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Diterpenoids from *Premna integrifolia*

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ABSTRACT

Premna integrifolia is an important constituent of famous herbal formulation "Dashmula" of Indian Ayurvedic system of medicines. The plant is known to possess hypoglycaemic, anti-inflammatory, antiarthritic and broad-spectrum antimicrobial activities due to the presence of several diterpenoids and spermine alkaloids in its decoction. In order to develop chemical markers for quality assurance of this herb in Ayurvedic formulation, we report here the isolation of three novel diterpenoids from the root bark of *P. integrifolia* namely 1β,3α,8β-trihydroxy-pimara-15-ene (**1**), 6α,11,12,16-tetrahydroxy-7-oxo-abieta-8,11,13-triene (**2**) and 2α,19-dihydroxy-pimara-7,15-diene (**3**). 1,3-Dihydroxy and 2-hydroxy diterpenes belong to a limited number of families and their isolation is also interesting from chemotaxonomic point of view. These diterpenoids were also evaluated for antibacterial activity.

1. Introduction

The genus *Premna* is distributed in tropical and subtropical Asia, Africa, Australia and the Pacific islands. *Premna integrifolia* Linn. (syn: *Premna serratifolia*), belonging to the family Verbenaceae, is one of the important constituents among ten herb formulations called "Dashmula", a favorite decoction of ten plants used in Indian system of medicine. The roots are astringent, stimulant, liver tonic, laxative, carminative, antibacterial and hypoglycaemic. The plant is also reported to possess antirheumatic, carminative, galactogenic, bechic, febrifuge, stomachic and anti-inflammatory activities (Husain et al., 1992). The decoction of roots is also used to treat gonorrhoea. Phytochemical studies on this plant showed the presence of beta-sitosterol and polyisoprenoid in leaves (Rao et al., 1984), and spermine alkaloids, aphilandrine and premnin in the stem bark (Basu and Dandiya, 1947; Dasgupta et al., 1984). Premnin is also known to decrease forces of contraction of heart and produces dilation of pupils (Chopra et al., 1956). Rajendran and Basha (2010) have recently described significant broad-spectrum antimicrobial activity in different extracts (*n*-hexane, chloroform, ethylacetate and ethanol) of the roots of *P. integrifolia*.

Continuing with our interest in phytochemical investigations in members of family Verbenaceae (Pandey et al., 2003, 2005; Tiwari et al., 2008), we report here the isolation and identification of three new diterpenoids (Fig. 1) together with beta-sitosterol and its glucoside from the methanolic extract of root bark of *P. integrifolia*. All the three diterpenoids were tested negative against *Staphylococcus aureus* (strains SA-96, SA-ATCC 29213 and SA-BKT). Occurrence of rare 1,3-dihydroxy and 2-hydroxy diterpenes is of chemotaxonomic importance and may serve as chemical marker for the quality control of this important Indian medicinal herb.

2. Results and discussion

Methanolic extract of root bark of *P. integrifolia* was fractionated by solvent partitioning using *n*-hexane, chloroform and *n*-butanol. The hexane and chloroform extracts were combined and subjected to column chromatography over silica gel to yield compounds **1**–**3**, beta-sitosterol and beta-sitosterol glucoside. The known compounds were identified by comparison of their spectroscopic and physical data with those reported in the literature.

Compound **1** was obtained as white crystalline solid. The molecular formula was determined as $C_{20}H_{34}O_3$ as inferred by the HRESIMS indicating four degrees of unsaturation. The IR spectrum showed absorption bands for hydroxyl group (3492 cm^{-1}) and a mono substituted double bond (2953, 1741, 970 cm^{-1}). The combined analysis of ^{13}C NMR and DEPT spectra revealed the presence of 20 carbon signals assigned to four methyls, seven methylenes, five methines and four quaternary carbons. The ^1H NMR spectrum showed signals for four methyl groups at δ 0.83, 0.85, 1.08, and 1.22. These were attributed to H-18, 17, 20, and 19 respectively. Two oxygenated methine groups in the molecule were established by resonances at δ 3.42 (dd, 3.3, 9.6 Hz) and 3.40 (brs) (Meragelman et al., 2003). The remaining unassigned oxygen atom in the structure of molecule **1** indicated the presence of a quaternary hydroxyl group. Three carbon resonances at δ 80.5, 75.4, and 75.7 for two tertiary and one quaternary carbinol carbon further confirmed the occurrence of three hydroxyl functionalities in the molecule.

