RESEARCH ARTICLE

Characterization, Antioxidant and Anticholinesterase Activity of Compounds Isolated from Alkaloid Extracts of *Citrus aurantifolia* Root Bark

Djélé Alette Edwige Zialé[1,2,*], Demel Axel Adou[1,3], Kohué Christelle Chantal N'gaman-Kouassi[1], Julia Deschamp[2], Nadia Bouchemal[2], Tony Lionel Palama[2], Marc Lecouvey[2], Janat Akhanovna Mamyrbekova-Békro[1], Yves-Alain Békro[1]

[1]Laboratory of Bio-Organic Chemistry and Natural Substances, Nangui Abrogoua University, 02 BP 801 Abidjan 02, Ivory Coast.
[2]Sorbonne Paris Nord University, CSPBAT Laboratory, CNRS UMR 7244, F-93017 Bobigny Cedex, France.
[3]Department of Science and Technology, Alassane Ouattara University, 01 BP 18 Bouaké 01, Ivory Coast.

Abstract: Xanthyletin (1), edulinin (2), 1-methyl-2-[(E)-(prop-1-enyl)]quinolin-4-one (3), 1,2-dimethylquinolin-4-one (4), lonchocarpol A (5), grandisinin (6), citracridone-I (7) and 5-hydroxynoracronycin (8) were isolated from alkaloid extracts of the root bark of Citrus aurantifolia. Their structures were characterized using spectral data (NMR, HRMS, and IR). Furthermore, compounds 2, 3, 4, and 6 known in the literature have been isolated for the first time in Citrus aurantifolia. However, compounds 1, 5, 7 and 8 are already known from the plant studied. The concentration required to reduce 50% of free radicals (RC_{50}) was carried out. Compounds 3 (RC_{50}= 185.836 µmol/L) and 4 (R_{C50}= 218.277 µmol/L) compared with vitamin C (RC_{50}= 17.033 µmol/L) showed antioxidant efficacy. The highest anticholinesterase activity was observed for compound 6 at 21.129 µM followed by compounds 3 and 4 at 251.130, 287.208 µM, respectively.

Keywords: *Citrus aurantifolia*, Alkaloids, Isolation, Antioxidant, Anticholinesterase.

Submitted: December 4, 2023. **Accepted:** May 19, 2024.

Cite this: Zialé DAE, Adou DA, N'gaman-Kouassi KCC, Deschamp J, Bouchemal N, Palama TL, Lecouvey M, Mamyrbekova-Békro JA, Békro YA. Characterization, Antioxidant and Anticholinesterase Activity of Compounds Isolated from Alkaloid Extracts of *Citrus aurantifolia* Root Bark. JOTCSA. 2024;11(3): 1227-36.

DOI: https://doi.org/10.18596/jotcsa.1395690

***Corresponding author's E-mail:** aletteziale22@gmail.com

1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative brain disorder and is considered the most common form of dementia in the elderly. According to a recent study, the number of patients with AD is on the rise, currently estimated at 26.6 million. This number is projected to quadruple by 2050 (1,2). However, it's important to note that brain aging appears to be a significant risk factor for developing AD (3). In fact, the first signs of Alzheimer's disease, which account for 75% of cases, typically manifest as problems with memory and cognitive function (3). Unfortunately, conventional therapies for Alzheimer's disease have shown limited effectiveness, largely due to the imprecise understanding of its underlying causes (2).

Free radicals (FR) and reactive oxygen species (ROS) are from metabolic processes or external sources. They generally come from oxygenated, nitrogenous, and sulfurous substances. The presence of unpaired electrons renders these chemical compounds unstable, and their reactivity plays crucial roles in cell signaling, gene expression, and ion transport (4). However, an excess of free radicals (FR) is highly detrimental to normal biological processes, affecting proteins, lipids, RNA, DNA, and carbohydrates in living matter. This is why the research for new natural antioxidants from plants is receiving particular attention due to their exceptional contribution to the fight against disease. Among these phytocompounds of interest, alkaloids could contribute to managing cellular damage due to oxidative stress and reducing the risk of chronic diseases due to their antioxidant potential (4,5).

Several studies have demonstrated the involvement of free radicals (FR) in Alzheimer's disease (AD). Indeed, AD is characterized by the accumulation of senile plaques, which generate free radicals, leading to damage to nerve cells (6). Neuronal death occurs mainly in basal forebrain areas, which are the main sources of cholinergic innervation, leading to acetylcholine deficiency in patients (7). Some plant extracts are known to improve cognitive faculties by protecting nerve cells. That's why it's important to intensify the research to provide more effective and inexpensive palliative care (8).

Citrus aurantifolia (Christm) Swingle belongs to the Rutaceae family. It can grow up to about 5 meters in height and thrive in warm subtropical or tropical regions. Widely utilized in West Africa, particularly in Côte d'Ivoire, it is esteemed for its nutritional qualities. Various parts of the plant are traditionally used for medicinal purposes: the leaves have antiseptic, antiviral, antifungal, and antimalarial properties; the fruit serves as a diuretic, anti-mosquito agent, and antimalarial remedy; and the root bark is utilized for addressing conditions such as diabetes, atherosclerosis, constipation, arthralgia, and indigestion. (9,10). The health benefits of C. aurantifolia are associated with a high content of biologically active compounds such as flavonoids, coumarins, limonoids, phenols, alkaloids, carotenoids, minerals, and vitamins (10,11).

The present study focuses on the phytochemical, anticholinesterase, and free radical scavenging of constituents isolated from the total alkaloid extracts of C. aurantifolia root barks.

2. MATERIAL AND METHODS

2.1. Biological Material

Acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel) (C3389-2KU), acetylthiocholine iodide (ATCI), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), galantamine hydrobromide ($C_{17}H_{21}NO_3$, HBr; 368.27 g/mol), stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), and ascorbic acid (vitamin C) were purchased from Sigma Aldrich (Poole, UK).

2.2. Plant Material

The root barks from *C. aurantifolia* were collected in Adiopodoumé (5° 20' 28'' North, 4° 7' 54'' West) in the south of Côte d'Ivoire. They were identified and authenticated by Professor Malan Djah François of Nangui Abrogoua University, Ivory Coast. A voucher specimen was deposited in the herbarium of laboratory of Bio-Organic Chemistry and Natural Substances of Nangui Abrogoua University, Côte d'Ivoire. After cleaning and air-drying, the root barks were ground.

2.3. Extracts Preparation

320 g of sample were extracted by maceration three times in methanol (3 x 1700 mL) for 72 hours under continuous stirring. The extracts were combined and evaporated under reduced pressure (218 mbar) at 40 °C. Then, 150 mL of H_2SO_4 (2% v/v, pH = 2) was added. The solution obtained was extracted with diethyl ether (4 x 100 mL), followed by the addition of NaOH (m/v, 25%) to adjust the pH to 9. The mixture was then successively extracted with chloroform, ethyl acetate (5 x 100 mL), and an ethyl acetate/methanol mixture (3/1). The extract was dried with anhydrous Na_2SO_4, and the filtrate was concentrated using a rotary evaporator (Büchi Rotavapor R-300 at 40 °C) to provide the CA1 (chloroform) and CA2 (ethyl acetate) extracts (12,13).

