

Isolation of biomolecules from the leaves of *Lecaniodiscus cupanoides* (Sapindaceae), a plant used in traditional medicine in Benin

ABSTRACT

The aim of the present study was to identify the structure of the bioactive molecules in the active ethyl acetate fraction of the hydroethanolic extract of *Lecaniodiscus cupanoides* (Sapindaceae), a Beninese plant used in the treatment of microbial infections. We prepared the hydroethanolic extract from powdered dried leaves. We fractionated the hydroethanol extract using the liquid-liquid extraction method with solvents of increasing polarity. The active ethyl acetate fraction obtained after bioguided fractionation of the hydroethanol extract on bacterial strains was purified by a series of atmospheric pressure column chromatographic methods coupled with thin layer chromatography. At the end of this purification process, three compounds, including a flavonoid and two fatty acids, were isolated and identified by interpretation of ¹H NMR, ¹³C NMR and mass spectrometry spectra. These were: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydrochromen-4-one; (E)-3-(3,4-dihydroxyphenyl)-2-(3-(3,4-dihydroxyphenyl) acryloyloxy) propanoic acid and (E)octadec -9- enoic acid (oleic acid).

The added value of this work lies in the fact that these three molecules have never before been identified in this plant, and therefore represent a potential avenue for the development of a therapeutic arsenal to combat microbial infections.

Key words : *Lecanioides cupanoides* (Sapindaceae) isolation, chromatographic methods, spectrometric analysis

INTRODUCTION

"*Lecaniodiscus cupanioides* is a 9-metre-high tree belonging to the Sapindaceae family and the genus *lecaniodiscus*. The tree is found in tropical Africa from Sierra Leone to Sudan, as well as in Angola, the south of the Democratic Republic of Congo and Uganda", (Burkil H. M. 2004) "This plant has many uses in traditional medicine. In the form of inhalation, the bark is used to treat headaches, sinusitis, otitis and eye and ear problems. The leaves are reputed to be antibacterial and rubefacient. They are applied to boils and bruises, but can cause burns if left on too long. The use of traditional medicine, in particular herbal remedies, has increased over the last few decades and many people now turn to them for the treatment of a variety of illnesses". (Pousset J.L. 2004). Herbal remedies therefore represent an alternative in primary care systems and a promising avenue for the development of improved traditional medicines. We wanted to characterise the molecules contained in the active ethyl acetate fraction

obtained after bioguided fractionation of the hydroethanol extract on bacterial strains of the plant used in traditional medicine.

MATERIAL AND METHOD

1- Plant material

The plant material consists of dried leaves of the *Lecaniodes cupaniodes* plant harvested in December 2021 in Abomey-Calavi (Benin) and identified in the National Herbarium of the University of Abomey-Calavi (Benin). We harvested 5kg of leaves. The leaves of the harvested plant were washed and then dried at room temperature in a ventilated room in the Pharmacognosy laboratory of the Faculty of Health Sciences/Pharmacy Faculty for a fortnight before being ground into powder using a plant grinder. The powder was stored at room temperature in a glass vial.

2- Méthods

2. 1- Préparation of crude extract

Extraction was carried out using dried leaves reduced to powder. We took 100g of powder and mixed it in 1000 ml of a hydroethanol mixture (40V/60V respectively) for 48 hours. After filtration on Whatman N°1 paper, the filtrates obtained were evaporated using a rotary evaporator R100 BUCHI at 40°C. The residues of this filtrate were dried in an oven for 48 hours at 40°C to obtain the dry extracts (Hougbebe et al 2014)

2.2- Liquid – liquid fractionation

Liquid-liquid extraction is used to transfer a solute from one liquid phase to another, immiscible liquid phase. It is based on the difference in affinity of the solute between two immiscible phases. The solution to be fractionated consists of the crude hydroethanol extract dissolved in 50 mL of distilled water. During fractionation, 500 mL of Cyclohexane Sigma Aldrich CAS 62610-50-8, Dichloromethane Sigma Aldrich CAS 75-09-2, Ethyl acetate anhydre 99,8% Sigma Aldrich CAS 141-78-6 and Methanol Sigma Aldrich CAS 67-56-1 were used successively. The various fractions collected were evaporated under reduced pressure at 40°C using a rotavapor R 100 BUCH

