

# PHYTOCHEMICAL AND BIOLOGICAL STUDIES ON *PODOCARPUS NERIIFOLIUS* D. DON: A HIMALAYAN CONIFER OF NEPAL

L. N. Gautam\*, S. Awale\*\*, S. K. Kalauni\*\*, K. Shrestha\*\*\* and M. B. Gewali\*

\*Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal

\*\*Toyama Medical and Pharmaceutical University, Sugitani, Toyama, Japan

\*\*\*Royal Nepal Academy of Science and Technology (RONAST), Khumaltar, Lalitpur, Nepal

**Abstract:** Brine Shrimp bioassay directed fractionation of *Podocarpus neriifolius* D. Don afforded a bioactive flavanol, dimethoxy aromadendrin, from chloroform fraction. In addition to this compound, other six compounds namely nonacosanol-10, hexadecanoic butyl ester,  $\beta$ -sitosterol,  $\beta$ -sitosterol-3-O- $\beta$ -D glucopyranoside, stigmastan-3, 5-diene-7-one and cholest-7-en-3-one were isolated from chloroform fraction. These compounds were separated by chromatographic techniques. Their structures were determined by using IR,  $^1\text{H-NMR}$ , COSY, HMBC, HMQC and Mass spectrums.

**Key words:** *Podocarpus neriifolius*; Dimethoxy aromadendrin; Brine Shrimp bioassay.

## INTRODUCTION

Among higher plants, conifers are considered among the most primitives. Some of the conifers are of great interest due to their use in Ayurvedic medicines as well as their phytochemical and biological significance. *Podocarpus neriifolius* D. Don. (Podocarpaceae), commonly known as "mount teak" and "thitmin" and locally known as "Gunsi" is an evergreen conifer grown in a sub-tropical region of central and eastern Nepal.<sup>1</sup> It is an endangered plant species. It grows at an altitude of 990- 1070 m attaining a height ranging 12-13 m. A decoction of the leaves of the species is used for the treatment of rheumatism and painful joints.<sup>2</sup> These medicinal values prompted us to undertake the detail chemical investigation of the plant and evaluation of biological activity. In course of the studies on *Podocarpus neriifolius*, we have isolated a potent cytotoxic compound, nagilactone-C<sup>3</sup> With a view to isolate further biologically active compound, we undertook phytochemical studies of *Podocarpus neriifolius*.

## MATERIALS AND METHODS

The fresh twigs were collected from Kirtipur of height 1372m during August 2003. Mrs. Nirmala Dawadi, (Senior Botanist, Department of Plant Resources, Ministry of Forest, Thapathali, Kathmandu, Nepal) identified the plant and this voucher herbarium specimen NP-100 was deposited at the National Herbarium and Plant Laboratory, Godawari, Nepal.

The twigs were shade dried and powdered. Thus obtained 1 kg of powder was placed in a well-ventilated enclosure and placed in a metallic percolator. This plant

material was extracted with ethanol (7  $\times$  200ml) at room temperature for 24 hours. The alcohol was drained off and repeated for three times. This ethanol extract was distilled under reduced pressure to yield semi solid product which was treated with water. This resultant aqueous extract was fractionated with hexane to get hexane-fraction (20 gm), with chloroform to get chloroform fraction (20gm) and finally with n-butanol to get n-butanol fraction (22 gm). Brine shrimp bioassay of different fractions was performed following Meyer's procedure.<sup>4</sup> After chloroform fraction ( $\text{LC}_{50} = 100\mu\text{g/ml}$ ) was found to be bioactive in Brine Shrimp Bioassay, it was subjected to purification. Repeated column chromatography as well as preparative TLC and afforded seven compounds.

M.Ps were determined in an electrical apparatus and were uncorrected. IR spectra were recorded on a Perkin Elmer 1310 IR Spectrophotometer using Nujol, and suitable dissolving solvents.  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ , HMBC, COSY, spectra were recorded with a Bruker WM 300 NMR spectrometer by using TMS as external reference and NMR-grade  $\text{CDCl}_3$  and  $\text{C}_5\text{D}_5\text{N}$  were used as solvent. EI-MS measurements were carried out on micro mass QUATTRO II triple quadruple mass spectrometer.

