated into various fractions with the help of a separating funnel by adding solvents of different polarity starting from less polar to more polar i.e. n-hexane (3 × 400 ml), $CHCl_3$ (3 × 400 ml), EtOAc (3 × 400 ml) and BuOH (3 × 400 ml) which yielded 160 g of n-hexane, 90 g of $CHCl_3$, 120 g of EtOAc, 145 g of BuOH and 130 g of aqueous fractions

The EtOAc fraction was subjected to repeated column chromatography and TLC in order to obtain the isolated compounds 1-3 (Figure 1). Their structures were established by comparing their spectral data and physical constants with data already present in literature [12,13].

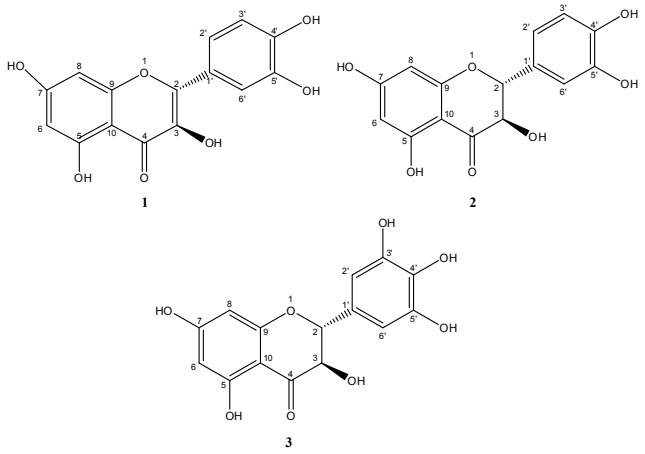

Figure 1. *Structures of isolated compounds (1-3) from EtOAc fraction of P. smithiana.*

Enzyme inhibitory assay

Anti-urease assay was performed according to the mentioned method [14] with slight modifications. First 5 µl of test compound, 25 µl (0.25 mg/ml) of enzyme were incubated at 37°C for 15 min. Then 55 µl of substrate (urea) was added and re-incubated at the same condition. After incubation, absorbance was measured at 630 nm, and data was recorded as pre read. Then 45 µL of phenol and 70 µL of alkali reagent was added to the mixture and incubated for 50 min. After incubation absorbance was measured at 630 nm and taken as after read. Thiourea was taken as positive control and methanol was taken as control.

Phosphodiesterase-I inhibition assay

In this assay, the PDE-I activity against snake venom (Sigma P-4631) (EC 3.1.4.1) was assayed using the reported method with some modifications [15]. Tris-HCl buffer 33 mM (pH 8.8), 30 mM Mg-Acetate was added as a cofactor with 0.000742 U of the enzyme as a final concentration using 96-well flat bottom plate as well as 0.33 mM bis (p-nitrophenyl)

phosphate (Sigma N-3002) as a substrate. EDTA (Ethylene Diamine Tetra Acetic acid) (E. Merck, Germany) was used as reference. After 30 min of incubation, the enzyme activity was monitored at 37°C on a microtiter plate reader spectrophotometer (Molecular Devices, USA) by following the release of p-nitrophenol from p-nitrophenyl phosphate at 410 nm. All the reactions were performed in triplicate, and the initial rates were measured as the rates of changes in the OD/min (optical density/minute) and used in subsequent calculations.

Carbonic anhydrase and α-chemotrypsin assay were performed following previously reported methods [9].

Molecular docking

The three dimensional (3D) X-ray crystallographic structure of urease enzyme (PDB ID: 4GY7) from jack bean was retrieved from Protein Data Bank (PDB) [16]. Geometry optimization and crystal structure refinement was carried out by utilizing Swiss pdb viewer v4.1.0 program [16]. The compounds (1-3) and standard thiourea structures were prepared for docking by using ChemSketch [17] and Avogadro's software [18]. Docking studies were carried out through AutoDock Vina [19] and initially method optimizations of the docking software were carried out.

PyRx tools was connected with AutoDock Vina [20]. Removal of solvent molecules, hydrogen addition and gasteiger charges calculation was carried out [21]. Furthermore, all the default parameters were maintained for docking through AutoDock Vina [22,23]. Interaction analysis of docked complexes was performed by LIGPLOT+ version v.1.4.5 [24] and PyMOL version 1.7.2 [25].

