FLAVONOIDS AND ANTIMICROBIAL ACTIVITY OF LEAVES OF *XYLOSMA LONGIFOLIUM*

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ABSTRACT

The methanolic extract of the leaves of *Xylosma longifolium* afforded a new flavonoidic compound named as Kaempferol-3- β -xylopyranoside-4'- α -rhamnoside along with Kaempferol, Quercetin, Kaempferol-3-rhamnoside, Quercetin-3-rhamnoside. Their structures were established on the basis of chemical and physical evidences (IR, ^1H NMR, and MS data). It was also screened for their antimicrobial activity.

Keywords: *Xylosma longifolium*, flacourtiaceae, flavonoids, antimicrobial activity.

INTRODUCTION

The genus *Xylosma*, a large genus of shrubs and trees is chiefly distributed in most of the tropical and subtropical regions. About four species are found in India.¹ It occurs as shady ravines of the western Himalayas from Kashmir to Kumaon and on low hills up to an altitude of 1,500 meter. The extract of *Xylosma longifolium* (Commonly known as "Khandhara") leaves is found to resemble opium in its action and is used with it for house pests and fences. It is used in Assam for intoxication, and also exhibits antispasmodic, dysentery, restlessness and insomnia.^{1,2} It is grown in India for its edible fruits.³ Medicinal importance of *X. longifolium* and scanty work on it encourages us to carry out the comprehensive investigation of the leaves of *X. longifolium*. Earlier investigation on this plant includes the isolation and characterization of β -sitosterol, β -amyirin, friedelin, olean-12-en-3 α -ol-28-oic acid 3 α -D-glucopyranoside⁴. Now we are reporting the isolation and characterization of a novel flavonoid kaempferol-3- β -xylopyranoside-4'- α -rhamnoside along with the known flavonoids kaempferol,⁵ quercetin,⁶ kaempferol-3-rhamnoside,⁷ quercetin-3-rhamnoside⁸ which have not been reported so far from this plant.

EXPERIMENTAL

General experimental procedure

The melting points were taken on a Koeffler block and are uncorrected. ^1H NMR spectra were recorded on Bruker Avance II 400 MHz with TMS as an internal standard. IR spectra were run on Shimadzu IR-408 Perkin-Elmer 1800 (FTIR). The MS were measured in both EI Mode and Jeol D-300 and FAB mode on Jeol SX 102/DA-6000 mass spectrometers.

Plant material

The leaves of *Xylosma longifolium* collected from Forest Research Institute, Dehradun in winter season and identified by Prof. Wazahat Hussain, Taxonomist, Department of Botany, Aligarh Muslim University, Aligarh, India.

Extraction and isolation

The leaves of *Xylosma longifolium* were dried under shade and crushed to make powder. The air dried powdered leaves (2.5 kg) were thoroughly extracted with light petroleum ether (60-80°C), benzene and methanol successively. The methanolic extract responded positively to color test for flavonoid. TLC examination of the methanolic extract in different solvent systems [benzene-pyridine-formic acid (36:9:5) and toluene-ethyl formate-formic acid (5:4:1)] showed it to be a complex mixture of five major compounds. They were tried to purify into individual components through column chromatography over silica gel. The column was eluted successively with benzene, benzene-ethyl acetate (9:1-1:1) mixture, ethyl acetate, ethyl acetate-methanol (9:1-1:1) mixtures and finally with methanol. The purity of the fraction was monitored by TLC examination and infrared spectral studies. The fractions which showed similar behavior on TLC and same IR spectra were mixed together. Repeated column chromatography of the fractions followed by fractional crystallization furnished five crystalline homogenous compounds, labeled as **XI-1**, **2**, **3**, **4** and **5**.

Compound XI-1

It was eluted with ethyl acetate-methanol (9:1) and crystallized from chloroform-methanol as yellow shining crystals (110 mg) m.p. 276-78°C. The

compound **XI-1** is identified as kaempferol by mixed m.p., and superimposed IR, ^1H NMR, UV data and comparison with authentic sample⁵.