2.4. Fractionation and Isolation

2.4.1. Compounds isolated from chloroform extract

The chloroform extract (CA1, 754.7 mg) was subject to column chromatography (CC), (length 45 cm, diameter 4 cm, height 14 cm), containing 40 g of silica gel 60 GF_{254} (Merck) with a gradient of petroleum ether (PE)/dichloromethane (DCM)/EtOH solvents by varying their proportions (7: 3: 0 to 0: 8: 2). Six fractions (F1 - F6) were collected according to their chromatographic profiles. After solvent removal from fractions F2 (46.7mg) and F4 (157.6 mg), respectively, two compounds **1** (20.7 mg) and **2** (56.7 mg) were obtained. Fraction F5 (68.2 mg) was fractionated on CC with a gradient of DCM/EtOH to give three subfractions (F5.1 - F5.3). Compound **3** (2.2 mg) was isolated from F5.2 on a preparative plate (PP) with DCM/MeOH (90:10). Fraction F6 (197.7 mg) was fractionated on CC with DCM/AcOEt (10 : 0 - 30 :70) to give two subfractions F6.1 and F6.2 (80.2 mg), F6.2 was further purified on CC to give compound **4** (3.4 mg).

2.4.2. Compounds isolated from ethyl acetate extract

The extract (CA2, 729 mg) was fractionated on CC with DCM/AcOEt (100: 0: to 0:100) to give 6 fractions (F1 - F6), F4 (160.6 mg) was purified on CC with a DCM/EtOH (100- 0 to 0-100) to give three subfractions (F4.1 - F4.3). Using HPLC-Prep, subfraction F4.2 (68 mg) yielded 2 fractions F4.2-1 and F4.2-2 (12.8 mg). Two compounds, **5** (2.6 mg) and **6** (3.4 mg) were isolated from F4.2-2 by PP (DCM/EtOH 10:0.5). The crystallization of F5 (250 mg) allowed the isolation of compound **7** (36.7 mg). Finally, fraction F6 (80.2 mg) was fractionated over CC (DCM/AcOEt) to give a single compound **8** (10.7 mg).

2.5. Structural Determination of the Isolated Compounds

The NMR spectra (^1H, ^{13}C, JMOD, DEPT, COSY, HSQC, HMBC, and NOESY) were performed in $CDCl_3$ (compounds **1-7**) and DMSO-d_4 (compound **8**) on a BRUKER AVANCE III spectrometer (400 MHz). HPLC-MS was performed on 1260_InfinityII with a reverse phase C18-AGILENT column at 30 °C. The Bruker maXis mass spectrometer in negative (ESI^-) or positive (ESI^+) mode was used for the high resolution mass spectrometry (HRMS) data. IR spectra were performed on the IR spectrometer (380 FT Nicolet from Thermo Fischer scientific). The

melting points of the compounds were determined with Stuart® SMP30.

2.6. Determination of Antioxidant Potential Against DPPH Radicals

The antioxidant power of compounds **1-8** was carried out using the method described by Blois (14). The extracts were diluted to final concentrations of 0.5; 0.25; 0.125; 0.0625; 0.03125; 0.0156; 0.0078 mg/mL in methanol. To 1 mL of each extract, 1.5 mL of DPPH solution (0.03 mg/mL) was added. The homogeneous mixture was incubated in the dark for 30 minutes. The absorbance (Abs) of the mixture obtained was measured at 517 nm. The blank consisted of 1.5 mL of DPPH solution, to which 1 mL of MeOH was added. A solution of ascorbic acid prepared in the same conditions as the tested samples was used as a reference. The tests were repeated three times for each sample. Equation (I) was used to determine the percentage reduction (PR) of the DPPH radical:

$$PR\ (\%) = [(A_b - A_e) / Ab] \times 100 \qquad (I)$$

A_b: Absorbance of the blank (1.5 mL of DPPH + 1 mL of MeOH)
A_e: Absorbance of the sample
The concentration required to reduce the DPPH radical by 50% (RC_{50}) was determined (8,15).

2.7. Anticholinesterase Activity

The percentage inhibition of acetylcholine (AChE) was determined according to the method described by Ellman et al (1961) (16). 50 µL of sample methanolic solution (0.5; 0.25; 0.125; 0.0625; 0.03125; 0.0156; 0.0078 mg/mL) was diluted to 1/4 with the buffer solution in 96 well plates. For 30 min at 37°C, 10 µL of AChE enzyme (0.22 U/mL in Tris-HCl buffer) was incubated, after which 20 µL of DTNB (3 mM in buffer) and 10 µL of ATCI (15 mM, H_2O millipore) were added. The resulting mixture was incubated at 18°C for 5 min in plates. Prepared under the same conditions, galanthamin and methanol were used as control and blank, respectively. The different absorbances were measured at 405 nm every 90 seconds for 6 minutes. The readings were taken three times. The anticholinesterase activity assays were performed three times for each sample. Equation (II) was used to determine the percentage inhibition (PI) of the enzyme.

$$PI\ (\%) = 100 - [(Ab\ extract / Ab\ control) \times 100] \qquad (II)$$

Ab = Absorbance

3. RESULTS AND DISCUSSION

3.1. Extraction Yields

The extraction yields are 0.812% (CA1) and 0.522% (CA2) with chloroform and ethyl acetate, respectively.

3.2. Structures of the Isolated Compounds

Eight compounds were isolated; compounds **1** to **4** are from chloroform extract, and compounds **5** to **8** are from ethyl acetate extract. The molecular structures of the isolated phytoconstituents were elucidated from spectral data (^{1}H, ^{13}C NMR, HRMS) and by comparison with those in the literature.

3.2.1. Nuclear magnetic resonance and IR data

Xanthyletin (1): white crystals; yield: 2.75%, melting point (m.p). 132 °C. The HRSM spectrum showed a molecular ion at m/z 251.0684 $[M + Na]^+$ giving the molecular weight at m/z 228 [M]. The ^{1}H NMR (400 MHz, $CDCl_3$, *δ*, ppm) showed the presence of doublets at 7.57 (1H, d, *J* = 9.5 Hz, *H*-4) and 6.22 (1H, d, *J* = 9.5 Hz, *H*-3) corresponding to the protons that can be attributed to the double bond conjugated to the carbonyl group. Two doublets at 6.34 (1H d, *J* = 9.9 Hz, *H*-6), 5.68 (1H, d, *J* = 9.9 Hz, *H*-7) and two singlets at 7.04 (1H, s, Ar-*H*) and 6.72 (1H, s, Ar-*H*) are in para position of dimethyl chromene. At 1.46 (6H, s, C*H*$_3$) two methyl groups resonate. The ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm) (Jmod) highlights 14 carbons including 6 quaternary carbons at 161.33 (*C*-2), 155.57 (Ar-*C*H), 143.47 (*C*-4), 118.64 (Ar-*C*H), 112.85 (Ar-*C*) and 77.86 (*C*-8). 6 tertiary carbons at 143.47 (*C*-4), 131.36 (*C*-7), 124.89 (Ar-*C*), 120.91 (*C*-6), 113.18 (*C*-3) and 104.55 (*C*-9) 77.86 (*C*-8), 104.55 (Ar-*C*). The two primary carbons which correspond to the carbons of the CH_3- groups resonate at 28.48 (*C*H_3). IR spectrum at (CH_2Cl_2, cm^{-1}): 1719 cm^{-1} reveals the carbonyl group (C=O). Anal. Calcd for $C_{14}H_{12}O_3$: C, 73.67; H, 5.30; O, 21.03. HRMS (m/z) $C_{14}H_{12}O_3$ (228 g/mol). The data acquired compared to those in the literature allow to determine the structure of the **Compound 1** (Figure 1) (17).

