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# Antioxidant Activity and a New Ursane-type Triterpene from *Vitellaria paradoxa* (Sapotaceae) Stem Barks

**Emmanuel Talla[1], Jean Noël Nyemb[1,2*], Alembert Tchinda Tiabou[3], Sandrine Gael Zambou Djou[1], Pierre Biyanzi[4], Laurent Sophie[5], Luce Vander Elst[5] and Joseph Mbafor Tanyi[2]**

*[1]Department of Chemistry, Faculty of Science, University of Ngaoundere, P.O.Box 454, Ngaoundere, Cameroon.*
*[2]Department of Organic Chemistry, Faculty of Science, University of Yaounde I, P.O.Box 812, Yaounde, Cameroon.*
*[3]Institute of Medical Research and Medicinal Plants Studies (IMPM), Ministry of Scientific Research and Innovation, P.O.Box 6163 Yaounde, Cameroon.*
*[4]Department of Food Science and Nutrition, National School of Agro-Industrial Sciences, University of Ngaoundere, Cameroon.*
*[5]Department of General, Organic and Biomedical Chemistry, Faculty of Medicine and Pharmacy, University of Mons, NMR and Molecular Imaging Laboratory, B-7000 Mons, Belgium.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors ET, JNN and ATT designed the study, wrote the protocol and managed the literature searches. Authors JNN and SGZD performed the chromatographic isolation. Authors LS and LVE performed the spectroscopic analysis. Authors SGZD, PB and JNN performed the antioxidant and statistical analysis. Author JNN wrote the first draft of the manuscript. Author JMT advised and managed the analyses of the study. All authors read and approved the final manuscript.*

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**Corresponding author: E-mail: nyembjeannoel@gmail.com;*

## ABSTRACT

**Aims:** The aim of the present study was to evaluate the total phenolics and flavonoids content of stem barks of *Vitellaria paradoxa* C. F. Gaertn., and identify its main chemical constituents.
**Methodology:** *V. paradoxa* stem barks were extracted by maceration with methanol. Preliminary phytochemical screening was performed on the crude methanol extract (CME). Besides, total polyphenols contents (TPC) and total flavonoids contents (TFC) contents were also evaluated using the Folin-Ciocalteu method and complexation with aluminum chloride respectively. The antioxidant activity was evaluated by DPPH (2.2-diphenyl-1-picrylhydrazyl) and FIC (ferrous ion chelating) assays. Chromatographic isolation of the crude methanol extract (CME) followed by the spectroscopic identification of the isolated compounds was performed adopting 1D NMR and MS techniques.
**Results:** All the compounds tested were found to be present in the CME of *V. paradoxa* stem barks. The extract was found to be rich in phenolics (18.48 ± 1.43 mgGAE.$g^{-1}$) and flavonoids (3.98 ± 0.44 mgGAE.$g^{-1}$). The CME showed high antioxidant activity as DPPH free radical scavenging and a low FIC activity. A new ursane type-triterpenoid named vitellaric acid (**4**) along with four known compounds (+)-catechin (**1**), (-)-epicatechin (**2**), betulinic acid (**3a**), and bassic acid (**3b**) were isolated from CME.
**Conclusion:** The results of preliminary phytochemical screening of the leaf extracts revealed the presence of phytochemicals which could be used as medical regimens. The study provides scientific evidence for the use of *V. paradoxa* stem barks for the treatment of diseases mainly those associated with oxidative stress due to reactive oxygen species. Results yield a new addition to the chemical literature of *V. paradoxa*, in addition it increases the importance of NMR and MS techniques in structure elucidation.


