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# Separation and characterization of phenolic compounds from *Terminalia ivoiriensis* using liquid chromatography-positive electrospray ionization tandem mass spectroscopy

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***Terminalia ivoriensis*** **A. Chev. (Combretaceae) is an Ivorian medicinal plant. There is little ethnobotanical and almost no chemical information available for this species. The aim of this study was to isolate phenolic compounds from *T. ivoriensis*. In this way, its ethyl acetate extract (Ea) was fractionated by silica gel column chromatography followed by Sephadex LH20 filtration. Elution solvents were methanol (MeOH), methylene chloride (Mc) and ethyl acetate (EtOAc). Analysis of the obtained fractions (F1-F4) by liquid chromatography coupled to positive electro-spray ionization tandem mass spectrometry (LC-ESI-MS/MS) afforded six known polyphenols: 1) 3,3'-di-O-methylellagic acid); 2) 3,7,8-tri-O-methylellagic acid; 3) Progallin A; 4) 3,3',4-O-Trimethyl-4'-O-β-D-glucopyranosylellagic acid; 5) Punicalagin and, 6) Punicalin. All these natural products were isolated here for the first time from this plant. They presented various biological activities among which were anti-inflammatory, antioxidant and anti-HIV activities.**

**Keywords:** *Terminalia*, *ivoriensis*, LC-ESI-MS, isolation, characterization, polyphenols, activity, anti-inflammatory, antioxidant, anti-HIV.

## INTRODUCTION

*Terminalia ivoiriensis* (also called Framire, Emery, Black afara and various other names) is a big tree belonging to the family *Combretaceae*. It is a large deciduous forest tree ranging in height from 15 to 46 m, branchless for up to 30 m, with diameter at breast height (dbh) of 2 to 4.75 m; bole clean, very straight with small buttresses and sometimes fluted. *T. ivoriensis* is widespread in Ivory Coast, Cameroon, Ghana, Guinea, Liberia, Nigeria, and Sierra Leone (Wadsworth, 1997). Its stem bark is used as antimalarial and tranquilizer agents (Lawal et al., 2010). This plant is also used in Ivorian traditional medicine for numerous diseases like cough, diarrhea, hypertension, diabetes and tooth decay. Concerning biological essay, its antioxidant and cytotoxic activities were reported by Ponou et al. (2010), and its antifungal activities were evaluated by Ouattara et al. (2013). *Terminalia* genus is a

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rich source of polyphenols like tannins and flavonoids (Zoubida et al., 2007; Beate et al., 2010; Manjuan et al., 2009). According to our knowledge, none of the phenolic compounds were isolated from *T. ivoiriensis*. We report here results of our phytochemical investigation of its stem bark.

## MATERIALS AND METHODS

### Generals

Fractionation and purification process were made using column chromatography. Silica gel 60 (230-400 mesh, Merck) and Sephadex LH20 were used as stationary phase. Analytical thin layer chromatography (TLC) was performed on percolated silica gel 60 $F_{254}$ plates (Merck) and detection was achieved by spraying with sulfuric vanillin, followed by heating 5 min at 105°C. Liquid chromatography coupled to mass spectrometry (LC-MS) was conducted using an Agilent LC-MSD ion trap mass spectrometer (Bruker Daltronics, Bremen, Germany) equipped with 1100 series HPLC and a Tosoh ODS-80Ts (150 × 4.6 mm, 5 µm) column. The ESI-MS was detected on an Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA).

### Plant material

The stem bark from *T. ivoriensis* was collected in July 2009, in its natural habitat in Ivory Coast, in the forest region near Abidjan (Southern Ivory Coast). Botanical determination was done by Pr. L. Aké Assi (Centre National de Floristique, Université de Cocody, Abidjan). Voucher specimens (n°754) were deposited at the Herbarium of the Centre National de Floristique (CNF). The stem bark was air dried and powdered; 2.3 kg were obtained.