Edulinin (2): white crystals; yield: 7.51%, m.p. 135°C. The HRSM spectrum ^{1}H NMR (400 MHz, $CDCl_3$, *δ*, ppm) in positive mode indicates a peak corresponding to m/z 314.1364 $[M + Na]^+$ which allow to determine the molecular weight at 291 [M]. The ^{1}H NMR (400 MHz, $CDCl_3$, *δ*, ppm) spectrum of phytocompound showed different signals at 7.85 (1H, d, *J* = 8.0 Hz, Ar-*H*), 7.60 (1H, dd, *J* = 8.6 and 7.1 Hz, Ar-*H*), 7.43 (1H, d, *J* = 8.5 Hz, Ar-*H*), 7.31 (1H, t, *J* = 7.6 Hz, Ar-*H*) indicating the protons of the aromatic ring. At 5.10 (1H, d, *J* = 3.4 Hz, O*H*) and 2.65 (1H, s, O*H*) spectrum showed OH groups. O-methyl and N-methyl resonate at 3.97 (3H, s, OC*H*$_3$) and 3.75 (3H, s, *NCH*$_3$). In addition, methylene and methyl protons resonated between 3.12-1.31 (1H, d, *J* = 13.7 Hz, *H*-a). The COSY spectrum showed a coupling between the protons *H*-5 (7.85 ppm) and *H*-6 (7.31 ppm); *H*-7 (7.60 ppm) pairs with *H*-8 (7.43 ppm) and *H*-6 (7.31 ppm). The protons *H* (OH1) at 5.10 ppm and *H* (OH2) at 3.59 ppm correlate with *H*-2' (3.59 ppm) and 6H (2Me) at 1.31 ppm, respectively. ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 166. 32 (*C*-2), 161.81 (*C*-4), 139.08 (Ar-*C*8a; 121.34 (C3), 117.88 (C4a), 73.11 (C3'), 130.91 (Ar-C7), 123.79 (Ar-C5), 122.76 (Ar-C6), 114.68 (Ar-C8), 79.48 (C2'), 62.40 (NCH_3), 30.24 (OCH_3), 27.87 (C1'), 25.84 (C3a)', 24.06 (C3' and C4'). The IR spectrum indicates the presence of an absorption band at 3415 cm^{-1} reflecting the existence of hydroxyl groups (OH). The band observed at 2919 cm^{-1} indicates an

aliphatic C-H valence vibration of the ethyl group (CH_3CH_2-). The absorption band at 1624 cm^{-1} corresponds to a deformation vibration characteristic of the carbonyl function (C=O). Anal. Calcd for $C_{16}H_{21}NO_4$: C, 65.96; H, 7.27; N, 4.81; O, 21.97. HRMS (m/z) $C_{16}H_{21}NO_4$ (291 g/mol); **Compound 2** (Figure 1) (18,19).

1-methyl-2-[(E)-(prop-1-enyl)]quinolin-4-one (3): yellow solid; yield: 0.29%, m.p. 125 °C. The mass spectrum (HRMS) showed a molecular weight at m/z 199.10 corresponding to the chemical formula $C_{13}H_{13}NO$. The ^{1}H NMR (400 MHz, $CDCl_3$, *δ*, ppm) spectrum showed different signals, at 8.45 (1H, d, *J* = 7.9, Hz, *H*-5), 7.66 (1H, dd, *J* = 7.9, 1.6 Hz, *H*-7), 7.49 ppm (1H, d, *J* = 8.6 Hz, *H*-8), 7.38 ppm (1H, t, *J* =7.6 Hz, *H*-6) which define the presence of an aromatic ring. Three olefin protons at 6.37 (1H, s, *H*-3); 6.47 (1H, d, *J* = 15.3, Hz, *H*-1') and 6.40 (1H, m, *H*-2'). The protons at 1.97 (3H, d, *J* = 6.3 Hz, *H*-3) and 3.75 (3H, s, NMe) correspond to the CH_3 and N-methyl groups, respectively. ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 178.22 (*C*=O), 152.26 (C-2), 141.65 (C8a), 126.95 (C5a), 132.26 (C7), 126.80 (C5), 123.46 (C6), 115.55 (C8), 136.38 (C2'), 125.46 (C1'), 108.91 (C3), 35.56 (N*C*H_3), 19.04 (*C*H_3). IR (CH_2Cl_2, cm^{-1}): 1595 (C=O), 1056 (NCH_3). Anal. Calcd for $C_{13}H_{13}NO$: C, 78.36; H, 6.58; N, 7.03; O, 8.03. HRMS $C_{13}H_{13}NO$ (199.10 g/mol); **Compound 3** (Figure 1) (20,21).

1,2-dimethylquinolin-4-one (4): brown solid; yield: 0.45%, m. p. 123 °C. ^{1}H and ^{13}C NMR (400 MHz, $CDCl_3$, *δ*, ppm) spectrums of Compound **4** are similar to those of compound **3** with the substitution of the (*E*)-prop-1-enyl group by methyl at 2.48 (3H, s, C*H*$_3$) and 22.36 (*C*H$_3$). IR 1622 (C=O), 1141 (N*C*H$_3$). Anal. Calcd for $C_{11}H_{11}NO$: C, 76.28; H, 6.40; N, 8.09; O, 9.24. HRMS $C_{11}H_{11}NO$ (174.09 g/mol); **Compound 4** (Figure 1) (20,22).

5,7-dihydroxy-2-(4'-hydroxyphenyl)-6,8-bis(3-methylbut-2-enyl)chroman-4-one (5): Yellow-green amorphous solid; yield: 0.1%, m.p. 216 °C. The mass spectrum produced in Q-TOF MS ESI in negative mode showed a molecular ion at m/z 407.1860 $[M+H]^-$. The ^{1}H NMR (400 MHz, $CDCl_3$, *δ*) spectrum of the phytocompound **(5)** showed at 12.33 ppm (1H, s, *OH*-5) and 6.38 ppm (1H, s, *OH*-7) the characteristic signals of the OH groups. The protons at 7.32 ppm (2H, d, *J* = 8.5 Hz, *H*-2' and *H*-6') and at 6.87 ppm (2H, d, *J* = 8.6 Hz, *H*-3' and *H*-5') correspond respectively to the protons in the ortho and meta position of the aromatic ring. A signal at 5.32 ppm (1H, dd, *J* = 12.5, 2.7 Hz, *H*-2); a singlet at 3.04 ppm (1H, dd, *J* = 17.1, 12.8 Hz, H3a) and at 2.79 ppm (1H, dd, *J* = 17.1, 3.1 Hz, *H*-3b). Signals resonating at 5.27 – 5.22 ppm (1H, m, *H*-2'') and 5.22 – 5.17 ppm (1H, m, *H*-2''') then at 3.34 ppm (2H, d , *J* = 6.9 Hz, *H*-1'') and 3.29 ppm (2H, d, *J* = 7.1 Hz, *H*-1'''). Furthermore, protons resonating at 1.71 ppm (s, *3H*-3a''), 1.81 ppm (s, *3H*-4''), 1.70 ppm (s, *3H*-4''') and 1.75 ppm (s , *3H*-3a''') are the methyl (*CH$_3$*-) and prenyl ((*CH$_3$)$_2$-C=CH-CH$_2$*-) groups. ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 196.70 (C=O), 162.48, 159.45, 157.88, 107.39, 106.56, 102.94 (Ar-*C*), 43.43 (*C*-3), 22.03 (*C*-1''), 21.39 (C-1'''), 155.99 (Ar-*C*-4'), 131.26 (Ar-*C*-1'), 134.90 (*C*-3''), 134.13 (*C*-3'''), 127.85 (Ar-*C*H-2' and Ar-*C*H-6'), 115.64 (Ar-*C*H-3' and Ar-*C*H-5'), 78.65 (*C*-2), 122.11 (*C*-2''), 121.89 (*C*-2'''), 26.00 (*CH$_3$*-3a''' and 3a''), 18.01 (*CH$_3$*-4'' and 4'''). IR (CH_2Cl_2, cm^{-1}): 3368 (OH), 2918 (Ar-CH), 1627 (C=O). Anal. Calcd for $C_{25}H_{28}O_5$: C, 73.51; H, 6.91; N, 8.09; O, 19.58. HRMS $C_{25}H_{28}O_5$ (408.17 g/mol); **Compound 5** (Figure 1) (23,24).

