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RESEARCH ARTICLE

PHYTOCHEMICAL STUDY AND EVALUATION OF THE ANTIOXIDANT ACTIVITIES OF LEAVES FROM *VERNONIAAMYGDALINA*

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Abstract

The aim of this study is to highlight the phytochemical composition and antioxidant potential of the ethereal extract of the leaves of *Vernoniaamygdalina*, a plant used in traditional medicine in Côte d'Ivoire.Phytochemical studies using TLC and LC-MS revealed, in addition to coumarins, flavonoids and phenolic acids. Determination of the antioxidant potential by TLC and spectrophotometry using the DPPH radical showed the antioxidant power of *Vernoniaamygdalina* leaves with a CR_{50} of $0.01995 \pm 0.2x10^{-5}$ % mg/mL.The many pharmacological properties of *V. amygdalina* would therefore depend on the coexistence of these phytocompounds.

Introduction:-

The end of the 20th century was marked by a number of major health crises (COVID-19, Ebola, Avian flu, etc.) affecting human beings.This upsurge in pathologies could be linked to global upheavals (production methods, new technologies, climate change, etc.) and oxidative stress (Akissi, 2021).Indeed, free radicals can have deleterious effects on many molecules, including proteins, lipids, RNA, DNA and carbohydrates (Kostova et al., 2006; Lü et al., 2010).Furthermore, oxidative stress-related diseases such as inflammatory conditions, cancers, diabetes, accelerated ageing and cardiovascular disease are public health issues (Adou et al., 2022).In this way, a great deal of research has been carried out to discover new bioactive compounds, particularly of natural origin, with diversified and unique chemical structures to tackle this upsurge.Moreover, plants remain one of the most promising sources of molecules in all areas of health, both for the treatment and prevention of certain pathologies.According to Newman and Cragg (2020), around 75% of medicines for the period 1981-2019 are of natural origin.In this context, *Vernoniaamygdalina*, a plant traditionally used in Côte d'Ivoire, was studied. The aim was to determine the phytochemical composition and antioxidant potential of a selective extract of *Vernoniaamygdalina* leaves.

Materials and Methods:-

Plant material

The leaves of *Vernoniaamygdalina* were collected in Anyama (5° 29′ 40″ north, 4° 03′ 06″ west, a town in the south of Côte d'Ivoire in the Autonomous District of Abidjan). The identification was confirmed by the late ASSY Jean of the Centre National de Floristique (CNF) in Abidjan (5° 20′ 11″ Nord, 4° 01′ 36″ Ouest), in accordance with

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existing herbaria. The various organs were dried under air conditioning (18°C) for two weeks, then ground using an electric grinder (Moulinex) to obtain the powders used for analysis.

Methods:-

Extraction

Coumarin extraction was carried out according to the method described by Grinkevitch and Safronitch (1993), with a few modifications. In a reflux heater, 30 g of plant powder were subjected to hydrolysis in HCl (2N, 100 mL) for 2 hr. After cooling, the pomace was dried in a fume hood for 48 h, then recovered in hexane (24 h).The pomace is then extracted with MeOH (100 mL) in a Soxhlet for 2 h. After removal of the solvent, the extracted mass is successively treated with KOH solutions at 0.5% (w/v) (90 mL) and 5% (w/v) (20 mL) for 1 h. The alkaline fraction obtained is treated with ethyl ether (3 × 50 mL) to remove undesirable compounds.After settling and separation, a 5% (v/v) HCl solution is carefully added to the alkaline phase up to pH= 2. The reaction mass was then treated with ethyl ether (3 × 100 mL). The diethyl ether fractions of the leaves were used for the rest of the study.

