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Contribution to the phytochemical and biological study of the methanolic extract of root bark of *Mezoneuronbenthamianum*Baill. : a medicinal plant from Ivory Coast

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ABSTRACT*:The identification of molecules in the methanolic extract of Mezoneuronbenthamianum root bark harvested in the middle of Ivory Coast was carried out by LC-MS. The corresponding results revealed the identification of six (06) phytocompounds, among which the pharmacological properties of five were highlighted. These are 2,4,6-Triallyloxy-1,3,5-triazine, 1,6-bis-O-galloyl-beta-D-glucose, epigallocatechin gallate, 1,4,6-tri-O-galloyl-beta-D-glucose, and resveratrol. Biological tests carried out on this extract have shown that it manifests not only significant anticoagulant and analgesic activities, but also relatively low toxicity. Therefore, the biological potential of methanolic extract from the root bark of M. benthamianum could be related to its phytochemical profile.*

KEYWORDS:*Mezoneuronbenthamianum, analgesic, anticoagulant, toxicity and LC-MS.*

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I. INTRODUCTION

Mezoneuronbenthamianum is a plant in the Caesalpiniaceae family. It is a climbing and thorny shrub that is widespread from Senegal to Nigeria. This plant is also present in Ivory Coast where it is found in all plant formations but preferably in secondary formations. Known in Nigeria as "Jenifinran" **[1]**, in Ivory Coast, *M. benthamianum* is called "Akobohuie" in Krobou; "Akpabawun" in Akyé; "Akoowué" in Baoulé and "Côlè" in Djimini. This plant is commonly used in folk medicine. In West Africa, it is used to treat malaise, wounds, urethral discharge, ulcers, skin infections, pain, fever, diarrhea, persistent coughs, and headaches **[1,2,3,4]**. In Ivory Coast, the fresh root of *M. benthamianum* is consumed in the form of a toothpick against sexual impotence **[5]**. Several studies have highlighted the pharmacological activities of the plant. The vine is used as an antiseptic, detergent and healer of torpid wounds **[6].** Extracts of root bark have been tested for its vasothoven, antioxidant, aphrodisiac and antibacterial properties **[7,8]**. Leaf extract has also shown antimicrobial effects **[9].** Thus, this study is part of a development strategy contributing to the valorization of medicinal plants in Ivory Coast. Thus, it will specifically evaluate the analgesic, anticoagulant and toxicological activities of the methanolic extract of the root bark of *M. benthamianum* on the one hand and contribute to the determination of the chemical composition of that extract on the other hand, using liquid chromatography coupled with mass spectrometry (LC-MS).

II. MATERIAL AND METHODS

MATERIAL

Plant material

The root bark of *Mezoneuronbenthamianum* was collected in July in Dabakala (8° 21′ 48" North, 4° 25′ 43" West), a town located in the middle of Ivory Coast. After authentication at the herbarium of the National Floristic Center (CNF) in Abidjan (5° 20′ 11" North, 4° 01′ 36" West), the bark was cleaned and dried first of all away from the sun for 2 days. Then, they were dried under air conditioning (18°C) for 7 days and in an oven (45°C) for 3 days. The dry bark was then ground into powder using an electric grinder (RETSCH, type SM 100). The resulting vegetable powder was used to prepare the extract to be analyzed.

Technical material, chemical and biological products

Technical material was composed of an electric shredder (RETSCH, type SM 100), a permanent stirrer, an oven, a rotary evaporator (BÜCHI Waterbath B-480), a centrifuge, a spectrophotometer, a Thermo Scientific OrbitrapExploris 480 mass spectrometer, EDTA tubes, a feeding probe, syringes and standard laboratory glassware. As for the chemical and biological products, they consist of methanol (80%), water, acetonitrile (CH_3CN), acetic acid (1%), physiological water, paracetamol, escin, sodium chloride and sheep's blood.

Animal material

For the biological tests *in vivo*, female mice of the species *Mus musculus* weighing between 22 and 32 g, albino of the Swiss breed and from IPCI (Institute Pasteur of Ivory Coast) were used.