Results and Discussion

Enzyme inhibition assay

A number of diseases are caused due to the over expression of enzyme urease and phosphodiesterase-I. *Helicobactor pylori* produces urease which causes gastric ulcer and urolithiasis while cardiovascular diseases and erectile dysfunction are associated with over expression of phosphodiesterase-I. New and novel discoveries are important to treat various ailment associated with hyperactivities of these enzymes [13,26]. In the present study three secondary metabolites; quercetin (1) dihydroquercetin (2) dihydromyricetin (3) were isolated from *Picea Smithiana* (wall) Boiss. The biological activities of these compounds are presented in Table 1.

Among these compounds, quercetin (1) and dihydroquercetin (2) showed selective inhibition against urease and were found inactive against CA-II and phosphodiesterase-I. On the other hand, dihydromyricetin (3) showed potent activity against urease and phosphodiesterase-I with IC_{50}=29.73 ± 1.22, 202.33 ± 1.46 µM, respectively, when compared with the standard inhibitors (Thiourea IC_{50}=21 ± 0.12, EDTA IC_{50}=277.69 ± 2.52 µM). Keeping in view the above results and discussion dihydromyricetin (3) can lead as potential chromosphere for hit to lead optimization.

Molecular docking

In silico drug designing plays an important role in the discovery of new inhibitors against the target. This process requires less time and is cost effective. Computational docking studies of the compounds 1-3 were carried out to analyse their binding pattern in the active site of the target (urease) enzyme. The docking studies were carried out by the AutoDock Vina. The docking procedure was optimized. Generally docking studies shows that, if a compound contribute lesser interaction energies then that compound has best activities against the targeted enzyme. It can be predicted from the compound that there are certain necessary features in the compounds which are responsible for their potency. The docking detail of compound 1-3 and reference thiourea are presented in the Table 2.

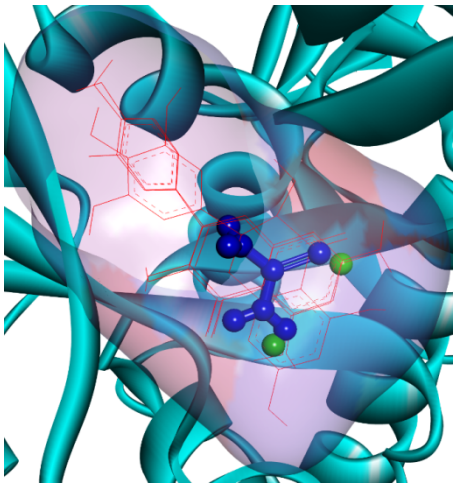

Figure 2. *The best docked conformation of compounds (1-3) (shown by red color sticks) along with superimposed standard thiourea (ball and sticks blue color) against the Urease enzyme (cyan color). These binding conformations were produced in the binding site, where metals (green dots nickel atoms) are already present in the active site of crystal structure.*

The best docked conformation of the compound 3 was analysed it was based upon the hydrogen and hydrophobic interactions. The docking energies of compound 3 are best than the reference thiourea and all other compounds. It means that there are certain features in the structure of this compound which are responsible for mediating the biological activities. The docked conformations of compounds 1-3 and reference thiourea are shown in the Figure 2. From the docking statistics, it is observed that the binding affinity of compound 3 is -8.5 kcal/mol (Autodock vina docking energies), which is best than the standard thiourea (-3.4 kcal/mol) (Table 2).

The interaction analysis revealed that, there are four hydrogen bonds formed by compound 3 in the binding site of urease enzyme, that hydrogen bond (2.84 Å) observed from Gly467 with the -OH group of compound 3 (Figure 3). Here, we also investigated other two hydrogen bonds from the His544,

distance of 2.05 Å and 2.95 Å with compound 3. The Asp632 in the binding pocket of urease enzyme also showed hydrogen bonding contact with a distance of 2.00 Å. The hydrophobic interactions which were also observed from the surrounding residues in the binding site of urease enzyme, these residues including His406, Thr440, Ala439, His491, Ile517, Tyr543 and Ala635 forms seven hydrophobic contacts with compound 3. The interaction profile of compounds 1 and 2 is shown in the Figures 4 and 5.

