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Phytomolecules of *Terminalia macroptera* stem bark extracts Guill et Perr (*Combretaceae*) and antibacterial activity

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Abstract
Today, the phenomenon of multidrug resistance is growing and makes inefficient use of many antibiotics on the market. Faced with this therapeutic impasse, the Scientific World will invest the field of plants, in search of new molecules. It is in this context that the study on *Terminalia maccroptera* was initiated.
The main objective of this study is to characterize the different phytomolecules of the 70% ethanolic extract of *Terminalia macroptera* by GC-MS and to identify the molecules susceptible to antibacterial activity.
Tri phytochemistry was performed using the tube reagent method for determining chemical components and gas chromatography-mass spectrometry (GC-MS) to characterize the existing phytomolecules in the extract.
The tri-phytochemical study showed the chemical groups: Poly-phenols, Flavonoids, saponins, tannins gallic, Cardiac glycosides, sterols and terpenes. The GC-MS has firstly confirmed the great chemical groups, and also identified 20 molecules accompanied by their mass spectra, their molecular weight and their chemical structures.
Among these molecules Beta-sitosterol is the most important in ethanol extract70%. It belongs to the family of triterpenes (known for their antibacterial activity). This molecule is therefore responsible for the antibacterial activity of our extract.

Keywords: terminalia macroptera- 70% ethanolic extract- phytomolecules- antibactérial activity

Introduction
Faced with the growing phenomenon of the emergence and re-emergence of diseases around the world, developing countries are the most vulnerable. Thus, infectious diseases are an important public health concern because of their frequency and severity in these countries [1]. The agents responsible for these infections are diverse and varied including fungi, bacteria, protozoa and viruses. To combat these microbial attacks, the scientific world has discovered numerous treatments to relieve patients [2]. However, the acquisition of these drugs is extremely difficult because of high costs, and makes access to medical care obsolete for the poor. This situation has led people to always resort to traditional medicine. Indeed, plants have been used since prehistory by humans for nutritional and therapeutic purposes and are the major source of drugs because of their high content of secondary metabolites [3, 4, 5]. In addition, the World Health Organization (WHO) estimated in 2007 that about 80% of the population in developing countries could be treated with plants [3, 5]. In 2001, several studies including those of Rate revealed that approximately 25% of prescription in the world for the drugs various to 60 and 70%
Antibacterial and anticancer substances are substances of natural origin [6]. Also, the frequent use of these molecules has led to the appearance of multi-resistant microorganisms, making treatments ineffective. With this constant, the scientific community has turned to natural substances including medicinal plants in the optics to find new molecules that will not only help fight against microbial diseases efficiently but also to value traditional medicine. Naturally, our choice was towards *Terminalia macroptera*, a *Combretaceae*, used by people in the north and central Ivory Coast, in the traditional treatment of gastroenteritis, dermatitis, hepatitis and other pathologies [2]. Also this plant deserves more research, because the combination of formulation based on *Terminalia macroptera* with antibiotics could prove useful to study. Indeed, corilagin (an ellagitanin) present in the leaves, activates the effect of beta-lactam antibiotics against resistant strains of methicillin [7]. The main objective of this study is to characterize by GC-MS the different phytomolecules of the 70% ethanolic extract

of *Terminalia macroptera* and to identify the molecule (s) susceptible to antibacterial activity.

Materials and Methods

Plant material

Plant Material It consists of bark *Terminalia macroptera* Guill. et Perr. (*Combretaceae*). These barks were collected in April 2016 in Niakara (north of Ivory Cost). Their authentication was performed by professor Ake-Assi of National Center Floristic (NCF), University Felix Houphouet Boigny of Cocody-Abidjan where a sample is retained.

Preparation of plants extracts

The stem barks of T. macroptera collected were washed cut and has been dried shelter powder by a type IKAMAGRCT grinder. According to the methods described by [8, 9], 100 g of plant powder have been macerated in 1 L of distilled water then homogenized under magnetic agitation for 24 hours at 25° C with a IKAMAG-RCT type agitator. The homogenate obtained, has been filtered successively two times through hydrophilic cotton (cotton wool) then once through whattman paper n°2. The volume of filtrate obtained is first reduced with a rot vapor Büchi at 60° C. Then, the rest of the filtrate is evaporated with a med center vent cell drying oven at 50° C to give a brown powder which is the aqueous extract. The same process was carried out by using ethanol 70% instead of distilled water to obtain ethanolic extract 70% [10]. The end, all the plant extracts obtained are kept in refrigerator until used for testing.

Phytochemical analysis

The phytochemical analysis of the different extract of Terminalia macroptera have based on the coloration and precipitation test [10, 11].

Test for alkaloids 0.5 g of extract was diluted into 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggen dorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggen dorff's reagent) was regarded as positive for the presence of alkaloids.

Test for polyphenols and tannins About 0.5 g of the extract was boiled into 10 ml of water in a test tube and then filtered. A few drops of 0.1% of ferric chloride was added and observed for brownish green or a blue-black coloration.

Test for terpenoids (Salkowski test) To 0.5 g each of the extract was added 2 ml of chloroform. Concentrated H2SO4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Test for glycosides: Extracts was treated with 2 ml of glacial acetic acid, add 1 drop of FeCl3 and 1 ml of concentrated H2SO4 appearance of brown coloration indicates the glycosides.

Test for flavonoids: Three methods were used to test for flavonoids. First, dilute ammonia (5ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1ml) was then added. A yellow coloration that disappears on standing indicates the presence of flavonoids. Secondly, a few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow coloration indicates the presence of flavonoids. Next, a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration indicates the presence of flavonoids.

Test for saponins To 0.5 g of extract was added 5 ml of distilled water in test tube. The solution was shaken and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken after which it was observed for the formation of an emulsion.

Fehling's test: Filtrates were hydrolysed with dil. HCL neutralized with alkali and heated with fehling's A and B solution. Formation of red precipitate indicates the presence of reducing sugars.