1,6-dihydroxy-3,5-dimethoxy-10-methyl-4-(3'-methylbut-2'-enyl)acridin-9-one (Grandisinin) (6): amorphous solid yellow-green; yield: 0.22%, m.p. 224.3°C. The ^{1}H NMR spectrum of compound **(6)** showed different signal clusters. Two doublets, which integrate for one proton each were observed, one at 8.02 ppm (1H, d, *J* = 8.8 Hz, *H*-8) and the other at 6.95 (1H, d, *J* = 8 .7 Hz, *H*-7) correspond to aromatic protons. An isolated aromatic signal proton at 6.38 ppm (1H,s,*H*-2); signals at 14.29 ppm (s, 1H, *H*-1') and 12.34 ppm (s, 1H, *H*-6') reveal the presence of two phenolic hydroxyl groups. The spectrum showed three singlets of 3H one at 3.56 ppm (3H, s, *N-Me*), and the other two at 3.92 ppm (3H, s, *O-Me5*), 3.90 ppm (3H, s , *O-Me3*) which each correspond to a methoxy group (*-O-Me*). Furthermore, the signals observed at 1.69 ppm (3H, s, *H*-13a), 1.79 ppm (3H, s, *H*-14) corresponding to methyl groups and protons at 3.46 (2H, d, *J* = 6.1 Hz, *H*-11), 5.23 ppm (t, *J* = 1.5 Hz, 1H, *H*-12) indicate the presence of a prenyl group *((CH$_3$)$_2$-C=CH-CH$_2$-).* ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 182.38 (C=O), 164.96, 163.42, 149.76, 109.30, 106.90 (Ar-*C*), 94.56 (Ar-*C*H), 154.54, 142.79, 135.9, 118.49 (Ar-*C*), 123.36, 111.60 (Ar-*C*H), 59.94, 56.14 (O*C*H_3), 47.66 (N*C*H_3), 131.98 (*C*-13), 123.96 (*C*-12), 26.33 (*C*-11), 25.85 (*C*H_3), 18.20 (*C*H_3-13a). IR (CH_2Cl_2, cm^{-1}): 1401 (C=C), 1627 (OH), 1020 (NCH_3), 1627 (C=O). Anal. Calcd for $C_{21}H_{23}O_5$: C, 68.28; H, 6.28; N, 3.78; O, 21.65. HRMS $C_{21}H_{23}NO_5$ (369.16 g/mol); **Compound 6** (Figure 1) (25).

6,10-dihydroxy-11-methoxy-3,3,12-trimethyl-3,12-dihydropyrano[2,3 c]acridin-7-one (citracridone) (7): Yellow solid; yield: 2.32%, m.p. 243 °C. The ^{1}H NMR spectrum indicated signals from benzene nuclei at 8.07 ppm (1H, d, *J* = 8.8 Hz, *H*-8); 6.99 ppm (1H, d, *J* = 8.8 Hz, *H*-9) and 6.27 ppm (1H, s, *H*-5). A signal at 14.25 ppm (s, 1H, *-OH*) revealed the presence of a phenolic hydroxyl group. The signals at 6.54 ppm (1H, d, *J* = 9.6 Hz, *H*-1) and 5.58 ppm (1H, d, *J* = 9.7 Hz, *H*-2) correspond to 2 H which can be attributed to a double bond conjugated to a carbonyl group (C=O). The singlets at 3.91 ppm (3H, s, *O-Me*) and 3.70 ppm (3H, s, *-N-Me*) correspond respectively to the -OMe and NMe group, and finally two methyl groups (*CH$_3$*-) resonating at 1, 52 ppm (6H s, *-CH$_3$*). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 181.63 (C=O), 154.50, 141.62, 135.88, 118.66 (Ar-*C*), 112.09, 123.58 (Ar-*C*H), 164.81, 161.22, 147.40, 106.94, 102.64 (Ar-*C*), 98.84 (Ar-*C*H), 90, 53 (*C*-3), C2

(124.84 ppm), 120.54 (*C*-1), 60.19 (O*C*H_3), 48.08 (N*C*H_3), 27.34 (*C*H_3). IR (CH_2Cl_2, cm^{-1}): 1401 (C=C), 1627 (C=O), 3407 (OH), 1096 (NCH_3). Anal. Calcd for $C_{20}H_{19}O_5$: C, 67.98; H, 5.42; N, 3.96; O, 22.64. HRMS $C_{20}H_{19}NO_5$ (353.13 g/mol); **Compound 7** (Figure 1) (26).

6,11-dihydroxy-3,3,12-trimethylpyrano[2,3-c]acridin-7-one (8): Red solide; yield: 1.42%, m.p. 256 °C. The ^{1}H NMR spectrum showed signals from a benzene ring at 7.67 ppm (1H, dd, *J* = 7.7, 1.7 Hz, *H*-8); 7.28 ppm (1H, dd, *J* = 7.7, 1.8 Hz, *H*-9); 7.23 (1H, t, *J* = 7.7 Hz, *H*-10). A signal from another benzene ring appears at 6.14 ppm (1H, s, *H*-5). A signal at 14.49 ppm (1H, s, -*OH*) revealed the presence of a phenolic hydroxyl group; the signals at 6.69 ppm (1H, d, *J* = 9.7 Hz, *H*-1) and 5.67 ppm (1H, d, *J* = 9.7 Hz, *H*-2) correspond to the 2 H of the double bond conjugated with the carbonyl group (C=O). A singlet at 3.75 ppm (3H, s, *N-Me*) and finally 2 (*CH*$_3$-) resonating at 1.47 ppm (6H s, *2CH*$_3$). ^{13}C NMR (DMSO-*d*$_4$): 181.37 (C=O), 163.71, 160.70, 148.74, 106.30, 101.99 (Ar-*C*), 97.00 (Ar-*C*H-5), 147.29, 136.69, 124.16 (Ar-*C*), 124.15, 115.25, 120.15 (Ar-*C*H), 124.16 (*C*-2), 120.55 (*C*-1), 76.75 (*C*-3), 48.08 (N*C*H_3), 26.78 (2CH_3). Anal. Calcd for $C_{19}H_{17}O_4$: C, 70.58; H, 5.30; N, 4.33; O, 19.79. HRMS $C_{19}H_{17}O_4$ (324.12 g/mol); **Compound 8** (Figure 1) (27).

