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Original Research Article

Inhibition of D – Amino Acid Oxidase by Chromatographic Fractions and Kaempferol-3-*O*-rutinoside Isolated from *Philenoptera cyanescens* Leaves

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ABSTRACT

Philenoptera cyanescens (PC) leaves are used ethno-medically for the treatment of mental disorder in Southwestern Nigeria. The crude extract and solvent fractions of the PC leaves have earlier been reported for *in vivo* antipsychotic properties in rodents. The D-amino acid oxidase (DAO) assay is an *in vitro* anti-psychotic model. However, there is little or no report on the inhibition of DAO by medicinal plants. This study investigates the *in vitro* antipsychotic activity of a bioactive compound, DCM and EtOAc chromatographic sub-fractions from *Philenoptera cyanescens* leaves. The inhibitory effect of the isolated compound, sub-fractions from DCM (a-j) and EtOAc (A-K), Risperidone and Haloperidol standards were tested on Pig Kidney D-amino acid oxidase enzyme (pkDAO), using D–Kynurenine as substrate. Percentage inhibition was measured in a fluorescence microplate reader with an excitation and emission wavelengths of 355 and 460 nm, respectively. Structure of isolated compound was elucidated using NMR and FT-IR analyses. PCDe (61.9%) at 0.2 mg/mL showed a higher percentage inhibition than Risperidone (43.2%) and Haloperidol (18.3%) among the PC DCM sub-fractions. Interestingly, PCEF gave 100% inhibition of the enzyme, when compared with other sub-fractions and standards. The ethyl acetate sub-fraction F showed best inhibitory activity, Kaempferol-3-*O*-rutinoside showed percentage inhibition of 13.7% comparable to the standard drug, Haloperidol. This compound has been reported earlier but isolated from this plant and tested on the antipsychotic property using D-amino acid oxidase for the first time.

***Keywords*:** D-amino acid oxidase, Inhibitory activity, *Philenoptera cyanescens*,Kaempferol-3-*O*-rutinoside, Antipsychotic agents

Introduction

Philenoptera cyanescens (Schum. & Thonn.) Roberty also called *Lonchocarpus cyanescens* (Schum. & Thonn.) Benth., is a member of the Leguminosae family.[1] It is a woody liane or climbing shrub, forming 2 – 3 m high in cultivation, it is commonly found in West Tropical Africa. The leaves of this plant are alternately arranged, young leaves turn bluish-black on drying. The flowers are bluish-black, fruits are flat with about 1 to 5 seed enclosed, pods are oblong and pointed at both ends.[1,2] *Philenoptera cyanescens* is locally called 'elu' in Yoruba land, Nigeria. In Senegal, the leaves are used as condiment eaten with couscous.[3] It is known for its various medicinal values, and has been reported for various pharmacological importance, among which are anti-diarrhea, anti-arthritis, anti-ulcer, anti-malaria, anti-diabetic and expectorant effective.[3-6] The root of this plant has been reported in treating skin rashes.[7] The seed oil of the plant is highly rich in potassium, iron and sodium.[8] It has been used traditionally in the treatment of psychotic disorder. The leaves, are combined with other ingredients and cooked as concoction and eaten by patients with psychosis in Nigerian ethnomedicine.[9]

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Oils from the leaf and stem of *Philenoptera cyanescens* have been reported to contain phytol, hexadecanoic acid, octadecenoic acid, alcohol among others. Phlobatannins, saponin, terpenoid, cardiac glycoside, steroids, tannins and flavonoids are some classes of secondary metabolites revealed in the preliminary phytochemical screening of this plant. Triterpenoids have been isolated from the ethyl acetate extract of the leaves of the plant[3]. Some phytophenolic constituents have also been isolated from other species of the genus of *Lonchocarpus* among which are 4′-prenyloxyvigvexin A (isoflavone) and dimethoxybitucarpin B (pterocarpan) from *Lonchocarpus bussei* leaves[10], lupeol, friedelin, stigmasterol, lupenone, chrysin, apigenin, 3,5,7,2',4'-pentahydroxyflavonol and stigmasterol 3-*O*-β-glucoside from *Lonchocarpus eriocalyx*[11], 2′,4′-dihydroxy-5′-prenylchalcone, isocordoin (chalcones), 8-prenylpinocembrin (flavanone) and 4-hydroxy-N-methylproline (alkaloid) from *Lonchocarpus cultratus*.[12]
The D-amino acid oxidase (DAO) (EC. 1.4.3.3) is an enzyme found in the glial cells of the brain. It is a Flavin Adenine Dinucleotide (FAD) containing enzyme, and is known to reduce the function of the N-Methyl D-Aspartate Receptor (NMDAR) by decomposing the D - serine in the glial cells.[13,14] Endogenous amino acid D - Serine is a co-agonist of the NMDAR,[15] its major function is to regulate the glutamatergic neurotransmission through this receptor. In recent times, the glutamate hypothesis has been linked to mental illnesses such as schizophrenia. This hypothesis suggests that mental illness (schizophrenia) may be related to the hypo functioning of the NMDAR. Madeira *et al.*[16] demonstrated that the activities of the D-amino acid oxidase, which decomposes D-serine are increased in the brain of post-mortem patients with schizophrenia. Hashimoto *et al.*[17] revealed that the concentration of serum D – serine level was highly reduced in schizophrenic patient. Therefore, drugs that will inhibit the enzyme D-amino acid oxidase could be an alternative in the treatment of mental illness. Song *et al*.[18] developed an *in vitro* fluorometric assay for

