^{13}C NMR ANALYSIS: TERPENOIDS, STEROIDS AND CAROTENOID FROM *DIOSPYROS SOUBREANA* (EBENACEAE)

ABSTRACT

Aims: The aim of current work was to identify secondary metabolites in the fractions of extracts of *Diospyros soubreana* using analysis of their ^{13}C NMR spectra.
Study design: A research project was undertook with *Diospyros soubreana*. The leaves of this plant are used in traditional medicine as hemostatic for wounds. In addition, no phytochemical study has been carried out on of this plant.
Place and Duration of Study: Leaves and bark of trunks of *D. soubreana* were collected in July 2014 in Agboville, in south-east of Côte d'Ivoire.
Methodology: A computerized procedure, based on ^{13}C NMR spectroscopy and using literature data, was developed to identify secondary metabolites in natural mixtures. The leaves and bark of trunk of *D. soubreana* were extracted with various solvents and the extracts were fractionated on silica gel.
Results: The computer assisted analysis of the ^{13}C NMR spectra of the fractions of chromatography led to identification of nine molecules: one monocyclic sesquiterpenoid lactone (**1**), five pentacyclic triterpenes (**2**, **3**, **4**, **5** and **6**), two sterols (**7** and **8**) and one carotenoid alcohol (**9**).
Conclusion: It has been shown that ^{13}C NMR is a powerful alternative tool for the identification of terpenoids, steroids and carotenoid in extracts of leaves and bark of trunk of various Diospyros species after a rapid fractionation step.

Keywords: Diospyros soubreana, terpenoids, steroids, carotenoid, ^{13}C NMR spectroscopy.

1. INTRODUCTION

In the last decades, many studies on *Diospyros* species have been conducted due to their medicinal and their economic importance. These studies focused on the chemical composition and biological activities of extracts and secondary metabolites. Chemical investigations have shown that *Diospyros* contained various bioactive compounds such as triterpenoids, naphtoquinones, naphthalene derivatives, flavonoids and coumarin derivatives [1].

Diospyros soubreana F. White (also called *Maba soubreana* A. Chev.) belonging to the family Ebenaceae, is a tropical tree found in West Africa from Côte d'Ivoire to South Nigeria. To the best of our knowledge, very few pharmacological and chemical data exist on this species. We earlier reported the isolation of three isocoumarins namely bergenin, norbergenin and 4-*O*-galloylnorbergenin from the leaves and the bark of trunks of this plant [1]. In continuation of the research on the chemical

constituents of this species, our investigation report for the first time some secondary metabolites which previously showed the biological activities in the treatment of numerous human diseases.. These compounds were identified by ^{13}C NMR spectroscopy which is a valuable tool for phytochemicals identification.

2. MATERIALS AND METHODS

2.1 General

The ^{13}C NMR spectra were recorded on a Bruker Avance 400 Fourier Transform spectrometer operating at 400 MHz for ^{1}H spectra and 100 MHz for ^{13}C using CD_3OD and Me_2CO-d_6 as deuterated solvents. The chemical shift was expressed in ppm from TMS (internal standard). The chromatography columns were performed on silica gel (Merck, 35-70 µm, 60-200 µm and 63-200 µm). Thin-layer chromatographies were carried out on aluminium plates coated with silica gel 60-F_{254} (Merck), and visualized with UV light (254 and 366 nm), then sprayed with vanillin-H_2SO_4 solution followed by warming.

2.2 Plant material

Leaves and bark of trunk of *D. soubreana* were collected in July 2014 in « Petit Yapo » forest, Agboville Department, south-east of Côte d'Ivoire. The plant samples were identified by a botanist of Centre National de Floristique (CNF), University Félix Houphouët-Boigny of Cocody-Abidjan, where voucher specimens are deposited. The samples were dried at room temperature, then ground. 100 g of leaf powder (DSF) and 120 g of trunk bark powder (DST) were obtained.