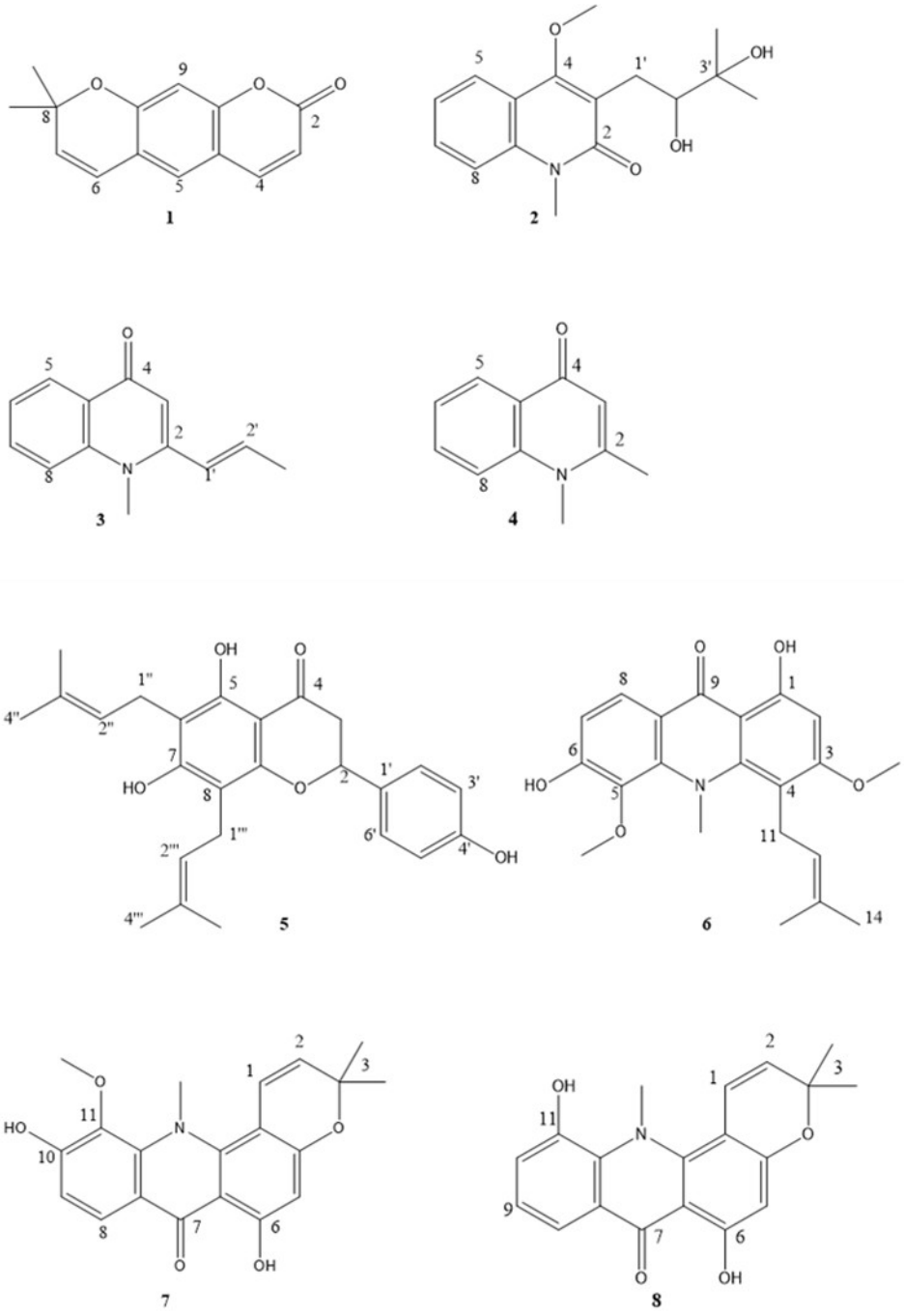

Figure 1: Structure of the compounds **1-8.**

3.3. DPPH Free Radicals Scavenging and Anticholinesterase Activity

The antioxidant potential against DPPH radicals and the anticholinesterase activity of compounds **1** to **8** were evaluated.

3.3.1. Antioxidant activity (DPPH radicals)

Figures 2 and 3 show overall that all the compounds present a reducing character of the DPPH radical.

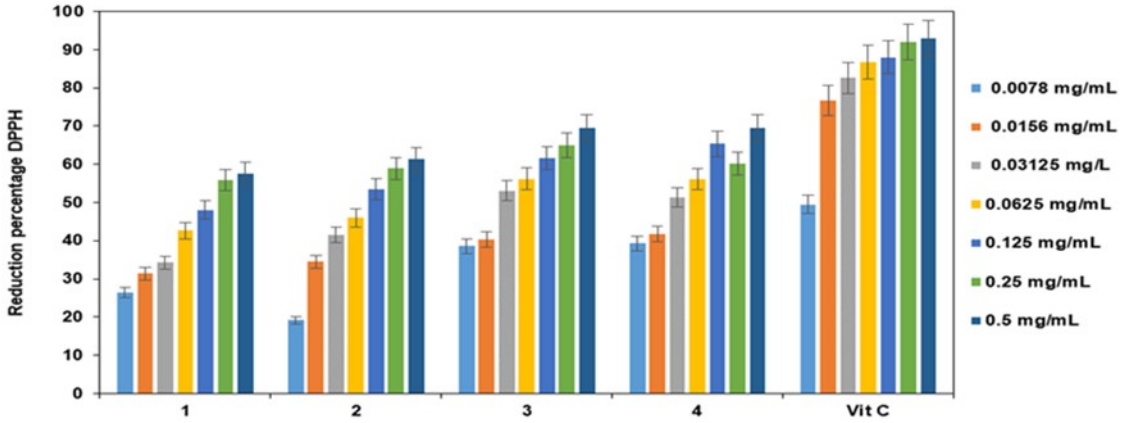

Figure 2: Reduction Percentage of the DPPH radical of the isolated compounds **1-4** and vitamin C.

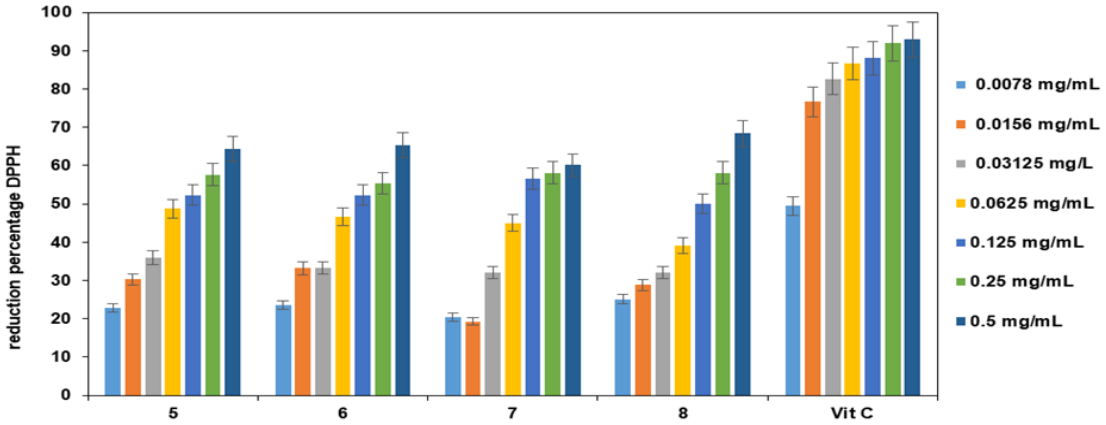

Figure 3: Reduction Percentage of the DPPH radical of the isolated compounds **5-8** and vitamin C.