inhibitors of DAO using D – Kynurenine as the D – amino acid substrate. The metabolism of D- Kynurenine can be *in vivo* or *in vitro* and it is metabolised by DAO in the brain to produce Kynurenic acid, which can emit fluorescence.

Scientific validation of the *in vivo* antipsychotic property of the crude and partitioned fractions of *Philenoptera cyanescens* against Apomorphine, Amphetamine as well as Ketamine has been reported.[19-21]Since the ethyl acetate (EtOAc) and dichloromethane (DCM) fractions of this plant were active *in vivo*, we continued to further isolate the active constituents from this plant. The present study aims on: (i) isolating constituents from the dichloromethane and ethyl acetate fractions of *Philenoptera cyanescens*, (ii) *in vitro* testing of eluted chromatographic pooled sub-fractions for the inhibition of D –amino acid oxidase using microplate fluorescence modified assay, as step towards bioassay guided discovery of antipsychotic drug(s).

Materials and Methods

General experimental procedure

Nuclear magnetic resonance spectral data (^{1}D and ^{2}D-NMR) were recorded on a Bruker Avance Neo 400 MHz and a Bruker Avance Neo 600 MHz spectrometer. Perkin Elmer FT-IR system spectrum BX spectrometer was used for the Infrared (IR) spectra, DAO enzymatic study was performed on a 96-well FLUOstar® Omega Microplate Reader, (BMG LABTECH GmbH, Ortenberg, Germany).Glass column (1000 x 30 mm), Scharlau Silica gel 60 (70 – 230 mesh size; Spain), were used for the Column Chromatography, TLC pre-coated plates (Merck, Germany), for pooling, and UV lamp was used to detect spots on TLC at 366 nm and 254 nm.

Chemicals and reagents

All solvents used were of analytical grade including; analytical *n*-hexane, dichloromethane, ethyl acetate and methanol. Enzyme pkDAO (Pig Kidney D-amino acid oxidase), D-Kynurenine substrate, co-factor FAD (Flavine Adenine Dinucleotide), Dimethyl sulfoxide (DMSO), Tris hydrochloride salt and Bouvine Serum Albumin (BSA) were purchased from Sigma®. Distilled water was further purified using membrane filter apparatus. Pooled sub-fractions were obtained from eluted DCM and EtOAc fractions of *Philenoptera cyanescens* leaves.

Plant collection

Fresh leaves of *Philenoptera cyanescens* were collected from Kajola, Ibadan, Oyo State, Nigeria between February and March, 2016. The plant was identified and authenticated by Dr. O. A. Osiyemi at the Forest Herbarium Ibadan (FHI), where voucher specimen was kept with voucher number FHI 109689.

Extraction and solvent-solvent partitioning

The method of Sonibare *et al*.[21] was used for the extraction process. Briefly, shade-dried whole plant (3 kg) was pulverised and exhaustively macerated using 20 L of methanol for 72 h. The methanol extract was filtered under pressure using a Buchner funnel connected to a vacuum pump and concentrated *in vacuo* at 40°C. The residue was re-extracted twice for optimum yield of the extract. The crude extract was dissolved in methanol: water (1:1) and partitioned into *n*-hexane, dichloromethane, ethyl acetate, butanol and aqueous fractions. The crude extract and partitioned fractions of *Philenoptera cyanescens* were stored in the refrigerator until ready for use. The dichloromethane and ethyl acetate fractions were further purified using column chromatography.

Fractionation and isolation

Column chromatography (CC) of DCM fraction (40 g) of *Philenoptera cyanescens* was performed on Silica gel 60 (mesh size 70-230, Merck), using gradient elution method, in increasing order of polarity (*n*-hexane; *n*-hexane: DCM; DCM: Ethyl acetate; Ethyl acetate: Methanol). The eluted sub-fractions were pooled together using analytical thin layer chromatography (TLC) on a Silica gel 60 F_{254} pre-coated plate, (20 × 20 cm). Observed spots were visualised under Ultraviolet (UV) light (254 and 365 nm), and similar spots were pooled together to obtain sub-fractions a – j.