Test for steroids and terpenoids: 9 ml of ethanol was added to 1 g each of the extracts and refluxed for a few minute and filtered. Each of the filtrates was concentrated to 2.5 ml in a boiling water bath. Distilled water, 5ml was added to each of the concentrated solution, each of the mixtures was allowed to stand for 1 h and the waxy matter was filtered off. Each of the filtrates was extracted with 2.5 ml of chloroform using a separating funnel. To each 0.5 ml of the chloroform extracts in a test tube was carefully added 1 ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface showed the presence of steroids. To another 0.5 ml each of the chloroform extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 min on a water bath. A grey color indicates the presence of terpenoids.

Gas chromatography coupled to the mass spectrometer (GC-MS)

3 mg of extract are homogenized in 1 ml of ethyl acetate contained in a test tube. The whole is sonicated for 30 minutes (ultrasonic bath), then spun on acrodix 0.2 microns. The filtrate obtained is evaporated to dryness at 80.degree. C. on a rotary evaporator. To the dried filtrate are added 100 µL of Bistrimethylsilyl trifluoroacetamide (BSTFA). The whole is incubated at 80.degree. C. for 45 minutes and then evaporated to dryness at 80.degree. C. on a rotary evaporator. The residue is then taken up in 1.5 mL of HPLC methanol, of which 1 µL is injected into the gas chromatograph. Silylated molecules are thus obtained.

The analysis of the silylated sample was performed on a gas chromatograph coupled to a Perkin Elmer mass spectrometer, model Clarus 680 CG / 600 SM, equipped with a high sensitivity quadrupole filter and software TURBOMASS version 5.4.2 for data exploitation. A fused silica capillary column was used (Rtx-5, 30 × 0.25 mm × 0.25 mm), with a stationary non-polar phase of 5% phenyl methylpolysiloxane. The carrier gas is helium with a flow rate of 1 mL / min. The injection was done in Splitless mode. The injection temperature is 280◦C and the oven temperature is programmed as follows: isotherm 65◦C for 2 min and increased at a rate of 6◦C / min up to 300◦C for a total duration of 62 min.

The ionization mode is the electronic impact. The ionization voltage is 70 electron volts at a temperature of 250 ° C. The

mass spectrometer is connected to a computer system managing a mass spectrum library (TURBOMASS version 5.4.2), and the identification of the constituents of the ethanolic extract was based on their mass spectrum. The mass spectra obtained are compared with those of the mass spectrum library.

Statistical analysis

The data are presented as mean ± S.E.M. All the data were analyzed by one-way ANOVA and differences between the means were assessed with Newman-Keuls Multiple comparison test. Differences were considered significant at $p < 0.05$. All analyses were carried out using Graph pad software, version 5.01 (USA).

Results

Phytochemical screening The phytochemical screening of T.macroptera stem bark extract, using different standard tests shown in table 1.revealed that the aqueous extract showed the presence of polyphenolic compounds, flavonoids, saponins, galic tannins, cardiotonic glucosids. but alkaloids, sterols, terpens, coumarins and cathechic tannins were absents. Also table 1, showed that ethanolic extract contains all the compound of aqueous extract with the presence of sterol and terpens**.**

Table 1: Phytochemical constituents of aqueous and ethanolic70% extracts of stem bark *T. macroptera*

Chemical class	Alc	Polyph	Flav	ST	TGal	TCat	Coum	Sap	Glc
Aqueous extract	-	+	+	-	+	-	-	+	+
Ethanolic70%e extract	-	+	+	+	+	-	-	+	+

Key: Alc: Alcaloides, Polyph: Polyphenols, Flav: Flavonoides, ST: Sterols &Terpens, TGal: Tanin Galique, TCat: Tanin Catechique, Coum: Coumarine, Sap : Saponosides, GlC : Glucosides cardiotoniques

Phytochemical screening The phytochemical screening of *T. macroptera* stem bark extract, using different standard tests shown in Table 1.revealed that the aqueous extract showed the presence of polyphenolic compounds, flavonoids, saponins, galic tannins, cardiotonic glucosids. but alkaloids, sterols, terpens, coumarins and cathechic tannins were absents. Also Table 1, showed that ethanolic extract contains all the compound of aqueous extract with the presence of sterol and terpens.

Chromatogram and mass spectra of different molecules from ethanol extract70%

The GC-MS chromatographic study of the 70% ethanolic extract gave the chromatogram of Figure. 1.

The peaks of the chromatogram of the ethanolic extract are compared with those of the reference compounds present in the data bank of the spectra library.

The analysis of the chromatogram reveals more than 30 separate compounds, 3 of which have been identified as major compounds, given the intensity of their peaks. These 3 compounds appeared at respective retention times (TR) of 24.72 min, 27.45 min and 41.75 min. In addition, the analysis of these major compounds according to the database of the library identifies them respectively to palmitic acid with an area (A) equal to 9.619 to 285 IU, to oleic acid (A = 18900540 IU). And beta-sitosterol (A = 39723684 IU) (Table II).

In addition, about 30 separate compounds were identified by comparing their mass spectra with those of the spectra library (Figs. 2-20). of the 20 compounds, 3 have been identified as phenolic compounds, which are:

Isoeugenol (TR = 15.42 min and A = 169737 IU)

3, 5-dihydroxy-1-methyl-4-methylbenzoate (TR = 19.56 min and A = 164172 IU),

-4 ((1E) 1-propenyl 4-hydroxy) 5-methoxyphenol (TR = 20.96 min and A = 250798 IU).

Also, 7 of the 20 compounds were identified as belonging to the family of fatty acids and their esters (R-COOH). It is also possible to classify the compounds in two subgroups according to the presence or absence of a double bond on their carbon chain structure (FIGS. 7, 8, 9, 10, 11, 12, and 13). The saturated fatty acid group thus obtained comprises: palmitic acid methyl ester (TR = 24.11 min and A = 1127120 IU), palmic acid (TR = 24.72 min and A = 9619285 IU) and stearic acid (TR = 27.77 and A = 3499047 IU). Then the group of unsaturated fatty acids which lists the following compounds: methyl ester linoleic acid (TR = 26.76 min and A = 998679 IU), methyl ester oleic acid (TR = 26.85 min and A = 1182073 IU), acid linoleic (TR = 27.37 min and A = 5765251 IU) finally oleic acid (TR = 27.45 min and A = 18900540 IU).