Phytochemical analysis by TLC

Thin-layer chromatographic (TLC) qualitative analysis of the ethereal extracts was carried out in accordance with the operating methods referenced in the bibliography (Dekker et al., 2003; Newman et al., 2003; Békro et al., 2007; Mamyrbékova-Békro et al., 2008; Cazes et al., 2010; N'Gaman et al., 2013).Deposits were made on resized chromatoplates (silica gel 60 F254, aluminum support, 20 × 20 cm, Merck). After migration in the solvent system toluene/chloroform/ethyl acetate (1/3/1/0.5), chromatograms were observed under UV at 254 and 365 nm, before revelation with basic lead acetate, 5% (w/v) methanolic KOH solution and ammonia.

Phytochemical analysis by LC-MS

Analysis was carried out on an HPLC chain (Agilent 1260 Infinity) coupled to a mass spectrometer (Q-TOF-MS Agilent 6530) equipped with an ESI source. A Sunfire® Waters C18 column with a length of 150 mm, a diameter of 2.1 mm and a particle diameter of 3.5 µm was used. The mobile phase consisted of a gradient of solvents A (H2O + 0.1% HCO2H) and B (ACN).A linear gradient was used for 41 min in the following proportions: 0-5% B (0-5 min), 5-95% B (5-15 min), 95-100% B (15-25 min), 100% B (25-30 min), 100-0% B (30-32 min) and 0% B (32-41 min). Sample injection volume and flow rate are set at 5 µL and 250 µL/min respectively. Data analysis is performed using Agilent MassHunter Workstation software.

Determination of antioxidant potential

The antioxidant power of ethereal coumarin extracts was tested following the method described by Takao et al . (1994). Deposits weré made on resized chromatoplates (silica gel 60 F254, aluminum support, 20 × 20 cm, Merck). After migration in the solvent system toluene / chloroform / ethyl acetate/ MeOH (1/3/1/0.5), chromatograms were dried and then treated with a methanolic solution of the stable radical DPPH (2 mg/mL).After an incubation time of 30 min, the phytoconstituents of extracts with potential free radical scavenging activity are revealed as pale yellow imprints on a violet background.The method of Blois (1958), adopted by Kabran et al. (2014), was used to measure the antioxidant activity of coumarin extracts.DPPH is solubilized in absolute MeOH to obtain a solution with a concentration of 0.3 mg/mL. Different concentration ranges (1; 0.5; 0.25; 0.1; 0.05; 0.025 and 0.01 mg/mL) of each extract are prepared in the same solvent. 1 mL extract and 2.5 mL methanolic solution of DPPH.After shaking, the tubes are placed in the dark for 30 min. The absorbance of the mixture is then measured at 517 nm against a blank consisting of 1 mL pure MeOH and 2.5 mL DPPH solution. Ascorbic acid (vitamin C) is the positive reference control. DPPH reduction percentages (%R) are calculated according to formula (1):

$$\%R = (A_b - A_e) / A_b \times 100 \quad (1)$$

Ab: absorbance of blank
Ae: absorbance of sample

The concentration required to reduce 50% of the DPPH radical concentration (CR_{50}) was determined using OriginPro 9.1 software (Etekpo et al., 2018; Tano et al., 2019).

Statistical analysis

A statistical study based on one-way analysis of variance (ANOVA) was carried out using OriginPro 9.1 software. The difference between means was considered significant at the 5% level. All experimental measurements were performed in triplicate and results expressed as means plus or minus standard deviations (Mean ± SD).

Results and Discussion:-

Phytochemical screening by TLC

The chromatograms were interpreted with reference to the literature (Wagner and Bladt, 1996; Dekker, 2003; Cazes, 2010). In view of the results obtained, the appearance of blue, yellow, orange and fluorescent blue coloration reflects the presence of coumarins in V. amygdalina leaf extracts (Table 1).In addition, the presence of flavonoids was detected by Neu's reagent in leaf extract. Indeed, this specific reagent reveals flavonoids in blue, yellow, green or orange (Wagner and Bladt, 1996). Dagnon et al. (2019) showed the presence of flavonoids in leaf extract.The results obtained are partly in line with the work of certain authors. Indeed, E. Canh Pham et al, 2024 showed that V. amygdalina leaves are known to be rich in phenolic acids, tannins and flavonoids.This entire study highlighted the absence of coumarin in the crude extract. Oluwaseun et al (2017) confirmed the presence of flavonoids, alkaloids, tannins and phenolic compounds in V. amygdalina organs.