Column chromatography (CC) of EtOAc fraction (45 g) of *Philenoptera cyanescens* methanol crude extract was performed on Silica gel 60 (mesh size 70-230, Merck), using gradient elution method, in increasing order of polarity (*n*-hexane; *n*-hexane: DCM; DCM: Ethyl acetate; Ethyl acetate: Methanol). The eluted sub-fractions were pooled together using analytical thin layer chromatography (TLC) on silica gel 60 F_{254} pre-coated plates, (20 × 20 cm). Observed spots were visualised under ultraviolet (UV) light (254 and 365 nm), and similar spots were pooled together to obtain sub-fractions A- K. Sub-fraction J yielded crystals, and was subjected to high performance liquid chromatography (HPLC). 1 mg/mL of crystals in methanol was prepared and filtered through 0.22 µm simple pure filter into 1 mL HPLC bottle. 20 µL of prepared sample was then injected into the HPLC injector and analysed using 1% acetic acid in water and 1 % acetic acid in acetonitrile mobile phase with flow rate of 0.6 mL at 270 nm wavelenght. The HPLC profile (Appendix 1) showed that it was not pure having a major fingerprint with other small peaks, and were further purified. About 3 mg of the crystals were pre-adsorbed in 12 mg of Silica gel and purified further with Silica gel of mesh size 230 – 400, column size 500 mm x 10 mm with solvent system, ethyl acetate: methanol: acetic acid (60:20:1), using isocratic elution method to obtain 41 sub-fractions, which were then pooled to 1 – 10. Sub-fractions 4 – 7 were pooled together again and subjected to preparative thin layer chromatography (PTLC). The pooled sub-fractions were dissolved in methanol, streaked on aluminium TLC plate and developed using the solvent system ethyl acetate: methanol: water (3: 2: 0.5) to obtain compound 1.

*Identification of compound **1** using spectroscopic techniques*

The TLC of compound **1** (Appendix 2), isolated from the ethyl acetate fraction of PC was structurally identified using Fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy. Specifically, Bruker Ascend 400 MHz spectrometer (Bruker Instruments Incorporation, Billerica, MA USA) was used for the ^{1}H and ^{13}C-NMR. Compound **1** was dissolved in deuterated methanol. The chemical shifts (δ) were expressed in parts per million (ppm), while tetramethylsilane (TMS) was used as internal standard. Both 1D-NMR (^{1}H-NMR, ^{13}C-NMR, Distortionless Enhancement by Polarisation Transfer (DEPT)), and 2D-NMR (COSY, HMBC, HSQC, & NOESY) were used for the identification of the compound.

D – Amino acid oxidase enzymatic assay

The method of Iwasa *et al.*[22] was used with little modifications in the D – amino acid enzymatic assay. 5 µL of 0.1 mg/mL of DAO in Tris buffer (pH 8.31) was carefully pipetted in a 96-well plate using a micropipette (2 – 20 µL). Thereafter, 6 µL of 200 µM FAD, 5 µL of 2.0 mg/ mL BSA in H_2O, 27 µL of 0.4 M Tris buffer (pH 8.31) and 5 µL of either Haloperidol, Risperidone (standards), pooled sub-fractions (a – j; A – K) or isolated compound was dissolved in DMSO and added, the reaction was mixed and incubated at 37°C for 20 minutes. After incubation, 5 µL of 7.0 mM D – Kynurenine substrate was added and incubated for another 60 min at 37°C. The reaction was mixed and the fluorescence of the solution was measured by a FLUOstar® Omega Microplate Reader, BMG LABTECH GmbH, Ortenberg, Germany, at an excitation and emission wavelength of 355 nm and 460 nm, respectively. The degree at which the sub-fractions inhibited DAO was expressed as a percentage according to the equation:

$$\% \text{ inhibition} = 100 - (\Delta I' / I) \times 100$$

Where; ΔI′ is the change in fluorescence intensity before and after adding D-Kynurenine, and I is the fluorescence intensity of blank sample (sample without substrate).

Statistical analysis

Data on the bioactivity assay was on the average of three replicate per sample analyzed, and data were calculated based on the formula;

$$\% \text{ inhibition} = 100 - (\Delta I' / I) \times 100$$

Results and Discussion

Column Chromatography of DCM and EtOAc fractions
Total number of 220 eluates (100 mL) were collected from the column chromatography of PC DCM fraction, while 230 eluates (100 mL) were collected from the column chromatography of PC EtOAc fraction (45 g), using solvent system of increasing polarity. The eluates were monitored with TLC and similar spots pooled into 10 and 11 sub-fractions for DCM and EtOAc fractions respectively. The detailed solvent system and yield of the pooled DCM and EtOAc sub-fractions were shown in Table 1 and 2, respectively.
Compound **1** was obtained as a yellow powder (2 mg). The identification was done using NMR and its FT-IR spectra. The IR revealed conjugated carbonyl at 1652 cm^{-1}, which is peculiar to flavonoid ring, an hydroxyl group at 3419 cm^{-1}, indicating the presence of an –OH group, aromatic C = C bonds at 1457 cm^{-1}, 1502 cm^{-1}, and 1598 cm^{-1}, as well as a CH_3 at 1365 cm^{-1} (Table 3).