With the exception of vitamin E (TR = 39.77 min and A = 4162491 IU) and diisooctyl phthalate (TR = 33.05 min and A = 261726 IU), all other compounds were identified as triterpenoid compounds. These are: stigmasterol (TR = 41.12 min and A = 6643677 IU), beta-sitosterol (TR = 41.75 min and A = 39723684 IU), beta-amyrin (TR = 42.16 min and A = 3282221 IU), lupeole (TR = 42.71 min and A = 6141556 IU), friedelin (TR = 44.50 min and A = 3411292 IU) and finally betulin (TR = 44.89 min and A = 4195372 IU).

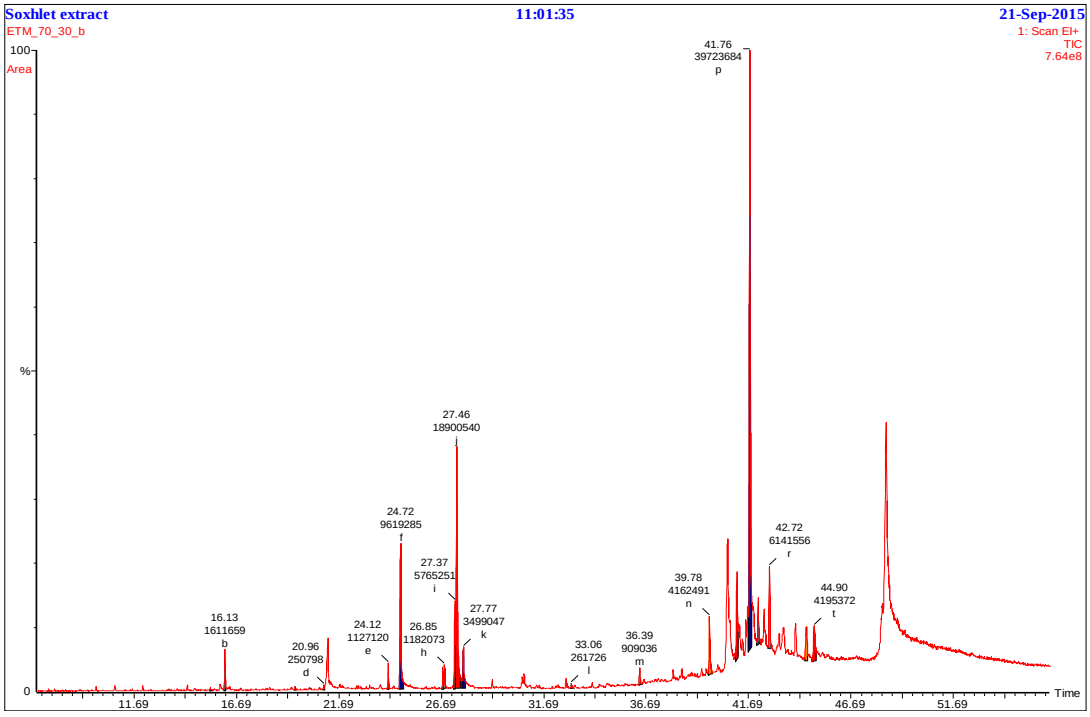

Fig 1: GC-MS chromatogram of 70% ethanol extract.

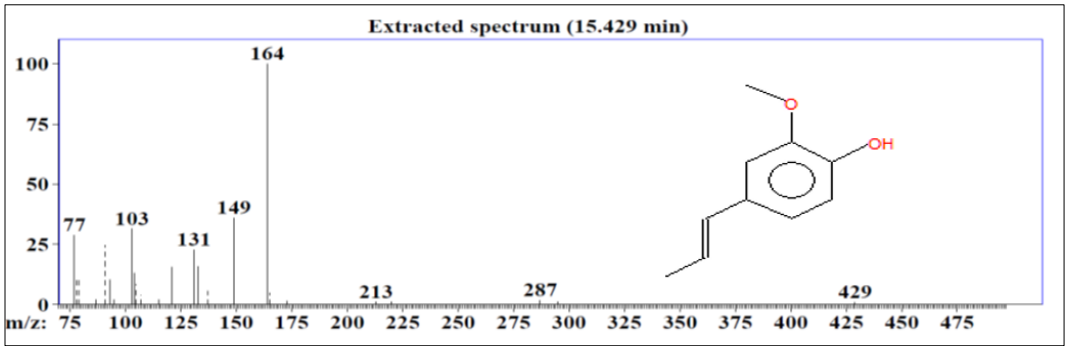

Fig 2: Mass spectrum and structure of isoeugenol.

