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In vitro anti-inflammatory activity of a new allelochemical from the bark of *Pithecellobium dulce* (Roxb.) Benth.

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Pithecellobium dulce (Roxb.) Benth. belongs to family Leguminosae which is commonly known as Vilayati imli or Jangle Jalebi in Hindi. It is a native of tropical America and cultivated throughout India. Its bark is reported to be used as astringent in dysentery and as febrifuge. It is also used in dermatitis and eye inflammation. A new allelochemical has been isolated from methanolic extract of the bark of this plant, along with two known compounds sinenestin (B), apigenin-7-O-glucopyranoside (C). The structure of a new allelochemical (A) has been characterized as 3,5,7,3′, 4′-pentahydroxy flavone-7-O-α-L-rhamnopyranosyl-3′-O-β-D-xylopyranosyl (1→3)–O-β-D-glucopyranoside, by various colour reactions, chemical degradations and spectroscopic analysis. *In vitro* anti-inflammatory assay of compound A shows significant anti-inflammatory activity against the denaturation of egg albumin protein.

Keywords: *Pithecellobium dulce* (Roxb.) Benth, Leguminosae, bark, allelochemicals (plant secondary metabolites), anti-inflammatory activity

Pithecellobium dulce (Roxb.) Benth.[1-6] belongs to family Leguminosae and commonly known as Vilayati imli or Jangle Jalebi in Hindi, it is a native of tropical America and cultivated throughout India. It is a medium-sized, thorny, evergreen tree. Its bark is used as febrifuge and it is also used in dermatitis and eye inflammation. Its leaves have been reported to possess astringent, emollient and antidiabetic properties[7]. The leaf and seed extracts of this plant have the potential to be used for an ideal eco-friendly approach for the control of mosquitoes[8]. Hydroalcoholoic fruit extract of this plant possesses potent anti-ulcer activity probably by acting as cytoprotective and anti-acid secretory agent[9].

Earlier workers have reported the presence of various active constituents from this plant *e.g.* N. P. Sahu *et al*.[10] have isolated 3-O- [β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl]- 28-O-[β-D-xylopyranosyI-(l→6)-β-D-glucopyranosyl]-echinocystic acid from the seeds of this plant. V. K. Saxena *et al*.[11] have isolated 3'-prenylapigenine-7-*O*-rutinoside from the stems of this plant. S. K. Nigam *et al*.[12] have isolated saponins from the seeds of this plant, echinocystic acid 3-O-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside, echinocystic acid and oleanolic acid 3-O-α-L-arabinopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranosides and 3-O-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranosides, oleanolic acid 3-O-α-L-arabinopyranosy-(1→2)-α-L-arabinopyranosyl-(1→6) -[β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside, and 3-O-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside. A. K. Yadav *et al*.[13] have isolated kaempferol-3-O-rhamnoside and quercetin from this plant. In the present paper we have reported the isolation and structural elucidation of a new compound (A) 3,5,7,3′,4′-pentahydroxy flavone-7-O-α-L-rhamno-pyranosyl-3′-O-β-D-xylopyranosyl (1→3) – O-β-D-glucopyranoside alongwith two known compounds sinenestin (B), apigenin-7-O-glucopyranoside (C), from methanolic extract of the bark of this plant. *In vitro* anti-inflammatory effect of isolated compound A against the denaturation of egg albumin protein showed good result.

Results and Discussion

Compound A has molecular formula $C_{32}H_{38}O_{20}$, m.p. 318-20°C, $[M]^+$ *m/z* 742 (FABMS). It gave positive Molisch and Shinoda tests[14-17] showing its flavonoidal glycosidic nature. Its IR spectrum showed absorption bands at 3440 cm^{-1} (-OH), 1690 cm^{-1} (>C=O α-β unsaturated), 1600 cm^{-1} (aromatic ring system) and 1083 cm^{-1} (glycosidic linkage). In