The reduction percentages (RP) of the compounds **1-4** from CA1 showed a good antioxidant profile (Figure 2). On the other hand, compound **1** reduces DPPH radical at 1096.49 µM with RP (55.86%), and at 2192.98 µM (57.55%). Compound **2** has reduction ability at the different concentrations tested with RP at 429.55 µM (53.50%), 859.11 µM (58.84%), and 1718.21 µM (69.45%)). Compounds **3** and **4** possess better ability to reduce DPPH radical with RP (53.08-69.45%) and (51.28 - 69.44%) at concentrations of 157.03 to 2512.56 µM.

The RP of compounds **5-8** from CA2 (Figure 9), showed RP > 50% at 306.24 to 1224.98 µM, with RP (52.31, 57.65 and 64.33%), (52.29, 55.44 and 65.30%), (56.57, 58.06 and 60.12%) and (54.07, 58.08 and 68.358%), respectively. The RP of vitamin C (49.49 - 88.49%), are higher than those of the isolated compounds at the tested concentrations. Thus, vitamin C is more effective than the samples tested at different concentrations against the DPPH radical. Therefore, we can admit that not only the presence of alkaloids but also coumarin and flavonoid contained in the extract of *C. aurantifolia* root bark would be at the origin of their antioxidant activity. In order to evaluate their antioxidant efficiency in scavenging the DPPH radical, the median reduction concentrations required to reduce DPPH radicals by up to 50% (RC_{50}) were defined (Table 1) (8,15).

Table 1: RC_{50} of compounds and vitamin C.

Compounds	1	2	3	4	5	6	7	8	Vit C
RC_{50} (µM)	271.936 ± 0.001	1336.769 ± 0.010	185.8361 ± 0.0001	218.277 ± 0.002	205.811 ± 0.007	306.6421 ± 0.0002	263.359 ± 0.011	478.217 ± 0.002	17.0331 ± 0.0002

Vit C: vitamin C; **1:** Xanthyletin; **2**: Edulinin; **3:** 1-methyl-2-[(E)-(prop-1-enyl)quinolin-4-one; **4:** 1-methyl-2-[(E)-(prop-1-enyl)quinolin-4-one; **5:** Lonchocarpol A; **6:** Grandisinin; **7:** Citracridone I; **8:** 5-hydroxynoracronycin

Table 1 shows that compounds **1**, **3**, **4**, **6**, and **7** have good antioxidant activity in a dose-dependent manner (28,29). Furthermore, compounds **3** and **4** show the highest antioxidant activity. The principle of antioxidant activity assessment is based on the ability of the compounds to give off hydrogen atoms. In fact, compounds 3 and 4 in Figure 1 show in positions 2' and 2, respectively, labile protons able to reduce the DPPH radical, which could justify their antioxidant capacity. The antioxidant potential compared to vitamin C shows the interest of the isolated compounds tested, since the oxidative phenomenon would be at the origin of oxidative stress, which is suspected to be responsible for pathologies (15,30). Therefore, the anticholinesterase potential of the isolated compounds was evaluated.

3.3.2. Evaluation of acetylcholinesterase (AchE) inhibitory activity

The acetylcholinesterase (AchE) inhibitory activity of compounds (**1-8**) was evaluated during 360 s according to the Ellman method (16). Variable percentages of inhibition (PI) were obtained (Figures 4 and 5).

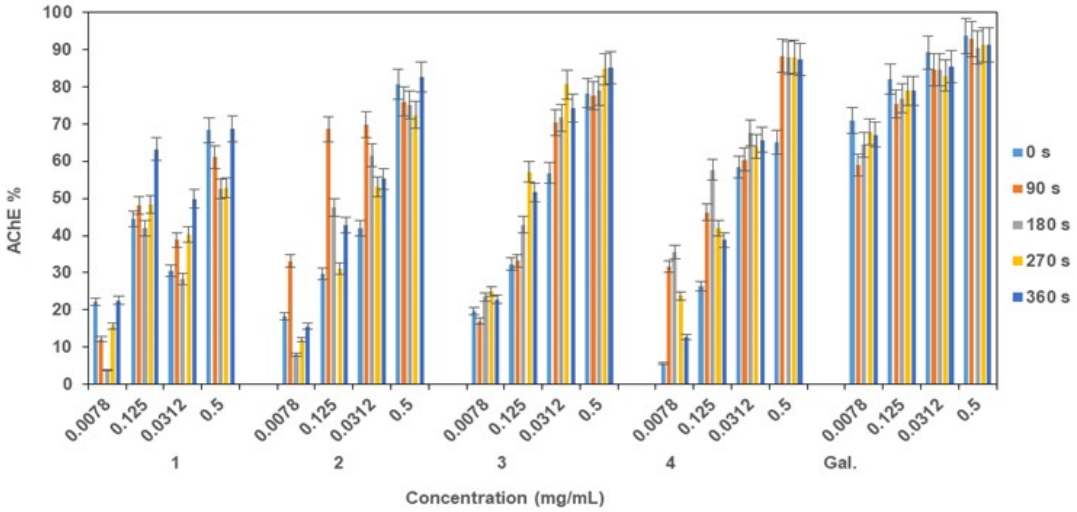

Figure 4: Anticholinesterase activity of compounds 1-4 and Galantamin.

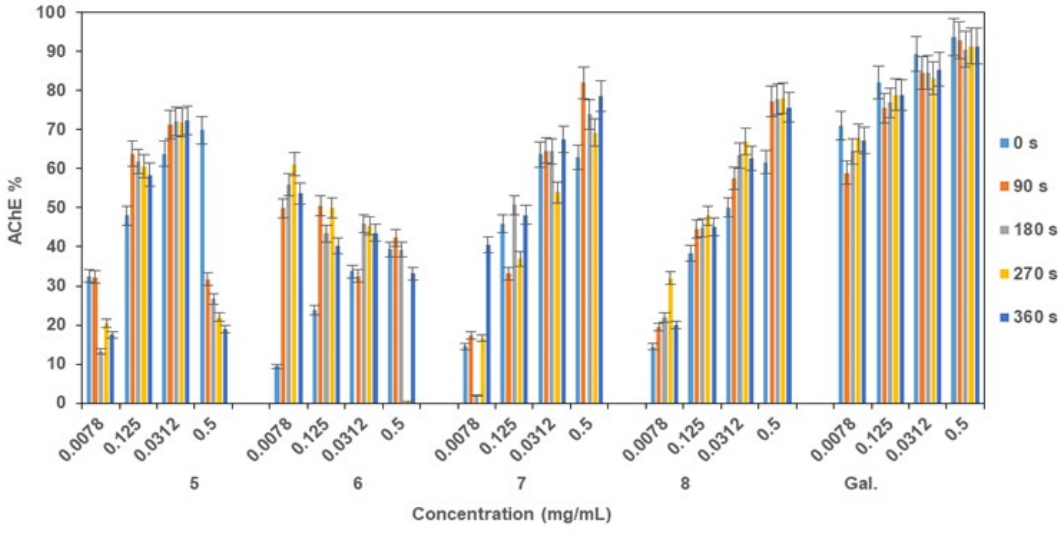

Figure 5: Anticholinesterase activity of compounds 5-8 and Galantamin.