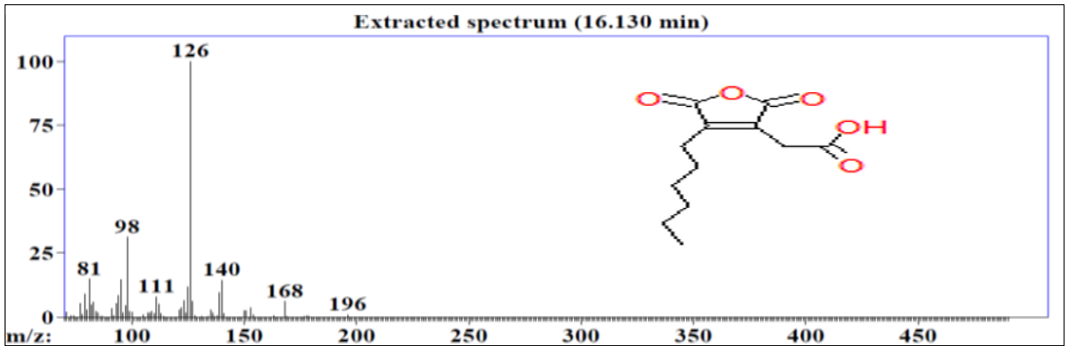

Fig 3: Mass spectrum and structure of 2, 5-dioxo-4-hexyl-3-acid furanacetic

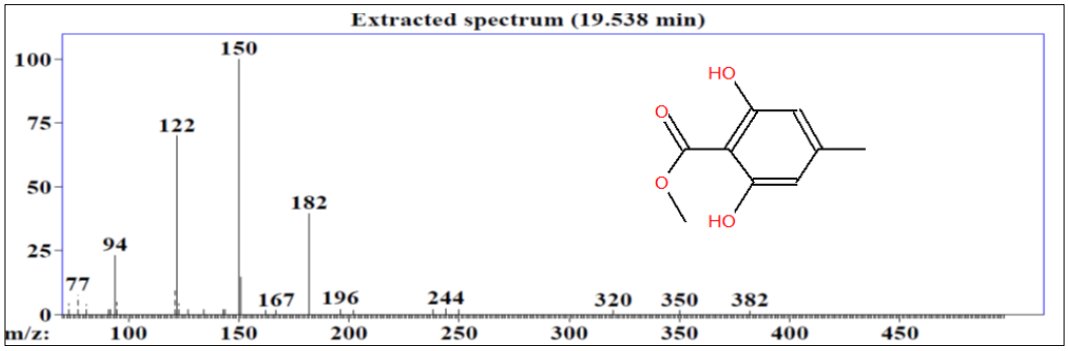

Fig 4: Mass spectrum and structure of 3, 5-dihydroxy-1-methyl-4-methylbenzoate.

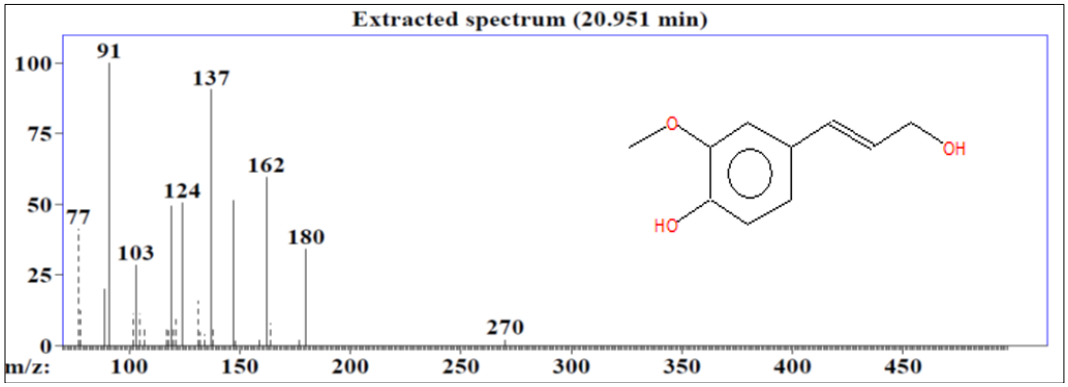

Fig 5: Mass spectrum and structure of 4 - ((1E) 1-propenyl 4-hydroxy) 5-methoxphenol.