*Identification of compound **1***
The ^{1}H- and ^{13}C-NMR spectra resonances clarified the aromatic and glycosidic nature of compound **1**. The ^{1}H- (MeOD - d_4) and ^{13}C-NMR (Table 4) spectra of the compound, which were assigned by various NMR experiments, showed signals ascribable to a Kaempferyl moiety {one methyl [δ 1.05 (3H, s, CH_3-6’’’)], six aromatic protons [δ 6.19 (1H, s, H-6, 6.86 (d, 1H, J = 6.0 Hz, H-3’ and H-5’ respectively), the spectrum of 1 displayed two ortho-coupled doublet resonances at δH 7.68 and 6.39 (each 2H, d, J = 6.0 Hz) assignable to H-2’/6’ and H-3’/5’ of ring B, respectively, indicating AA’BB’ spin system and indicated a p-substituted ring B. This further suggests the presence of a kaempferol aglycone unit, and a disaccharide glycoside moiety. The proton and carbon signals of the compound in the ^{1}H- and ^{13}C-NMR spectra were similar to those of kaemferol.[23]

The ^{13}C NMR revealed a carbonyl ketone at δ 177.9, confirming the conjugated carbonyl in the FT-IR spectra. This is in agreement with Hilal and Engelhardt[24]. The ^{1}H-NMR spectrum of isolated compound also exhibited a series of sugar signals at δ_H 3.43–4.48, with two anomeric protons at δ_H 5.10 (1H, brs, H-1″) and 4.82 (1H, d, J= 6.0 Hz, H-1‴). The DEPT result showed a CH_2 group at δ_C 68.3 attached to position-6’’ on the sugar moiety. The two anomeric carbons were found to resonate at δ_C 103.4 (C-1’’) and 101.0 (C-1’’’). The carbon signal at δ_C 16.5 assigned to a proton at δ_H 1.05 (CH_3 group at 6’’’), which confirms the rhamnose part of the glycoside[25]. The HSQC with DEPT-135 were used for the proton-carbon assignment. Comparison of ^{1}H-NMR, ^{13}C-NMR, DEPT-90, DEPT-135, HSQC and HMBC, with literature, identified compound **1** to be Kaempferol-3-*O*-rutinoside (Figure 1) with molecular formula $C_{27}H_{30}O_{15}$ and molecular mass of 594 g/mol.[26]

DAO Inhibitory Assay
Modification of assay
During reaction optimization, there was no precipitate observed in the solution of the enzymatic reaction after incubation at 60 min. Hence, Tris buffer and $ZnSO_4$ were not added and inhibition of enzyme was recorded 60 min after incubation and addition of substrate. Also, the volume of the reactant was reduced due to the size of the wells of the microplate available. The substrate and enzyme concentrations as well as reaction time were also optimized. It was observed that the fluorescence intensity increased with increase in substrate concentration except at 5.6 mM (Figure 2). Thus, substrate concentration of 7.0 mM was used during the study. Also, in the different volume of enzyme optimized (Figure 3), 5 uL of enzyme was used in the reaction, since fluorescence intensity was observed to be increased at this volume. The fluorescence intensity of the reaction time likewise increased with increase in time (Figure 4). Hence, incubation time was extended to 60 min after adding the substrate.

*Inhibition of DAO by Philenoptera cyanescens Dichloromethane, Ethyl acetate pooled sub-fractions and compound **1***
Song *et al*.[18] have established the production of Kynurenic acid from D – Kynurenine *in vitro*.They confirmed the use of D – Kynurenine as a substrate for D – amino acid oxidase (DAO) and also, verified that the assay can be used to test inhibitors of D – amino acid oxidase. Reports have also confirmed the fluorescence assay in the use of D – Kynurenine as a substrate in evaluating potential DAO inhibitors. [22, 27]

Table 1: Solvent system and Yield of *Philenoptera cyanescens* Dichloromethane Pooled sub-fractions

Pooled sub-fractions	Solvent system/ratio	Yield (mg)
A	n -Hex: DCM (1.5: 3.5)	54.5
B	n -Hex: DCM (1.0: 4.0)	80.8
C	n -Hex: DCM (1.5: 3.5)	1467.8
D	EtOAc (5.0)	112.7
E	EtOAc: MeOH (1.0: 1.5)	161.1
F	DCM : EtOAc (4.5:0.5)	1185.1
G	n -Hex : EtOAc : MeOH (0.5:1.5:1.0)	6632.0
H	DCM : EtOAc (3.0:2.0)	21501.7
I	DCM : EtOAc (3.0:2.0)	4527.1
J	DCM : EtOAc (3.0:2.0)	20835.5