Table 1:- Synopsis of the phytochemical composition of *V. amygdalina*.

Fraction	Rf	Color	Phytocompounds
	0,08	$Y^{b1,c1,d1,e1,e2}$; $Bf^{a2,b2}$; $B^{c2,d2}$	coumarins^{a,b,c,d}; 7-hydroxycoumarinor 6,7-dialcoxycoumarin^{a,b}/flavonoid^{e}
	0,12	B^{c2}	coumarin^{c}
	0,25	$Y^{a1,b1,c1,d1,e1}$; $Bf^{a2,b2,c2,d2,e2}$	coumarin^{a,b,c,d}; 7-hydroxycoumarin or 6,7-dialcoxycoumarin^{a,b}/ flavonoid^{e}
VA	0,43	Y^{a1}; B^{d2}	Coumarin^{a,d}
	0,52	B^{d2}	coumarin^{d}
	0,58	Or^{a1}; G^{b1}; $R^{a2,b2,c2,d2}$	NA^{c,d}; 6-hydroxy-7-alcoxycoumarin^{a,b}
	0,62	$Or^{c1,d1}$; Br^{c2}	coumarin^{c,d}
	0,92	B^{a2}	coumarin^{a}

Rf : retention factor ; B : blue ; Y : yellow ; Br :brown ; G : green ; Bf : blue-fluorescent ; Or : orange; a^2 : 365 nm ; b^1 : NH_3; b^2 : NH_3 at 365 nm: c^1 : $((AcO_2)_2Pb)$; c^2 : $((AcO_2)_2Pb)$ at 365 nm ; d^1 : KOH; d^2 : KOH at 365 nm; e^2 : Neu at 365 nm ; NA : not appreciated, VA: *Vernonia amygdalina*

LC-MS profile of the ethereal extract of V. amygdalina leaves

Positive-mode LC-MS analysis of the ethereal extract of *V. amygdalina* leaves revealed 20 peaks (Figure 1). Data on maximum absorbances and the masses of the various fragment ions (Table 2) helped to highlight the co-presence of coumarins, flavonoids and phenolic acids.

Coumarins areillustrated by peak 1, 5, 6, 7, 8, 10, 13, 14, 15, 16, 18 and 19. Peaks 1, 5, 7, 8, 10, 15, 16, 18 and 19 were attributed to auraptene, imperatorine, coumarin, aleuritine, 4,7,8-trihydroxy-6-methoxy-2-oxo-chroman-3-carboxylic acid and a furanocoumarin derivative respectively.Auraptene is characterized by a molecular ion at m/z 299 $[M+H]^+$ and daughter ions at m/z 163 $[M+H\text{-}C_{10}H_{16}]^+$ (loss of 2 isoprenic units); 135 $[M+H\text{-}C_{10}H_{16}\text{-}CO]$+ and 117 $[M+H\text{-}C_{10}H_{16}\text{-}CO\text{-}H_2O]^+$.Peak 5 was identified as imperatorine, with a molecular ion at m/z 271 [M+H]+ and daughter ions at m/z 201 $[M+H\text{-}C_5H_{10}]$+ (loss of 2-methylbut-2-ene); 163 (furanic ring breakage of a furanocoumarin); 135 and 117.Analysis of peak 16 identified 4,7,8-trihydroxy-6-methoxy-2-oxochroman-3-carboxylic acid. Indeed, this phytocompound is characterized by a molecular ion at m/z 271 $[M+H]^+$ and daughter ions at m/z 227 $[M+H\text{-}CO_2]^+$; 209 $[M+HCO_2\text{-}H_2O]^+$; 181 $[M+H\text{-}CO_2\text{-}H_2O\text{-}CO]^+$; 164 $[M+H\text{-}CO_2\text{-}2H_2O\text{-}CO]$+; 139 and 107.These proposals are based on a similar pattern of fragmentation pathways and UV spectra. Peak 6 indicates the presence of 3,4-dihydrocoumarin (m/z 149 $[M+H]^+$) with daughter ions at m/z 121 $[M+H\text{-}CO]^+$ and 105 $[M+H\text{-}CO_2]^+$.In addition, different fragmentations of the molecular ion corresponding to peak 13, giving m/z 157 $[M+H\text{-}CO]^+$, m/z 142 $[M+H\text{-}CO_2]$+ and m/z 125 $[M+H\text{-}CO_3]$+ suggest psoralen. Peak 14 showed under positive mode ionization a molecular ion at m/z 400 [M+H]+, fragmentation of this ion showed daughter ions at m/z 352, a base