UV-Vis spectrum, two bands at 329, 255 nm showed its flavonoidal skeleton. Its absorption bands at 389, 364 nm with $AlCl_3$ and 270, 371 nm with NaOAc and NaOMe showed the presence of –OH groups at C-3, C-5, C-7, C-4′ respectively[18]. In ^{1}H NMR spectrum, compound A showed three singlets at δ 15.05, 5.02, 5.05 and confirmed the presence of -OH groups at C-3, C-5 and C-4′ position. Four doublets at δ 6.09 (1H, d, J = 2.2H_Z,), 6.25 (1H, d, J = 2.1 H_Z), 7.69 (1H, d, J = 1.9 H_Z) and 6.67 (1H, d, J = 8.0 H_Z) were assigned to H-6, H-8, H-2′ and H-5′ position respectively and double doublet at δ 7.58 (1H, dd, J = 8.1, 1.9 H_Z), assingned for H-6′ position. The anomeric proton signals at δ 4.58 (1H, d, J = 1.6 H_Z), δ 5.21 (1H, d,J = 7.6 H_Z), δ 4.25 (1H, d, J = 7.2 H_Z), were assigned for H-1″, H-1‴, H-1⁗of L-rhamnose, D-glucose and D-xylose respectively. In ^{1}H NMR spectrum, coupling constant at J = 1.6 H_Z of H-1″ confirmed α-configuration of L-rhamnose while two coupling constants at J = 7.6 H_Z and J = 7.2 H_Z for the anomeric protons of D-glucose and D-xylose confirmed β-configuration of D-glucose and D-xylose[19].

In the mass spectrum of compound A, characteristic ion peaks at m/z 742 [M^+], 596[M^+-L-rhamnose], 464 [M^+-D-xylose], 284 [M^+-D-glucose], were found by subsequent losses from the molecular ion of each molecule of L-rhamnose, D-xylose, and D-glucose, revealing the presence of L-rhamnose at C-7 position and D-xylose attached with D-glucose at 3‴ position and D-glucose was linked to aglycone at C-3' position.

Acid hydrolysis of compound A with 10% ethanolic H_2SO_4 gave aglycone A-1, mol. formula $C_{15}H_{10}O_7$, m.p. 314-16°C, $[M]^+$ m/z 302 (FABMS), and sugar moieties. These were separated and studied separately. The aglycone A-1 was identified as 3,5,7,3′,4′-pentahydroxy flavone (Experimental Section).

The aqueous hydrolysate was neutralized with $BaCO_3$ and the $BaSO_4$ filtered off. The filtrate was concentrated and subjected to paper chromatographic examination (using Whatman filter paper No.1) and sugars were identified as L-rhamnose (R_f 0.35), D-xylose (R_f 0.26) and D-glucose (R_f 0.19), (Co-PC)[20].

Quantitative estimation[21] of sugars revealed that all the three sugars were present in equimolar ratio 1:1:1. Periodate oxidation[22] of compound A confirmed that all the sugars were present in the pyranose form. The position of sugar moieties in compound A were determined by permethylation[23] followed by acid hydrolysis which yielded methylated aglycone identified as 7,3'-dihydroxy-3,5,4'-trimethoxy flavone. This showed that glycosydation was involved at C-7 and C-3' positions of aglycone. The methylated sugars were identified as 2,3,4-tri-O-methyl-L-rhamnose [R_G 1.03], 2,4,6-tri-O-methyl-D-glucose [R_G 0.74], 2,3,4-tri-O-methyl-D-xylose [R_G 0.95] indicating that C-1″ of L-rhamnose was attached with −OH group at C-7 position of the aglycone and C-1⁗−OH of D-xylose was linked with C-3‴ −OH of D-glucose and C-1‴ of D-glucose was attached with −OH group at C-3′ position of aglycone. Therefore, inter glycosidic linkage (1→3) was found between D-xylose and D-glucose.

Enzymatic hydrolysis[24] of compound A with takadiastase enzyme liberated L-rhamnose indicating the presence of α-linkage between L-rhamnose and 3,5,7,3′,4′-pentahydroxy flavone-3′-O-β-D-xylopyranosyl (1→3)–O-β-D-glucopyranoside as proaglycone. Proaglycone on further hydrolysis with almond emulsin enzyme liberated D-xylose first followed by D-glucose which showed the presence of β-linkage between D-xylose and D-glucose. Thus the compound A was identified as 3,5,7,3′,4′-pentahydroxy flavone-7-O-α-L-rhamnopyranosyl-3′-O-β-D-xylopyranosyl (1→3)-O-β-D-glucopyranoside.