Key: n-Hex – n-hexane; DCM – Dichloromethane; EtOAc – Ethyl acetate; MeOH – Methanol

Table 2: Solvent system and Yield of *Philenoptera cyanescens* Ethyl acetate Pooled sub-fractions

Pooled sub-fractions	Solvent system/ratio	Yield (mg)
A	n -Hex : DCM/0.5 : 4.5	11.3
B	n -Hex : DCM/0.5 : 4.5	2.8
C	n -Hex : DCM/0.5 : 4.5	4.1
D	n -Hex : DCM/0.5 : 4.5	3.7
E	n-Hex : EtOAc/1.5 : 3.5	93.3
F	n-Hex : EtOAc/1.5 : 3.5	861.9
G	n-Hex : EtOAc/1.5 : 3.5	216.1
H	EtOAc : BuOH/3.5 : 1.5	2541.2
I	EtOAc : BuOH/3.5 : 1.5	3521.9
J	EtOAc : BuOH:H_2O/ 1.0 : 2.5 : 1.5	18050.3
K	EtOAc : BuOH:H_2O/ 1.0 : 2.5 : 1.5	155.1

Key: n-Hex – n-hexane; DCM – Dichloromethane; EtOAc – Ethyl acetate; MeOH – Methanol; BuOH – Butanol; H_2O – Water

Table 3: FT-IR Data of Isolated compound

S/N	Wave number (cm^{-1})	Characteristics	Interpretation
1	3419.61	Very intense and broad	Free -OH of an alcohol
2	2925.39	Medium intense and broad	-$CH_{stretch}$
3	1138.47, 1063.56, 1009.96	Sharp and medium intense peaks	-C-$O_{bending}$

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Table 4: ^{1}H-NMR (CD_3OD-*d*, 400 MHz) and ^{13}C-NMR (CD_3OD-*d*, 150 MHz) of compound 1

Carbon	^{1}H NMR (ppm)	^{13}C NMR (ppm), carbon type (Experimental)	^{13}C NMR (ppm), carbon type (Reported)**
2	-	165.1, Qc	165.1, Qc
3	-	134.2, Qc	134.5, Qc
4	-	177.9, Qc	178.8, Qc
5	-	161.5, Qc	161.7, Qc
6	6.19 (s, 1 H)	98.8, CH	99.9, CH
7	-	165.1, Qc	165.8, Qc
8	6.39 (s, 1 H)	93.6, CH	94.9, CH
9	-	157.1, Qc	158.3, Qc
10	-	104.1, Qc	104.8, Qc
1’	-	122.1, Qc	122.6, Qc
2’	7.68 (d, 1H, *J* = 6.0 Hz)	134.2, CH	132.4, CH
3’	6.86 (d, 1H, *J* = 6.0 Hz)	116.3, CH	116.1, CH
4’	-	161.5, Qc	160.4, Qc
5’	6.86 (d, 1H, *J* = 6.0 Hz)	116.3, CH	116.1, CH
6’	7.68 (d, 1H, *J* = 6.0 Hz)	134.2, CH	132.4, CH
1’’	5.10 (brs, 1H)	103.4, CH	104.6, CH
2’’	3.43 - 3.48, m	72.8, CH	72.8, CH
3’’	3.43 - 3.48, m	78.2, CH	75.8, CH
4”	3.43 - 3.48, m	70.8, CH	71.4, CH
5”	3.43 - 3.48, m	76.8, CH	77.2, CH
6’’	3.43 - 3.48, m	68.3, CH_2	68.6, CH_2
1’’’	4.82 (d, *J* = 6.0 Hz)	101.0, CH	102.2, CH
2’’’	3.20-3.86, m	72.6, CH	72.1, CH
3’’’	3.20-3.86, m	72.5, CH	72.3, CH
4’’’	3.20-3.86, m	74.3, CH	73.9, CH
5’’’	3.20-3.86, m	69.9, CH	69.8, CH
6’’’	1.05, s, 3H	16.5, CH_3	17.9, CH_3

Reported literature**(Dehsghani *et al*., 2017),Qc = quaternary carbon, m = multiplet, s = singlet, dd = double doublet, d = doublet, *J* = coupling constant (Hz), brs = broad singlet.