METHODS

Extraction

A mass of 50 g of powder was macerated in 375 mL of methanol (80%) for 24 h, at room temperature under permanent stirring. After vacuum filtration, the maceration was concentrated with a rotary evaporator (BÜCHI Waterbath B-480). Then, the concentrate was dried in an oven at 50°C for 48 hours. The crude methanolic extract obtained was stored in the refrigerator at 4°C in a hermetically sealed jar. It was subsequently used to perform analgesic, anticoagulant, toxicity and spectral analysis by LC-MS.

Analgesic test

The analgesic test of the methanol extract of *M. benthamianum* root bark was carried out according to the methods described by Koster *and al.*, **[10]** and Chatter *and al.*, **[11]**.

Three (3) batches of four (4) mice were made. The blank control batch received 0.1 mL of physiological water. The mice from the other two groups received respectively 0.1 mL of plant extract and 0.1 mL of paracetamol (reference analgesic) at 200 mg/kg BW. Half an hour after administration of the products (vegetable, paracetamol, physiological water), the animals received 0.1 mL of 1% acetic acid (CH3COOH) by intraperitoneal injection. The number of abdominal contortions was counted in each mouse for 30 min. The analgesic effect of the plant extract and paracetamol was evaluated by determining the percentage of inhibition of contortions according to the following formula:

$$\%\ \mathbf{d'inhibition} = \frac{\mathbf{Mtb} - \mathbf{Mext}}{\mathbf{Mtb}} \times \mathbf{100}$$

Mtb: Average number of contortions of mice from the white control group
Mext: Average number of contortions of mice treatedwithplantextract and paracetamol

Anticoagulant test

The study of anticoagulant activity was carried out following the method described by De Freitas and al. [12]. Different concentrations (5, 4, 3, 2 and 1 mg/mL) of the methanol extract of M. benthamianum and escin (triterpene saponin), were prepared in 0.85% NaCl physiological solution (m/v). The pure NaCl solution constituted the negative control while escin was used as a positive control. These different solutions were subsequently mixed in EDTA tubes with 25 µL of fresh blood from sheep fasted overnight. The mixture was homogenized, incubated for 30 min at 37°C and centrifuged at 3500 rpm for 10 min. Duringcentrifugation, erythrocyte lysis may or may not be observed. The absorbance of the supernatants was measured at 540 nm. The absorbance values as a function of the NaCl concentration enabled to draw a sigmoidal regression curve given by Boltzmann formula, by means of which the percentage of hemolysis of each extract was calculated according to the following formula:

$$\textbf{Hemolysis}\ (\%) = \frac{\mathbf{A1} - \mathbf{A2}}{\mathbf{1} + \mathbf{e}^{\frac{(\mathbf{S}-\mathbf{H50})}{\mathbf{ds}}}} + \mathbf{A2}$$