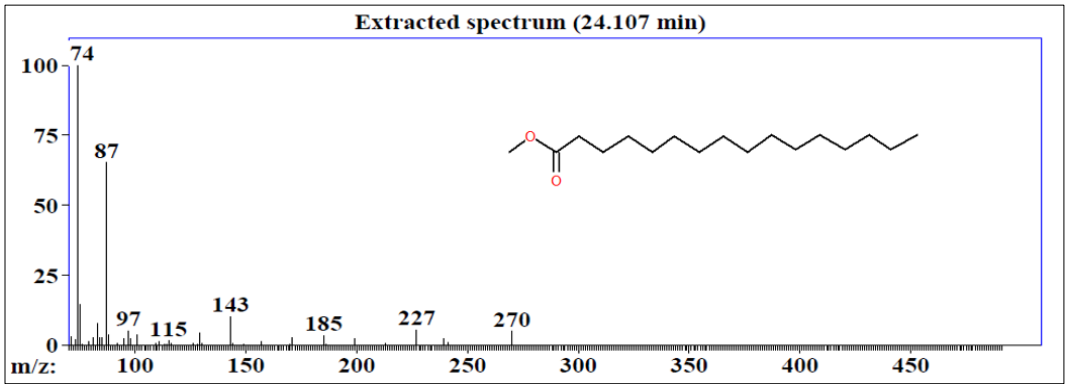

Fig 6: Mass spectrum and structure of the methyl ester of palmitic acid

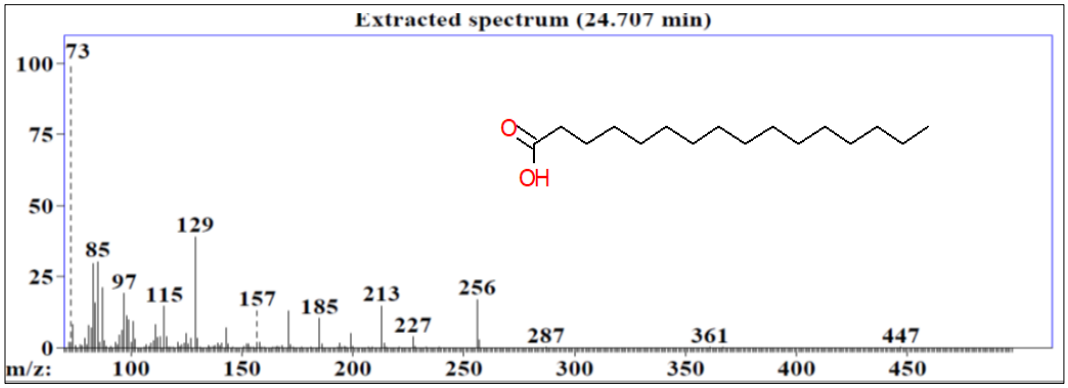

Fig 7: Mass spectrum and structure of palmitic acid

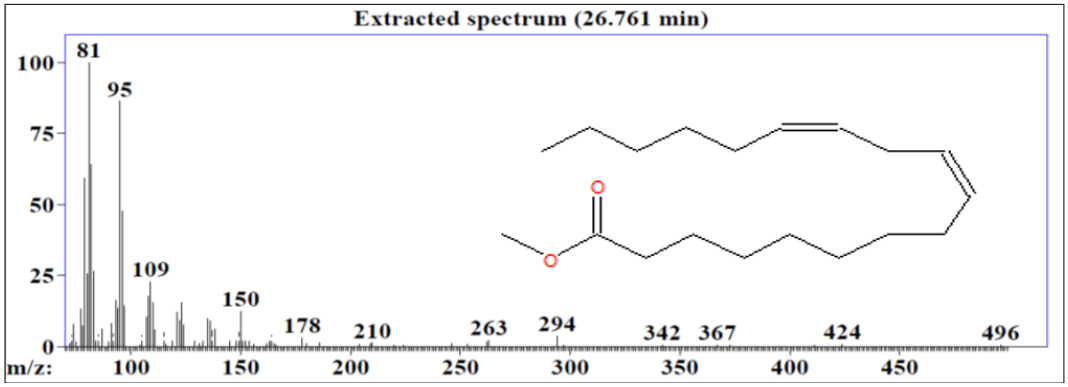

Fig 8: Mass spectrum and structure of the methyl ester of linoleic acid

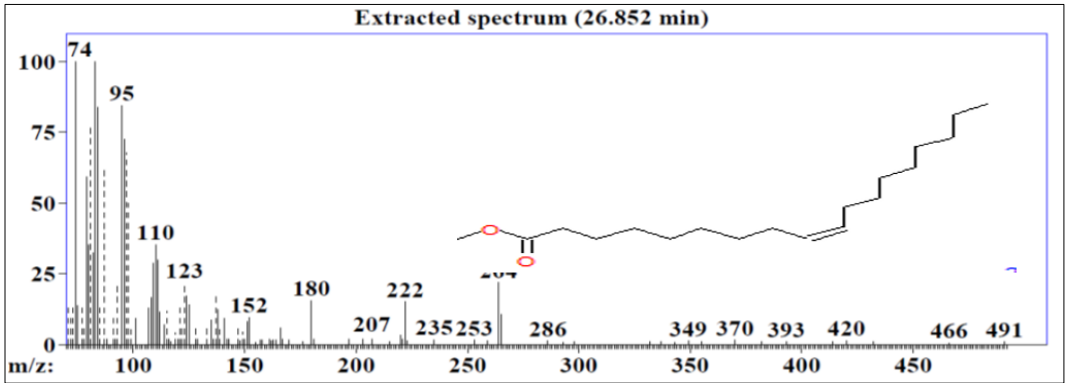

Fig 9: Mass spectrum and structure of the methyl ester of oleic acid

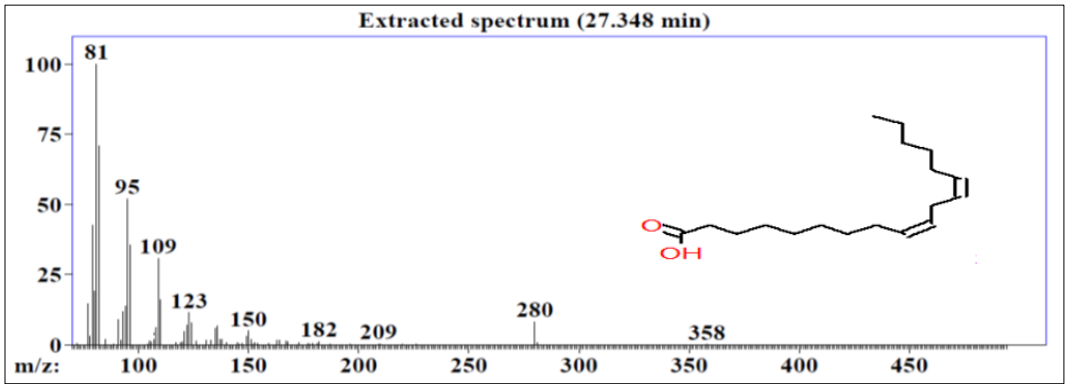