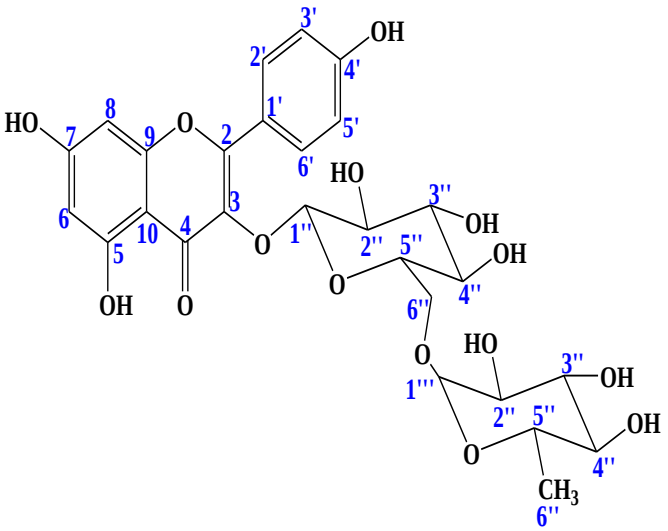

Figure 1: Kaempferol-3-*O*-rutinoside

It has been reported that first and second generation antipsychotics inhibited D-amino acid oxidase *in vitro*.[22,28] Chlorpromazine, Quetiapine, Sulpiride, Carbamazepine and Imipramine, are various antipsychotics used in the treatment of schizophrenia, epilepsy and depression. They inhibited DAO with a minimum inhibitory concentration (IC_{50}) of 65.8 ± 13.2 µM, 19.5 ± 2.6 µM, 85.4 ± 2.18 µM, 262.0 ± 53.4 µM, and 31.1 ± 7.03 µM, respectively.[22] In addition, Haloperidol, Risperidone, Olanzapine, Aripiprazole and Bonanserine inhibited human DAO *in vitro* with IC_{50} 13.4 ± 1.87 µM, 4.70 ± 0.17 µM, > 50 µM, 12.4 ± 1.38 µM, and 5.29 ± 0.47 µM, respectively.[28] Pooled sub-fractions from *Philenoptera cyanescence* dichloromethane and Ethyl acetate fractions, (PCD and PCE) at 0.2 mg/mL as well as the standards, (Haloperidol and Risperidone) at 0.1 mg/mL, inhibited Pig kidney D-amino acid oxidase enzyme *in vitro*. From the result of the *Philenoptera cyanescence* dichloromethane pooled sub-fractions (Figure 5), *Philenoptera cyanescence* dichloromethane sub-fraction e (PCDe), (61.9%) exhibited the highest percentage inhibition, PCDf (48.9%) and PCDg (49.6%) showed similar percentage inhibition comparable to the standard Risperidone (43.2%). Percentage inhibition obtained from PCDi (27.4%) and PCDj (17.6%) are comparable to that of Haloperidol (18.3%).The values obtained for the standards, Risperidone and Haloperidol are in comparism to previous study. Risperidone had a minimum inhibitory concentration of 4.70 ± 0.17 µM, and possesses strong human D – amino acid oxidase inhibitory effects,

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than Haloperidol which has minimum inhibitory concentration of 13.4 ± 1.87 µM[24]. In this study, we also observed that Risperidone had a higher percentage inhibitory effect (43.2%) than Haloperidol (18.3%). Interestingly, PCEF gave 100% inhibition of the enzyme among the ethyl acetate pooled sub-fractions, when compared with other pooled sub-fractions and standards. PCEG (53.4%), PCEH (83.1%) and PCEI (58.3%) showed higher percentage inhibition than the standards. However, Risperidone (43.2%) a second generation antipsychotic exhibited a close percentage inhibition to PCEG. Percentage inhibitions of PCEJ (24.5%) and PCEK (27.5%) are comparable to the first generation antipsychotic, Haloperidol (18.3%) (Figure 6). In addition, Kaempferol-3-O-rutinoside gave 13.7% inhibition of the enzyme (Figure 7), and this is comparable to Haloperidol. Generally, chromone derivatives have been shown to possess high inhibitory activity towards acetylcholinesterase enzymes and Dopamine agonist.[29-31]Acetylcholinesterase is responsible for the metabolism of acetylcholine. Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease are characterized by loss of basal forebrain cholinergic cells, and loss of dopaminergic neurons respectively, consequently leading to imbalance of acetylcholine and dopamine neurotransmitters. The presence of this compound may contribute to the antipsychotic property of *Philenoptera cyanescens* in Southwest Nigerian ethno-medicine.