On the basis of the above evidence, the structure of compound A was established as 3,5,7,3′,4′-pentahydroxy flavone-7-O-α-L-rhamnopyranosyl-3′-O-β-D-xylopyranosyl(1→3)-O-β-D-glucopyranoside. *In vitro* anti-inflammatory effect of compound A was evaluated against the denaturation of egg albumin. The results are recorded in Table I and Table II. From above results it was concluded that the compound A exhibited maximum % inhibition (122.5705%) of protein denaturation at 1000 µg/mL and Diclofenac sodium showed the maximum inhibition (120.0627%) at the same concentration. Thus at the same concentration, compound A showed more % inhibition of protein denaturation in comparison to

Table I — Effect of Diclofenac sodium on protein denaturation

	Absorbance of Control = 0.319	
Concentration of Diclofenac sodium in reaction mixture (µg/mL)	Absorbance of Diclofenac sodium	% Inhibition of Diclofenac sodium
50	0.365	14.42006
100	0.405	26.95925
250	0.440	37.93103
500	0.536	68.02508
1000	0.702	120.0627

Diclofenac sodium. Therefore compound A can be used as an anti-inflammatory active agent.

Experimental Section

All of the melting points were determined by Thermoelectric melting point apparatus and are uncorrected. The IR spectra were recorded on Simadzu 84005 FTIR spectrophotometer in KBr pellets, UV-Vis spectra were recorded on Systronics-2201 UV-Vis Double Beam Spectrophotometer in MeOH. NMR spectra were recorded on Bruker DRX-300 Spectrometer operating at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR using DMSO-d_6 as solvent and TMS as internal standard. The mass spectra were recorded on Jeol-SX (102) mass spectrometer.

Plant material

The bark of the plant was collected locally around Sagar region and taxonomically authenticated by Taxonomist, Department of Botany, Dr. H. S. Gour Central University, Sagar (M.P.) India. A voucher specimen has been deposited in the Natural Products Laboratory, Department of Chemistry of this University.

Extraction and Isolation

Air dried and powdered bark (5.0 Kg) of the plant was extracted with ethanol in a Soxhlet apparatus for 4 days. The ethanolic extract was concentrated and successively partitioned with chloroform, ethyl acetate, acetone, and methanol. The methanol soluble fraction was concentrated under reduced pressure by rotary vacuum evaporator to give brown viscous mass (3.50 g), which was subjected to TLC examination using silica gel-G and *n*-BAW (4:1:5) as solvent and I_2 vapours as visualizing agent. It showed three spots on TLC indicating it to be a mixture of three compounds A, B and C. These compounds were separated by column chromatography over silica gel using $CHCl_3$:MeOH (4:6) as eluent and studied separately.

Table II — Effect of Compound A on protein denaturation

	Absorbance of Control = 0.319	
Concentration of Diclofenac sodium in reaction mixture (μg/mL)	Absorbance of Compound A	% Inhibition of compound A
50	0.411	28.84013
100	0.445	39.49843
250	0.512	60.50157
500	0.611	91.53605
1000	0.710	122.5705

Study of Compound A

It was crystallized from acetone to give light brown crystals (1.15 g, Figure 1). It had mol. formula $C_{32}H_{38}O_{20}$. m.p.318-20°C. $[M]^+$ *m/z* 742 (FABMS). Anal. Found: C, 51.64; H, 5.10; O, 43.12. Calcd for $C_{32}H_{38}O_{20}$: C, 51.75; H, 5.16; O, 43.09%. UV-Vis: λ_{max} MeOH (nm) 329, 255, (+$AlCl_3$) 389, 364 (+NaOMe), 371 (+NaOAc) 270; IR (KBr): 3440, 1690, 1600, 1083 cm^{-1}; ^{1}H NMR (300 MH_Z, DMSO-d_6): δ 15.05 (1H, s, 3-OH), 5.02 (1H, s, 5-OH), 6.09 (1H, d, J = 2.2H_Z, H-6), 6.25 (1H, d, J = 2.1 H_Z, H-8), 7.69 (1H, d, J = 1.9 H_Z, H-2′), 5.05 (1H, s, 4′-OH), 6.67 (1H, d, J = 8.0 H_Z, H-5′), 7.58 (1H, dd, J = 8.1, 1.9 H_Z, H-6′), 4.58 (1H, d, J = 1.6 H_Z, H-1″), 3.08 (1H, m, H-2″), 3.47 (1H, m, H-3″), 3.38 (1H, m, H-4″), 3.85 (1H, m, H-5″),1.01 (3H, d, J = 6.2 H_Z, CH_3-6″), 5.21 (1H, d, J = 7.6 H_Z, H-1‴), 3.65 (1H, m, H-2‴), 3.12 (1H, m, H-3‴), 3.60 (1H, m, H-4‴), 4.05 (1H, m, H-5‴), 3.61 (1H, dd, J = 12.0, 2.1, H-6‴), 4.25 (1H, d, J = 7.2 H_Z, H-1⁗), 3.34 (1H, m, H-2⁗), 3.38 (1H, m, H-3⁗), 3.35 (1H, m, H-4⁗), 3.20 (1H, m, H-5⁗); ^{13}C NMR (75MH_Z, DMSO-d$_6$): δ 148.1(C-2), 135.4 (C-3), 176.1(C-4), 160.9(C-5), 98.2(C-6), 164.3(C-7), 95.1 (C-8), 157.5 (C-9), 104.5 (C-10), 120.8 (C-1′), 115.8 (2′), 145.4 (C-3′), 148.3 (C-4′), 115.4 (C-5′), 121.7 (C-6′), 101.2 (C-1″), 70.3 (C-2″), 72.1 (C-3″), 72.4 (C-4″), 71.8 (C-5″), 16.9 (C-6″), 102.9 (C-1‴), 74.8 (C-2‴), 75.5 (C-3‴), 70.6 (C-4‴), 76.1 (C-5‴), 66.6 (C-6‴), 99.8 (C-1⁗), 68.3 (C-2⁗), 72.2 (C-3⁗), 66.7 (C-4⁗), 72.5 (C-5⁗).