2.3 Extraction and isolation

100 g of leaves powder (DSF) were extracted by maceration with the mixture water/ethanol (70 : 30) for 24 h at room temperature. After filtration and the removal of the solvent under reduced pressure, a residue of 25.5 g was obtained. 15 g of this residue was suspended in ethanol 70% and extracted sequentially with increasing polarity solvents to give after evaporation 0.88 g of *n*-hexane (DSFH), 1.82 g of dichloromethane (DSFD) and 2.39 g of ethyl acetate (DSFA) extracts.

Powdered bark of trunks (120 g) were extracted in a Soxhlet apparatus, firstly with petroleum ether and then with dichloromethane, followed by maceration in ethyl acetate at room temperature. Extracts were filtered and concentrated under reduced pressure to give 0.8 g of petroleum ether (DSTPE), 1.2 g of dichloromethane (DSTD) and 9.7 g of ethyl acetate (DSTA) extracts.

388 g of the leaves dichloromethane extract (DSFD) was chromatographed on a silica gel column (63-200 µm) using a gradient of hexane/ethyl acetate (100 : 0 to 0 : 100) to give twelve fractions (FD1-FD12) along with 15-pentadecanolide (**1**) (89.7 mg). Fraction FD5 (54.8 mg) was constituted by the mixture α-amyrin (**2**), β-amyrin (**3**) and lupeol acetate (**5**). Fraction FD6 (40 mg) was successively purified by silica gel chromatography (63-200 µm), first with the gradient hexane/ethyl acetate (100 : 0 to 70 : 30) and then hexane/ethyl acetate (80 : 20) to furnish four fractions (FD61-FD64) according to their TLC profiles. Fraction FD62 (5.7 mg) was the mixture α-amyrin (**2**), β-amyrin (**3**) and lupeol (**4**) [2], while fraction FD63 (4.3 mg) was the mixture stigmasterol (**7**) [3] and β-sitosterol (**8**) [4].

A part of the extract DSFA (2 g) was subjected to a vacuum chromatography using a gradient of petroleum ether/ethyl acetate (100 : 0 to 0 : 100) then ethyl acetate/methanol (80:20) to provide six major fractions (FA1-FA6). The fraction FA4 (112.9 mg), treated by successive chromatographies on silica gel columns (35-70 µm and 60-200 µm) eluted with the gradient dichloromethane/ethyl acetate (100 : 0 to 40 : 60), then with dichloromethane/ethyl acetate (50 : 50) led to 7.1 mg of zeaxanthin (**9**) [5].

The extract DSTD (1.2 g) was chromatographed on a silica gel (63-200 µm) column (hexane/EtOAc, 100 : 0 to 0 : 100, then EtOAc/MeOH 90 : 10) to provide 35.7 mg of betulinic acid (**6**) [2].

3. RESULTS AND DISCUSSION

From the leaves and bark of trunks of *D. soubreana*, nine compounds were isolated and identified. The structures of these compounds (**Fig. 1**) were established by the main of ^{13}C NMR analysis and by comparison with literature data. Spectral data for the compounds (**1**-**9**) are proposed as **supporting information**. These molecules corresponded to one monocyclic sesquiterpenoid lactone (**1**), five pentacyclic triterpenoids (**2**, **3**, **4**, **5** and **6**), two ubiquitous sterols (**7** and **8**) and one carotenoid alcohol (**9**), all isolated for the first time from this plant. Pentacyclic triterpenoids [6,7] and phytosteroids [8] are class of compounds occurring widely in plants. Biosynthetically, these compounds have squalene as precursor [9]. Phytosterols are a family of more than 200 different compounds [8]; [8]; the richest source of phytosterols is composed of plant based foods stuff chiefly nuts, seeds, vegetable oils, cereals and legumes [10-12].

Although no biological activity tests were led to the isolated compounds (**1**-**9**), literature search revealed that these have diverse bioactivities. Indeed, α and β-amyrin which are found in leaves, barks and resins of various plants have shown anti-inflammatory [13-15], anti-microbial and other biological activities [7]. Their mixture prevents the impaired aortic vascular reactivity in high-fat diet-induced obese mice [16].