*Keywords: Vitellaria paradoxa; DPPH assay; FIC assay; phenolic compounds; flavonoids; triterpenoids.*

## 1. INTRODUCTION

Degenerative human diseases such as accelerative aging, cancer, diabetes, cardiovascular diseases, inflammation and neurodegenerative diseases (including Parkinson's diseases Alzheimer's diseases) have been recognized as being a consequence of free radical damage. Free radicals are species that contain unpaired electrons. Molecules are composed of electrons, which are present generally in pairs. However, under certain conditions such as in lipid peroxidation process that occur in human body, molecules may contain unpaired electrons. Free radicals are generally reactive in seeking other electrons to become paired. They are highly reactive metabolites which oxidize the constituents of the cell, and in particular, the membrane, thus accelerating its aging and destruction [1]. The most frequently encountered free radicals are the hydroxyl radical ($HO^{\bullet}$), the superoxide radical ($O^{2\bullet-}$), the nitric oxide radical ($NO^{\bullet}$) and the lipid peroxyl radical ($LOO^{\bullet}$) while non-free radical species principally being $H_2O_2$ and singled oxygen ($O_2$) [2,3]. This reactive oxygen species (ROS) are essential for production of energy, synthesis of biologically essential compounds, and phagocytosis, a critical process of the immune system. Nevertheless, they can induce some oxidative damages to biomolecules causing degenerative human diseases [3-6]. Almost all organisms are protected from free radical attack by defense mechanisms such as a preventive antioxidant system that reduces the rate of free radical formation, and another is a system to produce chain-breaking antioxidants that scavenge and stabilize free radicals. ROS can therefore be trapped and destroyed by the body's antioxidant systems, including superoxide dismutase, catalase and glutathione peroxidase. But due to some factors such as the change of environment, pollution, increasing stress, smoking, excessive exercise and/or dietary xenobiotics, the amount of free radicals can increase and become difficult to be controlled by these enzymes [7]. The resulting imbalance of free radicals versus antioxidant processes will cause the subsequent cellular damage which will lead to several diseases. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of free radical mediated diseases.

Several studies have been conducted to find the way to prevent or delay these diseases from the

beginning stage. Many natural products have been reported to contain large amounts of antioxidants that play a role in delaying, intercepting or/and preventing oxidative reactions [8] catalysed by free radicals. Medicinal plants are an important source of antioxidants [9]. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke [10]. This antioxydant activity may be mainly due to the presence of secondary metabolites like phenolics, flavonoids [11], phenolic acids and phenolic terpenes [12]. Flavonoids and phenolic acids are major classes of phenolic compounds, whose structure-antioxidant activity relationships in aqueous or lipophilic systems have been extensively reported [13]. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark [14]. The physiological and pharmacological activities of phenolic compounds may be derived from their antioxidant properties, which are related to their molecular structure [15]. Phenolics and flavonoids play a key role as antioxidants due to the presence of hydroxyl substituents and their aromatic structure, which enables them to scavenge free radicals [16]. Total phenolics content (TPC) and total flavonoids content (TFC) are therefore considered as an important index for evaluation of antioxydant activity.

Synthetic antioxidants like butylated hydroxyl-anisole (BHA), butylated hydroxyl-toluene (BHT), and propyl gallate (PG) are commercially available. However, they have several side effects like risk of liver damage and carcinogenic [17-20]. There is therefore a need for more effective, less toxic and cost effective anti-oxidants. Medicinal plants appear to have these desired comparative advantages, hence the growing interest in natural antioxidants from plants. Cameroon is blessed with rich floristic resources having particular reference to the antioxidant components from medicinal plants. One of these plants, *Vitellaria paradoxa* (Sapotaceae) is used traditionally to treat several diseases such as skin infections, diarrhoea, digestive disorders, dysentery, convulsions, cough, leprosy, malaria and breast cancer. Stem barks are used for hypertension, incurable wounds, jaundice, and hemorrhoids [21-23]. Shea butter is the fat extracted from the kernel of this plant; it was reported to contain high level of UV-B-absorbing triterpene esters [24]; its antioxidant properties have led to its use to protect the skin from sunburn, eczema and as a skin rejuvenator [25]. Analysis of the kernel revealed the presence of phenolic compounds such as gallic acid, catechin, epicatechin, epicatechin gallate, gallocatechin, epigallo-catechin, epigal-locatechin gallate as well as quercetin and transcinnamic acid [26]. Works on this plant are mostly focused on the fruit, kernel, seed and the fat from the seed [27-31].

