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Bioactive of Dioclimidazole from *Dioclea reflexa* Seeds

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Abstract: New secondary metabolites, dioclimidazole (1) was isolated from the chloroform extract of the seeds of *Dioclea reflexa* Hook (Fabaceae) through bioassay-guided fractionation. The efficacy of compound 1 was compared with huperzine A in an anticholinesterase assay where 1 showed a significant anticholinesterase activity *in vitro* of 8.0 HpAU at 20 µg/mL. Compound 1 (5 µg/mL) also gave an appreciable antibacterial activity *in vitro* against *Escherichia coli* and *Bacillus subtilis* to the same extent as 2.0 µg/mL of gentamycin sulfate. The MICs of Compound 1 on *Escherichia coli* and *Bacillus subtilis* are 0.1 and 0.5 µg/mL respectively.

Key words: Dioclimidazole · Dioclea reflexa · Anticholinesterase · HpAU

INTRODUCTION

Anticholinesterase agents as a group have received more extensive application as toxic agents in the form of agricultural insecticides and potential chemical warfare than as therapeutic agents [1]. Nevertheless, several members of this class of compounds are clinically useful. Physostigmine and Neostigmine are useful for glaucoma and myasthenia gravis treatment respectively [2,3]. Anticholinesterase catalyses the break down of acetylcholine into acetic acid and choline. It is found in nerve synaptic, neuromuscular junctions and in other tissues. The assay of this enzyme is clinically useful in designing drugs for the treatment of chronic liver disease, mania, stroke, post-traumatic brain syndromes and the cause of dementia all of which are gradually raising their ugly heads worldwide [4-7].

Higher plants are known to provide diverse range of secondary metabolites. However, despite the breadth of chemo-diversity, relatively few imidazole compounds have been reported from higher plants. Imidazole compounds possess an imidazole-glyoxaline-nucleus, similar to histidine. They belong to a small group of 13 alkaloids reputed from the rutaceae, euphorbiaceae and fabaceae families. Pilocarpine, isopilocarpine, pilosine, isopilosine and pilocarpidone obtained from *Pilocarpus jaborandi* finds great uses in ophthalmic and glaucoma treatment [8]. Lepidiline A and Lepidiline B an anti-cancer agents are among the imidazole alkaloids obtained from *Lepidium meyenii* [9].

The species *Dioclea reflexa* Hook belong to Fabaceae family sometime placed in Papilionaceae. The Fabaceae are herbs, vines, shrubs, trees and lianas found in both temperate and tropical areas. They comprise one of the largest families of flowering plants, numbering 630 genera and 18,000 species. *Dioclea reflexa* is native to west-central tropical Africa, south tropical Africa and southern America. The plant is locally known as "Agbaarin" in Yoruba, western Nigeria. It is propagated from seeds [10-12]. No previous phytochemical study of this species has been reported and there are no references for the chemical composition of its active secondary metabolite in the literature.

As part of ongoing survey of Nigerian flora and herbs, aimed at characterization of poorly studied species and identification of bioactive constituents, we have examined the chloroform extract of the seeds of *Dioclea reflexa*. The report of our findings is hereby presented.

MATERIALS AND METHODS

Instrumentation Mpts, uncorrected on a Fisher-Johns apparatus; UV-$CDCl_3$, on a Beckman DU-7 spectrophotometer; IR, KBr on a Perkin-Elmer 1600 FTIR

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spectrophotometer; ^{1}H and ^{13}C NMR, on a Varian VXR 500s spectrometer (^{1}H at 400 MHz, ^{13}C at 100 MHz) for solution in chloroform with TMS as internal reference; Low resolution CIMS: Finnigan 400 spectrometer; Low and high resolution FAB MS; Kratos MS 50 through peak matching on 2,5-dihydroxybenzoic acid (DHB) matrix with NaI addition. Open column liquid chromatography was run over Silica gel (Merck, 60-200 mesh).

Analytical TLC was performed on pre-coated Merck glass (Whatman K_6F Silica gel 60°A) and viewed under a UV lamp or iodine or sprayed with 5% phosphomolybdic acid in EtOH followed by heating. Healthy sheep was obtained from local market after certification.

Plant Material: The seed of *Dioclea reflexa* Hook was locally obtained in Kano State, Nigeria. It was botanically identified by B. S. Aliyu of Biological Sciences, Bayero University, Kano (BUK). Specimen of the plant is deposited in the herbarium of BUK (DF-00-31-00). The hard seeds were milled using Thomas model 4 Wiley mill and hermetically sealed in plastic bag for use.