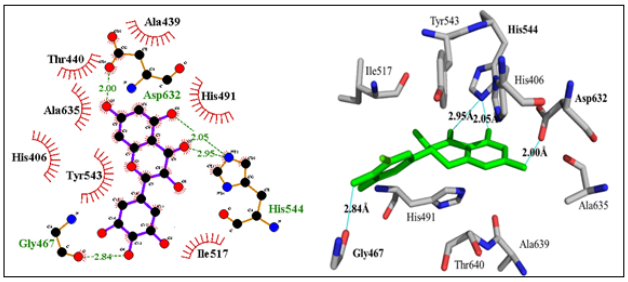

Figure 3. *The 2D (left) and 3D (right) interaction profile of compound 3 with the active site of urease enzyme. In the above 2D image half-moon shows the hydrophobic interactions while hydrogen bond is represented by dotted green lines with distance in Angstrom.*

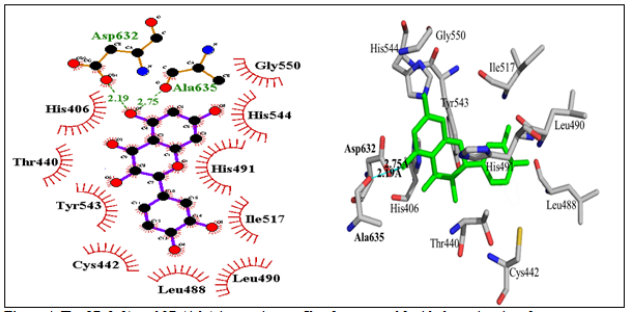

Figure 4. *The 2D (left) and 3D (right) interaction profile of compound 2 with the active site of urease enzyme.*

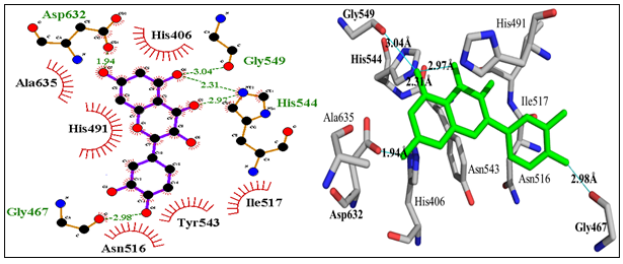

Figure 5. *The 2D (left) and 3D (right) interaction profile of compound 1 with the active site of urease enzyme.*

Quercetin (1)

Compound 1 was purified as colorless crystalline solid from EtOAc fraction of P. smithiana. The molecular formula of this compound was identified as $C_{15}H_{12}O_7$ (304.23) by EI-MS, ESI spectrum.

IR (KBr, *v*max in cm^{-1}) 3583, 1665, 2926, 1585 and 1465. UV λ_{max} (nm): 340, 265. 1H-NMR (600 MHz, MeOD) *δ*h: 5.90 (^{1}H, d, H-6, j=2.5), 5.92 (^{1}H, d, H-8, j=2.5), 6.62 (H, d, H-2' , j=11.5) and 6.64 (H, d, H-3'), 6.64 (H, s) respectively; ^{13}C-NMR (150 MHz, MeOD), *δc*: 85.4 (CH, C-2), 73.8 (CH, C-3), 198.5 (C, C-4), 165.5 (C, C-5), 97.6 (CH, C-6), 169.0 (C, C-7), 96.5 (CH, C-8), 165.0 (C, C-9), 102.1 (C, C-9), 129.3 (C, C-1'), 115.5 (CH, C-2'), 112.4 (CH, C-3'), 145.2 (C, C-4'), 147.7 (C, C-5') and 112.4 (CH, C-6') (Table 3). The HMBC correlations confirmed the position of all five hydroxyl group (OH) at 3, 5, 7, 4' and 5'. Based on spectroscopic data and comparing data with literature reported valve structure of compound 1 was identified as quercetin [12].

Dihydroquercetin (2)

Compound 2 was purified as colorless crystalline solid from EtOAc fraction of *P. smithiana*. The molecular formula of this compound was identified as $C_{15}H_{12}O_7$ (302.18) by EI-MS, ESI spectrum.