Acetylation of XI-1

The crystalline compound **XI-1** (20 mg) was acetylated by heating with pyridine (2 ml) and acetic anhydride (4 ml) on water bath for about 4 hours. It was cooled to room temperature and poured over crushed ice. The separated solid was filtered, washed with distilled water, dried and it was crystallized with chloroform-methanol, to yield yellow colorless needles of **XI-1Ac** m.p. 180-82°C. ^1H NMR (400 MHz, CDCl_3): δ : 2.3 (9H, s, 3 x OAc), 2.4 (3H s, 1 x OAc), 6.80 (1H, d, J=2.0 Hz, H-6), 7.0 (1H, d, J=2.0 Hz, H-8), 7.3 (2H, d, J=9.0 Hz, H-3', 5'), 7.7 (2H d, J=9.0 Hz, H-2' H-6'), Mass m/z: 286[M⁺], 258, 153, 134, and 121.

Compound XI-2

Further elution of the column with ethyl acetate-methanol in ratio (8:2) gave a fraction which on crystallization with chloroform-methanol yielded the compound **XI-2** as yellow shining crystals (120 mg) m.p., 311-12°C. Its identity as quercetin was established by comparison of its spectral data with an authentic sample⁶.

Acetylation of XI-2

The compound **XI-2** (20 mg) was heated with pyridine (1 ml) and acetic anhydride (2 ml) on water bath for about 4 hours. After usual work up by the procedure described earlier followed by crystallization chloroform-methanol, it afforded yellow colored crystals **XI-2Ac**, m.p. 194-95°C. ^1H NMR (400 MHz, CDCl_3): δ : 2.35 (12H, m, 4 x OAc), 2.38 (3H, s, 1 x OAc), 6.79 (1H, J=2.5 Hz, H-6), 7.21 (1H, d, J=2.5 Hz, H-8), 7.26 (1H, d, J=9.0 Hz, H-5'), 7.62 (q, J₁=2.5 Hz, J₂=9.0 Hz, H-6'), 7.70 (1H, d, J=2.5 Hz, H-2').

Compound XI-3

Elution the column with ethyl acetate-methanol (7:3) gave fraction which on crystallization with chloroform-methanol afforded yellow granular crystals (100 mg) m.p., 177-78°C. It gave positive Molish test and brownish green colour with alcoholic ferric chloride and identified *Kaempferol-3-rhamnoside* by comparison of spectral data with that of authentic sample⁷. Compound **XI-3** (20 mg) was acetylated with Ac_2O (3.0 ml) & pyridine (1.50 ml) by heating on water bath for two hrs. After usual workup followed by crystallization with chloroform-methanol afforded colorless needles, m.p. 159-60°C. ^1H NMR (400 MHz, CDCl_3): δ : 7.79 (2H, J=9.0 Hz, H-2', 6'), 6.90 (2H, d, J=9.0 Hz, H-3', 5'), 6.38 (1H, d, J=2.5 Hz, H-8), 6.20 (1H, d, J=2.5 Hz, H-6), 5.21 (1H, d, J=2.0 Hz, H-1'' rhamnosyl), 3.24-5.09 (4H, m, sugar protons), 2.32, 2.34, 2.45, (9H, each singlet, aromatic acetoxy), 1.9-2.12 (9H, m, aliphatic acetoxy), 0.87 (3H, d, J=6.0 Hz, rhamnosyl methyl).

Compound XI-4

The compound **XI-4** was eluted from the column with ethyl acetate-methanol (7:3) and crystallized from chloroform-methanol as yellow microscopic needles crystals (110 mg) m.p., 186-87°C. It was characterized as *Quercetin-3-rhamnoside* by mixed m.p., IR, ^1H NMR, and comparison with authentic sample⁸. It was acetylated by above mentioned procedure and crystallized with CHCl_3 -MeOH as colorless needles m.p. 302-303°C. ^1H NMR (400 MHz, CDCl_3): δ : 7.40 (1H, q, J=2.1 Hz, and 9.0 Hz H-6'), 7.20 (1H, d, J=2.50 Hz, H-2'), 7.10 (1H, d, J=9.0 Hz, H-5'), 6.70 (1H, d, J=2.1 Hz, H-8), 6.51 (1H, d, J=2.1 Hz, H-6'), 5.40 (1H, d, J=2 Hz, H-1''), 3.30-5.70 (sugar protons, m, H-2'', 3'', 4'', 5''), 2.52-2.90 (12H, m, OAc-3', 4', 5', 7), 1.90-2.21

(9H, m, OAc-aliphatic), 0.86 (3H, d, J=6.5 Hz, rhamnosyl methyls).