α-amyrin showed a wide spectrum of activity including anti-ulcer, anti-hyperlipidemic, anti-tumor and hepatoprotective [14]. It attenuates high fructose diet-induced metabolic syndrome in rats [17].

β-amyrin exhibited antibacterial [15], antinociceptive [19], antioxidant activities, and showed liver protection both in *in vitro* and *in vivo* studies [20].

Lupeol is known to have vast occurrence in many plants families [21] with rare reports in fungal and animals [22, 23]. This compound has been shown to possess antiprotozoal, anti-inflammatory, antimicrobial and antitumor activities. It also has cancer chemopreventive, cardioprotective, gastroprotective and hepatoprotective effects [21].

Lupeol acetate showed hypotensive [24], antimicrobial, anti-inflammatory, antimalarial, antituberculosis [25], and antinociceptive [26] activities. This molecule has antiarthritic effect [27]. It induced antifertility activity in male albino rats [28], neutralized *Daboia russellii* and *Naja kaouthia* venom [29] and ameliorates collagen-induced arthritis and osteoclastogenesis [30].

Betulinic acid, a pentacyclic triterpenoid of plant origin is widely distributed in the plant kingdom [31]; it has been isolated from various families such as Rhamnaceae, Myrtaceae, Paeoniaceae, Ebenaceae [32] and Euphorbiaceae [33]. This compound possesses many biological and medicinal properties including antibacterial, antinociceptive [32], antioxidant, anti-inflammatory, anthelmintic, antimalarial [32, 34], antitumor [31, 35] and anti-HIV (human immunodeficiency virus) [32] activities.

Stigmasterol, a sterol occurring in various medicinal plants, is used as a precursor in the synthesis of progesterone and as an intermediate in the biosynthesis of androgens, estrogens, corticoids and in the synthesis of vitamin D3. It has been described to possess antioxidant, anti-inflammatory, anti-osteoarthritic, antimutagenic, anti-hypercholestrolemic, CNS (central nervous system), hypoglycemic, thyroid inhibiting, cytotoxicity and antitumor activities [36].

β-sitosterol is a plant sterol distributed in a wide range of plant families including Apiaceae, Rosaceae, Tiliaceae, Solanaceae, Moraceae, Rubiaceae, Lamiaceae, Fabaceae, Polygonaceae, Rhamnaceae, Asteraceae, Acanthaceae, Cucurbitaceae, Gracilariaceae (marine algae), Thymelaeaceae, Vitaceae and Zingiberaceae. It appears to offer a number of health benefits and displayed many biological activities. Indeed, this molecule showed anti-inflammatory, antioxidant, hypocholesterolemic, analgesic, anthelminthic, anti-mutagenic, immunomodulatory, neuroprotection and antidiabetic activities. Additionally, β-sitosterol reduced the growth and spread of prostate cancer cells and induced apoptosis. It also has chemoprotective (or chemopreventive) and angiogenic effects. Otherwise, this compound is not considered as genotoxic or cytotoxic [37]. Furthermore, it is a neutralizing agent on viper and cobra venom [38] and displayed antibacterial, antifungal [39] and mosquito larvicidal [40] activities.

Zeaxanthin, one of the most common carotenoid alcohols found in nature, is a xanthophyll from higher plants, algae [41] and microbial sources [42]. It is the main pigment of yellow corn, *Zeaxanthin mays* L. (from which its name is derived). Zeaxanthin is naturally found in various dietary sources including corn, egg yolk, orange pepper, orange juice, honeydew, mango, avocado, and other vegetables and fruits. This compound is used as a feed additive and colorant in the food industry for birds, swine and fish. The pigment imparts a yellow coloration to the skin and egg yolk of birds, whereas in pigs and fish it is used for skin pigmentation. Zeaxanthin is used in the prevention of age-related macular degeneration, the leading cause of irreversible blindness in adults; it is protective against the oxidative damage causing cataract formation [43], has cancer-preventive properties [44] and inhibits macrophage-mediated LDL oxidation [45].