Compounds 1-4 from CA1 showed acetylcholinesterase (AChE) inhibitory activity (Figure 4). Indeed, compound 1 recorded percentages of inhibition (PI) ranging from 52.448% to 68.797% at 548.245 µM during 360 seconds. Compound 2 showed a PI of 72.366% to 82.61% at 1718.21 µM during 360 seconds; at 429.553 µM, the PI was greater than 55%. This result differs from that reported in the literature (19), which showed the low inhibitory capacity of 3-(2',3'-dihydroxy-3'-methylbutyl)-4-methoxy-1-methylquinolin-2-one (Edulinin) on AChE, with a percentage of 19.4% at a concentration of 0.1 mg/mL. Compounds 3 and 4 showed respective PIs of 85.088% and 87.273% at 2511.3 µM at 360 seconds; at 270 seconds, PIs of 84.727% and 87.998%; at 180 seconds, PIs of 78.917% and 87.850%; and at 90 seconds, PIs of 77.621% and 88.291%. In addition, this study was the first to

show the anticholinesterase activity of compounds 3 and 4.

Compounds **5-8** from CA2, have also recorded acetylcholinesterase (AchE) inhibitory activity (Figure 5). On the other hand, compound **5** showed AChE inhibition at 1224.97 µM (PI = 69.849%) at 0 s; 306.244 µM PI (63.847 - 72.457%) during the 360 s and 76.5612 µM PI (58.417 - 63.776%) from 90 to 360 s. Regarding compound **6**, it inhibits the enzyme at 21.129 µM with PI > 50%. As to compounds **7** and **8**, they have higher potential with PI (81.950, 77.210%) at 1415.9 µM, (67.470 and 66.927%) at 353.977 µM, respectively. In view of all the above, we noted that all the isolated compounds have good abilities to inhibit the enzyme (AChE). The compounds **3**, **4**, and **6** showed PI close to that of galanthamin, used as a standard (PI > 60%).
To show of the structure-activity relationships of the compounds, several structural features were identified in the general structure of the compounds studied: a) the alkaloids (**2, 3, 4, 6, 7** and **8**), b) coumarins (**1**) and c) flavonoid (**5**).

Compounds 2,3,4,6,7, and 8 from Citrus all feature an alkyl substituent on the nitrogen of the ring, which could justify the higher inhibition percentages (PI > 50) observed by these compounds. Indeed, Berkov et al. (2008), showed that the alkaloids N-allyl-nor-galanthamine and N-(14-methylallyl)-nor-galanthamin isolated from the leaves of Leucojum aestivum L demonstrated good inhibition of AChE. The presence of the substitution of the N-methyl derivative would be at the origin of the inhibitory activity of the two compounds (31). The best activity has been observed in sanguinin isolated from Galanthus woronowii. It is substituted at the N atom, but with a methyl group (32,33).

The results of a study carried out by Ryu et al (2012) on 20 flavonoids suggest that inhibitory flavonoids form a complex with AChE. The presence of a hydroxyl group, particularly in the A-ring of the flavonoid, as well as the double bond between C-2 and C-3, increases the enzyme's affinity (hydrogen bonds) and also enhances the AChE-inhibiting properties of flavonoids (34).

The traditional use of *Citrus aurantifolia* in the treatment of mental disorders could be justified by its antioxidant and anticholinesterase potential (11,35).

4. CONCLUSION

Chemical and biological studies of the root bark extracts from *C. aurantifolia* were performed. The structures of compounds were elucidated according to their spectral data (NMR, MS, and IR). The melting points were also determined. Additionally, this study identified eight known phytocompounds (**1-8**); among which compounds **2**, **3**, **4**, and **6** were isolated for the first time from the root bark of *C. aurantifolia*. The highest anticholinesterase activity was observed for compound **6**. The combined antiradical and anticholinesterase activities of compounds **3** and **4** could explain the various therapeutic properties attributed to *Citrus aurantifolia* root barks in traditional medicine.

5. CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

6. ACKNOWLEDGEMENTS

The authors thank the "Ministère de l'Enseignement Supérieur et de la Recherche Scientifique de Côte d'Ivoire (MESRSCI) for the financial support given to Djélé Alette Edwige Zialé for her PhD training at the Laboratoire CSPBAT of the Université Sorbonne Paris Nord, Bobigny (France). They also express their gratitude to the USPN for the access to the NMR Platform and to Professor Malan Djah François for the identification of the plant species studied.

7. REFERENCES

1. Brookmeyer R, Johnson E, Ziegler-Graham K, Arrighi HM. Forecasting the global burden of Alzheimer's disease. Alzheimer's Dement [Internet]. 2007 Jul 1;3(3):186–91. Available from: <URL>.

2. Ahue MK, Bekro JAM, Maurice T, Virieux D, Pirat JL, Bekro YA. Preliminary phytochemical screening and protective effect of Erythrina Senegalensis in an in vivo mouse model of Alzheimer's disease. J Med Pharm Allied Sci [Internet]. 2017 Mar 1;630–44. Available from: <URL>.

3. Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, et al. Global prevalence of dementia: a Delphi consensus study. Lancet [Internet]. 2005 Dec 17;366(9503):2112–7. Available from: <URL>.

4. Adou DA, guessan AHON, -Békro JAM, Békro YA. Evaluation of the antioxidant potential and aglycone coumarins contents of ethereal extracts of 4 medicinal plants from Côte d'Ivoire. J Pharmacogn Phytochem [Internet]. 2022 Nov 1;11(6):46–9. Available from: <URL>.

5. Aisa ER, Tukiran T. Comparison on Total Phenolics and Flavonoids and Antioxidant Activities of Methanol Extract of Horseshoe Crab (Tachypleus gigas) Eggs. J Turkish Chem Soc Sect A Chem [Internet]. 2023 Aug 30;10(3):787–96. Available from: <URL>.

6. El Kadmiri N, Hamzi K, El Moutawakil B, Slassi I, Nadifi S. Les aspects génétiques de la maladie d'Alzheimer (Revue). Pathol Biol [Internet]. 2013 Dec 1;61(6):228–38. Available from: <URL>.

7. Schliebs R, Arendt T. The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. J Neural Transm [Internet]. 2006 Nov 9;113(11):1625–44. Available from: <URL>.

8. Ziale DAE, Ngaman Kouassi KCC, Deschamp J, Bouchemal N, Palama TL, Lecouvey M, et al. Structural characterization and in vitro biological exploration of phytoconstituents isolated from a chloroform extract of Rauvolfia vomitoria (Apocynaceae) root bark from Côte d'Ivoire. J Pharmacogn Phytochem [Internet]. 2023 Jan 1;12(1):06–14. Available from: <URL>.

9. Ettebong E, Ubulom P, Etuk A. Antiplasmodial activity of methanol leaf extract of Citrus aurantifolia (Christm) Swingle. J Herbmed Pharmacol [Internet]. 2019 Sep 1;8(4):274–80. Available from: <URL>.

10. Sunday Enejoh O, Oladejo Ogunyemi I, Smart Bala M, Sotonye Oruene I, Musa Suleiman M, Folorunsho Ambali S. Ethnomedical Importance of Citrus Aurantifolia (Christm) Swingle. Pharma Innov J [Internet]. 2015;4(8):1–6. Available from: <URL>.

