SHORT REPORT

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Isolation of a Novel Indigoferamide-A from Seeds of Indigofera Heterantha Wall and its Antibacterial Activity

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Abstract: A new indigoferamide-A (**1**) along with three new source compounds dotriacontanoic acid (**2**), quercetin (**3**) and formononetin "4-hydroxy-4-methyl-2-pentanone" (**4**) were isolated from the seeds of *Indigofera heterantha* Wall. The structures of all these compounds were determined by using mass spectrometry, 1D-and 2D NMR techniques. The structure of the new indigoferamide-A was established by methanolysis as well as by GC-MS analysis.

Keywords: *Indigofera heterantha* Wall seeds; Indigoferamide-A; antibacterial activity.

1. Plant Source

In the ongoing phytochemical studies of medicinal plants from Pakistan, we investigate *Indigofera heterantha* Wall seeds (leguminosae). We report on the structure elucidation of the new Indigoferamide-A (**1**) **(Figure 1).** The seeds of medicinal plant *Indigofera heterantha* Wall were collected during the month of May, 2009 from Lower Dir in Northern areas of Pakistan. Taxonomic identification of the plant was done by Dr. Samin Jan, Associate Professor, Department of Botany, Islamia University, Peshawar, Pakistan. The voucher specimen (SJ-36) was deposited in the herbarium of Islamia University, Peshawar, Pakistan.

2. Previous Studies

Indigofera heterantha Wall, generally known as (Himalayan indigo) belongs to family leguminosae is a deciduous shrub distributed throughout the tropical region of the globe. In Pakistan, it has only 24 species [1]. Various chemical constituents such as triterpenes, steroids, alkaloids, flavonoids, lignin, acylphloroglucinols, saponins, tannins, quinines, cafffeic acid, gallic acid, "rutin, myricetin, quercetin and galangin" are reported from the genus Indigofera [2-4]. Some species of this genus contains nitro group containing compounds such as 3-nitropropionate and other toxic substances which acts as suicide inactivator of succinate dehydrogenase [2]. In our phytochemical investigation of the chemical constituents of *Indigofera heterantha* Wall, four compounds were isolated and characterized from the seeds of *Indigofera heterantha* Wall for the first time. Out of these four

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compounds one is new and the remaining three are reported as new source compounds. The new indigoferamide-A was tested for its biological activity.

3. Present Study

Air shade dried powdered seeds 22 kg were subjected to extraction (x 3) with 5% aqueous methanol for one week. The combined extract was concentrated under reduced pressure by a vacuum rotary evaporator, to obtained brownish residue F1 (2.29 kg), which was fractionated by using chloroform and water to yield F2 (41 g) of chloroform and F3 (1.6 kg) of water fraction. The chloroform fraction was partitioned into *n*-hexane and methanol fractions afforded FX1A (3 g) and FX1B (36 g) respectively, using soxhlet extractor. Water fraction was also partitioned with ethyl acetate (EtOAc), as a result FX3A (1 kg) of ethyl acetate fraction was obtained, which was further fractionated using ether: petroleum ether (2:1) and water to get three fractions, FX3AC (400 g), FX3AB (160 g) and residue fraction FX3AA (360 g). The fraction FX3AC was exposed to column chromatography on silica gel eluted with *n*-hexane- ethyl acetate; in increasing polarity to yielded sub fractions (A-F). The sub fractions (C-E) were combined based on TLC profile yielded 73 fractions. The sub fractions 41-64 were then mixed and chromatographed eluted with *n*-hexane-acetone in increasing polarity to obtain various fractions, Further separation and purification of these fractions using Prep TLC resulted in the isolation of four compounds including one new compounds and three known compounds.

Methanolysis of **1**: Compound **1** (3 m mole) was added to methanol (10 ml), then 2 ml HCl was added to the solution and refluxed the mixture for 2-3 hrs. Reaction was monitored by TLC, after completion of the reaction water and ethyl acetate was added to reaction mixture. Organic layer and aqueous layer were separated by using separating funnel; organic layer was evaporated and subjected to GC MS analysis.