## RESULT AND DISCUSSION

### 3, 5-dihydroxy 7, 4'-dimethoxy flavanone (7, 4' - dimethoxy aromadendrin)

The different fractions of the plant i.e. hexane, chloroform and butanol were subjected to the Brine Shrimp bioassay. On this bioassay, the chloroform fraction ( $\text{LC}_{50} = 100\mu\text{g/ml}$ ) proved to be most active. This chloroform

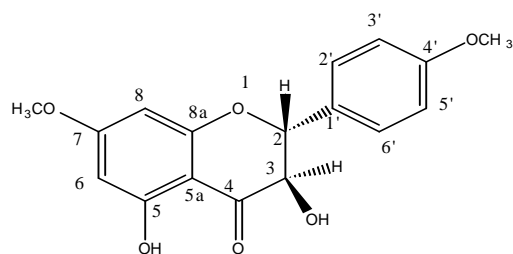
Author for Correspondence: L. N. Gautam, Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

fraction on repeated column chromatography and preparative TLC afforded a crystalline white compound whose LC<sub>50</sub> value was 43 µg/ml. This compound can be considered as a potent bioactive compound in the Brine Shrimp bioassay. This compound's structure was elucidated as follows.

The compound MP 139°C, R<sub>f</sub> 0.64 (1:4, methanol: chloroform), M<sup>+</sup> at m/z 316 corresponding to the molecular formula C<sub>17</sub>H<sub>16</sub>O<sub>6</sub> was a shiny needle shaped yellowish crystalline form. It gave positive test for flavanol.

IR absorption at 3370 cm<sup>-1</sup>, 1653 cm<sup>-1</sup>, 3005 cm<sup>-1</sup> were indicative of the presence of -OH,

-C=O and =C-H groups stretching respectively. The <sup>1</sup>H-NMR spectrum pattern was consistent with a 3, 4' and 7-oxygenated flavanol. The singlets at δ 3.90 and 3.70 were assigned for two methoxy groups attached to C-7 and C-4'. The doublets at δ 6.12 and 6.05 (J = 1.5Hz) were assigned as the protons of C-6 and C-8. The other doublets at δ 7.00 (J = 8.5Hz) due to the protons of C-3' and C-5' and the doublets at δ 7.50 (J = 8.5Hz) due to the protons of C-2' and C-6' were also seen in NMR spectra. The doublets at δ 4.60 and 5.10 (J = 11.5Hz) were assigned as the protons of C-3 and C-2 respectively and the coupling constant J = 11.5Hz suggested that these protons were in the trans position. The singlet at δ 3.5 was assigned as C-3 -OH group. The other singlet at δ 11.2 was due to OH group of C-5. It was deshielded by intra molecular H-bonding. <sup>13</sup>C-NMR values ranging from δ 56-196 indicated the presence of 17 carbons. The value at δ 196 was assigned as C=O group. The spectral evidences suggested the compound to be 3, 5-dihydroxy 7, 4'-dimethoxy flavanone (7, 4' - dimethoxy aromadendrin). This structure was confirmed by <sup>1</sup>H-<sup>1</sup>H COSY, HMBC and HMQC spectra. Furthermore, the proposed structure was supported by the mass fragmentation pattern which was found to be consistent with the structure proposed. Therefore the compound was identified as 3, 5-dihydroxy 7, 4'-dimethoxy flavanone (7, 4' - dimethoxy aromadendrin).<sup>5, 6, 7, 8.</sup>

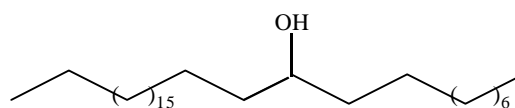


7, 4' - dimethoxy aromadendrin

#### Nonacosan-10-ol

It was isolated as white compound having MP 81°C, R<sub>f</sub> 0.7 (1:4 hexane: chloroform) and re-crystallized from ethyl acetate. The EI-MS mass spectrum showed M<sup>+</sup> at m/z 424 corresponding to the molecular formula C<sub>29</sub>H<sub>60</sub>O.

IR showed -OH (3350 cm<sup>-1</sup>) group. The <sup>1</sup>H-NMR spectrum had a triplet equivalent to six protons at δ 0.88 (J = 7.0Hz) which were assigned as two terminal methyl group and a broad singlet equivalent to 34 protons at δ 1.28 assigned as 17 methylene groups in an aliphatic chain. The remaining protons were found broad singlet at δ 1.38. Important clue for the structure was obtained from the mass spectrum. The peak at m/z 297 showed the loss of CH<sub>3</sub> (CH<sub>2</sub>)<sub>18</sub> group. The peak at m/z 157 indicated the loss of CH<sub>3</sub> (CH<sub>2</sub>)<sub>8</sub> CH (OH) group. Furthermore, the peak at m/z 139 was due to the loss of CH<sub>3</sub> (CH<sub>2</sub>)<sub>8</sub> C. This mass pattern was consistent with the fact that the -OH group at 10<sup>th</sup> position. The loss of series of 14 units showed it was long chain alcohol. Thus, the compound was identified as nonacosan-10-ol.<sup>9</sup>