From the above enzymatic assay results, D – Kynurenine substrate was used to investigate the pkDAO inhibitory effects of both first generation antipsychotic (Haloperidol) and second generation antipsychotic (Risperidone). Furthermore, dichloromethane and ethyl acetate chromatography pooled sub-fractions of *Philenoptera cyanescens* were investigated. The results showed that Risperidone possesses more inhibitory effect than Haloperidol. In support of this findings, second generation antipsychotics were found to possess strong inhibitory effect to human D-amino acid oxidase (hDAO) than first generation antipsychotics.[28]

Recently, more attention have been drawn to the use of D-amino acid oxidase enzyme as target drug for schizophrenia and the development of newer drugs that will inhibit DAO.[32,34] Antipsychotic drugs have been screened for their inhibitory effect on both hDAO and pkDAO.[22, 28]The current study has added to extant studies by reporting the bioassay guided activity of *Philenoptera cyanescens* leaves, and the results support the antipsychotic properties of this plant *in vivo.*

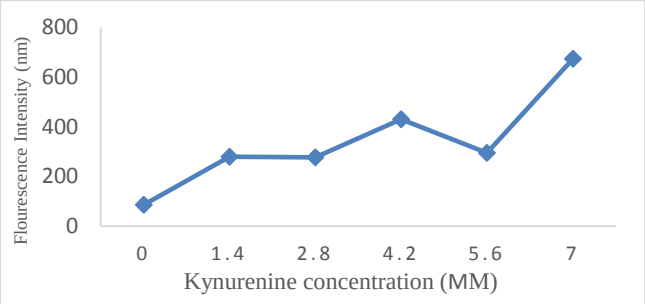

Figure 2: Effect of D-Kynurenine (substrate) concentration (mM) on the fluorescence intensity of the reaction process

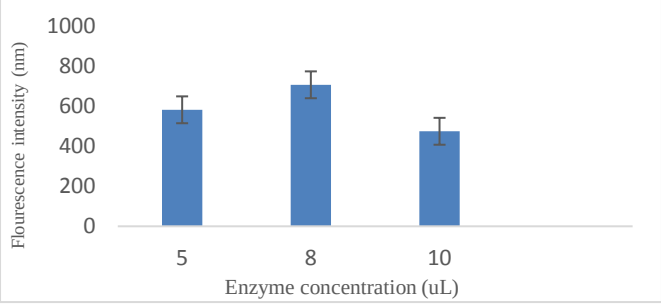

Figure 3: Effect of enzyme concentration on the fluorescence intensity of the reaction process

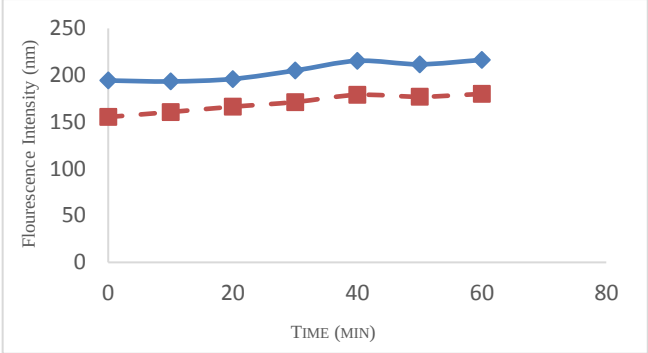

Figure 4: Changes in the fluorescence intensity at 355 nm excitation wavelength and emission wavelength of 460 nm as a function of time. A closed square with a dotted line and a closed trapezium with a solid line represent the fluorescence intensities of a sample with D- kynurenine in the presence or absence of inhibitor, respectively.

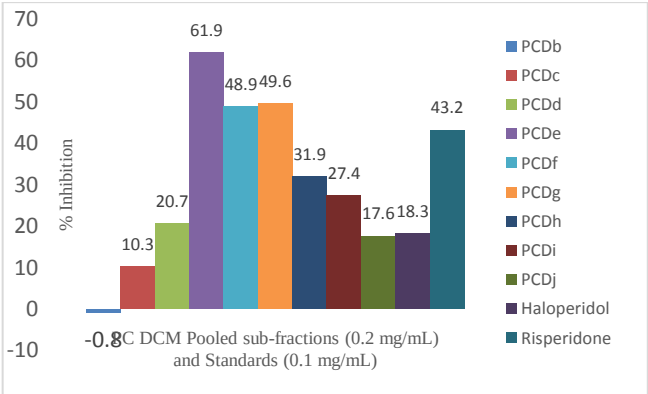

Figure 5: *In vitro* D-amino acid oxidase enzymatic assay results of pooled sub-fractions from *Philenoptera cyanescens* Dichloromethane fraction and standards. PCDb- *Philenoptera cyanescens* Dichloromethane sub-fraction b; PCDc- *Philenoptera cyanescens* Dichloromethane sub-fraction c; PCDd- *Philenoptera cyanescens* Dichloromethane sub-fraction d; PCDe - *Philenoptera cyanescens* Dichloromethane sub-fraction e; PCDf- *Philenoptera cyanescens* Dichloromethane sub-fraction f; PCDg- *Philenoptera cyanescens* Dichloromethane sub-fraction g; PCDh- *Philenoptera cyanescens* Dichloromethane sub-fraction h; PCDi- *Philenoptera cyanescens* Dichloromethane sub-fraction i; PCDj- *Philenoptera cyanescens* Dichloromethane sub-fraction j