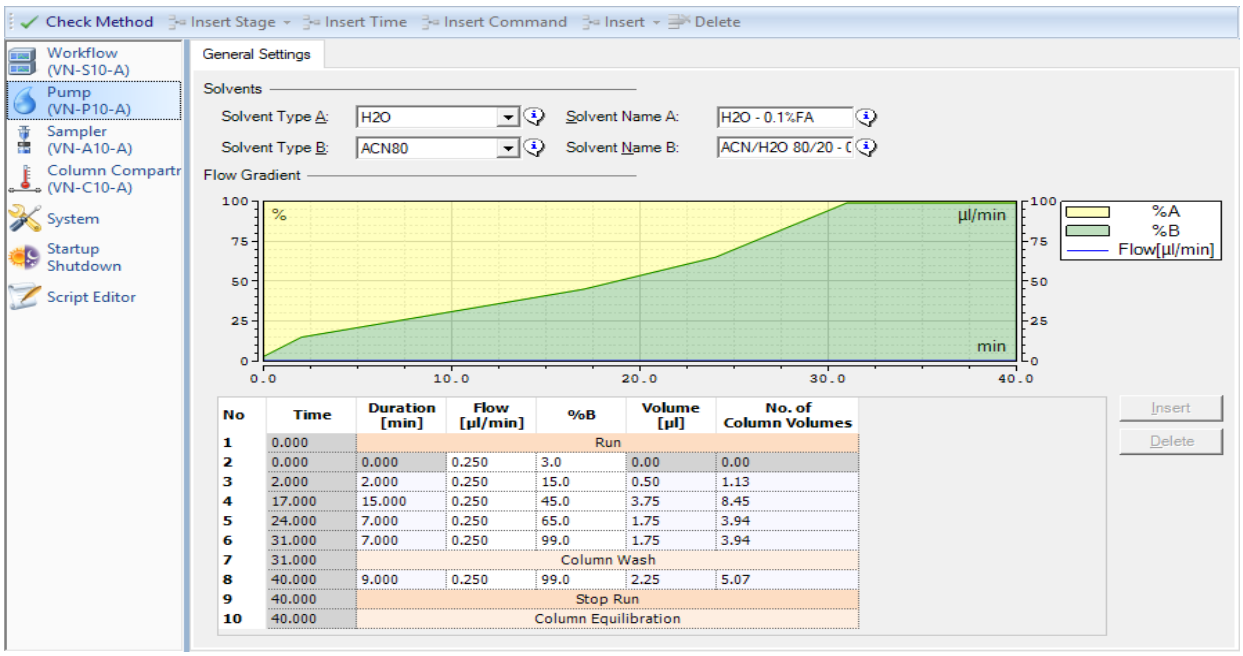
A1: Maximum average absorption values of the sigmoid curve;
A2: Minimum average absorption values of the sigmoid curve;
S: NaCl concentration;
H50: NaCl concentration that causes 50% of hemolysis;
ds: amplitude of the sigmoid curve of the transition between A1 and A2

Toxicity test

The toxicity study was realized in accordance with OECD Test Guideline 423 for chemical testing **[13]**. Mice in batches of three (3) were acclimated to the experimental environment in clean, well-ventilated cages for one week before experimentation. During this period, the animals were monitored daily to assess their mass and behaviour. Four (4) batches of these mice, previously fasted for 16 h, were administered 1 mL of *Mezoneuronbenthamianum* plant extract by gavage by administration of variable doses ranging from 300 to 2000 mg/kg bw. After administration of the extracts, the animals were observed for 14 days. The 50% lethal dose (LD50) was determined from the mortality rate of mice.

LC-MS analysis

The samplewasanalyzed in nanoLCMS (nLCMS) mode on a Thermo ScientificOrbitrapExploris 480 spectrometer in positive and negativeElectrospray modes. The crudemethanolicextract of *Mezoneuronbenthamianum*wassolubilized in the HPLC grade H_2O/CH_3CN (95/5) solvent mixture. The analyses wereperformed on a quantity of 300 ng of materialdeposited on the PepMapNeoC18 5**µ**m 300**µ**m x 5 mm / EASY - Spray column PepMapNeo 1500 bars, 75 µm x 150 mm, C18, 2 µm, 100 Å. Positive and negative mode analyses wereperformed over a 40-minute runwith the following instrumental and gradient parameters: CRMPO-PJ-P-480k-40min-100-1500-2000V-SL75-Gradient 2 - Trap and Elute – BackFlush.The interpretations for the positive and negative modes were carried out on a technical triplicate.



No	Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
1	0.000	Run				
2	0.000	0.000	0.250	3.0	0.00	0.00
3	2.000	2.000	0.250	15.0	0.50	1.13
4	17.000	15.000	0.250	45.0	3.75	8.45
5	24.000	7.000	0.250	65.0	1.75	3.94
6	31.000	7.000	0.250	99.0	1.75	3.94
7	31.000	Column Wash				
8	40.000	9.000	0.250	99.0	2.25	5.07
9	40.000	Stop Run				
10	40.000	Column Equilibration				

Figure 1 : Gradient used for analyzes in positive and negative Electrospray modes

Statistical analyzes

The analyzes of the measurements obtained during the anticoagulant test were carried out using the GRAPH PAD PRISM software. This software made it possible to determine H_{50} and dSparameters.

