World Journal of Chemistry 5 (1): 26-31, 2010
ISSN 1817-3128

Antioxidant and Anticonvulsant Alkaloids in *Crinum ornatum* Bulb Extract

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Abstract: Several alkaloids were isolated from *Crinum ornatum* Bulb extract out of which lycorine, crinamine, heamanthamine, hamayne and ornamine were fully characterized. Haemanthamine and hamayne are newly reported for this specie of plant. Also activity–directed fractionation identified lycorine, crinamine and haemanthamine as the antioxidant and anticonvulsant principles of the plant. The antioxidant activity (AA%) showed that apart from the crude ethanolic extract, which had the highest scavenging activity of 58.24%, lycorine at a dose of 1mg/ml is the most active in scavenging DPPH radical followed by haemanthamine, crinamine with hamayne having the lowest activity. Dose dependent anticonvulsant effect was observed for haemanthamine and lycorine. A 91.5% inhibition of shock was observed for the crude extract at 2mg/ml. Lycorine gave an average of 89.1% reduction of shock at the same concentration while haemanthamine gave 75.5%. The coincidence of antioxidant activity and protective effect against convulsion due to electroshock suggest that both free radical generation and lipid peroxidation may be involved in the anticonvulsant mechanism of *C. ornatum*.

Key words: *Crinum ornatum* · Antioxidant · Anticonvulsant · Alkaloids · Free - radicals

INTRODUCTION

The importance and use of plants cannot be over-emphasized. Plants are bio-renewable resources that will continue to furnish Scientist with new leads in drug production as well as provide the folklore remedies to some diseases. Several plants have been investigated for the presence of bio-active chemical constituents, based on the fact that they are being used traditionally. The *Crinum species* is also an interesting because they are being used in different parts of the world to treat various disorders. The bulbs of some *Crinum species* were used in Africa to treat urinary infections, coughs and colds, renal and hepatic conditions, sores, sexually transmitted diseases and backache, as well as to increase lactation in animal and human mothers [1]. Previous work done on the *Crinum species* reported the antibacterial and antifungal activities [2], antitumor and immunostimulating activities and antiparasitic activity [3] and insecticidal activity [4]. Alkaloids have been reported in *Crinum ornatum* bulb and they are lycorine, crinamine, ornamine, ornazidine and ornzamine [5]. Previous worked done [6] revealed that the crude extract of *C. ornatum* possesses antioxidant and anticonvulsant activities. This research, however focuses on the isolation of the active chemical constituents responsible for its antioxidant and anticonvulsant activities.

MATERIALS AND METHODS

Plant Materials: Fresh bulbs of *C. ornatum* were collected in June at Ife Road in Ibadan North Local Government Area of Oyo State, Nigeria. Specimens were identified and authenticated at Forestry Research Institute of Nigeria, Ibadan (No FHI 105367). The bulbs were air - dried and ground into fine powder.

Animals: Adult Wistar rats (males) weighing 160 - 220 g (14 weeks old) obtained from the animal House of the Department of Physiology, University of Ibadan were used. The animals were divided into two sets, one set for the experimental test and the other serving as control test. Five animals were used per group. The rats were fed with balanced livestock feed from Pfizer, plc and water was also given *ad libitum*.

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Extraction and Fractionation Procedures: 20kg of dried-powdered bulb *C. ornatum* was added to 50% ethanol and kept for 72 hrs before filtration. The filterate was decanted and concentrated with the aid of *Buchi Rotavapor* R110 under reduced pressure of 37°C. The crude extract was first basified with ammonia solution to pH 10. Ethylacetate was successfully added to basified aqueous solution of the crude extract in order to remove both alkaloidal and neutral components while butanol was added to the mother liquor to remove the polar fractions. The ethylacetate and butanol fractions were purified by Accelerated Gradient Chromatography (AGC) using solvent systems Hexane, EtOAc and Methanol and silica gel 60 F_{254}, 40-63 microns as adsorbent followed by thin layer chromatography. Eleven isolates were obtained out of which five were fully characterized by spectroscopic analysis. AGC of EtOAC fraction gave haemanthamine, further purification by preparative silica gel tlc using $CHCl_3$: MeOH (9:1) gave some other alkaloids while AGC of the Butanol fraction gave the major alkaloid lycorine. The compounds obtained were tested for anticonvulsant and antioxidant activities.