### Extraction and isolation

Air-dried and powdered stem bark (2.3 kg) from *T. ivoriensis* was extracted (by solid-liquid extraction method) at room temperature with methanol (3 × 8 L). All these three extracts were concentrated to 450 ml. This volume was completed to 500 ml with water. This methanol extract was washed three times with cyclohexane (3 × 500 ml) and four times with chloroform (4 × 500 ml). The insoluble phase in cyclohexane and chloroform was extracted with ethyl acetate (5 × 400 ml). The ethyl acetate extracts (Ea) were dried under vacuum, using a ROTAVAPOR (Büchi) and 5.41 g were obtained.

### Fractionation of Ea

A first fractionation (5.0 g of Ea) was performed on silica gel column chromatography with column size: diameter (d) = 5.3 cm and height (h) = 10 cm. Elution solvents were methylene chloride (Mc), ethyl acetate (EtOAc) and methanol (MeOH). Elution with the mixture Mc-EtOAc (50:50 v/v) gave fractions (1-23), which were taken together according to their TLC profile. These fractions were fatty acids. So, they were abandoned. Elution with the mixture ethyl acetate 100% and EtOAc-MeOH (98:02 v/v) gave fractions (24 to 40), among which 27 to 35 were taken together according to their TLC profile; fraction Ea1 (1.43 g) was in this way obtained. The last elution with EtOAc-MeOH (95:05 v/v) gave fractions (41 to 65), among which (43 to 50) were taken together according to their TLC profile; fraction Ea2 (1.12 g) was obtained.

Fraction Ea1 was then, fractioned on silica gel column chromatography with column size: diameter (d) =2.1 cm and height (h)=8 cm). Elution with the mixture EtOAc-MeOH (9:1 to 7:3, v/v) gave 20 fractions. Two fractions [2 to 8] and [14 to 18] were taken together respectively, according to their TLC profile. Both fractions were then purified respectively on Sephadex LH20 filtration gel. The elution solvent was MeOH. The first one [2 to 8] gave sample F1 (4 mg) and F2 (6 mg); the second one [14 to 18] gave one sample F3 (5 mg). Fraction Ea2 was also purified on Sephadex LH20 filtration gel in identical conditions; sample F4 (7 mg) was obtained.

### Analytical conditions of LC-ESI-MS method

Gradient elution was performed with water (with 0.05% acetic acid) (solvent A) and acetonitrile (solvent B) at a constant flow rate of 500 µl/min. An increasing linear gradient (v/v) of solvent B was used [t (min), % A]: [0, 70], [5, 55], [7, 54.5], [12.5, 53.5], [20, 52.5], [25, 51.5], [30, 35], [40, 00], [55, 70]. Detection was carried out at 273 nm. Every sample F1-F4 had been analyzed in the same conditions by LC-ESI-MS method. The analysis of their UV spectra and LC-MS chromatogram yielded six compounds F1 (1, 2), F2 (3, 4), F3 (5) and F4 (6). The MS/MS conditions used were adapted from previous works on the identification of phenolic compounds in vegetable residue samples (Sanchez-Rabaneda et al., 2003; Parejo et al., 2004 and Sanchez-Rabaneda et al., 2004).

## RESULTS AND DISCUSSION

A series of fractionations by chromatography of the ethyl acetate crude extract from *T. ivoriensis* yielded samples F1 to F4. These samples were further analysed by LC-ESI-MS method. The combination of silica gel column chromatography and Sephadex LH20 column chromatography was conducted to an appreciable separation. This was confirmed by a maximum of four peaks in the LC chromatogram of samples F1 to F4 (Figure 1). The LC-ESI-MS/MS is one of the methods commonly used for phenol identification in plants (Guodong et al., 2007; Sudawadee et al., 2009; Zoubida et al., 2007). To identify compounds in this study, we compare when possible sample peaks with those of available reference compounds analyzed under the same LC conditions, in order to compare their retention times, UV and mass data. Six phenolic compounds (1 to 6) were in these conditions identified (Figure 2).