III. RESULTS AND DISCUSSION

Analgesic test

Figure 1 highlights the effect of the methanolic extract of *Mezoneuronbenthamianum* root bark on abdominal contractions. The pain inhibition percentage of *M. benthamianum* extract is 47.92% while that of paracetamol is 100%.

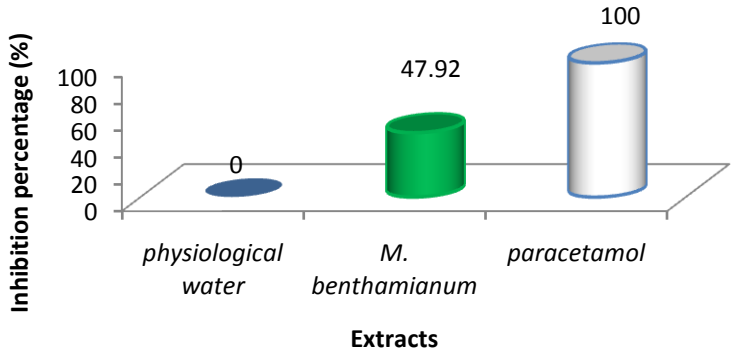


Figure 2 : Analgesiceffect of methanolicextract of *M. benthamianum*roots on abdominal contortions

Anticoagulant test

Figure 2 presents the anticoagulant effect of the methanolic extract of *M. benthamianum* root bark and the negative and positive controls. The hemolysis percentages of the root bark of *M. benthamianum* vary between 10.88±0.26 and 17.19±0.43% while those of escin oscillated between 21.6±0.84 and 41.13±0.97%. As for the negative control (physiological NaCl solution), it indicated a hemolysis percentage of 4.47%.

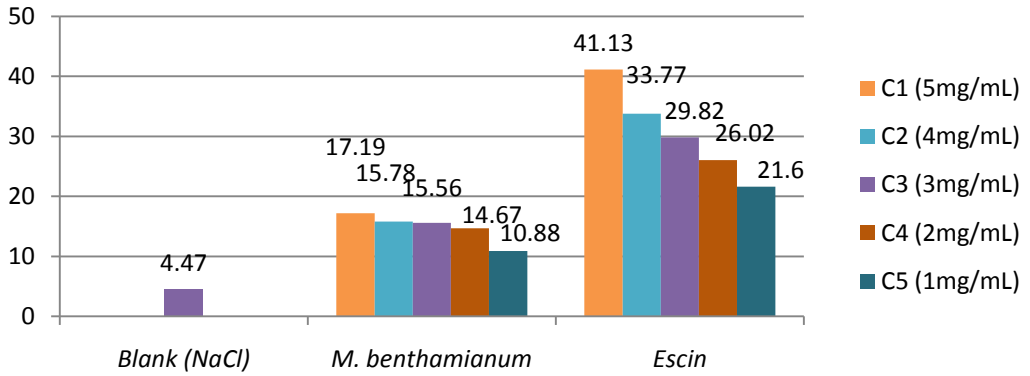


Figure 3: Anticoagulant effect of *M. benthamianum* extract, escin and NaCl at different concentrations

Toxicity test

The results of the toxicity test are recorded in Table 1. This table shows the mortality rate of mice at the different doses of *M. benthamianum*methanolic extract injected. The mortality rate was then used to determine thelethal dose (LD50) of the methanolic extract of the root bark of the plant studied, which is between 500 and 2000 mg/kg bw.