Indigoferamide-A (1) was obtained as light brown powder. The molecular formula $C_{43}H_{85}NO_5$ was deduced from HR-FABMS which showed pseudo molecular ion $[M+H]^+$ peak at *m/z* 696.6806 (calcd. for $C_{43}H_{86}NO_5$; 696.6760).Its optical rotation is $[\alpha]_D^{27}$: -16. The IR spectrum showed absorption bands at 3475 (OH), 3340 (amide group), 1664 (C = O), 2924, 2854 cm^{-1} (aliphatic) indicated an amide fatty acid [5]. The ^{1}H NMR spectrum (**Table 1**) of Indigoferamide-A **1** displayed three oxygenated methine multiplet signals at δ_H 3.55 (H-3), 3.52 (H-4) and 4.02 (H-2′), while a downfield nitrogenated methine multiplet resonated at δ_H 4.10 (H-2). The ^{1}H NMR spectrum also indicated oxygenated methylene resonated as a doublet at δ_H 3.74 (J = 5 Hz, H-1), and two terminal methyl protons triplet at δ_H 0.89 (J = 7 Hz, 6H) assigned to H-20 and H-23′. The characteristic resonances in the ^{1}H and ^{13}C NMR spectra **(Table 1)** at δ_C /δ_H 52.9/4.10 (C-2/H-2), 62.1/3.74 (C-1/H-1) and 176.9/8.56 (C-1′/N-H) [6]. The *E*-geometry of the double bonds was deduced by characteristic chemical shifts about δ_C 32.7-33.1 of the methylene carbons adjacent to the double bonds (δ_C 26–28 for Z-geometry) [7].

The HMBC **(Figure 3)** correlations of NH proton (δ_H 8.56) with carbonyl carbon (δ_C 176.9, C-1′) and a methine (δ_C 52.9, C-2) confirmed the amide linkage. The ^{1}H-^{1}H COSY **(Figure 3)** correlation of **(1)** can be demonstrated as H-22′/H-23′ then H-2′/H-3′/H-4′and H-1/H-2/H-3/H-4/H-5/H-6. The relative stereochemistry at chiral centers of the long chains were deduced by comparing ^{13}C NMR resonances with reported compounds [8-9]. The structure was further authenticated on the basis of mass fragments in the EIMS appeared at *m/z* 281 [M^+-$C_{23}H_{44}NO_5$], 368 [M^+-$C_{20}H_{39}O_3$], 223 [M^+-$C_{27}H_{55}NO_5$], 69 [M^+-$C_{38}H_{77}NO_5$] and 43 [M^+-$C_{40}H_{79}NO_5$] **(Figure 2)**.Thus the structure of indigoferamide-A **(1)** was charecterized as (*S*)-2-hydroxy-N-((2*S*,3*S*,4*R*,*E*)-1,3,4-trihydroxyicos-16-en-2-yl)tricosanamide (indigoferamide-A) **(Figure 1)**.

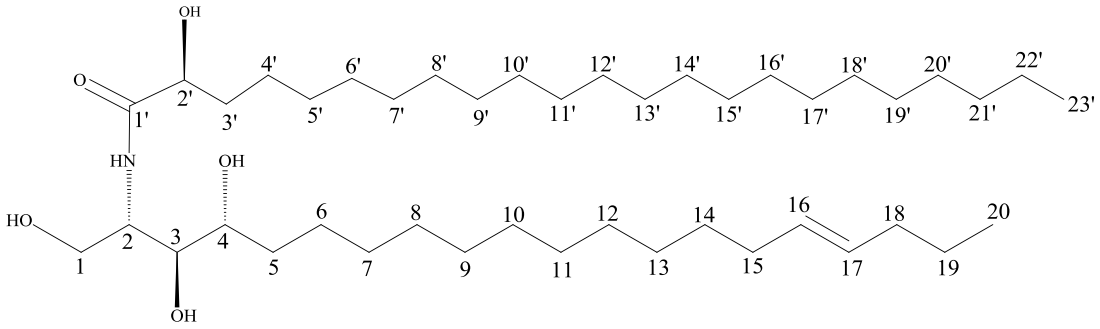

Figure 1. The chemical structures of compound **1**.