Compound XI-5 (I)

It was obtained from ethyl acetate-methanol (4:6-1:1) eluates and crystallized with methanol-chloroform as pale yellow granular crystals (110 mg) m.p. 265-67°C. Analysed for $C_{26}H_{28}O_{14}$: Calcd: C, 56.11; H, 5.03%; Found: C, 56.14; H, 5.07%, UV λ_{max} nm: 240sh, 269, 306sh, 345 (MeOH), 245sh, 312, 305sh, 340, 398 (AlCl₃), 277, 304, 340, 396, (AlCl₃/HCl), 281, 355, 370, (NaOAc), 279, 325sh, 365 (NaOAc/H₃BO₃), 255sh, 296, 355, 405, (NaOMe).

Acetylation of XI-5:

The crystalline glycoside (45 mg) was acetylated with dry Ac₂O/py (1:1) at room temperature for 48 hrs. After workup by the procedure described earlier, it was crystallized with chloroform-methanol as cream needles, m.p. 126-28°C. ¹H NMR (400 MHz, CDCl₃): δ : 6.80 (1H, d, J=2.2 Hz, H-6), 7.10 (1H, d, J=2.20 Hz, H-8), 7.89 (2H, d, J=8.50 Hz, H-2', 6'), 7.26 (2H, d, J=8.5 Hz, H-3', 5'), 5.15 (1H, d, J=9.50 Hz, H-1'' xylose), 5.60 (1H, d, J=1.2 Hz, H-1'' rham), 1.20 (3H, d, J=6.1 Hz, rham-CH₃), 3.77-5.60 (10H, m, gly-H), 2.45 (3H, s, OAc-5), 2.34 (3H, s, OAc-7), 1.98-2.20 (18H, m, aliphatic OAc), Mass m/z: M⁺ absent, 642 [M⁺-acetylated pentose + H]⁺, 628 [M⁺-acetylated hexose + H]⁺, 370 [M⁺-acetylated pentose-acetylated hexose + 2H]⁺, 286 [M⁺-614]⁺, 273 [(rham)Ac]₃⁺, 259 [(xyl)Ac]₃⁺, 153 [A₁+H]⁺, 121 [B₂]⁺.

Acid hydrolysis of XI-5:

The glycoside XI-5 was hydrolyzed with 2N HCl-MeOH (5 ml), (100 °C, refluxed for 2 hr). Water was added and the mixture was extracted with ethylacetate. The aqueous hydrolysate was neutralized with Ag₂CO₃, precipitate filtered off and filtrate was evaporated in vacuo giving a residue.

Identification of aglycone:

The aglycone in EtOAc fraction was crystallized from CHCl₃-MeOH as yellow needles, m.p. 280-81 °C and identified as Kaempferol by spectral and chromatographic comparison with authentic sample⁵. Analyzed for C₁₅H₁₀O₆: Calcd: C, 62.93; H, 3.49%; Found: C, 62.88; H, 3.45%. UV λ_{max} nm: 249sh, 267, 295sh, 330sh, 370 (MeOH), 260sh, 268, 300sh, 364, 421 (AlCl₃), 260sh, 267, 301sh, 365, 423 (AlCl₃/HCl), 260sh, 282, 316, 432 (NaOMe), 278, 301, 399 (NaOAc)

Identification of sugars:

The neutral hydrolysate was concentrated and chromatographed on whatman No.1 filter paper using n-BuOH-acetic acid-water (4:1:5) and EtOAc-Pyridine- H₂O (2:1:2) as solvent systems, employing the descending techniques. Authentic sugars were used as checks. The chromatograms were run for 24 hours and after drying at room temperature, they were sprayed with aniline phthalate and p-anisidine phosphate solutions. The chromatograms on drying at 100-05°C, showed the presence of only rhamnose (0.37, 0.28) and xylose (0.30, 0.22).