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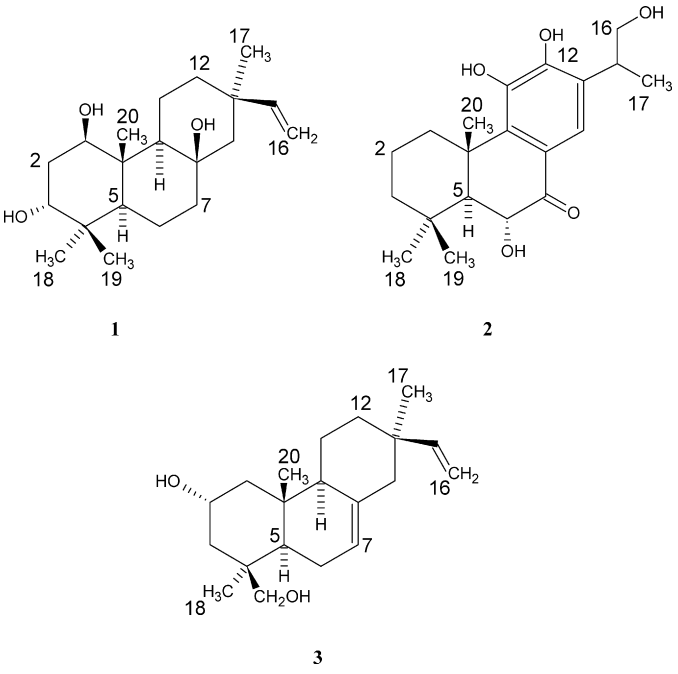

Fig. 1. Chemical structures of compounds **1**–**3**.

In the vinylidene region of ^{1}H NMR spectrum three mutually coupling ABX signals at δ 5.76 (dd, 10.8, 17.4 Hz), 4.90 (d, 17.4 Hz), and 4.84 (d, 10.8 Hz) revealed the presence of a vinyl group in the isolate (Thongnest et al., 2005). The long-range correlation signals of vinyl methine proton at δ 5.76 with δ 19.9 (C-11) and δ 36.5 (C-13) corroborate its substitution at C-13. The preceding evidences and the index of hydrogen deficiency confirmed that the molecule is a tricyclic diterpenoids with a vinyl group and three hydroxyl groups. The consistency of the chemical shifts with those of diterpenoids possessing similar partial structure, NOE correlations and analysis of ^{1}H–^{1}H vicinal proton coupling constants confirmed assignment of this diterpenoid as a pimarene. Three partial linkage moieties, –CH–CH_2–CH– (C-1 to C-3), –CH–CH_2–CH_2– (C-5 to C-7) and –CH–CH_2–CH_2– (C-9 to C-12) were established on the basis of ^{1}H–^{1}H COSY and HSQC spectras of **1**. Connection of these partial structures and the quaternary carbons to constitute a pimarene was based on cross-peaks of H_3-20 with C-1, C-5, C-9, C-10; H-5 with C-18, C-10, C-7 and H-11 with C-9, C-10, C-15, C-17 in HMBC spectra. In the ^{13}C NMR spectra a quite upfield signal at δ 11.2 was attributed to C-20 methyl. The upfield shift of this carbon can clearly be illustrated by the presence of a hydroxyl group at C-1 (Lorimer et al., 1997). This was further confirmed by the HMBC correlation observed between C-1 and H-20 and homonuclear COSY cross-signals observed between H-20 and H-1. The value at δ 80.5 was attributed to C-3 and the δ 75.7 to C-8 on the basis of informations extracted from 2D NMR experiments. The relative stereochemistry of compound **1** was deduced using *J* values and NOE experiment. The double doublet at δ 3.42 (3.3, 9.6 Hz) was assigned to H-1. The large coupling constant i.e. 9.6 Hz due to diaxial vicinal coupling ($J_{ax}^{H\text{-}1}$–$J_{ax}^{H\text{-}2}$), confirmed axial orientation of H-1. On exploring the published ^{1}H and ^{13}C NMR data for pimarene and ent-pimarene structures compound **1** showed a close resemblance with pimarene skeleton. Thus, on the basis of comparison of chemical shifts with pimarene diterpenes the relative stereochemistry of Me-20 was ascribed β axial. Since Me-20 is β axial and H-1 is α axial, hence the orientation of C-1 hydroxyl must be assigned as β equatorial. Furthermore the NOE cross-signals between H-1 and H-5 confirmed that these protons are in spatial vicinity and α oriented. A broad singlet at δ 3.40 attributed to H-3 designate this proton as equatorial in position, consequently the stereochemistry of H-3 was assigned as β. On reviewing the NOE correlation spectrum, no cross-peak was detected between H-3 and H-5 but a substantial signal was observed between H-3 and H-19. On the basis of above, stereochemistry of C-3 hydroxyl was defined as α axial.