Table 1 : Mortality rates of mice as a function of administered doses of *M. benthamianum*methanolextract

Batch mice		Batch 1	Batch 2	Batch 3	Batch 4
Dose administered (mg/kg PC)		300	300	2000	2000
Number deaths per day	1st day	0	0	0	1
	2nd day	1	0	0	0
	3rd day	0	0	0	0
	4th day	0	0	0	0

	5^{th}day	0	0	0	0
	6^{th}day	0	0	0	0
	7^{th}day	0	0	0	0
	8^{th}day	0	0	0	1
	9^{th}day	0	0	0	0
	10^{th} day	0	0	0	0
	11^{th}day	0	0	0	0
	12^{th}day	0	0	0	0
	13^{th}day	0	0	0	0
	14^{th}day	0	0	1	0
Mortality		1	0	1	2
Mortality rate (%)		33.33	0	33.33	66.66

LC-MS analysis

The chromatographic profiles of *Mezoneuronbenthamianum*extract in positive and negativeelectrospray modes are shown in Figures 4 and 5, respectively. A large number of compounds werehighlighted in each of the twochromatographic profiles of the extract.

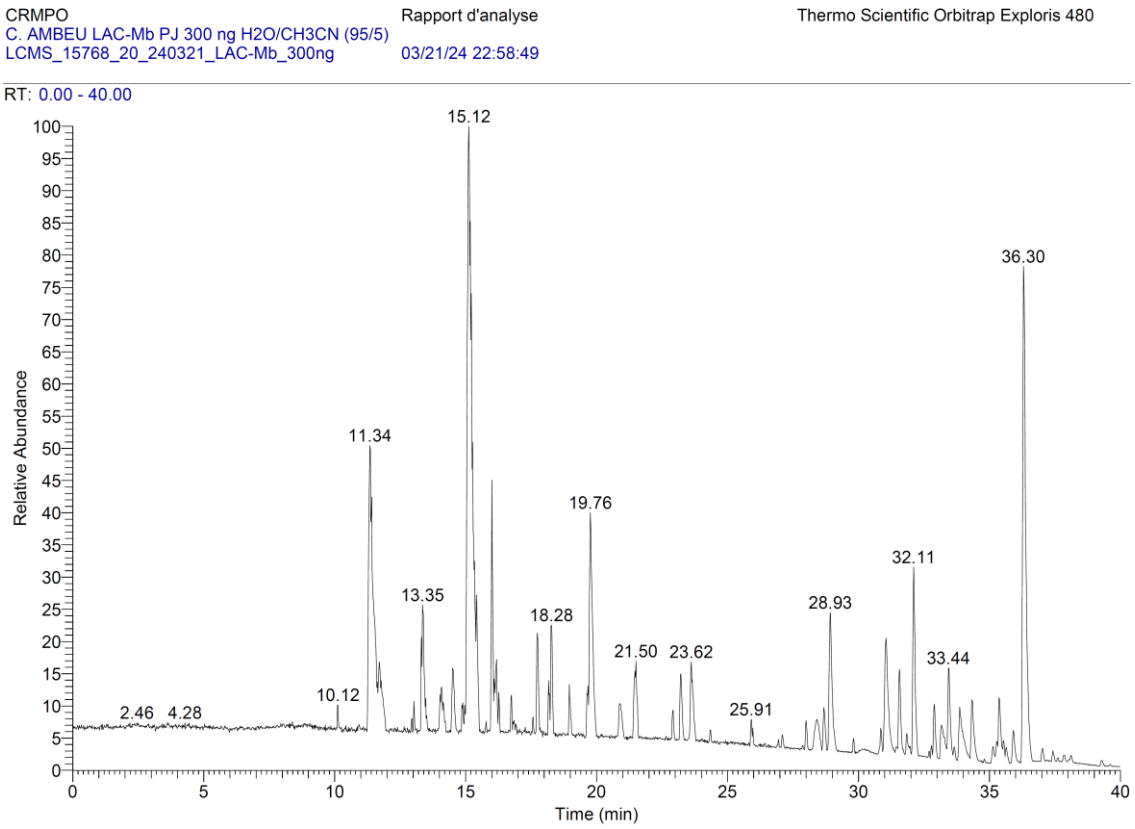


Figure 4 : Chromatographic profile of *M. benthamianum*extract in positive mode

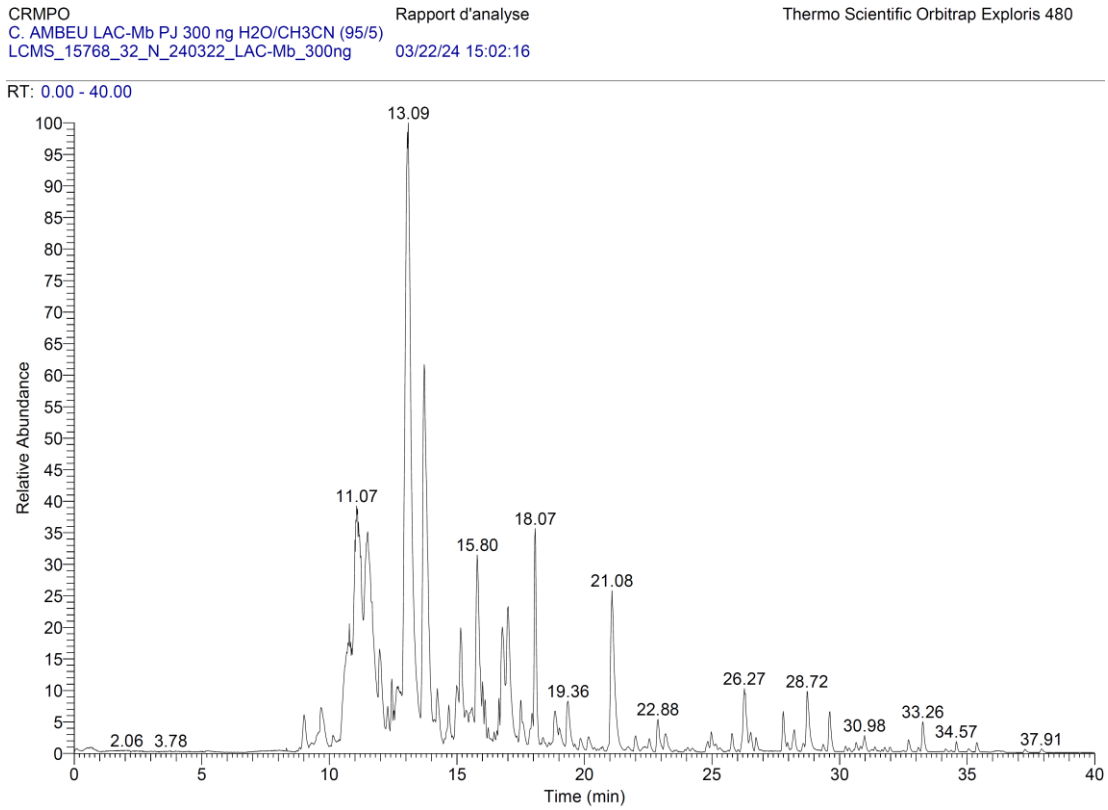


Figure 5 : Chromatographic profile of *M. benthamianum*extract in negative mode

The formulas of several ions contained in the sample have been highlighted (see supplementary data). The choice was made for the most intense ions, i.e. the most easily ionizable. The LCMS runs were performed at the highest experimental resolution of the Exploris 480 (480000 at mass m/z 200) in order to remove any ambiguity relative to the validation of the raw formula of each ion concerned. In addition, the proposed formulas were validated by modeling theoretical isotopic massifs at experimental resolution considering a maximum experimental error of 2 ppm. From these initial data, a second series of LCMSMS recordings was made in DDA (Data Dependent Acquisition) mode in both polarities in order to access as far as possible the fragmentation spectra of a large number of species. In order to validate the structure of potentially present compounds, the exploitation of these experiments was carried out by comparing the experimental MSMS spectra with those contained in two databases, MassBank (https://massbank.eu/MassBank/) and mzCLOUD (https://www.mzcloud.org/). Thus, six (6) compounds were identified in positive and negative electrospray modes (Tables 2, 3 and Figure 6). Information on these compounds is reported in Tables 2 and 3, and their molecular structures are shown in Figure 6.