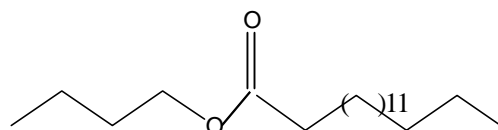


Nonacosan-10-ol

#### Hexa decanoic butylester

The compound was semisolid with MP above 175°C, having R<sub>f</sub> value 0.37 (1:7, methanol: chloroform). The mass spectrum showed M<sup>+</sup> at m/z 312 corresponding to the molecular formula C<sub>20</sub>H<sub>40</sub>O<sub>2</sub>.

The IR at 1750 cm<sup>-1</sup> was due to COOR group. In NMR, triplet protons at δ 0.90 (J = 10.5Hz, 6H) accounted for two methyl groups. The broad singlet peak at δ 1.3 accounted for 12 CH<sub>2</sub> groups (24-H). <sup>13</sup>C-NMR showed 7 peaks ranging from δ 14-80. The peak at δ 200 showed C=O group. Mass spectrum pattern afforded important clue for the structure. The base peak at m/z 73 was due to butoxy group. i.e. CH<sub>3</sub>(CH<sub>2</sub>)<sub>3</sub> O<sup>+</sup>. The other peak at 239 was due to loss of CH<sub>3</sub>(CH<sub>2</sub>)<sub>14</sub> COO group. The two peaks at m/z 257 and 116 were obtained from McLafferty rearrangement of the molecule. All the evidences suggested that the compound was hexa decanoic butylester.<sup>10</sup>

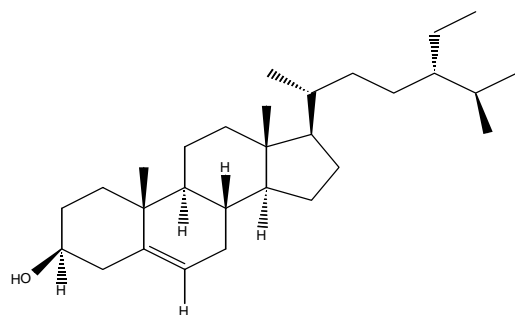


Hexa decanoic butyl ester

#### β-Sitosterol

It was isolated as white crystalline, MP 136°C, R<sub>f</sub> 0.32 (1:9, ethyl acetate: hexane). It gave positive Libermann-Burchard test indicating the compound to be sterol. The mass spectrum showed M<sup>+</sup> at m/z 414 corresponding to the molecular formula C<sub>29</sub>H<sub>50</sub>O.

All NMR, IR and mass spectral patterns were identical with that of β-Sitosterol.<sup>11</sup>

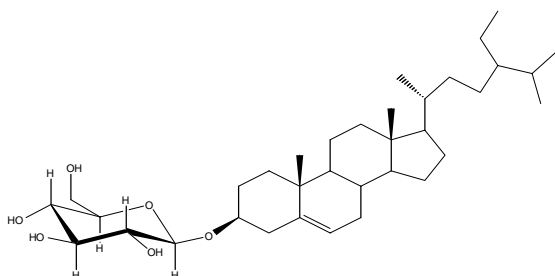


$\beta$ -Sitosterol

### $\beta$ -Sitosterol 3-O- $\beta$ -D glucopyranoside

It was white powder, MP above 280°C,  $R_f$  0.66 (1:4, methanol: chloroform).

$^1\text{H}$  NMR spectrum pattern was similar to that of  $\beta$ -sitosterol with some additional peaks relating to a carbohydrate moiety. The multiplet at  $\delta$  4.27 was assigned for the proton of C-3. Its deshielding may be due to attachment of  $\beta$ -O glucosyl moiety at C-3 carbon. The proton signals at  $\delta$  3.96, 4.03, 4.27, 4.52, 4.53, 5.02 in the deshielded region were assigned for respective C<sup>1</sup>- 5, C<sup>1</sup>-2, C<sup>1</sup>-3, C<sup>1</sup>-4, C<sup>1</sup>-6, and C<sup>1</sup>-1 protons of glucosyl moiety.  $^{13}\text{C}$ -NMR pattern was also similar to  $\beta$ -sitosterol with additional six peaks confirming the glucose ring. Furthermore, the structure was supported by  $^1\text{H}$ - $^1\text{H}$  COSY, HMBC and HMQC. All these spectral values were found to be consistent with  $\beta$ -sitosterol-3-O- $\beta$ -D glucopyranoside.<sup>12</sup>