GLC of TMSi ether of sugars:

The TMSi ether of sugars was obtained by taking 15 mg of sugar in dry pyridine (0.5 ml) and hexamethyldisilazane (0.2 ml) in a 10 ml round bottom flask. To this solution (0.2 ml) of trimethyl chlorosilane was added and flask was stoppered and allowed to stand at room temperature for 45 minutes. The solution was then dried and taken in heptane. The heptane soluble TMSi ether derivatives of sugar were then subjected to GLC (2% OV-1, column temp. 150-250°C, 10 min. dect. temp. 300°C, N₂ 50 ml/min) along with silyl derivatives of standard sugars (R_f 3.9, 3.8 min for rhamnose, 3.9, 4.5 min for xylose). The observed Rt-values were in agreement with those of an authentic sample of rhamnose and xylose.

Enzymatic hydrolysis of XI-5:

A mixture of compound XI-5 (100 mg) and α -rhamnosidase (contain α -pectinase) (10 mg) was incubated in (NH₄)₂SO₄-NaOAc buffer (pH 5.0) at 250°C for 30 hrs and then after addition of water, it was extracted with n-BuOH. The n-BuOH extract was chromatographed on silica gel column to give a partial glycoside, m.p. 254-56°C, identified as kaempferol-3-O xyloside. From the H₂O layer, L-rhamnose was identified by PC (n-butanol: acetic: water, 4:1:5).

Methylation of Partial glycoside:

CH₃I (1 ml) and Ag₂O (30 mg) were added to a solution of partial glycoside (30 mg) in DMF (3 ml). The mixture was stirred in dark at room temperature for 48 hours. The contents were filtered and the residue washed with little DMF. The filtrate was evaporated to dryness and the syrupy residue was hydrolysed with ethanol (25 ml). The alcohol was recovered and the syrupy residue was hydrolysed with 2N HCl. On usual workup it gave 3-OH, 5,7,4'-trimethoxy flavone (14 mg) m.p. 135-36°C. Analysed for C₁₈H₁₆O₆: calcd: C, 65.85; H, 4.87; Found: C, 65.91; H, 4.92%.

Quantitative estimation of sugars:

The anhydrous glycoside (25 mg) was hydrolysed by refluxing with 0.2

N HCl. After cooling over night, the aglycone was dried and weighed (11.5 mg), the ratio of aglycone to glycoside is 44.2% indicating the presence of two moles of sugar/mole of aglycone.

Antimicrobial Activity

The *in vitro* antimicrobial activity was carried out against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus subtilis* and *in vitro* antifungal activity was carried out against *Candida albicans*, *Fusarium oxysporum*, *Penicillium notatum*, *Aspergillus niger* and *Trichoderma viridae*. The agar well diffusion method^{9, 10} was used 0.1ml of diluted inoculum (10⁵CFU/ml) of test organism was spread on Mueller Hinton Agar plates (Hi-Midia Pvt. Ltd., Mumbai, India). The wells of 8mm diameter were punched into the agar medium and filled with 100 μ l of plant extract and some plant product of 1mg/ml concentration and solvent blank (DMSO) separately. The plates were incubated at 37°C overnight. The antibiotic (Chloramphenicol) and antifungal disc (Nystatin) of 30 mcg potency each were used in the system as positive controls. Zone of inhibition of bacterial and fungal growth around each well was measured in mm.