### 3,3'-di-O-methylellagic acid and 3,7,8-tri-O-methylellagic acid

In the LC chromatogram of sample F1 (Figure 1a), two peaks were observed at RT1= 13.736 min and RT2= 17.208 min respectively. These compounds were identified by comparison of their retention times (RT) with those of standard and confirmed by ESI-MS experiments. In the ESI-MS spectrum of 1, the ions $[M+H]^+$, $[M+Na]^+$ and $[2M+Na]^+$ gave characteristic peaks at 331.04531, 353.02710 and 683, 06383 m/z respectively. These data related to the molecular weight 330. 03788 g/mol and to

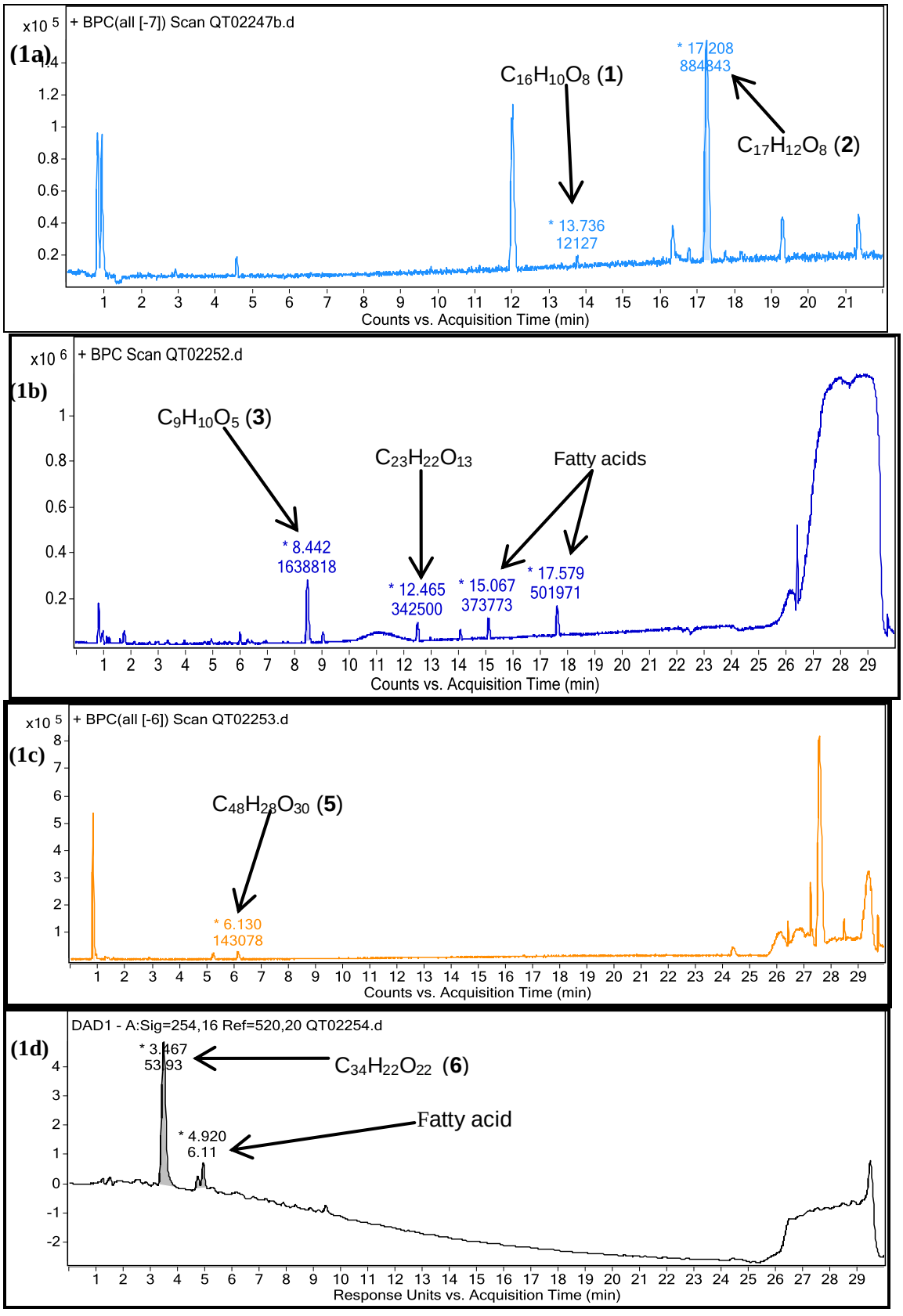


**Figure 1**. LC chromatogram of sample F1 - F3 (1a to 1c respectively) and UV spectrum of F4 (1d).

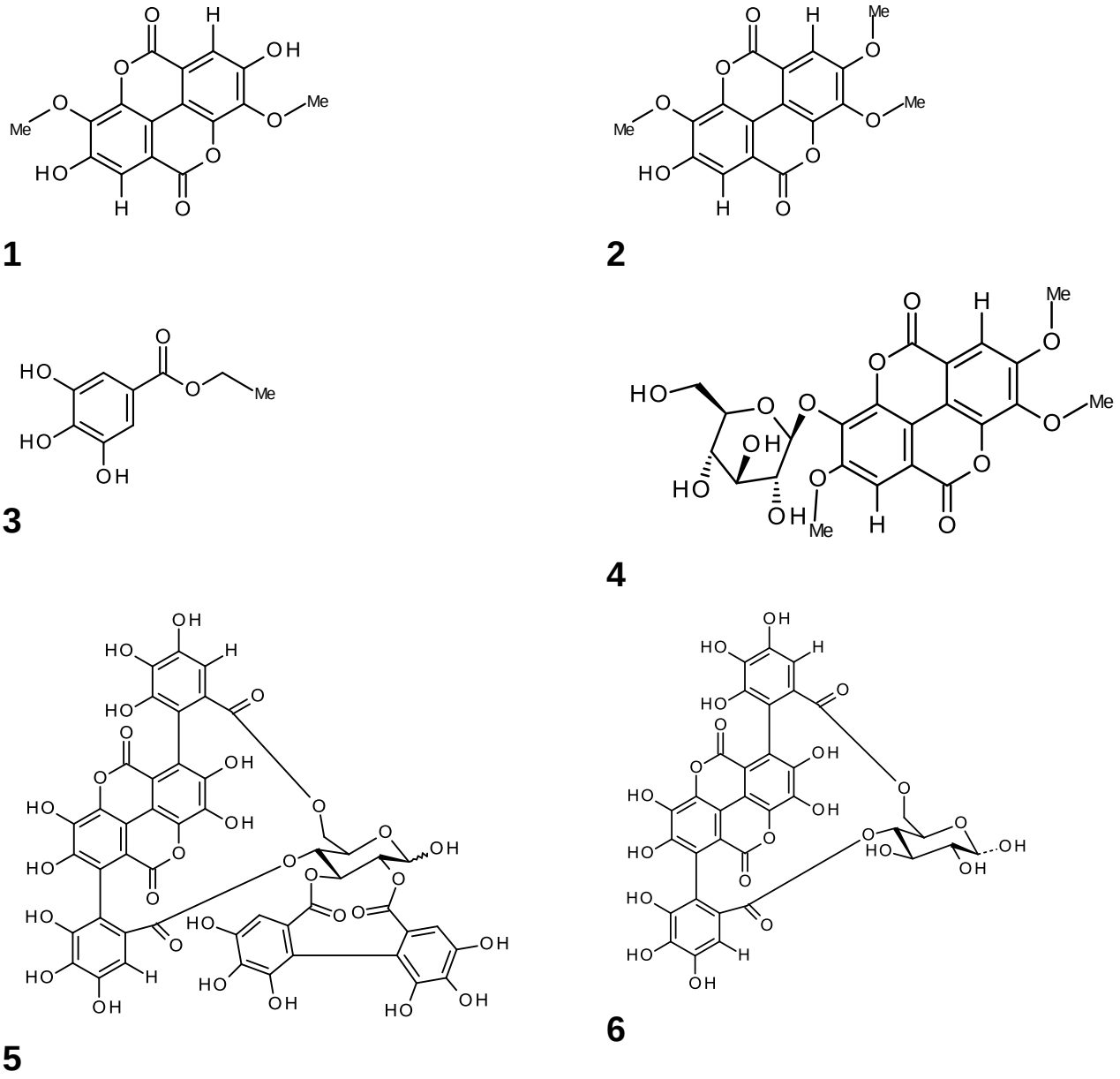

**Figure 2**. Structure of the isolated compounds from *T. ivoiriensis*: **1,** 3,3'-di-O-methylellagic acid; **2,** 2-Hydroxy-3,7,8-trimethoxychromeno[5,4,3-cde]chromene-5,10-dione; **3,** Progallin A; **4,** 3,3',4-O-Trimethyl-4'-O-β-D- glucopyranosylellagic acid; **5,** Punicalagin; **6,** Punicalin ().