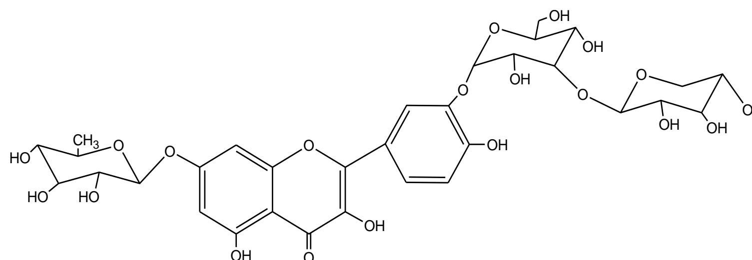

Figure 1 — Compound A

Acid hydrolysis of Compound A

Compound A (400 mg) was dissolved in ethanol (25 mL) and refluxed with 20 mL of 10% H_2SO_4 on a water bath for 7 h. The contents were concentrated and allowed to cool and residue was extracted with diethyl ether (Et_2O). The ether layer was washed with water and the solvent evaporated to dryness. The residue was subjected to column chromatography over silica gel using $CHCl_3$:MeOH (3:6) to give compound A-1, which was identified as 3,5,7,3′,4′-pentahydroxyflavone. The aqueous hydrolysate was neutralized with $BaCO_3$ and the $BaSO_4$ was filtered off. The filtrate was concentrated and subjected to paper chromatography examination using *n*- Butanol:Ethanol:Water (4:1:5) as a solvent and aniline hydrogen phthalate as detecting reagent. The sugars were identified as L-rhamnose (R_f 0.35), D-xylose (R_f 0.26) and D-glucose (R_f 0.19), (Co-PC).

Study of Compound A-1

It was analyzed for mol. formula $C_{15}H_{10}O_7$ (Figure 2). m.p.314-16°C. $[M]^+$ *m/z* 302 (FABMS). Anal. Found: C, 59.64; H, 3.39; O, 37.12. Calcd for $C_{15}H_{10}O_7$: C, 59.61; H, 3.33; O, 37.06%. UV-Vis: λ_{max} MeOH (nm) 335, 257 (+$AlCl_3$) 395, 374 (+NaOMe) 375 (+NaOAc) 274; IR (KBr): 3438, 1689, 1600 cm^{-1}; ^{1}H NMR (300 MH_Z, DMSO-d_6): δ 15.04 (1H, s, 3-OH), 5.06 (1H, s, 5-OH), 6.12 (1H, d, J = 2.4 H_Z, H-6), 6.35 (1H, d, J = 2.0 H_Z, H-8), 7.75 (1H, d, J = 1.11 H_Z, H-2′), 6.89 (1H, s, 4′-OH), 6.89 (1H, d, J = 8.1 H_Z, H-5′), 7.60 (1H, dd, J = 8.2, 1.9 H_Z, H-6′); ^{13}C NMR (75MH_Z, DMSO-d_6): δ 147.5(C-2), 136.2 (C-3), 177.3 (C-4), 163.8 (C-5), 99.4 (C-6), 166.3 (C-7), 94.6 (C-8), 158.2 (C-9), 106.5 (C-10), 122.7 (C-1′), 117.1 (2′), 146.5 (C-3′), 148.3 (C-4′), 116.7 (C-5′), 120.3 (C-6′).