RESULT AND DISCUSSION

The air dried powdered leaves of *Xylosma longifolium* (2.5 kg) after being defatted with light petroleum ether (60-80 °C) were thoroughly extracted with benzene and methanol successively. The methanolic extract gave a color test for flavonoid and showed a intricate mixture of five major compounds on TLC [benzene-pyridine-formic acid (36:9:5) and toluene-ethyl formate-formic acid (5:4:1)]. It was purified through column chromatography over silica gel. Elution of the column with different solvent system such as benzene: ethyl acetate (9:1-1:1) mixture, ethyl acetate, ethyl acetate-methanol (9:1-1:1) mixtures and methanol gave various fractions. The purity of the each fraction was monitored by TLC examination and infrared spectral studies. Appropriate fractions which showed similar behavior on TLC and IR spectra were pooled together. Repeated column chromatography of the fractions followed by fractional crystallization furnished five crystalline homogenous compounds, marked as XI 1-5. The compounds XI 1-4 were identified as Kaempferol,⁵ Quercetin,⁶ Kaempferol-3-rhamnoside,⁷ Quercetin-3-rhamnoside⁸ respectively, while the compound XI-5 was found to be a novel compound and characterized as Kaempferol-3- β -xylopyranoside-4'- α -rhamnoside which is discussed below.

The compound XI-5 was obtained from ethyl acetate-methanol (4:6-1:1) and crystallized with methanol-chloroform as pale yellow granular crystals (110mg) mp. 265-67°C. The glycosidic nature of XI-5 was evidenced by the positive Molish test obtained after hydrolysis and the formation of an osazone. Its was further confirmed by the ¹H NMR spectrum of the acetate of XI-5 as it showed two aromatic acetoxylys at δ 2.45 (3H) and δ 2.34 (3H) and six aliphatic acetoxylys at δ 1.98-2.20 (18H, 6 x OAc) indicating it to be diglycoside. The glycoside gave pink colour with Zn/HCl and red color on treatment with sodium amalgam followed by acidification¹¹ indicating its flavonone or flavone nature. A yellow color with Wilson boric acid reagent^{6, 12} and maxima at 240sh, 269, 306sh, 345 in the UV spectrum indicated it to be a flavonol glycoside. It gave brownish green color with FeCl₃ pointing out the presence of hydroxyl group at C-5. The IR spectrum showed strong absorption bands at 3450 (OH), 1665 (C=O), 2950 (C-H), 1645 (C=C, aromatic) and broad band at 1105-1000 cm⁻¹ supporting its glycosidic nature. A bathochromic shift of +43 nm in band I with AlCl₃ further confirmed the presence of a free 5-OH group. The presence of 7-OH group is confirmed by a bathochromic shift +12 nm in band II with fused NaOAc. Total acid hydrolysis of the glycoside with 2N HCl yielded equimolar mixture of L- rhamnose, and D-xylose (PC and GLC) and an aglycone, (1b) m.p., 280-81 °C which characterized as kaempferol (1b) by spectral and chromatographic comparison with authentic sample.¹³

- (a) R₁, R₂=H, R₃=rham, R₄=xyl.
 (b) R₁, R₂, R₃, R₄=H
 (c) R₁, R₂, R₃=H, R₄=xly.
 (d) R₁, R₂, R₃=CH₃, R₄=H

Acetylation of XI-5 with Ac₂O and pyridine afforded octa-acetate XI-5 Ac

m.p., 126–28°C. The ^1H NMR spectrum of **XI-5 Ac**, showed two *meta* coupled doublets at δ 6.80 and 7.10 ($J=2.20$ Hz) were attributed to C-6 and C-8 proton respectively, Two *ortho*-coupled doublets at δ 7.89 ($J=8.5$ Hz) and δ 7.26 ($J=8.5$ Hz) which corresponded to A_2B_2 pattern were assigned to C-2', 6', and C-3', 5' proton of B-ring respectively. The anomeric proton at δ 5.15 ($J=9.5$) and δ 5.60 ($J=1.2$ Hz) were assigned to H-1''' xylose (β -configuration) and H-1''' rhamnose (α -configuration) respectively. The rhamnosyl methyl appeared as a doublet at δ 1.20 (6.0 Hz). The remaining sugar protons were observed in the range δ 3.77–5.60. The mass spectrum of the acetylated glycoside (Scheme-I) was in agreement with the above assigned structure of the glycoside. The molecular ion as expected was not observed, the mass spectrum showed the presence of acetylated pentapyranoside, m/z 259 and acetylated hexopyranoside, m/z 273. The fragment ions observed at m/z 642 and m/z 628 accounted for the loss of acetylated pentopyranoside and hexapyranoside respectively from the molecular ion peak. The loss of both acetylated sugar moieties gave a fragment at m/z 370. The aglycone fragment was observed at m/z 286. A retro Diels-Alder fragmentation pattern was observed at m/z 153 and 121 leading to fragments $[A, +H]$ and B_2^{+} which supported the two hydroxyl groups in ring-A and one hydroxyl group in ring-B. Other prominent sugar fragment ions appeared at m/z 213, 171, 153, 111, 97, 96.