2.3- Purification and isolation

"Fractionation of the ethyl acetate extract and its sub-fractions was carried out using the atmospheric pressure column (APC) liquid-solid chromatography technique. The stationary phases used were successively R P 18 silica gel (40-63µm), normal silica gel (60 PF 254) and Sephadex LH 20 gel. These different gels were solubilised in methanol (30g in 150 mL methanol) and then poured into a glass column. The eluent is under atmospheric pressure, enters at one end and exits at the other. It can be a single solvent for conditioning or a mixture of solvents for the different gradients". [13] The chromatographic partition conditions for the ethyl acetate fraction and its sub-fractions are shown below

Dichlorométhane :	100%
Dichlorométhane – Acétate d'éthyle :	90 – 10
Dichlorométhane – Acétate d'éthyle :	70- 30

Acétate d'éthyle - MeOH : 50 – 50
Acétate d'éthyle - MeOH : 20 – 80
MeOH 100 %
Elution : 200 mL of solvent gradient

Collection rate : 5mL/tube of 1 goutte / seconde

Deposition : 200 mg of solubilised fraction in 10 mL MeOH

"The sub-fractions obtained were analysed by thin layer chromatography using the solvents ethyl acetate/MeOH/H₂O(v/v/v) 81-11-8 as the mobile phase and 10% alcoholic potash as the developer, which revealed the presence of phenolic acids, flavonoids and quinones" (Bruneton 1999). "The sub-fractions grouped together after analytical TLC were subjected to further fractionation on CPA. Fractions at the end of purification were passed over an SPE column with Sephadex gel for exclusion chromatography, which separates compounds based on size and molecular weight" (Houngbeme et al, 2015). Figures 1 and 2 below describe the stages of two major sub-fractions collected and from which we were able to isolate the molecules

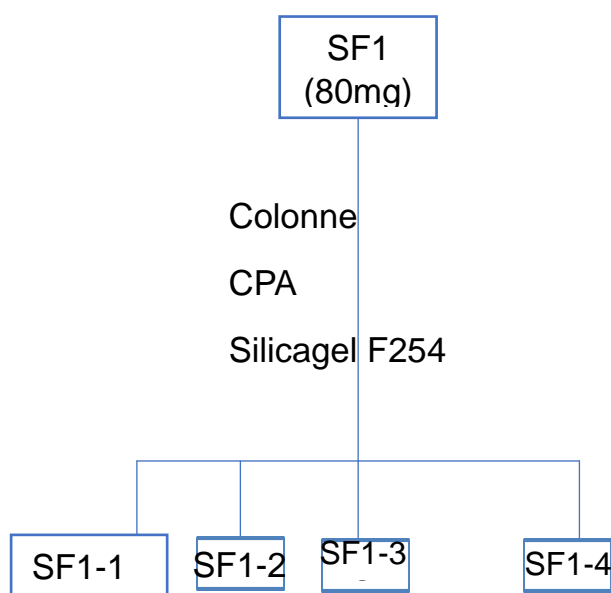


Figure 1 : Diagram showing the purification stages of the sub-fraction SF1

The selected S/F1 fraction was further purified on F 254 silica gel to give 4 sub-fractions SF1-1; SF 1-2; SF 1-3; SF 1-4.

The SF 1-1 sub-fraction revealed two well-separated spots at UV 254 nm. We therefore applied preparative TLC to scrape off the majority spots. The scraped majority spots were then separated by Exclusion Chromatography (Figure 2). Spot 1 gave compound M3 and spot 2 gave 2 compounds M4 and M5.