IR (KBr, *v*max in cm^{-1}) 3580, 1664, 2925, 1583 and 1460. UV λ_{max} (nm): 335, 260. ^{1}H-NMR (600 MHz, MeOD) $^{\delta}$h: 4.86 (^{1}H, d, H-2, j=12.5), 4.49 (^{1}H, d, H-3, j=12.7), 5.90 (^{1}H, d, H-6, j=2.5), 5.92 (^{1}H, d, H-8, j=2.5), 6.62 (H, d, H-2', j=11.5) and 6.64 (H, d, H-3'), 6.64 (H, s) respectively; ^{13}C-NMR (150 MHz, MeOD), *δ*c: 157.3 (C, C-2), 132.4 (C, C-3), 80.5 (C, C-4), 165.5 (C, C-5), 97.6 (CH, C-6), 169.0 (C, C-7), 96.5 (CH, C-8), 165.0 (C, C-9), 102.1 (C, C-9), 129.3 (C, C-1'), 115.5 (CH, C-2'), 112.4 (CH, C-3'), 145.2 (C, C-4'), 147.7 (C, C-5') and 112.4 (CH, C-6') (Table 4). The HMBC correlations confirmed the position of all five hydroxyl group (OH) at 3, 5, 7, 4' and 5'. Based on spectroscopic data and comparing data with literature reported valve structure of compound 2 was identified as dihydroquercetin [12].

Dihydromyricetin (3)

Compound 3 was purified as light yellow powder from EtOAc fraction of *P. smithiana*. The molecular formula of this compound was identified as $C_{15}H_{12}O_8$ (320.24) by EI-MS, ESI spectrum.

IR (KBr, *v*max in cm^{-1}) 3590, 1660, 2924, 15, 88 and 1460. UV λ_{max} (nm): 345, 270. ^{1}H-NMR (600 MHz, MeOD) *δ*h: 4.86 (^{1}H, d, H-2, j=12.5), 4.49 (^{1}H, d, H-3, j=12.7), 5.90 (^{1}H, d, H-6, j=2.5), 5.92 (^{1}H, d, H-8, j=2.5), 6.64 (2H, H-2') and 6.64 (^{2}H, s, H-6') respectively; ^{13}C-NMR (150 MHz, MeOD), *δ*c: 85.4 (CH, C-2), 73.8 (CH, C-3), 198.5 (C, C-4), 165.5 (C, C-5), 97.6 (CH, C-6), 169.0 (C, C-7), 96.5 (CH, C-8), 165.0 (C, C-9), 102.1 (C, C-9), 129.3 (C, C-1'), 109.1 (CH, C-2'), 148.7 (C, C-3'), 135.1 (C, C-4'), 148.7 (C, C-5') and 109.1 (CH, C-6') (Table 5).

The HMBC correlations confirmed the position of all five hydroxyl group (OH) at 3, 5, 7, 3', 4' and 5'. Based on spectroscopic data and comparing data with literature reported

valve structure of compound 3 was identified as dihydromyricetin [13].

Table 1. *Enzymes inhibitory activities of secondary metabolites of Picea smithiana (wall) Boiss.*

Compounds	Ureases		Phosphodiesterase-I		CA-II
	% Inhibition (0.5 mM)	IC_{50} ± S.E.M. (µM)	% Inhibition (0.5 mM)	IC_{50} ± S.E.M. (µM)	% Inhibition (0.5 mM)
Quercetin (1)	75.54	208.87 ± 2.11	35.39	-	10.12
Dihydroquercetin (2)	79.27	202.87 ± 2.01	47.45	-	14.76
Dihydromyricetin (3)	92.28	29.73 ± 1.22	78.33	202.33 ± 1.46	24.55
Standard	Thiourea	Thiourea	EDTA	EDTA	ACZ
	98.12	21 ±0.12	80.33	277.69 ±2.52	89.44

CA-II: Carbonic Anhydrase-II, IC_{50}: Minimum Inhibitory Concentration; SEM: Standard Error of Mean; µM: Micro Molar; mM: mili Molar; EDTA: Ethylene Diammine Tetraacetic Acid; ACZ: Acetazolamide.