Fig 10: Mass spectrum and structure of linoleic acid

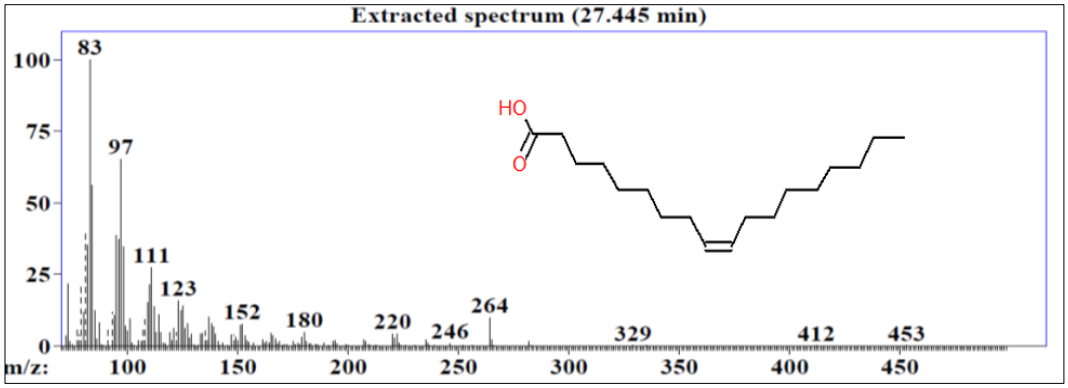

Fig 11: Mass spectrum and structure of oleic acid

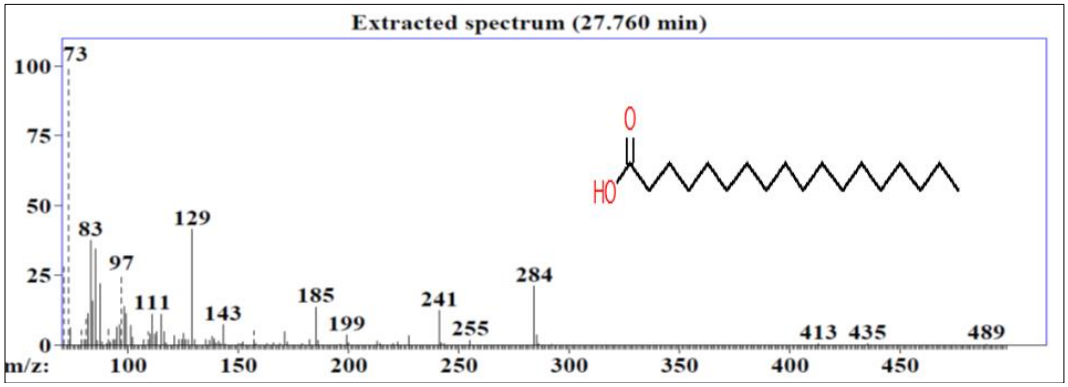

Fig 12: Mass spectrum and structure of stearic acid

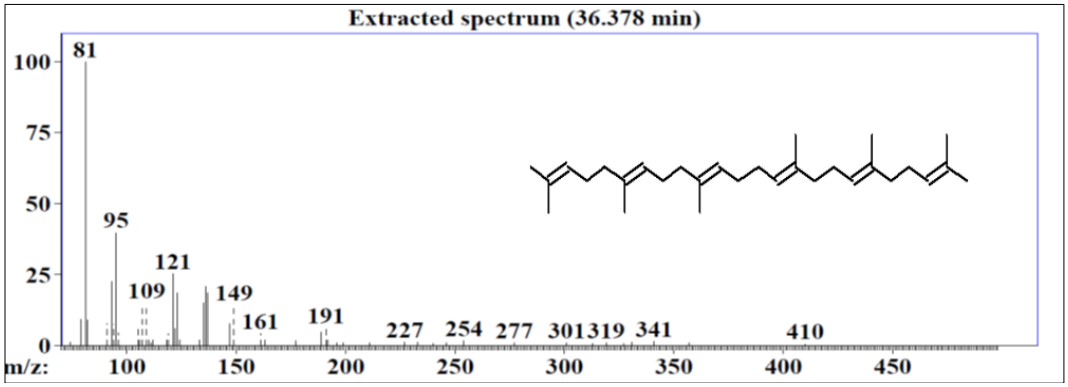

Fig 13: Mass spectrum and structure of squalen

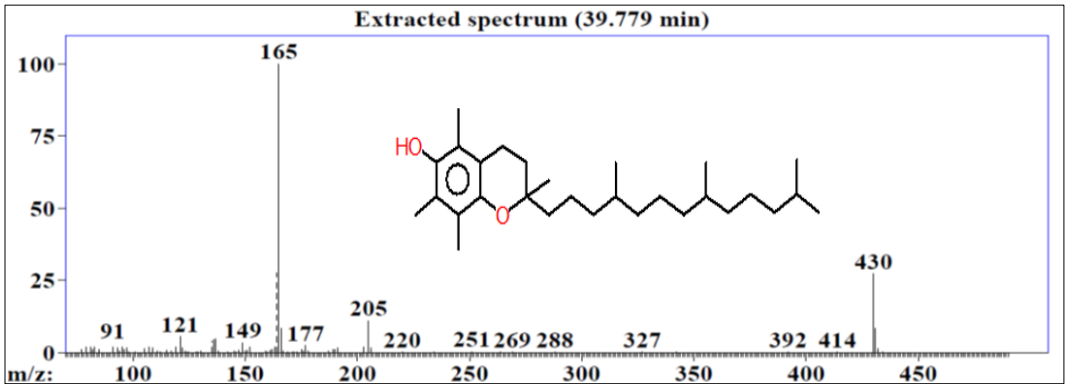

Fig 14: Mass spectrum and structure of vitamin E.

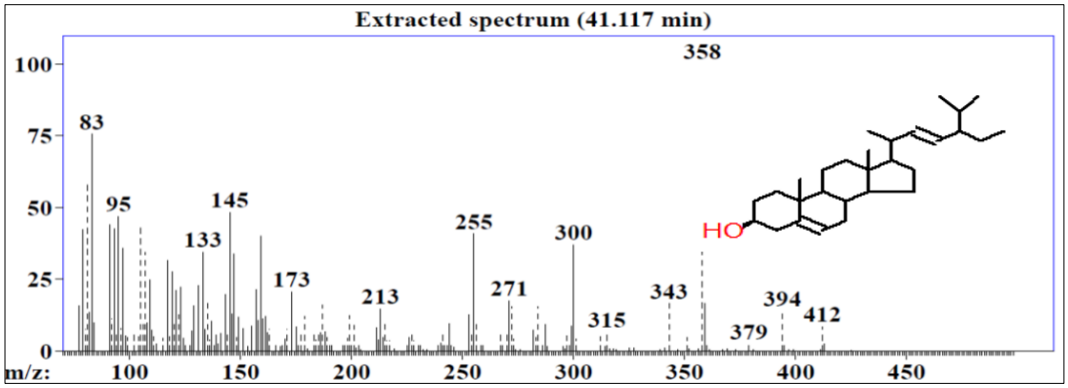

Fig 15: Mass spectrum and structure of stigmasterol.