Permethylation of Compound A

Compound A (30 mg) was dissolved in DMF(25 mL) and treated with MeI (5 mL) and Ag_2O (15 mL) in round bottomed flask fitted with air condenser and refluxed for 24 h and the contents were filtered and washed with DMF. The filtrate was concentrated under reduced pressure and hydrolysed with 10% ethanolic H_2SO_4 for 4-5 h to give methylated aglycone identified as 7,3'-dihydroxy-3,5,4'-trimethoxy flavone and the aqueous hydrolysate obtained after the removal of aglycone was neutralized with $BaCO_3$ and the $BaSO_4$ filtered off. The filtrate was concentrated and subjected to paper chromatography examination over Whatman filter paper No.1 using *n*-Butanol:Ethanol:Water (5:1:4) as a solvent and aniline hydrogen phthalate as detecting reagent. The methylated sugars were identified as 2,3,4-tri-O-methyl-L-rhamnose [R_G 1.03], 2,4,6-tri-O-methyl-D-glucose [R_G 0.74], 2,3,4-tri-O-methyl-D-xylose [R_G 0.95].

Enzymatic Hydrolysis of Compound A

Compound A (25 mg) was dissolved in MeOH (20 mL) and hydrolysed with an equal volume of takadiastase enzyme. The reaction mixture was allowed to stay at RT for two days and filtered. The hydrolysate was concentrated and subjected to paper chromatography examination using *n*-Butanol:Ethanol:Water (4:1:5) as a solvent and aniline hydrogen phthalate as a spraying reagent which showed the presence of L-rhamnose (R_f 0.35). The proaglycone was dissolved in MeOH (20 mL) and further hydrolysed with equal volume of almond emulsin enzyme at RT as usual procedure yielded aglycone identified as 3,5,7,3′,4′-pentahydroxy flavone and sugars were identified as D-xylose (R_f 0.26) and D-glucose (R_f 0.19) (Co-PC).

Study of Compound B

It was crystallized from methanol to give white amorphous powder (0.350 g, Figure 3). It had mol. formula $C_{20}H_{20}O_7$. m.p.175–77°C. $[M]^+$ *m/z* 372 (FABMS). Anal. Found: C, 64.49; H, 5.38; O, 30.10. Calcd for $C_{20}H_{20}O_7$: C, 64.51; H, 5.41; O, 30.08%. UV-Vis: λ_{max} (nm) 325, 284, 213 nm; IR (KBr): 1643, 1600, 1590, 1473, 1379, 1213, 870 cm^{-1}; ^{1}H NMR (300 MH_Z, DMSO-d_6): δ 6.81 (1H, s, H-3), 7.15 (1H, s, H-8), 7.59 (1H, s, H-2′), 7.16 (1H, d, J = 8.1 H_Z, 5′-H), 7.72 (1H, d, J = 1.09 H_Z, H-6′), 3.74 (3H, s, 5-OCH_3), 3.76 (3H, s, 6-OCH_3), 3.92 (3H, s, 7-OCH_3), 3.80 (3H, s,3′-OCH_3), 3.85 (3H, s, 4′-OCH_3); ^{13}C NMR (75MH_Z, DMSO-d_6): δ 169.7 (C-2), 95.9 (C-3), 187.1 (C-4), 153.5 (C-5), 129.1

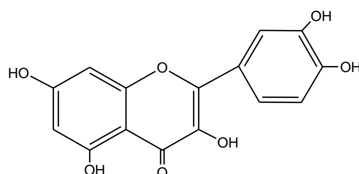

Figure 2 — Compound A-1

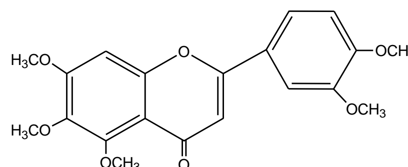

Figure 3 — Compound B

(C-6), 156.3 (C-7), 98.5 (C-8), 154.1 (C-9), 106.2 (C-10), 129.2 (C-1′), 114.7 (2′), 149.5 (C-3′), 147.8 (C-4′), 117.1 (C-5′), 119.4 (C-6′). Thus it was identified as Sinensetin by comparison of its spectral data with reported literature values[25,26].