peak at m/z 191 due to loss of the glycosyl unit and m/z 175 suggesting that this compound may be an isomer of scopoline.

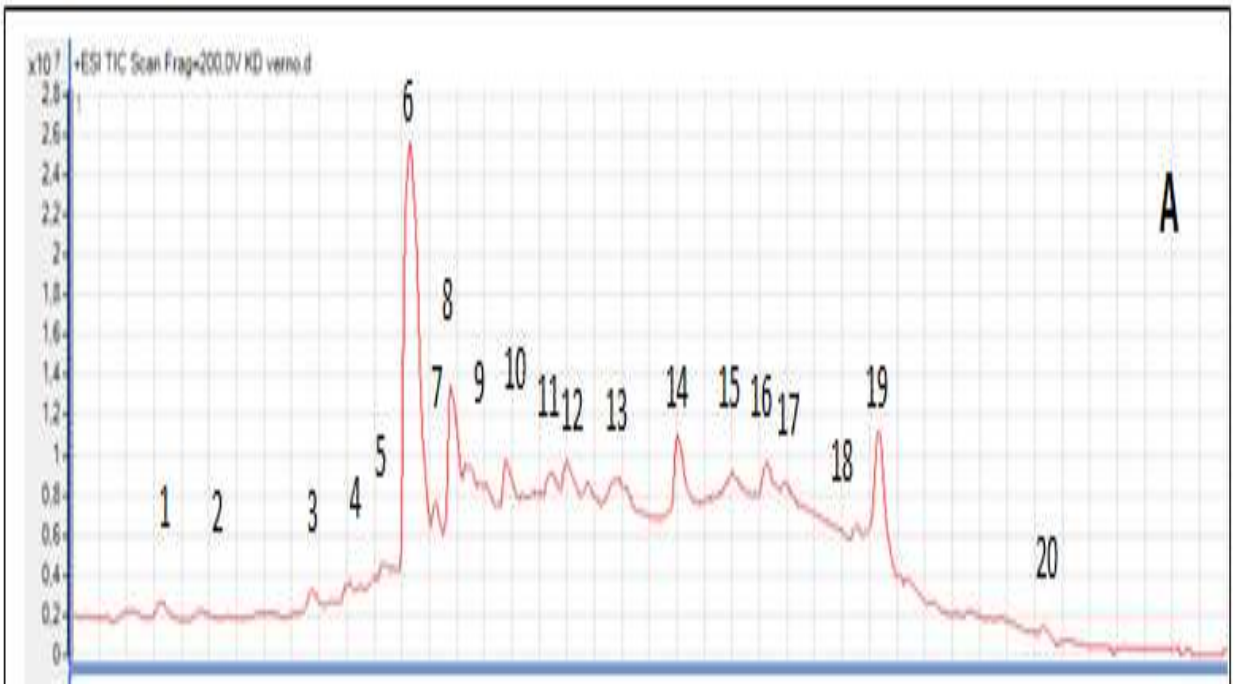

Figure 11:-Mass Spectrum of the ether extract of *V.amygdalina* leaves.