11. Amat-ur-Rasool H, Symes F, Tooth D, Schaffert LN, Elmorsy E, Ahmed M, et al. Potential Nutraceutical Properties of Leaves from Several Commonly Cultivated Plants. Biomolecules [Internet]. 2020 Nov 15;10(11):1556. Available from: <URL>.

12. de Andrade JP, Giordani RB, Torras-Claveria L, Pigni NB, Berkov S, Font-Bardia M, et al. The Brazilian Amaryllidaceae as a source of acetylcholinesterase inhibitory alkaloids. Phytochem Rev [Internet]. 2016 Feb 22;15(1):147–60. Available from: <URL>.

13. Assohoun K, Kabran GRM, Bekro JAM, Virieux D, Pirat JL, Bekro YA. Total alkaloids and in vitro antioxidant activity of Crinum jagus L. (Amaryllidaceae) organs from Côte d'Ivoire. Int J Green Herb Chem [Internet]. 2020 Oct 31;9(4):451–63. Available from: <URL>.

14. Blois MS. Antioxidant Determinations by the Use of a Stable Free Radical. Nature [Internet]. 1958 Apr;181(4617):1199–200. Available from: <URL>.

15. Etekpo SD, N'Gaman-Kouassi K, Yao T, Mamyrbekova-Bekro J, Bekro Y. Phytochemical, Antioxidant and pharmacological study of (SM.) JACQ.- FEL. (Melastomataceae). J Pharm Biol Sci [Internet]. 2022 Jul 28;10(1):37–43. Available from: <URL>.

16. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol [Internet]. 1961 Jul 1;7(2):88–95. Available from: <URL>.

17. Amaral JC, da Silva MM, da Silva MFGF, Alves TC, Ferreira AG, Forim MR, et al. Advances in the Biosynthesis of Pyranocoumarins: Isolation and 13 C-Incorporation Analysis by High-Performance Liquid Chromatography–Ultraviolet–Solid-Phase Extraction–Nuclear Magnetic Resonance Data. J Nat Prod [Internet]. 2020 May 22;83(5):1409–15. Available from: <URL>.

18. Noulala CGT, Ouete JLN, Atangana AF, Mbahbou GTB, Fotso GW, Stammler HG, et al. Soyauxinine, a New Indolopyridoquinazoline Alkaloid from the Stem Bark of Araliopsis soyauxii Engl. (Rutaceae). Molecules [Internet]. 2022 Feb 7;27(3):1104. Available from: <URL>.

19. Yang Z duo, Zhang D bo, Ren J, Yang M jun. Skimmianine, a furoquinoline alkaloid from Zanthoxylum nitidum as a potential acetylcholinesterase inhibitor. Med Chem Res [Internet]. 2012 Jun 4;21(6):722–5. Available from: <URL>.

20. Nguyen NT, Dang PH, Vu NXT, Le TH, Nguyen MTT. Quinoliniumolate and 2 H -1,2,3-Triazole Derivatives from the Stems of Paramignya trimera and Their α-Glucosidase Inhibitory Activities: In Vitro and in Silico Studies. J Nat Prod [Internet]. 2017 Jul 28;80(7):2151–5. Available from: <URL>.

21. Yasir M, Tripathi MK, Singh P, Shrivastava R. The Genus Glycosmis [Rutaceae]: A Comprehensive Review on its Phytochemical and Pharmacological Perspectives. Nat Prod J [Internet]. 2019 Mar 18;9(2):98–124. Available from: <URL>.

22. Rádl S, Obadalová I. Utilization of Aromatic Denitrocyclization Reaction for the Synthesis of 3-Unsubstituted 1,4-Dihydroquinolin-4-one Derivatives. Collect Czechoslov Chem Commun [Internet]. 2004 Apr;69(4):822–32. Available from: <URL>.

23. Ribeiro AB, Abdelnur PV, Garcia CF, Belini A, Severino VGP, da Silva MF das GF, et al. Chemical Characterization of Citrus sinensis Grafted on C. limonia and the Effect of Some Isolated Compounds on the Growth of Xylella fastidiosa. J Agric Food Chem [Internet]. 2008 Sep 10;56(17):7815–22. Available from: <URL>.

24. Tahara S, Katagiri Y, Ingham JL, Mizutani J. Prenylated flavonoids in the roots of yellow lupin. Phytochemistry [Internet]. 1994 Aug 10;36(5):1261–71. Available from: <URL>.

25. Tian-Shung W, Chang-Sheng K, Furukawa H. Acridone alkaloids and a coumarin from Citrus grandis. Phytochemistry [Internet]. 1983 Jan 1;22(6):1493–7. Available from: <URL>.

26. Ye Y, Xu G, Li DL. Acridone alkaloids and flavones from the leaves of Citrus reticulata. Nat Prod Res [Internet]. 2022 Jul 18;36(14):3644–50. Available from: <URL>.

27. Segun PA, Ismail FMD, Ogbole OO, Nahar L, Evans AR, Ajaiyeoba EO, et al. Acridone alkaloids from the stem bark of Citrus aurantium display selective cytotoxicity against breast, liver, lung and prostate human carcinoma cells. J Ethnopharmacol [Internet]. 2018 Dec 5;227:131–8. Available from: <URL>.

28. Bissim SM, Kenmogne SB, Tcho AT, Lateef M, Ahmed A, Ngeufa Happi E, et al. Bioactive acridone alkaloids and their derivatives from Citrus

aurantium (Rutaceae). Phytochem Lett [Internet]. 2019 Feb 1;29:148–53. Available from: <URL>.

29. Tjahjandarie TS, Pudjiastuti P, Saputri RD, Tanjung M. Antimalarial and antioxidant activity of phenolic compounds isolated from Erythrina cristagalli L. J Chem Pharm Res [Internet]. 2014;6(4):786–90. Available from: <URL>.

30. Favier A. Intérêt conceptuel et expérimental dans la compréhension des mécanismes des maladies et potentiel thérapeutique.

31. Berkov S, Codina C, Viladomat F, Bastida J. N-Alkylated galanthamine derivatives: Potent acetylcholinesterase inhibitors from Leucojum aestivum. Bioorg Med Chem Lett [Internet]. 2008 Apr 1;18(7):2263–6. Available from: <URL>.

32. Sarikaya BB, Kaya GI, Onur MA, Bastida J, Somer NU. Phytochemical investigation of Galanthus woronowii. Biochem Syst Ecol [Internet]. 2013 Dec;51:276–9. Available from: <URL>.

33. Ortiz JE, Garro A, Pigni NB, Agüero MB, Roitman G, Slanis A, et al. Cholinesterase-inhibitory effect and in silico analysis of alkaloids from bulbs of Hieronymiella species. Phytomedicine [Internet]. 2018 Jan 15;39:66–74. Available from: <URL>.

34. Ryu HW, Curtis-Long MJ, Jung S, Jeong IY, Kim DS, Kang KY, et al. Anticholinesterase potential of flavonols from paper mulberry (Broussonetia papyrifera) and their kinetic studies. Food Chem [Internet]. 2012 Jun 1;132(3):1244–50. Available from: <URL>.

35. Loizzo MR, Tundis R, Bonesi M, Menichini F, De Luca D, Colica C, et al. Evaluation of Citrus aurantifolia peel and leaves extracts for their chemical composition, antioxidant and anti-cholinesterase activities. J Sci Food Agric [Internet]. 2012 Dec 16;92(15):2960–7. Available from: <URL>.