Free Radical Scavenging Activity: 3.94mg of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), a stable radical was dissolved in methanol (100ml) to give a 100µM solution. To 3.0ml of the ethanolic solutions of DPPH was added 0.5 ml of the ethanolic extract (from stock solution 0.1g in 100ml). The decrease in absorption at 517nm of DPPH was measured 10min later. The actual decrease in absorption induced by the test compound was calculated by subtracting that of the control. The percentage inhibition was also calculated. All tests and analyses were run in triplicates and the results obtained were averaged. This analysis was carried out for the crude ethanolic extract and some of the pure compounds lycorine, crinamine, haemanthamine and hamayne with doses ranging from 1.0mg/ml to 125µg/ml [7].

Anticonvulsant Activity:

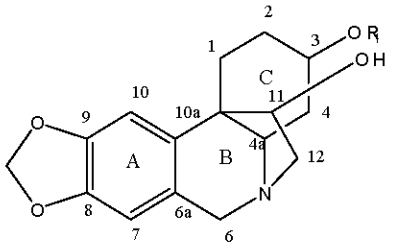
Electrical Stimulation Test: Anticonvulsant screening was conducted using the Maximal Electroshock Test (MEST) [8]. Adult Wistar rats (males) weighing 160 - 220 g (14 weeks old) obtained from the animal House of the Department of Physiology, University of Ibadan were used. The animals were divided into two sets, one set for the experimental test and the other serving as control test. The rats were grouped five per cage. Each animal served as its own control being placed on stimulator to enable it adapt to it. The pure compounds obtained were separately dissolved into 2.5% Tween 80. Doses ranging from 0.5 to 2mg/ml were injected interperitoneally into the animals. The Ealing Kymograph with in - built stimulator (Student Kymograph Basic Stimulator) was used to measure the degree of shock or convulsive seizures by considering the hind limb tonic extension (HLTE). Abolition of HLTE was considered as protection from electroshock. The grading was based on the percentage of animals protected from electro seizure. Response against time at each applied voltage and the number of animals protected at each concentration were noted. Tween 80 was used for the control animals. The results were noted at 30min, 1hr and 2hrs intervals [9].

RESULTS AND DISCUSSION

A total of 11 isolates were obtained out of each 5 compounds were fully characterized. The UV spectra taken in ethanol (99.8%) for 0.01%w/v of the fractions were determined with the aid of HEλ1OS α UV-Visible spectrophotometer. The samples were scanned between 190 to 315nm. The NMR (^1H, ^{13}C, COSY and HETCOR) spectra of the pure compounds were determined using a 200MHz machine for 10% (w/v) solutions in deuteriochloroform, DMSO-d_6 or deuteriomethanol. Pulse irradiation technique employed was FT NMR at ambient temperature. The ^1H NMR signals of the alkaloids appeared at δ 1.5 - 8.5 (δ scale relative to TMS). Vmax (cm^{-1}) from IR data also confirmed the structures. Atmospheric chemical ionization (APCI) positive (+) mass spectrum of the partially purified sample showed the molecular weights of the major alkaloids and some minor components. The compounds are Haemanthamine, Crinamine, Hamayne, Lycorine and Ornamine.

Haemanthamine was identified by its uv, ir, ^1H nmr, ^{13}C nmr and hector [1, 10]. Crinamine was identified by its uv, ir, ^1H nmr [1, 3], ^{13}C nmr and ms. Hamayne was identified by its uv, ir, ^1H nmr, [11]. Lycorine was identified by its uv, ir, ^1H nmr and ms [1, 3, 10]. Ornamine was identified by its uv, ir, ^1H, nmr and ms [5]. Of these compounds, Haemanthamine and Hamayne are being reported newly in the bulb of *C. ornatum.*

Structures of the new compounds reported here are as shown in Figure 1.



