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ISOLATION OF BIOACTIVE ALKALOIDS FROM *GENTIANA OLIVIERI* AND ITS NON-TOXIC EFFECT

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Abstract

From the chloroformic extract of *Gentiana olivieri* plant, two alkaloids gentianine and gentianadine were isolated and their structures established on the basis of spectral data and by comparison with the known compounds. Brine shrimp (LC_{50}) bioassay of the crude extract and the pure compound was found to be non-toxic.

Introduction

Gentiana olivieri Griseb plants were collected from the mountains of Quetta near Hazar Ganji in April 1991. The plant is used in folk medicine from centuries in the Asian countries. Much of the early isolation work of alkaloids was done with the aid of ammonia (Akramov & Samatov, 1969). In this paper we report the isolation of two alkaloids by using modern techniques which was quite rapid and yielded comparatively larger amounts. So far most of the compounds isolated were reported without any biological testing. The present report describes the cytotoxic effect of the crude extract and pure alkaloids.

Materials and Methods

Extraction and purification of alkaloids: Ten kg of air dried plant of *Gentiana olivieri* was grinded and soaked in ethanol for two weeks. The ethanolic extract was evaporated to a gummy material. The gum was dissolved in 5% HCl and the acidic solution was extracted with chloroform. The acidic aqueous layer was then basified with ammonia solution to pH 8.0, and further extraction was carried out with chloroform. The chloroform soluble fraction obtained was evaporated to obtain a rust colored material (18 gm.). The mixture was then subjected to TLC on silica gel (GF 254, 0.2 mm) precoated plates using chloroform-hexane-ethanol (5:4:1) to check the number of alkaloids present.

Ten gm of this extract was then loaded on a silica gel column and elution was carried out first with hexane and then with increasing polarities of hexane - chloroform and chloroform - ethanol and finally ethanol only. The fraction eluted with chloroform - hexane (20:1) appeared as a pure white fraction clearly separated out. The TLC of this fraction on silica gel plate showed a major compound along with two minor spots. The upper minor spot was also separated on preparative TLC. The major compound was

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further purified with hexane and isolated as a white amorphous solid (3.6 gm.) which gave orange color on reaction with Dragendorff's reagent. These pure compounds were identified with the help of UV spectrum, IR spectrum, High resolution electron mass spectrum (HRMS), ^{1}H-NMR and ^{13}C-NMR and optical activity.

Cytotoxic assay: Brine shrimp (LC_{50}) bioassay method was used to test the efficacy and cytotoxicity of the pure compound and crude extracts of *Gentiana olivieri*. Three fractions of *G. olivieri* which were ethanolic, aqueous and pure compound were subjected to simple organisms as shrimp nauplii as suggested by Meyer *et al.*, (1962) and Mc Laughlin (1990). Concentrations of 10 μgs/ml, 100 μgs/ml and 1000 μgs/ml of each sample were taken separately in vials containing 5 ml of brine or sea water and ten shrimps with 3 replicates. The survivors were counted after 24 hours and the data calculated to estimate lethal concentration for 50% population (LC_{50}) with 95% confidence intervals for statistically significant comparison of potencies.

Results and Discussion

Alkaloids from *G. olivieri*

1. Gentianine: Two compounds were separated from the chloroform extract of *G. olivieri* after running the column with increasing polarities of the solvent. The major compound gentianine was isolated in significant amount whereas gentianadine the minor compound was separated in a very little amount. These fractions were identified with the help of UV, IR, Optical density, HRMS and NMR. Compound (1) was identified as gentianine, while compound (2) was gentianadine. Gentianine Fig. 1 and 2 was optically inactive and could not be resolved. The UV spectrum of the compound showed maximuim absorption at 218 nm, indicating the presence of a pyrimidine ring.