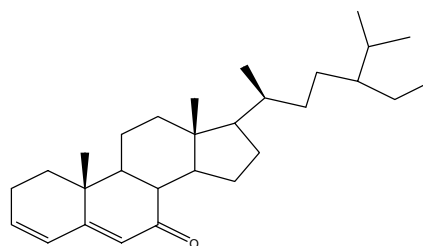
$\beta$ -Sitosterol 3-O-  $\beta$ -D glucopyranoside

### Stigmasta-3, 5-dien-7-one

This white crystalline compound MP 189°C,  $R_f$  0.42 (EtOAc: Hexane 1:9) gave positive Libermann Burchard test for sterols. Mass spectrum showed  $M^+$  ion at 410 corresponding to molecular formula  $\text{C}_{29}\text{H}_{46}\text{O}$ .

The  $^1\text{H}$  NMR showed six methyl groups at  $\delta$  0.88, 1.35, 1.32 (d,  $J = 6.4$  Hz); 1.09(d,  $J = 6.4$  Hz); 1.05 (d,  $J = 6.4$  Hz) and 0.89 (t,  $J = 7.3$  Hz), which were assigned respectively for the protons of C-18, C-19, C-21, C-26, C-27 and C-29. The hydrogens at  $\delta$  5.10 (dd,  $J = 8.9, 15.1$  Hz); 5.15 (d,  $J = 8.7$  Hz) and 5.4 (s) were assigned for olefinic protons.  $^{13}\text{C}$ -NMR showed 21 different carbons. The peak at  $m/z$  269 was assigned to the loss of side chain of ring D. The peaks at 174 and 236 were

due to rupture pair of bonds of ring C at 8, 14-and 11, 12-bond. Similarly, the peak at 134 was assigned from retro-Diels–Alder cleavage of ring B. Thus, the compound was identified as stigmasta-3, 5, diene-7-one.<sup>13</sup>

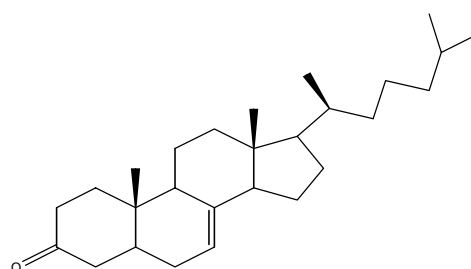


Stigmasta-3, 5-dien-7-one

### Cholest-7-ene-3-one

The compound white crystalline, MP199°C;  $R_f$  0.34( $\text{CHCl}_3$ : MeOH, 9:1) gave positive test of sterol. Mass spectrum showed  $M^+$  ion at 384 corresponding to molecular formula  $\text{C}_{27}\text{H}_{44}\text{O}$ .

$^1\text{H}$  NMR exhibited 5 methyl signals at  $\delta$  1.32 (d,  $J = 6.4$  Hz); 1.09(d,  $J = 6.4$  Hz); 1.05 (d,  $J = 6.4$  Hz), 0.88, and 1.01 which were assigned as C-27, C-26, C-21, C-18 and C-19 methyl protons. The methylene protons seen in range  $\delta$  1.7 to 2.02. The hydrogen at  $\delta$  5.1 (dd,  $J = 8.7, 15$  Hz) was assigned for olefinic proton.  $^{13}\text{C}$ -NMR values ranging from  $\delta$  14.56 to 150.93 indicated the presence of 26 carbons. The value at  $\delta$  200 was showed -C=O group. The fragment at  $m/z$  271 represents the removal of side chain from ring D. Similarly the peak at  $m/z$  95 was indicative of the rupture of 5, 6-and 9, 10-bond occurring via retro-Diels–Alder cleavage. The peak at  $m/z$  230 was due to fission of 13, 17-and 14, 15-bond. These evidences made us to conclude the compound as Cholest-7-ene-3-one.



Cholest-7-ene-3-one

To sum up, the Brine shrimp bioassay directed fractionation of the plant afforded a bioactive compound 3, 5-dihydroxy 7, 4'-dimethoxy flavanone (7, 4'-dimethoxy aromadendrin) from chloroform fraction. In addition to this bioactive compound, other six compounds: nonacosanol-10, hexadecanoic butyl ester,  $\beta$ -sitosterol,  $\beta$ -sitosterol-3-O-  $\beta$ -D glucopyranoside, cholest-7-en-3-one and stigmasta-3, 5-dien-7-one were also isolated from this plant.

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