The aim of our study was to investigate the probable antioxidant effects of crude methanolic extract (CME) of *V. paradoxa* stem barks, to determine its total phenolics content (TPC) and total flavonoids content (TFC) using the Folin-Ciocalteu method and complexation with aluminum chloride respectively, and to isolate bioactive constituents of this plant. The qualitative phytochemical constituents of extracts were also examined.

## 2. MATERIALS AND METHODS

### 2.1 General Experimental Procedures

A Bruker AV-500, the Avance AV-300, Avance AV-400, and Avance AV-600 spectrometers, operating at 500 MHz, 300 MHz, 400 MHz and 600 MHz were used for $^{1}H$ NMR, while a Bruker AV-500 spectrometer operating at 125 MHz was used for $^{13}C$ NMR, were used for experiments with chemical shifts given in ppm. The spectra were run using $CDCl_3$, DMSO-$d_6$ and acetone-$d_6$ as solvents and TMS as internal standard. ESI-MS spectra (ionization voltage 3 kV) were measured on a Q-TOF Ultima spectrometer (Waters), while the low resolution electron impact mass spectra (EI-MS) were recorded on a Finnigan MAT 312 mass spectrometer. Column chromatography (CC) was performed on silica gel normal phase 60 (Merck, 63-200 µm) with step gradients of n-hexane-EtOAc and EtOAc-MeOH as eluents. Analytical Thin layer chromatography (TLC) was carried out on silica gel precoated plates F-254 Merck (20 x 20 cm). Detection of the spots was achieved under UV light (254 and 365 nm) and by spraying with 50% sulfuric acid followed by heating at 105℃. The absorbance in the experiments was read on a Rayleigh Vis-723N spectrophotometer. DPPH (Aldrich, 95%) was used as free radical donor. Folin-Ciocalteu reagent (Sigma, 2 N) and $Na_2CO_3$ (Labosi, 99.5%) were used to determine TPC, while aluminum chloride hexahydrate (JHD, 97%), were used for TFC quantification. Ferrozine reagent (Cambrian, 99%) and Ferrous sulfate heptahydrate (Vetec, 99%) were used for the FIC assay. Ascorbic acid (Riedel-De Haen, 99.7%), Quercetine (Sigma, ≥ 95%), Gallic acid

(Sigma-Aldrich, 97.5-102%), ethylenediaminetetracetic acid (EDTA; Vetec, 99%), and were used as standards. All organic solvents used for the tests were upgrade. Water used was distilled. All solutions were used on the day of preparation.

## 2.2 Plant Material

The stem barks of *V. paradoxa* were collected in May 2014 in Ngaoundere, Adamawa Region of Cameroon and identified by Mr. Nana Victor, plant taxonomist at the National Herbarium of Yaounde (Cameroon) where a voucher specimen (50216/HNC) is deposited for further verification. The stem barks were washed, cut in small pieces, and dried at room temperature for 3 weeks. The dried barks were ground into uniform powder to increase the surface area of the sample for extraction.

## 2.3 Extraction and Isolation

Powder of dried barks of *V. paradoxa* (500 g) was extracted at room temperature with methanol (2.5 L) by maceration for 48 hours. The obtained extract was filtered through Whatman No. 1 filter paper and evaporated to dryness with a rotary evaporator at 45℃ under reduced pressure, yielding a brown extract (81.54 g). This crude methanol extract (CME) was used for further investigation, isolation of compounds, potential antioxidant properties and total phenolics determination.