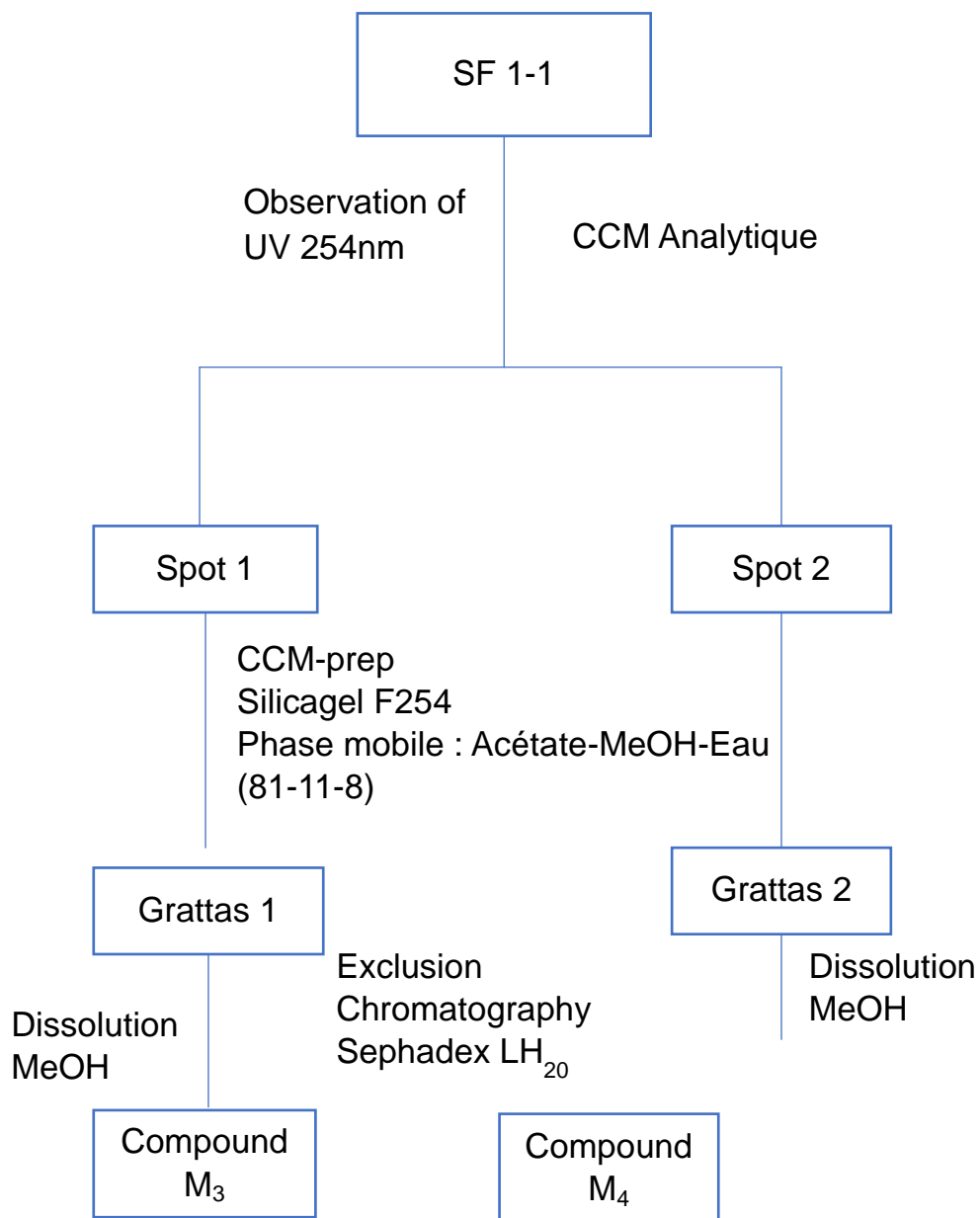


Figure 2 : Diagrams showing the steps in the purification of the sub-fraction SF1-1