Conclusion

It was found that *Philenoptera cyanescence* dichloromethane sub-fraction e, *Philenoptera cyanescenc* ethyl acetate sub-fraction F and H (PCDe and PCEF, PCEH) may contain the active principle(s) responsible for the bioactivity of *Philenoptera cyanescens* ethyl acetate and dichloromethane fractions. Kaempferol-3-*O*-rutinoside (identified from *Philenoptera cyanescens* ethyl acetate sub-fraction J) was shown to inhibit D-amino acid oxidase, which is responsible for the hypo functioning of the NMDA receptor in the brain by decomposing D – Kynurenine. The mechanism of action of this compound may be related to D-amino acid oxidase inhibitory activity. The effect of the identified compound and the other constituents of the chromatographic sub-fractions could contribute to the ethno-medicinal use of *Philenoptera cyanescens* leaves in treating mental illness among Southwest people of Nigeria.

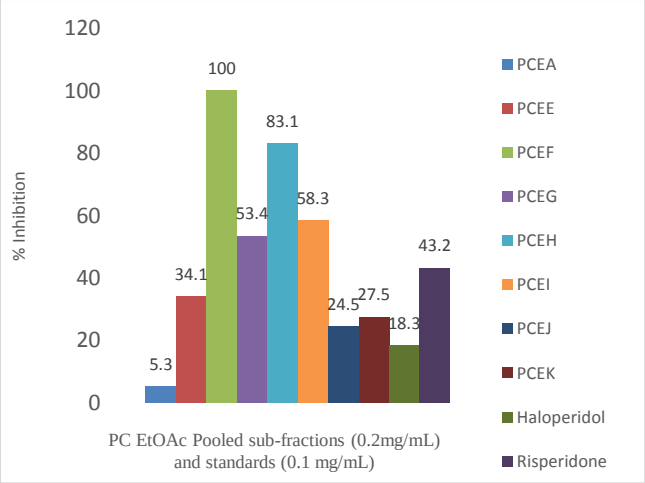

Figure 6: *In vitro* D-amino acid oxidase enzymatic assay results of pooled sub-fractions from *Philenoptera cyanescens*Ethyl acetate fractionand standards.PCEA- *Philenoptera cyanescens* Ethyl acetate sub-fraction A; PCEE- *Philenoptera cyanescens* Ethyl acetate sub-fraction E; PCEF- *Philenoptera cyanescens* Ethyl acetate sub-fraction F; PCEG - *Philenoptera cyanescens* Ethyl acetate sub-fraction G; PCEH- *Philenoptera cyanescens* Ethyl acetate sub-fraction H; PCEI- *Philenoptera cyanescens* Ethyl acetate sub-fraction I; PCEJ- *Philenoptera cyanescens* Ethyl acetate sub-fraction J; PCEK- *Philenoptera cyanescens* Ethyl acetate sub-fraction K

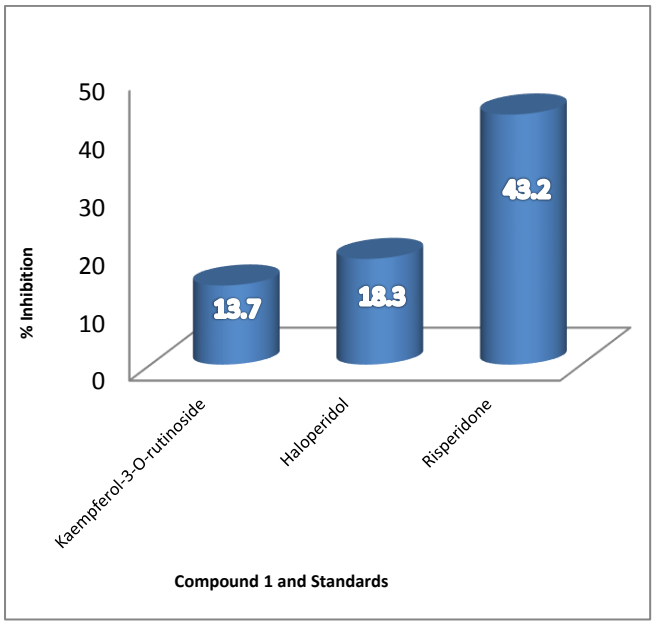

Figure 7: *In vitro* D-amino acid oxidase enzymatic assay result of Kaempferol-3-*O*-rutinoside (0.2 mg/mL) isolated from *Philenoptera cyanescens* ethyl acetate sub-fraction J obtained from ethyl acetate fraction of *Philenoptera cyanescens* leaves and standards (0.1 mg/mL).

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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