Me-20 showed an intense signal in correlation with H-11 axial (δ 2.40 ddd, 13.5, 6.9, 3.6 Hz) in COSY spectra suggesting the position of H-11 axial as β. The other proton of C-11 correlated with H-17, hence appraised the α orientation of C-17 methyl group. This was further confirmed by an intense *W* correlation signal observed between H-12 β and H-17 in homonuclear COSY spectrum. Based on these observations the structure of compound **1** was assigned as 1β,3α,8β-trihydroxy-pimara-15-ene.

Compound **2** was obtained as pale yellow crystalline solid and the molecular formula was determined as $C_{20}H_{28}O_5$ on the basis of high resolution mass as well as by elemental analysis. The IR spectrum of **2** showed absorption bands at 3494 and 1675 suggesting the existence of hydroxyl and conjugated carbonyl group. It also showed UV absorption maxima at λ_{max} 236 nm and 289 nm. The ^{13}C NMR and DEPT spectrum was consistent with a diterpene structure. The ^{13}C NMR spectrum exhibited 20 signals which were attributed to four methyls, four methylenes, four methines, seven quaternary and one carbonyl carbon. A downfield quaternary signal at δ 200.6 indicated the presence a ketone moiety in the molecule. The presence of an aromatic ring was confirmed by completely substituted carbon signals at δ 150.1, 149.0, 143.9, 138.9 and 130.1 assignable to C-12, 11, 9, 8 and 13, respectively (Rodríguez, 2003). On the basis of the presence of a carbonyl and aromatic moiety as inferred by ^{1}H and ^{13}C NMR data and unsaturation degree of seven as derived by HRMS results, it was apparent that compound **2** has tricyclic diterpenoid skeleton.

The ^{1}H NMR spectrum of **2** showed three methyl singlets at δ 1.17, 1.20 and 1.58. These were attributed to Me-19, Me-18, and Me-20, respectively. In the aromatic region only one signal was observed at δ 7.46 ascribable to a penta substituted aromatic ring. The presence of an oxygenated isopropyl group was evidenced by the ^{1}H signals at δ 1.35 (3H, d, 6 Hz), 3.77 (2H, d, 7.5 Hz) and 3.10 (1H, m). The isopropyl group was substituted on the strength of long-range correlation of isopropyl methine protons with aromatic carbons at δ 150.1 (C-12) and 130.1 (C-13). Appearance of a signal at δ 4.70 in ^{1}H NMR spectrum suggested the presence of carbinol carbon. Location of carbonyl group was fixed as C-7, on the basis of cross-peaks of C-7 with H-5, H-6, and H-14. Furthermore ^{13}C chemical shift of C-8 also confirms it in conjugation with an aromatic ring. A ^{13}C signal at δ 67.7 was accredited to hydroxyl methylene carbon which was in gem position with the methyl signal at δ 15.5. The remaining two oxygen atoms were considered as hydroxyl groups. These were substituted in benzene ring as evidenced by downfield quaternary aromatic signals at δ 149.0 and 150.1. The proton and carbon signals were assigned explicitly to its diterpenoids framework using amalgamation of HSQC, HMBC, ^{1}H–^{1}H COSY and NOESY experiments.

The relative stereochemistry of isolate **2** was determined through analysis of *J* values and correlations observed in NOESY spectrum. The large vicinal coupling constant of H-5 and H-6 ($J_{ax}^{H\text{-}5}$–$J_{ax}^{H\text{-}6}$), suggested that H-5 and H-6 both were in diaxial position. An intense COSY correlation signal between H-5 and H-6 further confirmed the above interpretation. Literature appraisal indicated that the chemical shift of C-20 depends markedly on its orientation and from the analysis of the ^{1}H and ^{13}C spectrum of isolate **2**, it has been deduced that C-20 methyl values bear close resemblance to abietane type skeleton (Arihara et al., 2004; Qina et al., 2007). Vicinal coupling constants and NOE cross-peaks also sustained the above assumption. On the basis of the above evidences, structure of