RESULTS

1- Yield of fractions

Table 1 : Summary of the yield of the fractions

Plant matériel	Extract	Mass	Yield
Crude extract (20g)	Extract C ₆ H ₁₂	0,25 g	1,25%
	Extract CH ₂ CL ₂	0,29 g	1,45%
	Extract AcOEt	3,58 g	17,9%
	Extract MeoH	3,4 g	17%
	Extract Aqueous	9,25 g	46,2%

2- Collection of sub-fractions

The different sub-fractions are summarised in the following table:

Table 2 : Distribution of tubes collected after TLC and revelation

Sub - fractions	Tubes collected	Mass obtained (mg)
S/F1	T 1-13	80
S/F2	T 14- 20	50
S/F3	T 21-40	20
S/F4	T 41 -60	14
Total / yield		119/59,5%

3- Structure of the isolated molecules

By processing the information from the various spectra, we were able to elucidate the structure of the isolated compounds.

Spectrométric data of compound M₃

- ❖ **MS (m/z):** 287,003 g/mol ([M+H]⁺) ; 286,003 ([M+H]⁺-H) ; 270,003 ([M+H]⁺ - OH) ; 190,003 ([M+H]⁺ - C₆H₅O₂).
- ❖ **¹H NMR (CD₃OD, 400MHz, δ en ppm):** δ 15,08 (s, H-énol) ; δ 6,88 (t, H-éthylène); δ 6,70-6,21 (H-benzénique); δ 5,03 (d, OH-aromatique).
- ❖ **¹³C NMR (CD₃OD, 100MHz, δ en ppm):** δ 161,5 (C=O-cyclique); δ 159,08-147,28 (-C-OH aromatique); δ 122,8-115,9 (-CH benzène dihydroxylé); δ 105,6 (C-énolique); δ 103,6 (C-benzène hydroxylé).

Compound M₃ absorbs UV light at a wavelength of 365 nm. It reacts with the NEU reagent with a yellowish fluorescence, suggesting that its structure is based on the flavonoid typ

The protons of the A ring of a flavonoid appear on a ¹H NMR spectrum between 6 and 6.5 ppm (Houngbèmè et al., 2015, Tokoudagba et al., 2022).

The presence of a peak at δ 172-186 ppm on the ^{13}C NMR spectrum (peak of the C_4 carbonyl group) of the C ring, allows us to say that the C ring has a $\text{C}=\text{O}$ double bond. Thus the structure of compound M_3 would be based on the structure in Figure 3 below

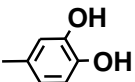
Figure 3 : Basic structure of compound M_3

Taking into account the information provided by the interpretation of the ^1H NMR spectrum, the ethylene hydrogen, the aromatic protons at δ 6.70-6.21 ppm and the $-\text{OH}$ protons of the phenolic groups at δ 5.03 ppm, we can assign the compound M_3 the probable structure shown in Figure 4 below:

Figure 4 : Probable structure of compound M_3

The molecular weight obtained by the positive impact mode $[\text{M}+\text{H}]^+$ gave 287.003 g/mol, corresponding to a molecular weight of $\text{M}=286.003\text{g/mol}$, which is close to that of the molecule proposed in Figure 3. The appearance of certain major peaks including the fragments $([\text{M}+\text{H}]^+-\text{H})$; $([\text{M}+\text{H}]^+-\text{OH})$ on the one hand, and $([\text{M}+\text{H}]^+-\text{C}_6\text{H}_5\text{O}_2)$ indicating the loss of the

cycle B ortho disubstitué par $-\text{OH}$:

Oc1ccccc1O

secondly, justifies the structure proposed for compound M_3 in Figure 3.

In order to confirm the structure of compound M3, we carried out a Chemdraw simulation. The values of the chemical shifts of the protons and carbons in the experimental spectra of the compound are close to or equal in places to the values shown in the spectra of the same type obtained by chemdraw simulation. Compound M3 is therefore consistent with the claimed structure. The final structure of the isolated compound M3 is shown in Figure 5 below:

Figure 5 : molecular structure of compound M₃

Spectrometric data for compound M₄

❖ **MS (m/z):** 283,012 ([M+H]⁺); 268,012 ([M+H]⁺ - CH₃); 254,012 ([M+H]⁺ - CH₂CH₃); 240,012 ([M+H]⁺ - CH₂-CH₂-CH₃); 238,012 ([M+H]⁺ - COOH).