Table 2:-Synopsis of the LC-MS profile of the phytochemical composition of the ethereal extract of *V. amygdalina* leaves.

Pic	R_t(min)	λ_{max} (nm)	Molar Mass	MS (m/z) Ion $[M+H]^+$	MS/MS Ion	Molecular formula	Phytocompounds
1	3,29	226 285 315	298	299	163,137, 135,117	$C_{19}H_{22}O_3$	Auraptene
2	4,71	205 226	298	299	283,190, 145,117	$C_{16}H_{10}O_6$	Irolone
3	8,60	227 260 295	270	271	253,235, 154,137,117,109	$C_{15}H_{10}O_5$	Apigenin
4	10,12	215 235	270	271	255,180,163,149,119	$C_{16}H_{14}O_4$	Alpinetin
		280	212	213	195,167	$C_{13}H_8O_3$	Urolithin
5	11,33	227 280	270	271	201, 163, 135, 117, 103	$C_{16}H_{14}O_4$	Imperatorine
		330	180	181	163, 145, 103	$C_9H_8O_4$	CinnamicacidderivativeFragesine
6	12,29	235 280	370 148	371	353,269 121,105	$C_{21}H_{22}O_6$ $C_9H_8O_2$	3,4-dihydrocoumarin

				149			
7	13,26	210 240 285	270 241	271 242	163,117,103	$C_{16}H_{14}O_4$	Coumarin NI
8	13,76	215 240 265	416 198	417 197	400,369,264,234,146 179, 161, 119, 101	$C_{21}H_{20}O_9$ $C_9H_8O_5$	Aleuritin 2,4,5-trihydroxycinnamiqueacid
9	14,37	210240285	266	267	138,107	$C_8H_8O_4$	NI
10	15,78	210 245 270	270	271	149, 135, 117, 103	$C_{16}H_{14}O_4$	Furanocoumarinederivative
11	17,40	210250315	315	316	153		Dihydroxybenzoichexosideacid
12	18,01	250 315	280	281			NI
13	18,77	215285	341 186	342 187	325,311,281,268 157,142,125	 $C_{11}H_6O_3$	dimethoxylflavanon Psoralene
14	19,93	250 285 310 210245345	400 270	401 271	352,191,175 163, 135, 117, 103	 $C_{16}H_{14}O_4$	Scopolineisomer furanocoumarinderivative
15	22,05	210 260	270 120	271 121	163, 135, 117, 103	$C_{11}H_{10}O_8$	4,7,8-trihydroxy-6-methoxy-2-oxo-chroman-3-carboxyliqueacid NI
16	24,03	210 250 270	270	271	149,135,117,103	$C_{16}H_{14}O_4$	furanocoumarinderivative
17	25,29		642	643			NI
18	25,90	210 250 270	596 270	597 271	149, 135, 117, 103	$C_{26}H_{44}O_{15}$ $C_{16}H_{14}O_4$	NI furanocoumariederivative
19	28,53 28,53	210 255	656 270	657 271	201,135,117,103	 $C_{16}H_{14}O_4$	NI Imperatorine
20	29,39	210 250 270	610 568	611 569	466,303,177,122	$C_{27}H_{30}O_{16}$	Rutine NI