R_1 = ß Me Haemanthamine
R_1 = H Hamayne

Fig. 1: Structures of Haemanthamine and Hamayne

Haemanthamine is a brown resinous substance, R_f 0.42 (Silica gel $F_{254,}$ EtOAc:MeOH 8:2), it is an alkaloid, soluble in water, methanol, chloroform, ethylacetate, hexane and acetone. UV [EtOH]nm (log ε): 240.0 (3.10), IR (KBr) V_{max} cm^{-1} 3423 (NH stretch), 2943.4 (C-H Aromatic stretch); 2870.5 (C-H Aliphatic stretch), 1703.2 (C=O Stretch), 1496.3 (C-H Aliphatic bending), 1387.1 C=N, 1247.2 (C-O Unconjugated), 1125.6 (C-O Very strong), 997.6 (C-H Aromatic 1 adjacent free H) and 742.5 (C-H Aromatic out of plane bend). ^{1}H NMR (200MHz; $CDCl_3$): δ 0.84t, 1.21s, 2.15 (1H, m, J=10, H-3), 3.35 (3H, s, OMe), 3.70 (1H, d, J = 16, H-6a), 3.92 (1H, q, J = 5, H-11), 4.30 (2H, d, J =16, H -6b) 4.82s, 5.82 (2H, s, -OCH_2O-) 6.18 (1H, dd, J = 5, H-2), 6.40 (1H, s, H-7), 6.80, (2H, s, H-10), ^{13}C NMR (50MHz, $CDCl_3$): δ 178.008, 146.895(C-8), 146.521 (C-9), 135.431 (C-2), 134.968 (C-10a), 125.671 (C-6a), 124.133 (C-1), 107.129 (C-7), 103.515 (C-10), 101.181 (-OCH_2O-), 79.836 (C-11), 76.273 (C-3), 66.242 (C-4a), 63.257 (C-6), 60.726 (C-12), 56.043 (-OCH_3), 50.511 (C-10b), 29.742 (C-4), Hetcor ($CDCl_3$) results show the following ^{1}H values and corresponding ^{13}C values: 6.18 (H-2) (135.431), 6.18 (H-2) (124.133), 6.45 (H-7) (107.129), 6.80 (H-10) (103.515), 5.82 (-OCH_2O-), (101.136) 3.70 (H-6a) (80.215), 3.70 (H-6a) (76.305), 3.35 (OMe) (66.333), 3.35 (OMe) (63.381), 3.60, 4.30 (H-6b) (61.347), 3.35 (OMe) (56.058) 2.15 (H-3) (30.303).

Crinamine is a colourless needle like crystallline substance. R_f 0.45 (Silica gel $F_{254,}$ EtOAc:MeOH 8:2). It is soluble in methanol, chloroform, ethylacetate, hexane, acetone and sparingly soluble in water. UV [EtOH]nm (log ε): 225.0, 293.0 (1.878, 2.139) IR (KBr) V_{max} cm^{-1}. 3440.3, (O-H stretch). 2992.1, (C-H Aromatic stretch), 2037.6 (O-CH Aliphatic stretch), 1697 (C=O stretch) 1490.4 (-H Aliphatic bending) 1320.1 C=N, 1235.1 (-O Uncojugated) 1119.4 (-O Very strong) 979.5 (-H Aromatic 1 adjacent free H) 864.1 (C-H Aromatic 2 adjacent free Hs), 736.3 (C-H Aromatic (out of plane bend). ^{1}H NMR (200MHz; $CDCl_3$): δ 1.220s, 2.30 (1H, m, J = 10, H-4), 2.50 (1H, s, H-4), 3.20m., 3.40 (3H, s, OMe), 3.70 (1H, d, J = 17, H-6a), 4.00 (1H, t, J=7.5, H-11), 4.30 (1H, d, J=17, H-6b), 5.90 (2H, s, -OCH_2O-), 6.25 (1H, s, H-1), 6.50 (1H, s, H-7), 6.80 (2H, s, H-10), ^{13}C NMR (50MHz, $CDCl_3$): δ 146.759 (C-8/9), 135.923 (C-10a), 126.709 (C-6a), 123.996 (C-2), 107.129 (C-7), 103.447 (C-10), 101.136-(-OCH_2O-), 80.215 (C-11), 77.934 (C-1), 77.297, 76.661, 76.305 (C-3), 66.333 (C-5), 63.681 (C-6), 61.347 (C-12), 56.058 (OCH_3), 50.496 (C-10b), 30.3 (C-4).