¹H NMR (CD₃OD, 400MHz, δ en ppm): δ 10,96 (s, H-carboxylique); δ 2,19 (t, H-méthylène); δ 1,60 (q, H-méthylène); δ 1,38-1,27 (q, H-méthylène); δ 1,35 (q, H-méthylène); δ 5,34 (t, H-éthylénique); δ 2,03 (t, H-méthylène); δ 1,38-1,27 (q, H-méthylène); δ 0,90 (t, H-méthyle).

❖ **¹³C NMR (CD₃OD, 100MHz, δ en ppm):** δ 179,5 (C-carboxyle); δ 36,7 (-CH₂ aliphatique); δ 27,1 (-CH₂ aliphatique); δ 31,0-30,4 (C-méthylène aliphatique); δ 131,0 (C-éthylène aliphatique); δ 23,9 (C-méthylène aliphatique); δ 14,6 (C-méthyle)

The molecular weight obtained by the positive impact mode [M+H]⁺ gave 283.012 g/mol, corresponding to a molecular weight M=282.012g/mol, approximately equal to that of a

monounsaturated C18 fatty acid. The appearance of the fragments ($[M+H]^+ -COOH$) and ($[M+H]^+ -CH_3$) confirms the "fatty acid" function of the compound.

The absence of a chemical shift in the proton NMR spectrum between 6 and 7 ppm shows that it has no aromatic protons (Houngbèchè et al., 2015; Harbone, 1993, Fatondji et al., 2010) and therefore no aromatic ring. The M4 molecule would be an aliphatic molecule. The presence of the carboxylic proton that appeared as a singlet at δ 10.96 ppm and the strongly shielded methyl proton δ 0.90 ppm, again justify that the compound is a fatty acid. The unsaturated nature of this fatty acid is corroborated by the appearance on the proton spectrum of two ethylenic protons at δ 5.34, each appearing as a triplet by coupling.

The NMR spectrum of carbon 13, shows the presence of a strongly unshielded carbon appearing at δ 179.5 ppm which is the carbon of a carboxyl group. The chemical shifts observed correspond well to the different aliphatic carbons in the skeleton of a fatty acid, oleic acid.

In view of all these spectral interpretations, we assigned the skeleton below to the probable structure of compound M₄

Fig 6 : structure of compound M₄

To confirm the molecular structure, we simulated the spectra using Chemdraw. The values of the chemical shifts of the protons and carbons in the experimental spectrum of the compound are very close and do not differ significantly from the values shown in the analogous spectra obtained by Chemdraw simulation. Compound M₄ therefore corresponds well to the oleic acid structure

Figure 7 : structure of the isolated compound (M₄)

Spectrometric data for compound M₅

- ❖ **MS (m/z):** 361,34 g/mol ($[M+H]^+$); 360,34 ($[M+H]^+-H$); 344,34 ($[M+H]^+-OH$); 316,34 ($[M+H]^+-COOH$); 201,34 ($[M+H]^+ - 159$); 202,34 ($[M+H]^+ - 158$); 131,34 ($[M+H]^+ - 229$)

- ❖ **¹H NMR (CD₃OD, 400MHz, δ en ppm):** δ 11,01 (s, H-carboxylique); δ 7,62 (s, H-C=C); δ 6,89-6,42 (H-aromatique); δ 5,02 (s, HO-aromatique); δ 3,18 et δ 2,90 (s, H-méthylénique).

- ❖ **¹³C NMR (CD₃OD, 100MHz, δ en ppm):** δ 173,2 (-COOH); δ 165,98 (ester -COO); δ 147,18-144,48 (-C-OH aromatique); δ 129,2 (-CH aromatique substitué); δ 117,2-113,5 (-CH aromatique non substitué); δ 38,01 (-CH₂).