A portion (50 g) of the methanol extract was subjected to column chromatography over silica gel (300 g). Step gradient elution was conducted with hexane–EtOAc (1:0 → 0:1) and EtOAc–MeOH (1:0 → 1:1) to yield 9 series of fractions ($F_1$-$F_9$) according to their TLC profile using the mixtures of Hex/EtOAc and EtOAc/MeOH as eluent. The fractionnation of the methanolic extract lead to the isolation of 4 compounds (Fig. 3). (+)-catechin, (**1**, 6 mg), (-)-epicatechin (**2**, 40 mg), a mixture of betulinic acid, bassic acid (**3a** and **3b**, 5 mg), and 3-O-*β*-(*p*-hydroxy-*cis*-coumaroyl)-2-methoxy-9-homo-olean-12-ene-28-oic acid (vitellaric acid) (**4**, 6 mg) were obtained with Hex/EtOAc (7:3; 6.5:3.5; 5.5:4.5 and 4.5:5.5).

**(+)-catechin:** White powder, $^1$H-NMR (acetone-$d_6$, 400 MHz) *δ* in ppm: 6.12 (*d*, H-2, *J* = 6.4 *Hz*), 4.93 ( *d*, H-3, *J* = 6.4 *Hz*), 2.79 (*m*, 2H-4), 7.30 (*m*, H-6), 7.32 (*m*, H-8), 7.44 (*ov*, H-2'), 7.43 (*ov*, H-5'), 7.46 (*ov*, H-6'), 8.03 (*s*, 7-OH), 8.01 (*s*, 5-OH), 7.56 (*s*, 3´-OH), 7.45 (*s*, 4´-OH), 7.23 (*s*, 3-OH).

**(-)-epicathechin:** Brown yellowish powder, TOF-MS-ESI+: (5.45e$^3$) *m/z* 291.2 $[M+H]^+$; 893.2 $[3M+Na]^+$ corresponding to $C_{15}H_{14}O_6$. $^{13}$C NMR (DMSO-$d_6$, 125 MHz) *δ* (ppm): 78.02 (CH-2); 64.87 (CH-3); 28.17 ($CH_2$-4); 156.2 (C-5); 95.0 (CH-6); 156.5 (C-7); 94.0 (CH-8); 155.7 (C-9); 98.3 (C-10); 130.6 (C-1´); 117.9 (CH-2´); 144.5 (C-3´); 144.4 (C-4´); 114.7 (C-5´); 114.8 (CH-6´); $^1$H-NMR (DMSO-$d_6$, 500 MHz) *δ* (ppm): (4.78; H-2); (4.01; H-3); (2.61; 2.72; 2H-4); (5.91; H-6); (5.72; H-8); (6.90; H-2´); (6.68; H-5´); (6.67; H-6´).

**Betulinic acid:** White powder, LREI-MS: (70 ev, direct inlet) *m/z* (rel. int.): 219.0 (41.6), 207.0 (40.1), 203.0 (14.0), 189.0 (78.0), 175.1 (9.8). $^1$H NMR ($CDCl_3$, 600 MHz): 0.73, 0.80, 0.92, 0.94, 0.95 and 1.67 (*s*, each 3H, H-24, H-23, H-27, H-25, H-26 and H-30), 4.58 and 4.71 (*bsr,* 2H-29, 1H each for terminal isopropenyl group), 3.17 (*dd,* $J_{ax,ax}$ = 12.0 *Hz*, $J_{ax,\ eq}$ = 4.8 *Hz*, H-3 due to geminal OH coupling), 2.97 (*ddd*, *J* = 15.6 *Hz*, *J* = 10.8 *Hz*, *J* = 4.8 *Hz*, H-19*β*).