Haymane is a yellow substance, R_f 0.63, (Silica gel $F_{254,}$ EtOAc: MeOH 8:2) It is soluble in water, methanol, chloroform, ethylacetate, hexane and acetone. UV [EtOH]nm (log ε): 224.0, 289.0 (1.786, 0.941), IR (KBr) V_{max} cm^{-1} 3417.6 (O–H Stretch), 2055.8 (C-C stretch), 1654.5 (C=O stretch), 1423.4 (C-H Aliphatic bending), 1101.1 (C-O very strong), 1009.9 (OCH weak) 821.6 (CH Aromatic 2 adjacent free H), 705.9 (C-H Aromatic out of plane bend). ^{1}HNMR (200MHz; $CDCl_3$): δ 1.30 s, 2.40 (2H, m, J=10, H-4), 4.00 (1H, m, J=7.5, H-11), 5.60 (2H, s, H-6a/6b), 5.98 (2H, s, -OCH_2O-), 6.60 (1H, s, H-7), 6.80 (1H, s, H-10).

Lycorine is a creamy shining crystal R_f 0.71 (Silica gel $F_{254,}$ EtOAc: MeOH 8:2). It is soluble in ethylacetate, hexane and acetone, but sparingly soluble in water, methanol and chloroform. UV [EtOH]nm (log ε): 238.0, 290.0 (2.398, 2.344). IR (KBr) V_{max} cm^{-1} 3429.8 (O-H stretch), 20197 (C-C stretch), 1660.6 (C=O stretch), 1502.5 (C=C conjugated), 1356.7 (C=N), 1111.1 (C-O very strong), 973.6 (C-H Aromatic 1 adjacent free H), 845.8 (C-H Aromatic 2 adjacent free H), 742.5 and 675.5 (C-H Aromatic out of plane bend). ^{1}H NMR (200MHz; DMSO-d_6): δ 2.20t, 2.50q, 3.30t, 3.70s, 4.00d, 4.25 (1H, s, OH-2), 4.90 (1H, s, OH-1), 5..20 (1H, s, H-7), 5.35 (1H, s, H-10), 5.90 (2H, s, -OCH_2O-), 6.65 (1H, s, H-8), 6.85 (1H, s, H-11). The chemical shift values of the inter-nuclear correlated protons (COSY Experiment) gave the following chemical shift of carbon atoms and corresponding number of ^{1}H attachments, 2.20t (3H), 2.50q (2H), 3.30t (2H), 4.00d (2H + 2H), 4.25s (2H), 4.89s (2H), 5.00s (2H), 5.30s (2H) 5.80s, (1H) 6.65s (1H), 6.80s (1H).

Ornamine is a brown crystalline substance. R_f 0.49 (Silica gel $F_{254,}$ EtOAc:MeOH 8:2). It is soluble in water, methanol, chloroform, ethylacetate, hexane and acetone. UV [EtOH]nm (log ε):220.0, 263.0 (1.092, 0.632). IR (KBr) V_{max} cm^{-1} 3455.7. (O-H stretch). 2068 (C-C stretch), 1703.2 (C=O stretch), 1121.3 (C-O very strong), 1016.1 OCH weak. 730.4 (C-H Aromatic out of plane bend). ^{1}HNMR (200MHz; CD_3OD): δ 1.12m, 1.90s, 3.35s, 4.60s, 5.00t, 5.90t, 6.30m, 6.90d, 7.90s, 8.50s.