the molecular formula $C_{16}H_{10}O_8$ (cal. 330.03761 g/mol). This compound was previously isolated and identified from *T. bellerica* (Beate Pfundstein et al., 2010), from the roots of *Euphorbia hylonoma (*Zengjun et al., 2011) and in aqueous extract of *Casearia sylvestris* SW (Saulo et al., 2008). In the ESI-MS spectrum of 2, the ions $[M+H]^+$, $[M+Na]^+$, $[2M+NH_4]^+$ and $[2M+Na]^+$ gave characteristic peaks at 345.06129, 367.04288, 706.14023 and 711.0959 m/z respectively. The molecular formula deduced was $C_{17}H_{12}O_8$ (cal. 344.05326). This compound was also previously isolated from *E. hylonoma* (Zengjun et al., 2011).

**Progallin A and 3,3',4-O-Trimethyl-4'-O-β-D-glucopyranosylellagic acid** (**4**).

The LC analysis of sample F2 yielded compounds 3 and 4. In their LC chromatogram (Figure 1b), four peaks were observed at RT 8.442, 12.485, 15.067 and 17.579 min.

Analysis of the ESI-MS data of peaks at RT 15.067 and 17.579 min showed that they were fatty acids. Only compounds at RT 8.442 and 12.485 min were phenols. They were identified by comparison of their retention times (RT3= 8.442 min and RT4= 12.465 min) with that of standard and confirmed by ESI-MS experiments. In their ESI-MS spectra, characteristic ion peaks were observed. In the ESI-MS spectrum of 3, the ions $[M+H]^+$ and $[M+Na]^+$ are observed