Otherwise, 15-pentadecanolide, isolated from *Angelica archangelica* L. roots, appears to possess minimal irritancy, allergenicity and acute toxicity [46].

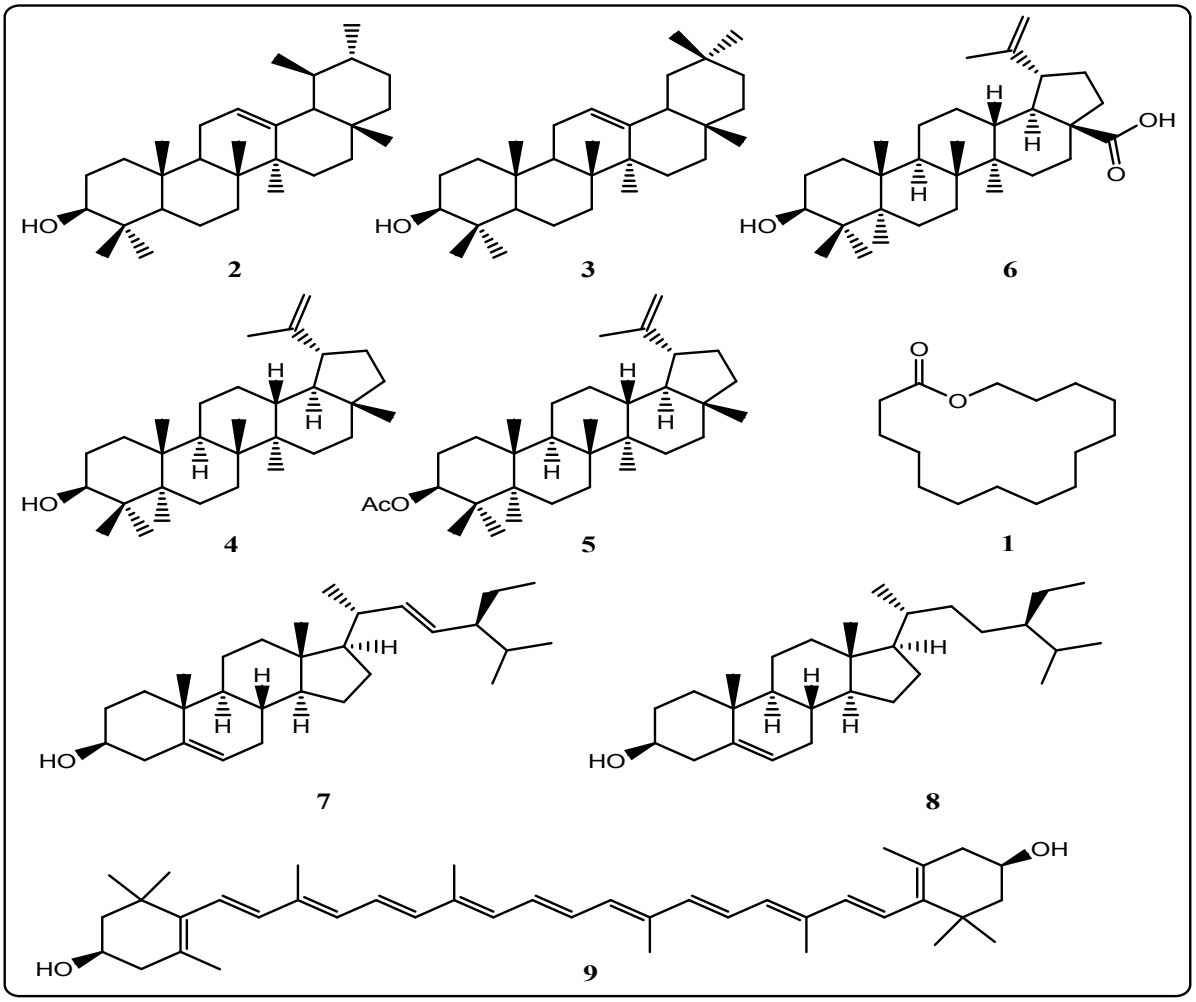

FIG. 1: STRUCTURES OF ISOLATED COMPOUNDS FROM *DIOSPYROS SOUBREANA*

4. CONCLUSION

This study undertook with the leaves and stem barks of *D. soubreana* led to the isolation and identification of nine compounds, all isolated for the first time from this species. Their structures have been established using ^{13}C NMR spectroscopy. These molecules belong to various chemical groups including terpenoids, steroids and carotenoids. The literature review carried out on them made it possible to demonstrate multiple biological activities.