The methylene dioxy aryl chromophore and isolated double bond of the lycorine and crinine-type alkaloids were displayed. They exhibited maximum near λ 240nm and another maximum around λ 280nm - 290nm when no other saturation was present. The extinction value of the longer wavelength maximum of lycorine and crinine type alkaloids are generally greater except in cases where there is a C-7-methoxyl group. The proton NMR results showed that the alkaloids in general contain both unsaturations and an oxygen function group in ring C for haemanthamine and crinamine, the chemical shifts of the signals of the aromatic hydrogen at C-7 and C-10 are typical signals of aryl hydrogens adjacent to oxygen functions. The 10-proton signal occurred as a sharp singlet and was located at consistently lower field than the 7-proton signal. Furthermore, the latter was slightly shorter and broader than the former, indicating a weak splitting arising from coupling with one or both of the benzylic hydrogen. The assignment of the lower-field signal to the 10-hydrogen was made on the basis of the effect on its chemical shift associated with hydrogenation of the 1,2 - double bond. The major differences revealed in the ^{1}H NMR spectra of the haemanthamine and crinamine series are associated with the signals resulting from the 1-and 2-protons. The 1-, 2- and 3-hydrogens of the haemanthamine series, gave rise to an ABX pattern. The olefinic spectral region showed six peaks, though one or more peaks were obscured in some spectra, usually by overlapping with other signals. The pair of resonance lines located at the low-field end of the olefinic hydrogen multiplet is a one-proton doublet associated with the l-hydrogen. The remaining four lines are a doublet of doublets resulting from H-2 coupling with H-1 and with H-3. The olefinic hydrogen pattern in the crinamine showed marked differences from the haemanthamine analogues. In the ^{1}H NMR specrum of crinamine itself, the observed signal for both hydrogens was a broadened singlet.

Furthermore, ^{1}H NMR spectra of the dihydro-products revealed a diamagnetic shift of the low-field aryl hydrogen signal. The methylenedioxy-group was characterized in the pure compounds by a sharp two-proton signal in the range 5.82-5.9. In a delayed COSY experiment emphasizing long-range coupling, hydroxyl groups at δ 4.25 and 4.89 displayed cross peaks with the signals at δ 4.20 and 4.80 for lycorine. The singlet at δ 5.90 corresponding to the -$0CH_20$- functional group exhibited cross-peaks with the proton-singlet occurring at δ 5.80. HETCOR was used to decide on the orientation of the ^{13}C - ^{1}H nuclei of haemanthamine. These correlations as observed for individual H and C at the corresponding δ values further confirmed the structure of a 5, 10b ethanophenanthridine type alkaloid. Interestingly, the singlet at δ 5.82 corresponding to the protons of the methylenedioxy functional group showed correlation with the carbon at δ 101.13. The atmospheric chemical ionization positive mass spectrum of the partially purified sample shows the presence of atleast nine alkaloids with molecular ion at m/z 390, 331, 329, 301, 287, 285, 265, 210 and 204. These values also confirmed the molecular weights of the isolated compounds.

Free Radical Scavenging Activity: Scavenging activity of ethanolic extract on DPPH is shown in Table 1. The antioxidant activity (AA%) showed that apart from the crude ethanolic extract, which had the highest scavenging activity of 58.24%, lycorine at a dose of 1mg/ml is the most active in scavenging DPPH radical followed by haemanthamine, crinamine with hamayne having the lowest activity. Hence, extract of *C. ornatum* decolourised DPPH due to their hydrogen donating activity [12].

Reduction in absorbance is due to the pairing of the odd electron of the radical indicating the ability of the compounds to scavenge free radicals. These results indicate that lycorine, haemanthamine, crinamine and the ethanolic extract are free radical inhibitors, primary antioxidants that react with free radicals.