at m/z 199.06209, 221.04408 respectively; that allowed the molecular formula $C_9H_{10}O_5$ (cal. 198.05285). In that of 4, the ions $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[2M+Na]^+$ are observed at m/z 507.11665, 524.14286, 529.0985 and 1035.20587, respectively. In this way, the molecular formula $C_{23}H_{22}O_{13}$ (cal. 506.10611) was deducted. Compound 3 was previously isolated and identified from the acetic ether part of the leaves of *Phyllanthus emblica* L (Zhong et al., 2011), and from *Polygonum capitatum* (Liu et al., 2008). Concerning compound 4, it was previously isolated from *Camptotheca acuminate* (Zhang et al., 2004).

**Punicalagin**

The LC chromatogram of sample F3 showed one peak (Figure 1c) at RT5= 6.130 min. From the LC chromatogram was extracted its ESI-SM spectrum which showed two characteristic ion peaks $[M+NH_4]^+$ and $[M+Na]^+$ at m/z 1102.10253 and 1107.05847 respectively. These data allowed the molecular formula $C_{48}H_{28}O_{30}$ (cal. 1084.0667). *Punicalagin* was previously isolated and identified from two genus of *Terminalia*: *T. chebuta and T. horrida* (Beate et al., 2010) and from *Pomegranate husk* (Aqil et al., 2012).