Table 2. *Docking statistics of compounds 1-3 and reference thiourea against the urease enzyme.*

Compounds	Hydrogen bonding residues and distance		Hydrophobic interacting residues	Binding affinity (Kcal/mol)
1	Gly467 His544 Gly549 Asp632	2.98 Å 2.31 Å and 2.97 Å 3.04 Å 1.94 Å	His406, His491, Asn516, Ile517, Tyr543 and Ala635	-8.4
2	Asp632	2.19 Å	His406, Thr440, Cys442, Leu488, Leu490, His491, Ile517, Tyr543, His544 and Gly550	-8
	Ala635	2.75 Å		
3	Gly467 His544 Asp632	2.84 Å	His406, Thr440, Ala439, His491, Ile517, Tyr543 and Ala635	-8.5
		2.05 Å and 2.95 Å		
		2.00 Å		
Thiourea (standard)	-		-	-3.4

Table 3. *^{1}H-NMR and ^{13}C-NMR spectra of quercetin (1).*

C. No.	δC	δH (mult, J=HZ)	Types	HMBC
1	-	-	-	-
2	85.4	-	C	C-2, C-1′, C-6′
3	73.8	-	C	C-4, C-1′, C-4, C-10
4	198.5	-	C	-
5	165.5	-	C	-
6	97.6	5.90 (^{1}H, d, 2.5)	CH	C-6, C-5, C-7, C-8
7	169	-	C	-
8	96.5	5.92 (^{1}H, d, 2.5)	CH	C-8, C-7, C-9C-10
9	165	-	C	-
10	102.1	-	C	-
1′	132.7	-	C	-
2′	115.5	6.62 (H, d,11.5)	CH	C-1′, C-3′, C-4′
3′	112.4	6.64 (H, d,11.5)	C	C-1′, C-4′, C-5′
4′	145.2	-	C	-
5′	147.7	-	C	-
6′	112.4	6.64 (H, s)	CH	C-6′, C-5′, C-1′

Table 4. *^{1}H-NMR and ^{13}C-NMR spectra of dihydroquercetin (2).*

C. No.	δC	δH (mult, J=HZ)	Types	HMBC
1	-	-	-	-
2	157.3	4.86 (^{1}H, d, 12.5)	CH	-
3	132.4	4.49 (^{1}H, d, 12.7)	CH	-
4	80.5	-	C	-
5	165.5	-	C	-
6	97.6	5.90 (^{1}H, d, 2.5)	CH	C-6, C-5, C-7, C-8
7	169	-	C	-
8	96.5	5.92 (^{1}H, d, 2.5)	CH	C-8, C-7, C-9C-10
9	165	-	C	-
10	102.1	-	C	-

1′	132.7	-	C	-
2′	115.5	6.62 (H, d,11.5)	CH	C-1′, C-3′, C-4′
3′	112.4	6.64 (H, d,11.5)	C	C-1′, C-4′, C-5′
4′	145.2	-	C	-
5′	147.7	-	C	-
6′	112.4	6.64 (H, s)	CH	C-6′, C-5′, C-1′

Table 5. ^{1}H-NMR and ^{13}C-NMR spectra of dihydromyricetin (3).

C. No.	δC	δH (mult, J=HZ)	Types	HMBC
1	-	-	-	-
2	85.4	4.86 (^{1}H, d, 12.5)	CH	C-2, C-1′, C-6′
3	73.8	4.49 (^{1}H, d, 12.7)	CH	C-4, C-1′, C-4, C-10
4	198.5	-	C	-
5	165.5	-	C	-
6	97.6	5.90 (^{1}H, d, 2.5)	CH	C-6, C-5, C-7, C-8
7	169	-	C	-
8	96.5	5.92 (^{1}H, d, 2.5)	CH	C-8, C-7, C-9C-10
9	165	-	C	-
10	102.1	-	C	-
1′	129.3	-	C	-
2′	109.1	6.64 (H, s)	CH	C-2′, C-3′, C-4′
3′	148.7	-	C	-
4′	135.1	-	C	-
5′	148.7	-	C	-
6′	109.1	6.64 (H, s)	CH	C-6′, C-5′, C-1′

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