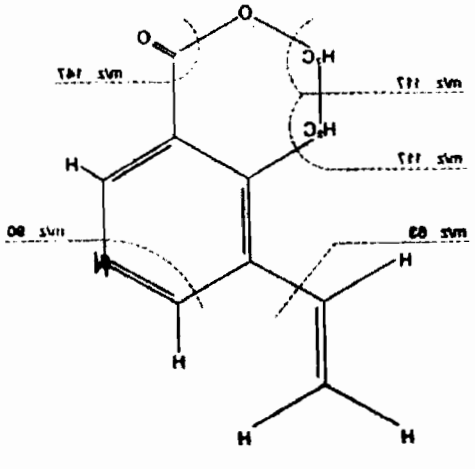

Fig. 1 Structure of gentianine

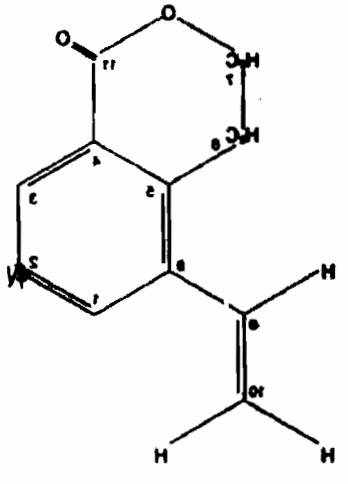

Fig. 2. Fragmentation pattern of gentianine

The IR spectrum included intense absorptions at 1715 cm^{-1} (δ-lactone) at 1630 cm^{-1} (conjugated double bond) and 1585 cm^{-1} (pyridine group). Thus the IR spectrum indicated the presence of an unsaturated lactone and the nucleus.

The high-resolution electron-impact mass spectrum of the alkaloid showed the molecular ion peak at m/z 175.0619 corresponding to the formula $C_{10}H_9O_2N$ (calculated 175) indicating the presence of seven double bond equivalents in the molecule. The other major fragments were at m/z 147 (C_9H_9ON, 17.30), m/z 117 (C_8H_7N 50.83), m/z 90.0473 (C_7H_6, 20.61) and m/z 63 (C_5H_3, 4.71). The HRMS peak at m/z 147.0 (C_9H_9ON) was due to the loss of (-C=O) carbonyl group from the molecular ion. An important peak at m/z 117.0 is in accord with the composition of C_8H_7N resulted from the loss of (-CHO) formyl group from the molecular ion. A fragment at m/z 90.0473 having the composition C_7H_6 was consistent with the loss of nitryl group (H-C=N). The last peak at m/z 63.0325, with the composition C_5H_3 was attributed to cleavage of vinyl group.

The ^{1}H-NMR spectrum ($CDCl_3$, 100MHz)δ of the alkaloid displayed two singlets each integrating for the protons at 8.85 and 9.16 which were assigned to C-3 and C-1 respectively. The spectrum further showed to doublets at 5.61 and 5.8 which were assigned to C-10 (J_{ab}=2.00 Hz). Two triplets centered at 3.1 and 4.6 of pyridine group corresponded to C-6 (J_6=8.00) and C-7 (J_7=6.00). A double doublet at 6.7 was due to the vinylic proton and was assigned to C-8. The vinyl proton H_a is coupled with H_b by a typical geminal coupling J_{ab}=2.00 Hz. It is also coupled to H-7 with a constant J_{ac}=4.00 Hz by a complex splitting called dd. Similarly the coupling for the protons H-6 and H-7 lying at ortho position is 8.00 Hz and 6.00 Hz, respectively.

The ^{13}C-NMR ($CHCl_3$, 100MHz)δ spectra of the alkaloid were also analysed and the result so obtained was applied for the structural determination and identification of the compound. 151.4 (s,C-1), 150.4 (s,C-3), 121 (C-4), 148 (C-5), 25.2 (t,C-6), 67.9 (t,C-7), 131.1 (q,C-8), 127.8 (C-9), 120.8 (d,C-10), 164 (C-11).

Comparison of the above mentioned spectroscopic data with the published reports (Govindachari, 1957; Kubota & Tomita, 1961; Rakhmatullaev & Yunosov, 1972; Baileul *et al.*, 1977) established the compound to be 4-(2-Hydroxy ethyl)-5- vinyl nicotinic lactone commonly known as gentianine or erythricine.

Although gentianine is an already reported compound, but the present purification and isolation was done by an entirely new method not reported before. This method was quite rapid and yielded gentianine in comparatively larger amounts.

Spectral data

UV (MeOH) \max 218 (loge 4.38)

IR ($CHCl_3$)νmax cm^{-1}: 1715 (δ lactone), 1630 (conjugated double bond), 1585 (pyridine), 1120 and 1040 (C-O) HRMS m/z (rel. int.): 175.0619 ($M^+C_{10}H_9NO2$), 147.0681 (C_9H_9NO, 17.30), 117.0570 (C_8H_7H, 50.83), 90.0473 (C_7H_6, 20.61) and m/z 63.023 (C_5H_3, 4.71).

^{1}H-NMR ($CDCl_3$, 400MHz)δ: 9.16 (1H, s, H-1), 8.85(1H, s, H-3), 3.1(2H, t, J6,7=8.00Hz, H-6), 4.6(2H, t, H-7), 6.7(1H, dd, J8, 10α=4.00), 5.8(2H, d, J10α, ß=2.00 Hz, H-10), 5.61(2H, d, H-10).

^{13}C-NMR($CHCl_3$, 100MHz)δ: 151.4 (s, C-1), 150.4 (s, C-3), 121 (C-4), 148 (C-5), 25.2 (t, C-6), 67.9 (t, C-7), 131.1 (q, C-8), 127.8 (C-9), 120.88 (d, C-10), 164 (C-11).