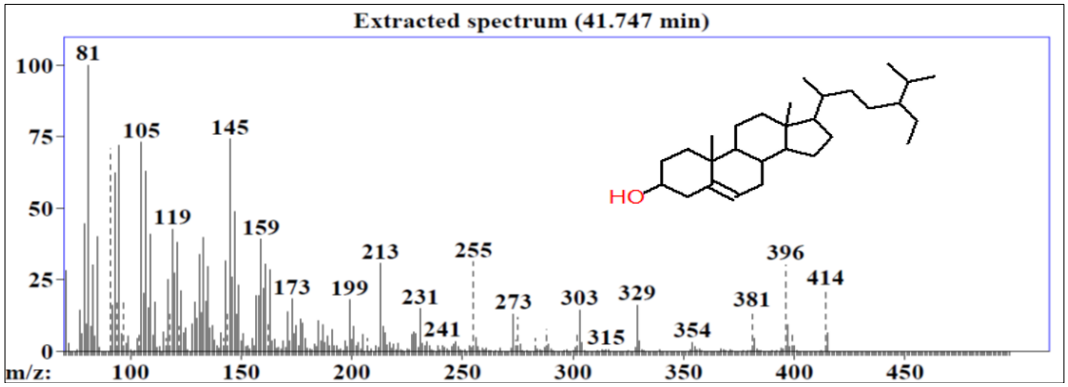

Fig 16: Mass spectrum and structure of beta-sitosterol

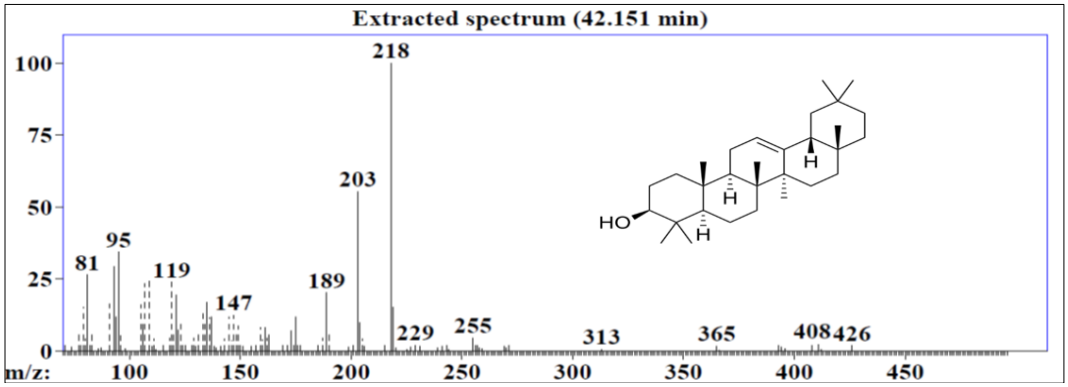

Fig 17: Mass spectrum and structure of beta-amyrin.

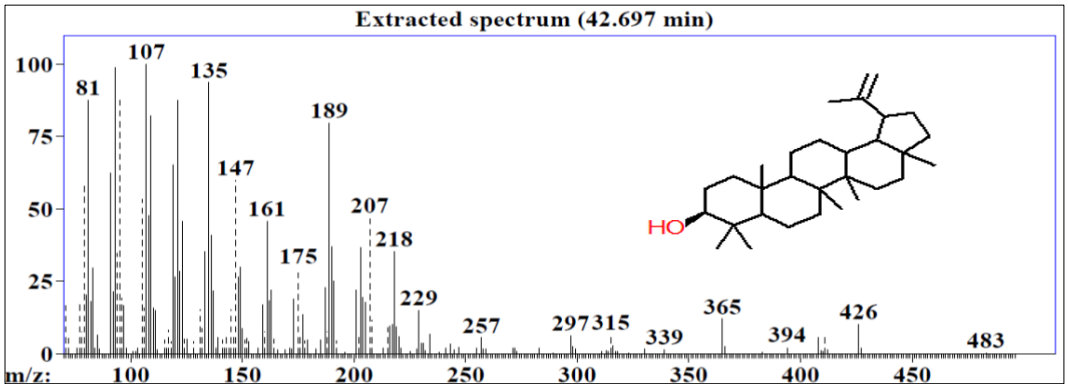

Fig 18: Mass spectrum and structure of lupole

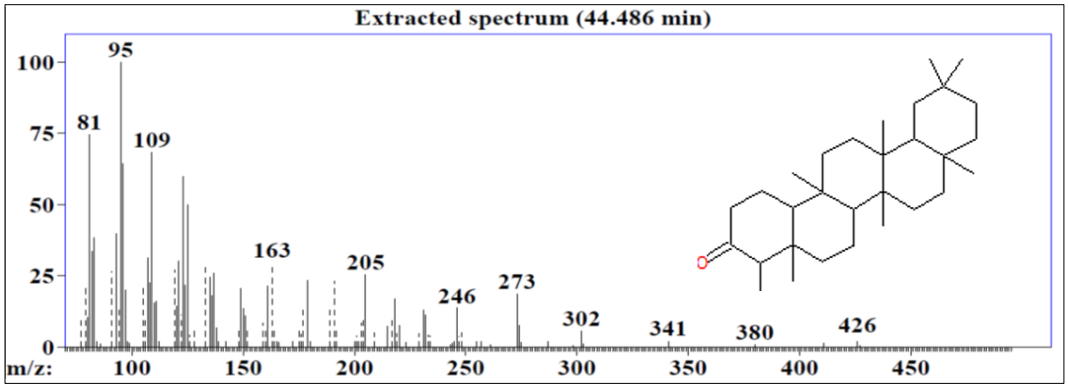

Fig 19: Mass spectrum and structure of Friedelin

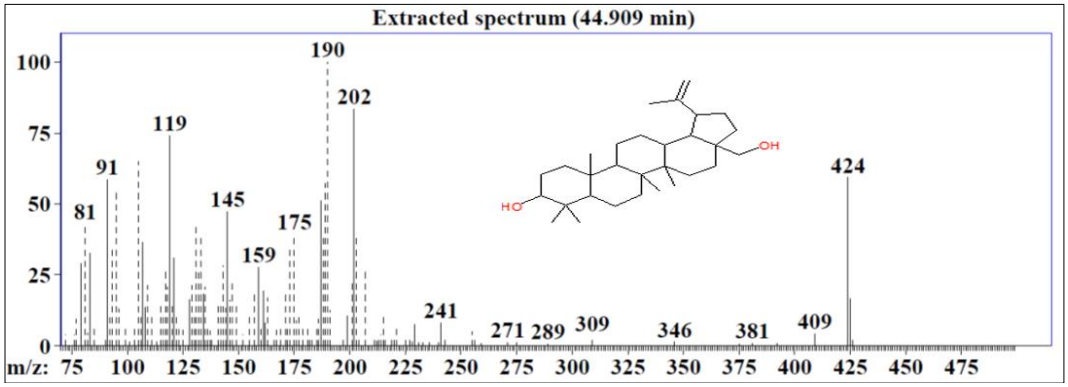

Fig 20: Mass spectrum and structure of betulin

Represtiveness of the different molecules in the ethanol extract70%, analyzed To the GC-SM