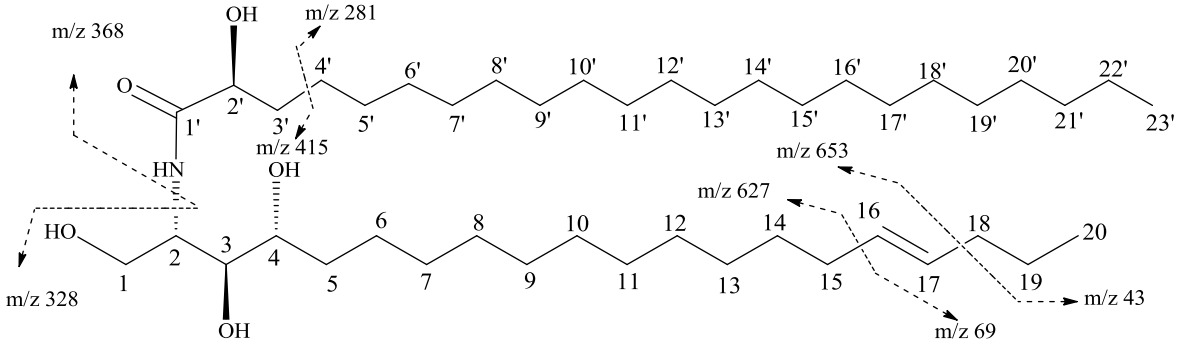

Figure 2. Key EI MS fragmentation in compound **1**.

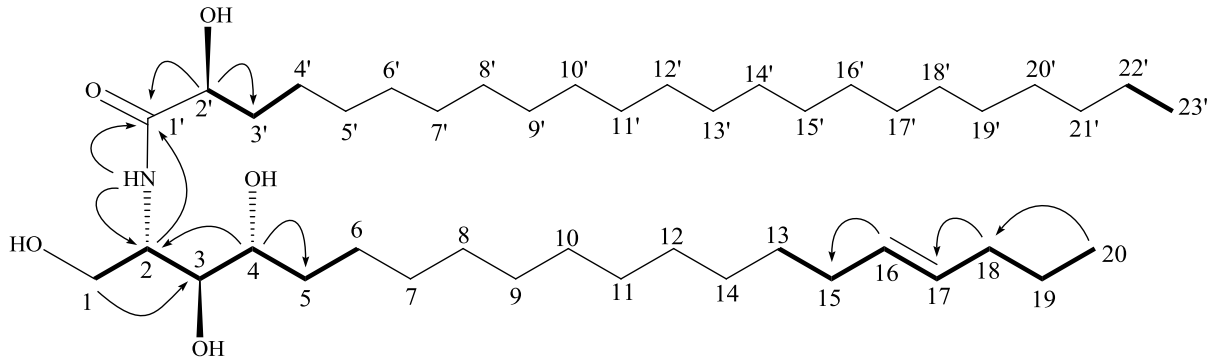

Figure 3. Key HMBC correlations in compound **1**

Compound (2) Dotriacontanoic Acid

Compound **(2)** was isolated as white crystalline solid. The EI-MS showed the molecular ion $[M]^+$ peak at *m/z* 480 for $C_{32}H_{63}O_2$. The IR spectrum displayed absorption bands at 3410 and 1705 corresponding to hydroxyl and carbonyl groups respectively. The ^1H and ^{13}C NMR spectral data of **(2)** were identical to the reported value for dotriacontanoic acid [10].