Table 1: Antioxidant Activity (AA%) of the ethanolic extract and some of the pure extracts*

	Crude Ethanolic Extract	Haemanthamine	Crinamine	Lycorine	Hamayne
1.0mg/ml	58.24	8.82	1.76	14.70	1.18
500μg/ml	27.65	3.53	1.18	11.76	0.59
250μg/ml	13.53	1.76	0.59	8.24	0.59
125μg/ml	6.47	1.18	0	5.88	0

*The reduction in A_{517} of DPPH (100μM) caused by the ethanolic extract and some of the pure extracts was measured in triplicate after 10min

**Each value represents the mean ± standard deviation of triplicate analysis.P = 0.05 compared with control Student's t - test

The crude extracts showed better activity than the pure compounds but an experiment was carried out to show if there is a synergy between these compounds. Various concentrations of lycorine, haemanthamine and crinamine were combined. The results obtained showed that lycorine, haemanthamine and crinamine at 1mg/ml each gave a % inhibition of 59.85. Also at 1mg/ml, 1mg/ml and 0.5mg/ml respectively gave a % inhibition of 55.3. And at 1mg/ml, 0.5mg/ml and 0.5mg/ml respectively gave a % inhibition of 50.1. The results showed that combining lycorine, haemanthamine and crinamine enhanced their antioxidant activity.

Anticonvulsant Activity: Dose dependent anticonvulsant effect was observed for haemanthamine and lycorine. A 91.5% inhibition of shock was observed for the crude extract at 2mg/ml. Lycorine gave an average of 89.1% reduction of shock at the same concentration while haemanthamine gave 75.5%. The response of haemanthamine however decreased to zero at the lowest concentration of 0.5mg/ml.

The anticonvulsant activity of the crude extract and lycorine at a dose of 2mg/ml in HLTE of MEST suggests that this fraction possess anticonvulsant activity for the treatment of generalized tonic - clonic and partial seizures [8, 13].

CONCLUSION

The results of this study showed that, attempts to isolate and characterize the antioxidant and anticonvulsant principles of *Crinum ornatum*, eleven isolates were obtained out of which five compounds were fully characterized and reported. However this work has been able to isolate two other amaryllidaceae alkaloids in addition to lycorine, crinamine, ornamine, ornazamine and ornazidine which previously reported. The new alkaloids are haemanthamine and haymane. Both are crinine type alkaloids. Interestingly, the research has also been able to identify the presence of a diterpene in the bulb of *Crinum ornatum*. Further work will be needed to fully characterize this compound. Lycorine is a 5, 10b - ethanophenanthridine type alkaloid. Haemanthamine, crinamine and haymane are crinine type alkaloids. The close R_f values of haemanthamine and crinamine reflect a similiar degree of basicity. Haemathamine and crinamine gave spectra that closely resembled each other since they are stereoisomers.

The results obtained are in agreement with other workers. In the free radical scavenging activity, the tested compounds showed appreciable activity. Reduction in absorbance occurred since DPPH abstract the phenolic hydrogen of the electron donating molecule indicating antioxidant activity [7, 14].

The coincidence of antioxidant activity and protective effect against convulsion due to electroshock suggest that both free radical generation and lipid peroxidation may be involved in the anticonvulsant mechanism of *C. ornatum*. This study has provided further insight into the chemistry of *C. ornatum* growing in Nigeria. New antioxidant compounds have been isolated from this plant. The results of this study demonstrate that *C. ornatum* crude extract and lycorine have potent anticonvulsant activity. Since these compounds are effective in the MEST, these compounds can be effective in anticonvulsant therapy that involves tonic - clonic and partial seizures. The results also suggest that the extracts of *C. ornatum* could be considered for use as antioxidants. It was also concluded that differences in antioxidant activities of the alkaloids are independent on the relationship between their c hemical structures and reactive oxygen species. Development of anticonvulsants from *C. ornatum* may produce natural antiepileptic drugs. Its use at larger doses should however be monitored for its potent toxicity. Further studies will be conducted to determine the mechanism of action of the alkaloids and compare their mode of action to some tranquilizers like phenothiazines, benzodiazepines and barbiturates. These drugs are used as anticonvulsants and antipsychotics [15]. Also, more *in vivo* assays are also essential to characterize them as biological antioxidants. This study has therefore justified the use of this plant in traditional medicine.

AKNOWLEDGEMENT

The authors will like to thank Professor Robert T. Rosen of the Center for Advanced Food Technology, Cook College Rutgers. The State University of New Jersey for running the atmospheric chemical ionization (APC1) mass spectrum of the sample.

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