Compound M₅ absorbs UV light at a wavelength of 365nm and shows a yellow coloration with the NEU reagent. The compound is thought to belong to the flavonoid or phenolic acid class.

The ¹³C NMR spectrum shows two strongly unshielded peaks at δ 173.2 ppm and 165.98 ppm. These two signals correspond respectively to the carbons -COOH (carboxylic acid) and -COO (ester). In addition, on the same spectrum we observe carbons at δ 147.18-144.48 ppm which correspond to phenolic carbons (aromatic -C-OH). Taking these observations into account, we can say that compound M₅ is a phenolic acid also containing an ester function in its structure.

The ¹H NMR spectrum, shows a proton at δ 11.01 ppm which is a proton of the most deblinded carboxyl group; aromatic protons at δ 6.89-6.42 ppm (Houngbèmè et al., 2015, Tokoudagba et al., 2022); protons that appear singlet at δ 5.02 (HO-aromatic). The presence of protons at δ 7.62 indicates the presence of an alkene bond (H-C=C-H). On the basis of the proton and carbon NMR spectrometric data, added to the family of the molecule, we have assigned compound M5 the probable structure shown in Figure 7 below:

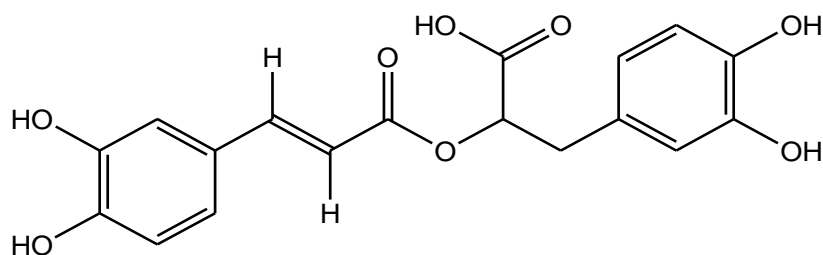


Figure 8 : Structure probable of compound M₅

The molecular mass obtained in positive electrospray mode [M+H]⁺ gave 361.34 g/mol, corresponding to a molecular molar mass M=360.34g/mol. This value corresponds to the calculated mass (M=360g/mol) for the structure proposed in Figure 1. The appearance of some major peaks, including the fragments ([M+H]⁺-H); ([M+H]⁺ -OH) on the one hand, and ([M+H]⁺-COOH) on the other, reinforces the NMR data for the proposed molecule.

The melting temperature of the compound was also determined to complement the spectrometric analyses. Measurement of a small quantity of the compound's crystals on a Kofler bench gave, on average (n=8), T_f=174.12 ±0.06 °C. This value lies within the 171-175°C range corresponding to the melting point of rosmarinic acid, the structure of which corresponds to that shown in Figure 8.

As with the other compounds, we simulated the spectra using Chemdraw. The values of the chemical shifts of the protons and carbon in the simulation spectra and the experimental spectra were compared. These values are close to, and in some places equal to, the values shown in spectra of the same type obtained by chemdraw simulation.

Based on interpretations of spectrometric analyses, knowledge of the melting point, and comparisons with spectra recorded by simulations, the structure of compound M5 is that of rosmarinic acid, a phenolic acid presented in figure 8 below:

Figure 9: Molecular structure of compound M₅ (Acide rosmarinique)

CONCLUSION

We isolated three molecules from the active ethyl acetate fraction of the hydroethanolic extract of the dried leaves of *Lecanioides cupanoides* (Sapindaceae): one flavonoid and two fatty acids. This fraction, rich in flavonoids and oleic acid, could be an alternative in the development of a therapeutic arsenal to combat microbial diseases

ACKNOWLEDGEMENTS

We would like to thank the **FAFABE Foundation** for its financial support for this project.

CONFLIT OF INTEREST

The authors of this manuscript declare that there is no conflict of interest.