**Punicalin** (**6**)

On the LC chromatogram of sample F4 (Figure 1d), no peak was observed. But, its UV spectrum (at 280 nm) exhibited two peaks at RT 3.467 and 4.920 min. The analysis of their ESI-SM data showed that compound at RT 4.920 min was a fatty acid and that at RT 3.467 min a phenol. This last one was identified by comparison of its retention time (RT6= 3.467 min) with that of standard and confirmed by its ESI-MS data. From its LC chromatogram was extracted the ESI-SM spectrum. The characteristic ions $[M+Na]^+$, $[M+NH_4]^+$ and $[M+H]^+$ gave the peaks at m/z: 783.07294, 800.09879 and 805.05478 respectively. The molecular weight was M= 782.06565 g/mol with the formula $C_{34}H_{22}O_{22}$ (cal. 782.06039). Compound 6 was previously isolated from *T. horrida* (Beate Pfundstein et al., 2010) and from *pomegranate husk* (Honghao et al., 2010; Wang et al., 2013).

Among the six phenolic compounds identified, 1, 3, 5 and 6 were previously isolated from *Terminalia* genus. The others (2 and 4) had never been isolated from *Terminalia* genus but, from another plant. However, all these six polyphenols were isolated here for the first time from *T. ivoriensis*. Most of these isolated compounds (1, 2, 4, 5 and 6) belong to tannin's group, a sub-family of polyphenols. Chemically, tannins are complex substances, which usually occur as mixtures of polyphenols that are difficult to separate because they do not crystallize.

Complex tannins are generally considered to have risen from simple polyphenols by polymerization. Tannins are one of the many types of secondary com-pounds found in plants. They are oligomeric compounds with multiple structural units with free phenolic groups. They are soluble in water, with exception of some high molecular weight structures, and are able to bind proteins forming insoluble or soluble tannin-protein complexes. Many extracts with high tannin content are used to promote wound healing.

**Biological activity**

Concerning the biological properties of the isolated compounds, various activities were found in literature. As it is known, polyphenols are recognized for their antioxidant activities. Concerning our isolated compounds, it had been reported antioxidant activities of 1, 3, 4, 5 and 6 (Zengjun et al., 2011; Zijia et al., 2008; Wang et al., 2013). These results agree with previous antioxidant essay realized on crude extract from *T. ivoriensis* (Ponou et al., 2010).

Antimicrobial activity was reported for 3, 7, 8-tri-O-methylellagic acid against Gram-positive bacteria and Gram-negative bacteria (Kuete et al., 2007). Among the isolated compounds, *Punicalagin* and *Punicalin* presented most activities. *In vitro* antiproliferative, apoptotic and antioxidant activitieis of *Punicalagin* were reported (Aqil et al., 2012). Frequently, *Punicalagin* and *Punicalin* showed similar activities. Their anti-hepatotoxic activity on carbon tetrachloride ($CCl_4$)-induced toxicity in the rat liver was evaluated by Lin et al. (1998) and a high activity was found for both compounds. Anti-inflammatory activity was evaluated by Lin et al. (1999) and a good activity was obtained. Inhibitory activity of *Punicalin* was evaluated on HIV-1 reverse transcriptase by Martino et al. (2004) with high activity. Antiviral activity of *Punicalagin* toward human enterovirus 71 *in vitro* and *in vivo* was also evaluated by Yajun et al. (2012) with high activity.

**Conclusion**

From the ethyl acetate crude extract of *T. ivoriensis,* six polyphenols were isolated and characterized for the first time. This result was obtained through LC-ESI-MS methods

method. These natural products exhibited various biological activities. Most of these activities have confirmed the traditional use of this plant.

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