To appreciate the representativity of the different molecules in the ethanol extract, stigmasterol was arbitrarily chosen as the base molecule. The abundance of each compound is determined relative to the area of stigmasterol (A / 6643677). Thus, two groups of compounds are obtained:

The group of compounds that are more represented than stigmasterol (A / 6643677> 1). These are: palmitic acid (1,448), oleic acid (2,845) and beta-sitosterol (5,979). The value 5.979 approximately 6 indicates that beta-sitosterol is 6 times more abundant than stigmasterol in the ethanolic extract studied. Then, the group of compounds less represented than stigmasterol (A / 6643677 <1), which gathers all the other identified compounds. (Table II)

Table 2: Summary of the results of the CG-SM chrommatogram and comparison the area of different molecules compared to that of stigmasterol

N°	Retention time: TR (min)	Area: A (UI)	Area /6643677	Name of molecule
a	15,429	169 737	0,026	Isoeugénol
b	16,130	1611659	0,243	2,5-dioxo 4-hexyl 3-acide furanacétique
c	19,538	164 172	0,025	3,5-dihydroxy 1-méthyl 4-méthylbenzoate
d	20,951	250798	0,038	4-((1E) 1-propenyl) 4-hydroxy 5-méthoxyphenol
e	24,107	1127120	0,170	Méthyl ester acide palmitique
f	24,707	9 619 285	1,448	Acide palmitique
g	26,761	998 679	0,150	Méthyl ester acide linoléique
h	26,852	1182073	0,178	Méthyl ester acide oléique
i	27,348	5765251	0,868	Acide linoléique
j	27,455	18900540	2,845	Acide oléique
k	27,760	3 499 047	0,527	Acide stéarique
l	33,058	261 726	0,039	Diisooctyl phthalate
m	36,378	909036	0,137	Squalène
n	39,779	4162491	0,627	Vitamine E
o	41,117	6643677	1,000	Stigmastérol
p	41,747	39723684	5,979	β-Sitostérol
q	42,151	3282221	0,494	β-Amyrine
r	42,697	6141556	0,924	Lupeole
s	44,486	3411292	0,513	Friedéline
t	44,909	4195372	0,631	Bétuline

Discussion

To understand the origin of the activity of the extracts (aqueous and ethanolic) of T. macroptera, a triphytochemical study was carried out on these extracts. This study reveals that the aqueous extract contains polyphenols, flavonoids, gallic tannins, saponins and cardiotonic glycosides. While the ethanol extract contains in addition to listed compounds, sterols and terpenes.

All these chemical groups according to the literature have antibacterial activities. Polyphenols containing tannins and flavonoids are known for their toxicity to microorganisms [12] and their antibacterial activity [13]. The work of Vincken *et al*. [14] has shown that saponosides isolated from the medicinal plant possess antibacterial properties. Sterols and terpenes are also endowed with antibacterial activity [13].

The gas chromatography coupled to the mass spectrometer revealed several peaks. More than thirty molecules were separated and three of them appear as majority compounds. But only the compound corresponding to the retention time peak TR = 41.74 min is the most predominant. This compound is named beta-sitosterol and would be responsible for the antibacterial activity of the ethanolic extract. Indeed the beta sitosterol is a molecule belonging to the family of triterpenes and according to the literature, triterpenes are known for their antibacterial activity [15]. Several research studies have shown the existence of triterpene molecules with antibacterial activities. This is the case of glycosidehydroxyimberbic acid, isolated from Combretum imberbe [16]. In addition to sterols and terpenes, other compounds have been identified by comparison of their mass spectrum with that of the library, linked to mass spectroscopy. These compounds would identify themselves mainly to three groups including the group of polyphenols, that of fatty acids and their esters and finally the group of triterpenes. In addition, the antibacterial activity of the ethanolic extract of *T. macroptera* is probably due to the presence of betulin. Indeed according to Tang *et al* [17, 18], betulin inhibits the in vitro growth of *S. aureus* and *E. coli*.

This study also revealed the presence of phenolic compounds in the ethanolic extract of *T. macroptera*. The antibacterial activity of these compounds has already been demonstrated in the literature. Indeed, phenols cause enzymatic inhibition by interaction with bacterial proteins [19] and toxicity by intercalating into membrane phospholipids and thus destabilize the plasma membrane of bacteria [20]. This would explain the interesting antibacterial activity of the studied extracts of this plant. These results further confirm those obtained with the triphytochemical study by the tube staining method with the ethanolic extract. Moreover, the different mass spectra reveal that the molecular masses are less than 1000 daltons indicating that the molecules of the ethanolic extract are therefore small molecules.

Conclusion

The phytochemical sorting of the extracts studied made it possible to highlight polyphenols, flavonoids, gallic tannins, sterols, saponosides and cardiac glycosides. In addition, analysis by mass spectrometry-coupled chromatography also made it possible to highlight twenty (20) molecules from the ethanol extract 70% of *T. macroptera*. It is also noted that the phytomolecules identified belong mainly to the family of triterpenes and polyphenols. This further confirms the antibacterial activity of ethanol extracts 70% of our plant and therefore its use in the treatment of pathologies microbial infection in Ivory Coast and elsewhere.

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