Anticholinesterase Assay: Extracts, partitioned fractions, bulked chromatographic eluents and pure compound (5, 10, 20 µg/mL) were tested for cholinesterase enzyme inhibition according to standard protocols [13,1,14] with huperzine A as positive control *in vitro*. A calibration (straight-line) curve was plotted for % ChE inhibition Vs huperzine A amount which obeys Beer Lambert's law. The percentage inhibition of sheep liver ChE of standard huperzine A (values obtained are the mean of three observations) was also determined using the same approach. For sake of convenience and comparison, attempts were made to normalize the degree of inhibition of various fractions to standard huperzine A unit. One huperzine A unit (HpAU) is equivalent to ChE inhibition at 1 µg amount in standard calibration curve.

Antibacterial Assay: Antimicrobial activity was by disc diffusion method [15,16].

Determination of (MICs): MIC was determined following the method recommended by the NCCL (2003a) [17] standard. The agar dilution method was used to determine the MICs of dioclimidazole. The microorganism stains were tested at a final inoculum concentration of 10^4 c.f.u. per drop and incubated at 35°C for 18 h. The *in vitro* sensitivity of the test stains to dioclimidazole was obtained based on manual interpretation and no visible growth of organisms was recorded as the endpoint MIC.

Extraction and Isolation: The plant material (2000 g) was exhaustively extracted by percolation with 99% EtOH (5 L) at room temperature for a week. The percolate was evaporated to dryness on a rotary evaporator at 35°C to give F1 residue. The residue F1 [30 g, 1.3 HpAU at 20 µg/mL] was defatted through maceration with n-hexane (3 X 200 mL) to obtained n-hexane extract F2 [09.1 g, 1.1 HpAU at 20 µg/mL] after being concentrated in vacuum. The remaining F1 (after being defatted) was extracted with chloroform (3 X 200 mL) by further maceration. The chloroform extract was concentrated to obtained F3 residue [15.3 g, 3.0 HpAU at 20 µg/mL].

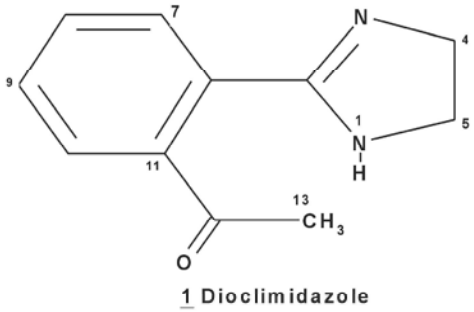
The F3 fraction registered a moderate degree of ChE inhibition at all concentration tested when compared with huperzine A (Table 1). A portion of this $CHCl_3$ soluble residue F3 (4.0 g) was fractionated in an open column Silica gel (120 g) eluted with n-hexane: $CHCl_3$ gradient. Eluants were analyzed on TLC, pooled into 16 fractions and each subjected to anticholinesterase assay. Fraction 5 (F*5) [175 mg, 5.3 HpAU at 20 µg/mL], eluted with n-hexane: $CHCl_3$ (2:1) was further fractionated in Silica gel pencil column. The pencil column was eluted with n-hexane: toluene: chloroform (4:1:1).

The column yielded a yellow precipitate which was purified by re-crystallizing in 2-propanol. This produced a cream bioactive compound dioclimidazole [30 mg, 8.0 HpAU at 20 µg/mL]. This dioclimidazole (5 µg) partially inhibited the growth of *Escherichia coli* and *Bacillus subtilis* to the same extent as 2.0 µg of gentamycin sulfate. Dioclimidazole, a cream precipitate is a new natural compound. Efforts to isolate a single crystal of the precipitate for X-ray crystallography were unsuccessful.

Dioclimidazole 1: M.pt. 196-198°C
UV: λmax ($CHCl_3$) 251 nm.
IR: (KBr) cm^{-1} 2980, 1680, 1430, 890-750.
CIMS: M/e 190.2, 189.2, 188.1, 121.3, 69, 42.9.
^{1}H NMR: (400 MHz, $CHCl_3$): see Table 3.
^{13}C NMR: (100 MHz, $CHCl_3$): see Table 3.

RESULTS AND DISCUSSION

The isolation of dioclimidazole was guided by *in vitro* anticholinesterase and antibacterial assays using repetitive open column chromatography to separate bioactive chloroform extract [4.0 g, 3.0 HpAU at 20 µg/mL].The purified dioclimidazole was obtained as a cream precipitate (M.pt. 196-198 °C) which gave a positive nitrogen test when subjected to Lassaigne's test.



1 Dioclimidazole

Fig. 1: Dioclimidazole (1)

Table 1:.*In vitro* anticholinesterase assay of *Dioclea reflexa* fractions.