Compound (3) Quercetin

Quercetin was isolated and purified as yellow crystalline solid. The EI-MS revealed molecular ion $[M]^+$ peak at *m/z* 302 corresponding to molecular formula $C_{15}H_{10}O_7$. The IR spectrum showed absorption bands at 3500 (OH), 1662 (C=O) and 1614 (C=C) cm^{-1}. The UV spectrum exhibited maximum absorption at 257 nm indicated the presence of aromatic system in (**3)**.The NMR spectral data of **(3)** indicated its flavonoid nature, in which ring A protons resonated at δ_H 6.25 (d, *J* = 1.8 Hz, H-6) and 6.51 (d, *J* = 1.8 Hz, H-8) with characteristic signals 5,7-dihydroxylated pattern, while 3′,4′-dihydroxylated pattern of ring B protons shown at δ_H 7.03 (d, *J* = 2 Hz, H-2′), 7.62 (d, *J* = 9 Hz, H-5′) and 7.72 (1H, dd, *J* = 9, 2 Hz, H-6′). The ^{13}C NMR (BB and DEPT) spectra showed the presence of 15 carbons including five methine and one carbonyl and nine other quaternary carbons, All the spectral data were identical with the reported value for **(3) [**11-12].

Compound (4) Formononetin

Compound **(4)** was isolated as yellow powder. The EI-MS showed the molecular ion $[M]^+$ peak at *m/z* 272 corresponding to molecular formula $C_{16}H_{12}O_4$. The IR absorption bands at 3148 and 1640 cm^{-1} indicated the presence of aromatic -OH and carbonyl groups. The UV spectrum showed maximum absorption at 248 nm indicating the presence of conjugated system. The spectral data of **(4)** indicated the presence of a singlet at δ_H 8.15 (H-2), and 7-hydroxylated pattern for ring A, where *meta*-coupled doublet at δ_H 6.81 (1H, d, *J* = 2 Hz, H-8), a doublet at 8.05 (1H, d, *J* = 8.4 Hz, H-5) and a doublet of doublet at δ_H 6.79 (1H, dd, *J* = 8, 2 Hz, H-6) and a 4'-methoxylated ring B protons signals resonated at δ_H 7.41 (dd, *J* = 9, 2 Hz, H-2', H-6') and δ_H 6.91 (dd, *J* = 9, 2 Hz, H-3', H-5'), including a singlet at δ_H 3.79 for the methoxy protons present C-4′.

5. Antibacterial activity

5.1. Preparation of LB media

18 g of trypton was added to 1800 ml of the distilled water and then 9 g of the sodium chloride and 18 gm of the yeast extract were added. The pH 7 of the media was maintained by adding a few drops of 2 M sodium hydroxide (NaOH) solution and sterilized using autoclaved at 121 ^{0}C for 15 minutes. After sterilization of the LB media both the Gram +ve (*B. subtillis*) and Gram –ve (*E. coli*) bacterial strains were transferred in to two sterile test tubes containing 6 ml of the LB media using sterile wire from their respective agar plates and then were placed in the shaker at 37^{o}C for approx. 24 hours.

6. Antibacterial activity of new Indigoferamide-A

The indigoferamide-A (**1**) isolated from the seeds of *I. heterantha* was assayed for its anti-bacterial activity. The bacterial growth was high at low concentration and was decreased with the increase of concentrations. In graph (A) 1μg/ml of the test sample was used against *E. coli and B. subtillis* which showed high bacterial growth, while the bacterial growth decreases with the increase of concentration of test sample from 10 μg/ml in graph (B), 50 μg/ml in graph (C), 100 μg/ml in graph (D), 200 μg/ml in graph (E) and 500 μg/ml in graph (F) respectively. The bacterial growth inhibition is maximum at 500 μg/ml concentration. The data was obtained as absorbance vs time and were then plotted in graphical form.

From the results of graphs (A), (B), (C), (D), (E) and (F) (**Figure 3**) of the test sample concluded that the growth of bacteria is higher at low concentration as compaired to standard antibiotic carbanicillin graph H, while bacterial growth inhibition at higher concentration graphs (F) is comparable to that of standard carbanicillin. These results proved that the new isolated indigoferamide-A can be used as a potent antibacterial agent.

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Supporting Information

Supporting Information accompanies this paper on: www.acgbup.org/RNP

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