NI : Not Identified

Five (5) flavonoids were identified in the ethereal extract of *V. amygdalina*. These were irolone (peak 2); apigenin (peak 3); alpinetin (peak 4); dimethoxyflavanone (peak 13) and rutin (peak 20). Indeed, irolone (peak 2) is characterized by a molecular ion at m/z 299 $[M+H]^+$ and daughter ions at m/z 283 $[M+H-H_2O]^+$; 190 $[M+H-H_2O-C_6H_6O]^+$; 145 $[M+H-H_2O-C_6H_6O-CH_4O_2]^+$ and 117 $[M+H-H_2O-C_6H_6O-CH_4O_2-CO]^+$.Apigenin (peak 3) and rutin (peak 20) were revealed by molecular ions at m/z 271 $[M+H]^+$ and 611 $[M+H]^+$ respectively, and the fragmentation of these ions is similar to that obtained in Table 2. The fragmentation of peak 4 suggests alpinetine at m/z 271($[M+H]^+$). The fragment at m/z 255 $[M+H-CH_3]^+$corresponds to CH_3 loss.Ten, the fragment at m/z 180 $[M+H-CH_3-C_6H_6]^+$ (loss of a benzene group), followed by fragments at m/z 163 $[M+H-CH_3-C_6H_6-H_2O]^+$and 149 $[M+H-CH_3-C_6H_6-2H_2O]^+$. Finally, the fragment at m/z 119 $[M+H-CH_3-C_6H_6-2H_2O-CO]^+$ indicates the loss of carbon monoxide. Peak 13 features a molecular ion at m/z 342 $[M+H]^+$. Its UV profile (absorption bands at 215 and 287)

suggests a flavanone as reported by Portet et al. (2008). Fragments at m/z 325 [M+H-CH_3] and m/z 311 [M+H-$2CH_3$] reveal the subsequent loss of 2 CH_3-, indicating a methoxylated compound. Fragmentation of the m/z 311 ion yielded a fragment ion at m/z 281, originating from the loss of a CO molecule. The absence of significant H_2O loss indicates a flavanone skeleton (Portet et al., 2008). Thus, peak 13 would be a dimethoxyflavanone.Among the compounds identified in the ethereal extract of *V. amygdalina*, several phenolic acid derivatives such as cinnamic acids (peaks 5 and 8) and a benzoic acid (peak 11) were detected. In addition to phenolic acids, fragésine (a lignan) is highlighted by peak 6. Indeed, fragmentation of fragesin showed a molecular ion at m/z 371 $[M+H]^+$ and a daughter ion at m/z 353 [M+H-H_2O] (Gao et al., 2015).

Antioxidant activity of ethereal leaf extracts

A compound's antioxidant activity is explained by its ability to resist oxidation. In the DPPH TLC test, compounds with antioxidant activity show up as active yellow-pale fingerprints on a violet background in the chromatograms. In the presence of free-radical scavengers (or reducers), the violet DPPH radical is reduced by H-transfer to its reduced form, yellow 1,1-diphenyl-2-picrylhydrazine (DPPH-H) (Takao et al., 1994).It should also be remembered that DDPH also acts as a developer of free-radical scavenging phytocompounds. Ethereal extracts of v. amygdalina leaves contain coumarins capable of reducing (or scavenging) DPPH. The anti-free radical properties of these extracts were therefore revealed by TLC. The frontal ratios (Rf) of the compounds corresponding to the said activity zones have been recorded in Table 3.

Table 3:- Rf of different antiradical phytocompounds in ethereal leaf extracts against DPPH.

Retention factor (Rf)	
VA	0,08 ; 0,13 ; 0,25 ; 0,42 ; 0,52 ; 0,92

Comparing the chromatographic profiles of the phytochemical screening and those of the DPPH TLC test, we found that these zones of anti-free radical activity were mainly those of coumarins. Indeed, coumarins are known for their remarkable antioxidant powers (Lin et al., 2008).

Spectrophotometric antioxidant profile of ethereal leaf extracts

Figure 2 shows the results of the antioxidant activity evaluation test, which compares the DPPH-reducing concentrations of the various samples studied with those of the reference antioxidant, Vitamin C. Leaf extracts from the fractions tested showed DPPH reduction rates of over 50% from 0.03 mg/mL.

Analysis of variance (ANOVA) applied to the mean of the percentages of reduction at 0.25 mg/mL gives a value of $P < 0.05$. The differences between the percentage reduction values are therefore significant. In addition, VA ethereal extracts showed good anti-radical (or reducing) activity close to that of vitamin C (96.558 ± 0.002%) at the same concentration. Comparing our results with those reported by N'guessan (2013) in a study of ethyl acetate and n-butanol extracts of *V. amygdalina*, we find that the reduction percentages are relatively close.