Fraction NO	Concentration µg/mL	HpAU*	[l]Remark
F1	5	-	Trace of inhibition
	10	1.0	
	20	1.3	
F2	5	-	Trace of inhibition
	10	-	
	20	1.1	
F3	5	-	Good inhibition at all concentration.
	10	2.5	
	20	3.0	
F*5	5	1.2	Good inhibition at all concentration.
	10	3.6	
	20	5.3	
Dioclimidazole	5	2.6	Very Good inhibition at all concentration.
	10	5.1	
	20	8.0	

* Means of three measurements.
HpAU= Huperzine A unit deduced from Standard graph.
[l]Remark on cholinesterase inhibition.

Table 2: Antimicrobial results.

	Zone of inhibition (mm)[a] 5 µg/mL						
Microorganism	Dioclimidazole	F*5	F3	F2	F1	Standards(2.0 µg/mL)[b] gentamycin	MIC(µg/mL) Dioclimidazole
Bacillus subtilis ATCC 6633	20.8	10.4	14.2	10.2	8.6	8.1	0.5
Escherichia coli ATCC 11230	30.1	14.0	15.8	11.2	9.8	30.4	0.1

[a] Values including diameter of the filter paper disc (6.0 mm), are means of three replicates.
[b] Gentamycin.

Table 3: ^{1}H NMR and ^{13}C NMR Assignment of Compound 1 in DMSO

Position	δH (mult.)	J (Hz)	δC	HMBC (δH/C)
1	4.3, bs		-	5,4
2	-		160, s	
3	-		-	
4a				
b	3.61, m			
3.50, m		49.5, t	5, 2	
5a				
b	3.51, m			
3.46, m		51.2, t	4	
6	-		136.6, s	
7	7.69, dd	7.5, 1.5	128.6, d	6, 8, 11
8	7.52, ddd	7.5, 7.2, 1.4	131.1, d	11, 9
9	7.20, ddd	7.2, 1.5, 8.5`	130.7, d	11, 10, 7
10	7.80, dd	8.5, 1.4	127.8, d	11, 12
11	-		140.2, s	
12	-		190.3, s	
13	2.4, s		25.1, q	12,11, 10

It also gave positive iodoform test suggesting a CH_3CHO- or CH_3CHOH moiety. The infrared and ultraviolet spectra indicated the presence of conjugated system. The fingertip signals on IR spectrum also indicated the presence of benzene system. The IR of dioclimidazole also exhibited bands characteristic of conjugated ketones carbonyl (1680 cm^{-1}) and amines (1480 cm^{-1}).

The standard ^{13}C NMR spectrum as well as polarization transfer (DEPT) experiment of dioclimidazole depicted eleven carbon atoms with 11 non-exchangeable protons bonded directly to carbon. The ^{13}C NMR (DEPT) showed resonance for four quaternary carbons, four methines, two methylenes and one methyl group giving an attached proton formula of $C_{11}H_{11}$.

One important piece of evidence for the structural elucidation came from the high resolution exact mass measurement of 1, providing a protonated molecule of m/z 188, indicating a molecular formula of $C_{11}H_{12}N_2O$ (Cal. 188.3, found 188.1). This molecular formula possesses a double bond equivalent of seven, which was consistent with the exact mass measurements.

The ^{1}H-^{13}C 2D NMR shift correlated measurement (HSQC) of dioclimidazole showed the presence of one proton (δ 4.3, H_{-1}) which is not directly attached to any carbon. This fact suggested the presence and attachment of heteroatom like nitrogen. This fact further supported the Lassaigne's test earlier conducted.

The ^{13}C NMR spectrum contained one signal in the aromatic region due to imidazole moiety. The ^{1}H NMR spectrum contained four aromatic proton signals at δ 7.80 (1H, dd, J=8.5, 1.4 Hz, H_{-10}); δ 7.69 (1H, dd, J=7.5, 1.5 Hz, H_{-7}); δ 7.52 (1H, ddd, J= 7.5, 7.2, 1.4 Hz, H_{-8}); and δ 7.20 (1H, ddd, J=8.5, 7.2, 1.5 Hz, H_{-9}) that were directly coupled to ^{13}C signals at δ127.8, 128.6, 131.1 and 130.7 respectively. A ^{1}H-^{13}C 2D NMR shift correlated measurement (HMBC) showed long range coupling between H_{-13} and C_{-12}/ $C_{-11,}$ C_{-6} and C_{-10} which indicated the joining and orientation of CH_3CO- moiety. Dioclimidazole as interesting as it appeared, showed a significant activity both on anticholinesterase [8.0 HpAU at 20 µg/mL]; and has better zones of inhibition in antibacterial assay when compared with 2.0 µg/mL gentamycin . The MICs of Compound 1 on *Escherichia coli* and *Bacillus subtilis* are 0.1 and 0.5 µg/mL respectively using agar dilution method. (Table 1 and 2).

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