Enzymatic hydrolysis of the parent glycoside **XI-5** gave conclusive evidences of the position of attachment of two sugars and nature of their linkage. Hydrolysis of **XI-5** with α -rhamnosidase (containing α -pectinase) gave L-rhamnose indicating α -nature of sugar and a partial glycoside of (**1c**), which gave a bathochromic shift of +60 nm with NaOMe in band I without a decrease in intensity (absent in glycoside). Thus showing that C-4' hydroxyl which was glycosylated in **XI-5** had become free. The partial glycoside of **XI-5** was identified as kaempferol-3-xyloside (**1c**), m.p., 224–26°C by UV diagnostic shift reagents and co-chromatography with authentic sample.¹⁴ Methylation of partial glycoside (**1c-5c**) followed by hydrolysis with 2N HCl gave a partial methyl ether (**1d**), m.p.134–35°C characterized as 3-OH, 5,7,4'-trimethoxyflavone (kaempferol 5,7,4'-trimethyl ether) by spectral and chromatographic comparison with authentic sample.¹⁵ The methylated sugar were identified as 2,3,4-tri-O-methyl-xylose by SiO₂ TLC according to Petek.¹⁶ This finally established that L-rhamnose was α -linked at C-4' while D-xylose was β -linked at 3-position. Quantitative estimation of sugar by Somogyi copper micro method¹⁷ indicated the presence of two moles of sugar/mole of aglycone.

On the basis of above results, compound **XI-5** was identified as *Kaempferol-3- β -xylopyranoside-4'- α -rhamnoside*. To the best our knowledge it is being reported for the first time.

gram positive and fungal strains. The aqueous extract showed high activity against *Staphylococcus aureus* (IAO-SA-22) and *Candida albicans* (IAO-109) and moderate activity against *Bacillus subtilis* (MTCC-121) and *Trichoderma viridae* (lab isolate). The alcoholic extract found to show high activity against *Staphylococcus aureus* (IAO-SA-22) and *Candida albicans* (IAO-109) and moderate activity against *Bacillus subtilis* (MTCC-121), *Salmonella typhimurium* (MTCC-98), *Aspergillus brassicola*.

Table.1 Antimicrobial activity of *Xylosma longifolium* aqueous and alcoholic extracts.

Stains	Aqueous extract	Alcoholic extract	Standards
<i>Staphylococcus aureus</i> (IAO-SA-22)	11	14	20
<i>Escherichia coli</i> (K-12)	--	--	22
<i>Salmonella typhimurium</i> (MTCC-98)	--	7	18
<i>Bacillus subtilis</i> (MTCC-121)	10	9	16
<i>Candida albican</i> (IAO-109)	11	15	20
<i>Fusarium oxysporum</i> (lab isolate)	--	--	20
<i>Aspergillus niger</i> (lab isolate)	--	--	25
<i>Trichoderma viridae</i> (lab isolate)	7	--	16
<i>Aspergillus brassicola</i>	--	8	18

Low activity (1-5), moderate activity (6-10), high activity (11-15), very high activity (16-20), no activity (--)

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Scheme1. Mass fragmentation pattern of the compound **XI-5 Ac.**

The antimicrobial activity of aqueous and alcoholic extracts of *Xylosma longifolium*, extract was investigated against bacterial and fungal strains. It was seen that all the extracts exhibited a low, moderate or high activity against