Table 2: Possible compounds in *M. benthamianum* extract in positive mode

Retention time (min)	**Exact mass of ion $[M+H]^+$**	**Formula of compound**	**Main fragments of the $[M+H]^+$ ion observed in MSMS**	**Database m/zCLOUD**	**Possible compounds**
13.35	479.0820	$C_{21}H_{18}O_{13}$	-	461.0716, 309.0605, 153.0181	3,4-Di-O-galloylshikimic acid
33.44	250.1186	$C_{12}H_{15}N_3O_3$	81.0699, 79.0542, 69.9923, 56.0131	-	2,4,6-Tris(allyloxy)-1,3,5-triazine

Table 3: Possible compounds in *M. benthamianum* extract in negative mode

Retention time (min)	**Exact mass of ion $[M-H]^-$**	**Formula of compound**	**Main fragments of the ion $[M-H]^-$ observed in MSMS**	**Database m/zCLOUD**	**Possible Compounds**
11.07	484.0859	$C_{20}H_{20}O_{14}$	423.0569, 331.0671, 313.0565, 271.0461, 241.0356, 211.0249, 193.0143, 169.0143, 125.0244	331.0666, 313.0561, 271.0456, 241.0349, 211.0245, 169.0139, 125.0241	1,6-bis-O-galloyl-beta-D-glucose

13.72	457.0776	$C_{22}H_{18}O_{11}$	305.0667, 169.0142, 125.0243	-	Epigallocatechine gallate
13.72	635.0890	$C_{27}H_{24}O_{18}$	591.0986, 483.0760, 465.0671, 423.0566, 313.0566, 169.0143	591.0986, 483.0774, 465.0668, 439.0877, 423.0565, 331.0667, 313.0561, 295.0456, 169.0139	1,4,6-tri-O-galloyl-beta-D-glucose
21.08	227.0714	$C_{14}H_{12}O_3$	185.0607, 143.0501	-	Resveratrol

3,4-Di-O-galloylshikimic acid

2,4,6-Tris(allyloxy)-1,3,5-triazine

1,6-bis-O-galloyl-beta-D-glucose

Epigallocatechinegallate

1,4,6-tri-O-galloyl-beta-D-glucose

Resveratrol

Figure 6: compounds identified in *M. benthamianum* extract

IV. DISCUSSION

The present study consisted of analgesic, anticoagulant, toxicity tests and LC-MS spectral analysis of the methanolic extract of *M. benthamianum* root bark. The analgesic test showed that the extract significantly inhibited pain (47.92±0.34%) compared to paracetamol (100±0.00%). This pharmacological suitability of *M. benthamianum*root bark could be justified by its phytochemical composition. This organ contains sterols,

terpenes, saponins, flavonoids, coumarins, tannins, phenolic acids and alkaloids **[3,17]**.Several studies have shown the analgesic effects of some of these secondary metabolites. This is the case for terpenes [**18**], flavonoids [**19**], saponins [**20**], alkaloids **[21]** and coumarins **[22]**. These bioactive phytocompounds inhibit the release of mediators (serotonin, acetylcholine, histamine, bradykinin, substance P and prostaglandins ($PGE_{2\alpha}$, $PGF_{2\alpha}$)) involved in the pain process. Similarly, the anticoagulant activity of that extract observed at a haemolysis percentage of 17.19±0.43% is also due to the co-presence of those secondary metabolites. Indeed, bioactive phytocompounds, such as saponins and coumarins, have anticoagulant properties that are widely described in several studies **[20, 23, 24]**. The manifestation of the anticoagulant effect of the methanol extract of *M. benthamianum* root bark could explain its use in the traditional treatment of hemorrhoidal pathology **[17]**. Concerning the toxicity test, the treated mice showed some clinical signs such as increased heart rate, breathing difficulties and seizures. However, the animals that survived returned to a normal appearance in the following days. In addition, the death of a few mice was observed after administration of doses between 300 and 2000 mg/kg bw. As a result, according to the OECD guideline **[13]**, *M. benthamianum* root bark would be classified as Category 4, with an LD_{50} ranging from 500 to 2000 mg/kg bw. Thus, according to the Hodge & Sterner toxicity scale **[25]**, *M. benthamianum* root bark had low toxicity, thus the justification for its recurrent use in traditional medicinal practice. Positive and negative analysis of the methanolic extract of *M. benthamianum* root bark resulted in the structural identification of six compounds. This result subsequently led to the search for their potential biological activities. A systematic review of the literature to support the biological activity of 3,4-Di-O-galloylshikimic acid did not lead to conclusive results. However, the other five molecules identified contain many pharmacological properties. These are 2,4,6-Triallyloxy-1,3,5-triazine, resveratrol, epigallocatechin gallate, 1,4,6-tri-O-galloyl-beta-D-glucose and 1,6-bis-O-galloyl-beta-D-glucose ; most of them are polyphenols and thus of great therapeutic interest currently.