REFERENCES

- 1- Bruneton J. (1999) Pharmacognosie. Phytochimie et Plantes médicinales ;4^{ème} édition ; Edition médicale internationale ; Paris ; 364-1043
- 2- Burkil H. M. Useful plants of west tropical Africa 2004
- 3-Kossi Jean Marie D. Tokoudagba, Gouton Alban. Houngbèmè, Ayidé C. Ahouansou, Urbain C. Kasséhin. Gabin A. Assanhou and Fernand A. Gbaguidi. Structural Identification of Isolated Molécules of *Parkia biglobosa* (Mimosaceae) a Plant Used in Traditional Medecine In Bénin World Journal of Pharmacy And Pharmaceutical Sciences 2022, 11 (5) 49-57
- 4- Kossi Jean Marie D. Tokoudagba, Ayide C. Ahouansou and Fernand A. Gbaguidi
Phytochemical study of antimicrobial activity of fractions of hydro ethanolic extract of *lecaniodiscus cupanioides* (Sapindaceae) World Journal of Pharmaceutical Research 2022,11(5) 51 – 62
- 5- Kossi Jean Marie D. Tokoudagba and Fernand A. Gbaguidi
Phytochemical study and evaluation of the antioxydant activity of fraction to the extract of *lecaniodiscus cupanioides* (Sapindaceae) International Journal of Sciences Academic Research 2022 3 (2) 3456 - 3460
- 6- Kossi Jean Marie D. Tokoudagba, Ayide C. Ahouansou, Fernand A. Gbaguidi
Phytochemical and Antibacterial Activity of Fractions of Hydroethanolic Extracts of *Parkia biglobosa* (Mimosaceae) and *Carissa edulis* (Apocynaceae)] International Journal of Science and Academic Research 2021, 2(12) 3241-3245
- 7- Fatondji H. R, Gbaguidi F, Kpoviéssi S, Sonounameto E, Lagnika L, Ambaliou S, Moudachirou M, Poupaert J, Accrombessi G: Synthèse, caractérisation et études de propriété antimicrobiennes de la semicarmazone et de thiosemicarbazone de la carvone. J.Soc.ouest-Afr.Chim ; 2010.030 :11-17
- 8- Houngbèmè A.G, Ganfon HMY, Medegan S, Yèhouènou B, Bambola B, Gandonou C, Gbaguidi FA : Antimicrobial activity of compounds from *Acanthospermum hispidum* DC and *Caesalpinia bonduc* (L.) ROXB: Beninese plants used by healers against HIV-associated microbial infections. Journal Applied Pharmaceutical Science Vol.5(06) :2015
- 9- Houngbeme A. G. C. Gandonou. B Yehouenou. SDS Kpoviessi D Sohounhloue. M. Moudachirou and F. Gbaguidi Phtochemical analysis, toxicity and antibactériel activity of benin medicinal plants extracts used in the traitement of sexually transmitted infections associated with hiv/aids. Int J Pharm Sci Res 2014 ; Vol 5(08) ; 073-081

10- Portet B. : Recherche bioguidée de molécules antipaludiques d'une plante guyanaise : *Piper hostmanianum* var. *berbicense*. Thèse de doctorat. 2007. Université de Toulouse.

11- Pousset Jean – Louis Plantes médicinales africaines -Utilisation pratique 2004

12- Rajput A.P and Rajput T.A : Isolation of stigmasterol and betasitostérol from chloroform extract of leaves of *corchorus fascicularis* Lam. International Journal of biological chemistry; 2012. 6(4): 130-135

13. Kossi Jean Marie D. Tokoudagba, Gouton Alban. Hounbèmè1, Ayidé C. Ahouansou, Urbain C. Kasséhin, Gabin A. Assanhou and Fernand A. Gbaguidi. STRUCTURAL IDENTIFICATION OF ISOLATED MOLECULES OF PARKIA BIGLOBOSA (MIMOSACEAE) A PLANT USED IN TRADITIONAL MEDICINE IN BENIN. WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES. Volume 11, Issue 6, 49-57 (2022)