The presence of anti-inflammatory compounds such as lupeol, lupeol acetate, α-amyrin, β-amyrin, β-sitosterol and stigmasterol in the leaves may justify the use of this part of the plant to treat wounds. In addition, this plant could be a potentially source of antioxidant compounds. This study is worth pursuing in order to isolate other bioactive compounds. Moreover, biological tests deserve to be done to highlight other pharmacological potentialities.

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SUPPORTING INFORMATION

Description of isolated compounds

• **15-pentadecanolide** (**1**) 89.7 mg

Colorless liquid

$C_{15}H_{28}O_2$, 240 g/mol

^{13}C NMR δ 169.6 (C-1), 33.9 (C-2), 24.0 (C-3), 26.3 (C-4), 26.7 (C-5), 27.1 (C-6/C-7/C-8/C-9/C-10/C-11), 26.7 (C-12), 23.2 (C-13), 25.4 (C-14), 64.2 (C-15).

• **α-amyrin** (**2**)

Colorless solid

$C_{30}H_{50}O$, 426 g/mol

^{13}C NMR δ 39.7 (C-1), 27.4 (C-2), 79.0 (C-3), 39.7 (C-4), 55.2 (C-5), 18.0 (C-6), 34.2 (C-7), 40.8 (C-8), 47.9 (C-9), 37.1 (C-10), 23.5 (C-11), 124.4 (C-12), 139.5 (C-13), 42.8 (C-14), 28.1 (C-15), 26.9 (C-16), 33.3 (C-17), 61.6 (C-18), 39.6 (C-19), 39.6 (C-20), 31.9 (C-21), 41.7 (C-22), 29.3 (C-23), 15.6 (C-24), 15.6 (C-25), 15.9 (C-26), 23.3 (C-27), 28.1 (C-28), 17.4 (C-29), 22.7 (C-30).

• **β-amyrin** (**3**)

Colorless solid

$C_{30}H_{50}O$, 426 g/mol

^{13}C NMR δ 38.7 (C-1), 27.2 (C-2), 79.0 (C-3), 38.8 (C-4), 55.1 (C-5), 18.3 (C-6), 32.6 (C-7), 39.7 (C-8), 47.7 (C-9), 37.1 (C-10), 23.6 (C-11), 121.7 (C-12), 145.1 (C-13), 41.7 (C-14), 26.1 (C-15), 26.9 (C-16), 32.5 (C-17), 47.2 (C-18), 46.8 (C-19), 31.1 (C-20), 34.7 (C-21), 37.1 (C-22), 28.1 (C-23), 15.5 (C-24), 15.6 (C-25), 16.8 (C-26), 26.0 (C-27), 28.4 (C-28), 33.3 (C-29), 23.5 (C-30).

• **Lupeol** (**4**)

Colorless solid

$C_{30}H_{50}O$, 426 g/mol

^{13}C NMR δ 38.7 (C-1), 27.4 (C-2), 79.0 (C-3), 38.7 (C-4), 55.2 (C-5), 18.3 (C-6), 34.2 (C-7), 40.8 (C-8), 50.4 (C-9), 37.1 (C-10), 20.9 (C-11), 25.1 (C-12), 38.0 (C-13), 42.8 (C-14), 27.4 (C-15), 35.5 (C-16), 43.0 (C-17), 48.2 (C-18), 47.9 (C-19), 151.0 (C-20), 29.7 (C-21), 40.0 (C-22), 27.9 (C-23), 15.4 (C-24), 16.1 (C-25), 15.9 (C-26), 14.5 (C-27), 18.0 (C-28), 109.3 (C-29), 19.3 (C-30).