2. **Gentianidine**: The uppermost band separated on preparative TLC was identified with the help of UV, IR, mass spectrum and NMR. The UV spectrum of the compound (2) Fig. 3 and 4 showed maximum absorption at 273 nm which is typical of a pyrimidine ring. The IR spectrum of gentianadine includes intense absorption at 1730 cm^{-1} indicating the presence of δ-lactone. A minor peak at 1600 cm^{-1} was due to the pyrimidine ring.

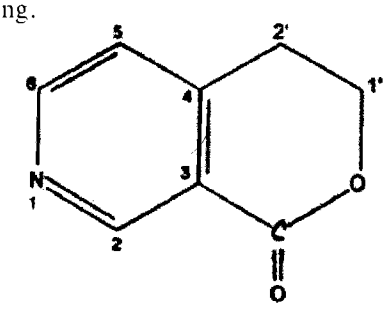

Fig. 3. Structure of gentianine

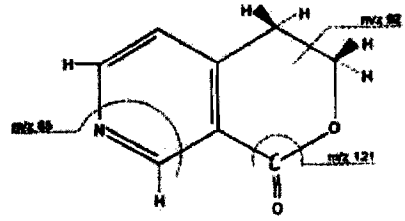

Fig. 4. Fragmentation pattern of gentianine

The ^{1}H-NMR spectrum ($CDCl_3$; 100MH2) of the compound gentianadine displayed two-protons at 5.48 and 6.96 ppm which were assigned to the adjacent methylene groups of a lactone ring (-CH_2-CH_2-O-), at the position of C-1 and C-2, respectively. In addititon to the signal in the strong-field region, weak-field signals were also present. One proton singlet present at 0.88 ppm was ascribed to C-2 of pyridine ring and one-proton doublet at 1.37 ppm corresponds to C-6. The spectrum further showed a one-proton quadruplet at 2.81 ppm which relates to C-5.

H-5 and H-6 not only exhibit spin-spin coupling but also form a system of the AB type (JAB=6.0 Hz). The spectrum also shows the interaction of H5 and H2 with a small constant (J=1.0 Hz). The high resolution electron impact mass spectrum (HRMS) of the compound gentianadine showed the maximum peak corresponding to the molecular ion at m/z (M^+149) having the formula $C_8H_7NO_2$ indicating the presence of six double bond equivalents in the molecule. The peak at m/z 121.00 (C_7H_7NO) arises due to the loss of carbon mono oxide (-CO). The expulsion of a formyl group results in the formation of a peak at m/z 92.028 (C_6H_6N). An important peak is formed at m/z 65 by the loss of a nitryl group (H-C=N) which leads to the formation of an ion having the melecular formula of (CH_5).

Spectral data

UV (MeOH) \max nm(logs): 243(3.48)

IR ($CHCl_3$) max cm^{-1}: 1730 (δ lactone), 1595 (pyridine).

HRMS m/z (rel. int. %): 149.147 ($M^+C_8H_7HO_2$), 121.00 (C_7H_7HO 52.83), 92.028 (C_6H_6N, 23.15), 65.043 (C_5H_5, 6.73).

^{1}H-NMR ($CDCl_3$, 400MHz)δ: 5.48 (2H, t, H-1), 6.96 (2H, t, H-2), 0.88 (1H, s, $J_{2,5}$ = 1.0Hz, H-2), 1.36 (1H, d, J5, 6=6.0Hz, H-6), 2.81 (1H, q, H-5).

Table 1. Brine shrimp bioassay of gentianine and ethanolic and aqueous extracts of *Gentiana olivieri*.

Type of extract	10 μg/ml		100 μg/ml		1000 μg/ml		Concentration LC_{50} μg/ml
	Alive	Dead	Alive	Dead	Alive	Dead	
1. Ethanolic	30	0	30	0	30	0	>1000
2. Aqueous	30	0	30	0	30	0	>1000
3. Gentianine	30	0	30	0	30	0	>1000

LC_{50}: Lethal concentration (for 50% population)

Brine shrimp toxicity bioassay: Brine shrimp (LC_{50}) bioassay method was used to testify the cytotoxic effect of the pure isolated alkaloid gentianine, and the ethanolic and aqueous extract of *Gentiana olivieri*. Since the plant was purified for the development of drug, therefore, it was necessary to find that its concentrations used do not produce any lethal effect on any of the thirty nauplii by all three concentrations tested.

None of the nauplii died after 24 hours (Table 1). The LC_{50} calculated was found to be greater than 1000. Thus, *G. olivieri* is non-toxic and could safely be given to the experimental animals for further investigations.

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