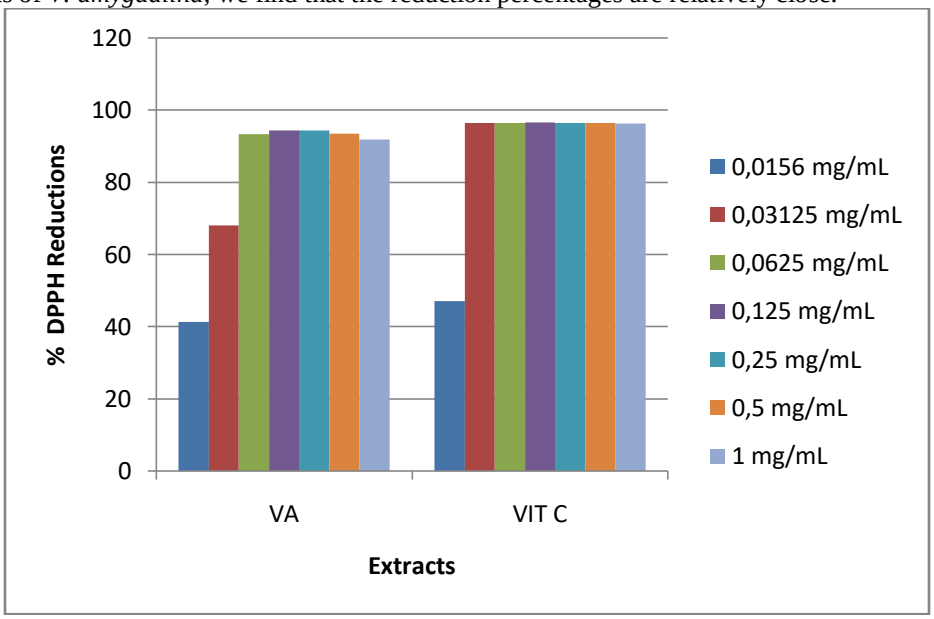

Figure 2:-Synopsis of the quantified antioxidant activity of *Vernonia amygdalina* ethereal leaf extracts.

VA: ethereal extract of *V.amygdalina*leaves; VIT C: vitamin C

The concentrations of ethereal leaf extracts required to reduce 50% of the DPPH concentration were determined graphically. The lower the CR_{50}, the higher the antioxidant activity. It should be noted that vitamin C was used as the reference antioxidant. The CR_{50} values determined are shown in Table 4.

Table 4:- CR_{50} values (mg/mL) for ethereal leaf extracts.

	Extrait	
	VA	**VIT C**
CR_{50} (mg/mL)	0,01995 ±0,2x10^{-5}%	0,016150 ±0,3x10^{-6}%

In view of the CR_{50} values presented in Table 4, the ethereal extract of V. anygdalina leaves shows remarkable DPPH radical reduction capacities, compared with that of vitamin C. Extracts with CR_{50} values close to those of VA vitamin C (0.01995 ± 0.2x10^{-5} % mg/mL).

Conclusion:-

The present study focused on the phytochemical and antioxidant analysis of an ethereal extract from the leaves of *Vernoniaamygdalina* from Côte d'Ivoire. Phytochemical screening by TLC revealed the presence of coumarins and flavonoids in the selective extract of *V. amygdalina* leaves. In addition, LC-MS/MS analysis enabled structural characterization of phytoconstituents such as auraptene, imperatorine, coumarin, aleuritine, apigenin, scopoline and others. From the qualitative TLC test to the quantitative DPPH radical scavenging spectrophotometry test, it emerged that the ethereal extract of *V. amygdalina* showed a good antioxidant profile. Moreover, the antioxidant capacities revealed are close to those of vitamin C. The phytochemical approach thus provides a rational explanation for the use of *V. amygdalina* leaves in endogenous and popular therapeutic practices in Côte d'Ivoire.

Competing interests

Authors have declared no competing interests exist.

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