• **Lupeol acetate** (**5**)

Colorless solid

$C_{32}H_{52}O_2$, 468 g/mol

^{13}C NMR δ 38.4 (C-1), 27.4 (C-2), 80.6 (C-3), 38.4 (C-4), 55.3 (C-5), 18.2 (C-6), 34.1 (C-7), 40.8 (C-8), 50.3 (C-9), 37.0 (C-10), 20.9 (C-11), 25.0 (C-12), 38.0 (C-13), 42.8 (C-14), 27.4 (C-15), 34.5 (C-16), 42.9 (C-17), 48.2 (C-18), 48.0 (C-19), 150.9 (C-20), 29.8 (C-21), 39.9 (C-22), 27.9 (C-23), 16.5 (C-24), 16.1 (C-25), 15.9 (C-26), 14.5 (C-27), 18.0 (C-28), 109.3 (C-29), 19.2 (C-30), 173.7 (C-31), 21.4 (C-32).

• **Betulinic acid** (**6**) 35.7 mg

White solid

$C_{30}H_{48}O_3$, 456 g/mol

^{13}C NMR δ 38.3 (C-1), 27.3 (C-2), 79.0 (C-3), 38.8 (C-4), 55.3 (C-5), 18.2 (C-6), 34.3 (C-7), 40.6 (C-8), 50.5 (C-9), 37.1 (C-10), 20.8 (C-11), 25.4 (C-12), 38.3 (C-13), 42.4 (C-14), 30.5 (C-15), 32.1 (C-16), 56.2 (C-17), 46.8 (C-18), 49.2 (C-19), 150.4 (C-20), 29.7 (C-21), 37.0 (C-22), 27.9 (C-23), 15.3 (C-24), 16.0 (C-25), 16.1 (C-26), 14.6 (C-27), 180.0 (C-28), 109.7 (C-29), 19.3 (C-30).

• **Stigmasterol** (**7**)

Colorless solid

$C_{29}H_{48}O$, 412 g/mol

^{13}C NMR δ 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.2 (C-4), 140.7 (C-5), 121.7 (C-6), 31.8 (C-7/C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.6 (C-12), 42.2 (C-13), 56.8 (C-14), 24.3 (C-15), 28.9 (C-16), 55.9 (C-17), 12.0 (C-18), 19.4 (C-19), 40.5 (C-20), 21.1 (C-21), 138.3 (C-22), 129.2 (C-23), 51.2 (C-24), 31.8 (C-25), 21.2 (C-26), 18.9 (C-27), 25.4 (C-28), 12.2 (C-29).

• **β-sitosterol** (**8**)

Colorless solid

$C_{29}H_{50}O$, 414 g/mol

^{13}C NMR δ 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.8 (C-7/C-8), 50.1 (C-9), 36.5 (C-10), 21.0 (C-11), 39.7 (C-12), 42.2 (C-13), 56.7 (C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-19), 36.1 (C-20), 18.7 (C-21), 33.9 (C-22), 26.0 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), 18.9 (C-27), 23.0 (C-28), 11.9 (C-29).

• **Zeaxanthin** (**9**) 7.1 mg

Reddish orange powder

$C_{40}H_{56}O_2$, 568 g/mol

^{13}C NMR δ 37.1 (C-1/C-1'), 48.4 (C-2/C-2'), 65.1 (C-3/C-3'), 42.5 (C-4/C-4'), 126.1 (C-5/C-5'), 136.5 (C-6/C-6'), 125.5 (C-7/C-7'), 138.5 (C-8/C-8'), 135.7 (C-9/C-9'), 131.3 (C-10/C-10'), 124.9 (C-11/C-11'), 137.7 (C-12/C-12'), 136.4 (C-13/C-13'), 132.5 (C-14/C-14'), 130.0 (C-15/C-15'), 30.2 (C-16/C-16'), 28.7 (C-17/C-17'), 21.6 (C-18/C-18'), 12.8 (C-19/C-19'/C-20/C-20').