- 2,4,6-Triallyloxy-1,3,5-triazine is a natural product with antimicrobial activity whose bioactivity has been validated by molecular docking of four antibacterial proteins **[26]**.
- Resveratrol is a polyphenol of the stilbene class found in some plants and also in some fruits **[27]**. It is a substance induced by environmental or pathogenic stress and intended to contain the damage of the pathogen locally **[28]**. It has an antiproliferative effect on cultured cancer cells **[29]** and decreases inflammation in macular degeneration, an age-related vision pathology **[30]**. Resveratrol is also known as an insulin-sensitizing agent and resistance to weight gain, which also mimics the benefits of physical activity on the remodeling of muscle fibers while increasing resistance to exercise **[31**].
- Epigallocatechin gallate is a flavanol formed by esterification of epigallocatechin and gallic acid. It is the most abundant flavanol in tea and is known to be a powerful antioxidant **[32,33]**. It plays a chemopreventive role in ovarian cancer through the regulation of molecular pathways that induce the stem phenotype of ovarian cancer stem cells **[34]**.
- 1,4,6-tri-O-galloyl-beta-D-glucose and 1,6-bis-O-galloyl-beta-D-glucose are two compounds called galloylglucoses. Galloylglucoses that belong to the phenolic acid derivatives and the subclass of gallotannins, are composed of halves of glucose and gallic acid by esterification or O-acylation, and can be divided into mono-, di-, tri-, tetra-, penta-galloylglucose and so on, depending on the number of gallic acid groups. They are considered as a natural defense against herbivores. Over the past few decades, studies have revealed the many biological properties of these compounds. They contain antioxidant, antitumor, anti-inflammatory, antibacterial properties and cardiovascular protective effects, among other properties **[35]**. Thus, the presence of these different organic compounds in the root bark of *M. benthamianum* could also justify its common use in traditional medicine.

V. CONCLUSION

In order to enhance the value of the Ivorianflora in general and in particular to provide a rational response to the traditional use of *Mezoneuronbenthanianum*, biological investigations (analgesic, anticoagulant and toxicityactivity) and chemical investigations (LC-MS analyses) werecarried out on the rootbark of the said plant. Biological tests showedthat the rootbark of *M. benthamianum*showed a significantanalgesiceffect (47.92%) compared to paracetamol (100±0.00%). The non-negligiblehemolytic power of thisorganwasnotedwithlevelsbetween 10.88±0.26 and 17.19±0.43%. The lethal dose of between 500 and 2000 mg/kg bwprovesthat the rootbark of *M. benthamianum*is of lowtoxicity and thereforedoes not presentany major danger to the body. The phytochemicalanalysis by LC-MS of the methanolextract of the rootbark of *M. benthamianum*highlighted the presence of six phytocompounds, amongwhichpharmacologicalproperties of five werehighlighted. These are 2,4,6-Triallyloxy-1,3,5-triazine, resveratrol, epigallocatechin gallate, 1,4,6-tri-O-galloyl-beta-D-glucose, and 1,6-bis-O-galloyl-beta-D-glucose. On the one hand, the biological and phytochemical analyses carried out on *M. benthamianum*constitute a contribution to the valorization of the Ivorianspecies and on the other hand, justifyits use in traditionalmedicinal practice againstsome pathologies.

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