Study of Compound C

It was crystallized from acetone to give light yellow crystals (0.410 g, Figure 4). It was analyzed for mol. formula $C_{21}H_{20}O_{11}$. m.p.232-34°C. $[M]^+$ *m/z* 448. Anal. Found: C, 56.29; H, 4.52; O, 39.20. Calcd for $C_{21}H_{20}O_{11}$: C, 56.25; H, 4.50; O, 39.25%. UV-Vis: λ_{max} MeOH nm 354, 258, 414, 389, 273, 394; IR (KBr): 3445, 1685, 1600, 1085 cm^{-1}; ^1H NMR (300 MH_Z, DMSO-d_6): δ 6.72 (1H, s, H-3), 6.40 (1H, d, *J* = 2.1 Hz, H-6), 6.78 (1H, d, *J* = 2.0 Hz, H-8), 7.88 (2H, d, *J* = 9.4 Hz, H-2', H-6'), 6.11 (2H, d, *J* = 8.6 Hz, H-3', H-5'), 5.06 (1H, d, *J* = 7.3 Hz, H-1"), 3.12-3.21 (3H, m, H-2", H-3", H-4", H-5"), 3.52 (1H, dd, *J* = 11.2, 6.4 Hz, Ha-6"), 3.60 (1H, dd, *J* = 11.2, 1.4 Hz, Hb-6"); ^{13}C NMR (75 MH_Z, DMSO-d_6): δ 162.1 (C-2), 122.6 (C-3), 187.6 (C-4), 160.8 (C-5), 98.1 (C-6), 165.6 (C-7), 95.8 (C-8), 158.6 (C-9), 105.1 (C-10), 125.5 (C-1'), 128.3 (C-2'), 116.6 (C-3'), 162.5 (C-4'), 115.6 (C-5'), 129.3 (C-6'), 100.4 (C-1″), 72.9 (C-2″), 77.1 (C-3″), 70.4 (C-4″), 77.2 (C-5″), 62.1 (C-6″). It was identified as Apigenin 7-O-β-glucopyranoside by comparison of its spectral data with reported literature values[27].

In-Vitro anti-inflammatory activity of Compound A by inhibition of albumin denaturation method

Different concentrations of compound A (50, 100, 250, 500, 1000 µg/mL) was prepared. The reaction mixture (I) consisting of 2 mL of 1% albumin solution (Hen's egg), 3 mL of phosphate buffer saline (*p*H 6.4) and 4 mL of different concentrations of compound A (50, 100, 250, 500, 1000 µg/mL) were prepared separately. Similarly, reaction mixture (II) consisting of 2 mL of 1% albumin solution (Hen's egg), 3 mL of phosphate buffer saline (*p*H 6.4) and 4 mL of Diclofenac sodium (reference drug) of different concentrations (50, 100, 250, 500, 1000 µg/mL) were also prepared. Similar volume of double-distilled water served as control. Then the reaction mixtures I and II were incubated at 37°C for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm by Systronics-2201 UV-Vis double beam spectrophotometer[28,29].

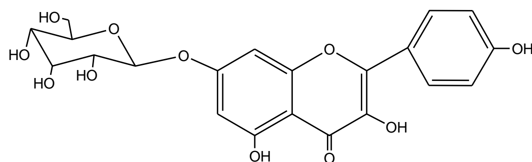

Figure 4 — Compound C

The percentage inhibition of protein denaturation was calculated by using the following formula:

% Inhibition = 100 × [Vt/Vc – 1]

Where,

Vt = Absorbance of test sample

Vc = Absorbance of control.

Conclusion

The phytochemical analysis of methanolic extract of the bark of *Pithecellobium dulce* (Roxb.) Benth. showed the presence of a new allelochemical (A) identified as 3,5,7,3′,4′-pentahydroxy flavone-7-O-α-L-rhamnopyranosyl-3′-O-β-D-xylopyranosyl (1→3)-O-β-D-glucopyranoside along with two known compounds Sinenestin (B) and Apigenin-7-O-Glucopyranoside (C). The isolated compound A possessed potent *in vitro* anti-inflammatory effect against the denaturation of protein. So it can be used as a source of anti-inflammatory agents.

Acknowledgements

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