**Bassic acid:** white powder, LREI-MS: (70 ev, direct inlet) *m/z* (rel. int.): 248.0 (14.0), 219.0 (0.2), 203.0 (14.0), 189.0 (78.0), 187.0 (20.8), 147.0 (12.1), 105.0 (49.2), 133.1 (44.1). $^1$H NMR ($CDCl_3$, 600 MHz): H 5.09 (1H, *m*, H-2), 5.02 (1H, *m*, H-3), 5.85 (1H, *m*, H-6), 5.62 (1H, H-12), 3.01 (*d*, *J* = 4.8 *Hz*, H-18), 4.05 and 3.47 (1H each, *brs*, H-23), 0.97, 0.94, 0.93, 0.91, 0.80, and 0.73 (3H each, *s*, H-24, 25, 26, 27, 29, and 30).

**Vitellaric acid** (3-O-*β*-(*p*-hydroxy-*cis*-coumaroyl)-1*β*-2*α*-19α-23-tetrahydroxy-urs-5,12-dien-28-oic acid): White powder, TOF-MS-ESI+: (3.12e$^3$) *m/z* (rel. int.): 663.3 $[M+H]^+$ (4.7), 553.3 (6.3), 537.3 (27.5), 532.3 (58.8), 515.3 (100) 489.3 (4.5), 439.3 (6.1), 391.3 (11.3), 336.4 (6.1), 310.3 (5.0), 233.1 (4.3), 162.8 (4.4); LREI-MS: (70 ev, direct inlet) *m/z* (rel. int.): 248.1 (3.1), 246.1 (1.2), 205.1 (7.3), 203.1 (8.7), 201.1 (17.9), 189.0 (11.1), 187.1 (14.2), 175.1 (7.2), 173.1 (16.8), 147.1 (38.3), 145.1 (47.2), 133.0 (28.6), 131.0 (28.8), 119.2 (34.2), 93.0 (38.4), 91.0 (39.9), 77.0 (12.8). $^1$H NMR (acetone-$d_6$, 300 MHz) and $^{13}$C NMR (DMSO-$d_6$, 125 MHz) data see Table 3.

## 2.4 Phytochemical Screening

Qualitative phytochemical screening was carried out to investigate the various classes of natural compounds present in the extracts. This was carried out using standard procedures to identify

the constituents as described by Trease and Evans [32] and Harborne [33].

#### 2.4.1 Alkaloids

In about 5 mL of dissolved extract, few drops of 2% sulphuric acid were added and the mixture was distributed in two tests tubes.

- **Mayer's Test.** The first tube was treated with Mayer's reagent (potassium mercuric iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.
- **Dragendroff's Test.** The second one was treated with Dragendroff's reagent (solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids.

#### 2.4.2 Test for anthraquinons

- **Borntrager's test.** To 0.5 mg of the extract, 5 mL of chloroform was added and shaken for 5 minutes. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. A pink violet or red colour in the lower layer indicates the presence of anthraquinons.

#### 2.4.3 Flavonoids

- **Alkaline reagent test.** In a few amount (5 mL) of dissolved extract in the appropriate solvent, few drops of sodium hydroxide solution (10%, w/v) were added. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.
- **Lead acetate test.** 5 mL of dissolved extract were treated with few drops of alkaline lead acetate solution. Formation of yellow colour precipitate indicates the presence of Flavonoids.

#### 2.4.4 Phenolic compounds

- **Ferric Chloride Test.** 5 mL of dissolved extract was treated with few drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

#### 2.4.5 Saponins

- **Froth Test:** About 0.5 g of the extract was shaken with 10 mL of distilled water and then heated to boil. Frothing (appearance of creamy miss of small bubbles) shows the presence of saponins.
- **Foam Test:** 0.5 g of extract was shaken with 10 mL of water. If foam produced persists for 10 minutes it indicates the presence of saponins.

#### 2.4.6 Steroids

- **Liberman-Burchard's test.** To 0.5 g of the extract 2 mL of acetic anhydride was added with 2 mL of sulphuric acid. The colour change from violet to blue or green in samples indicates the presence of steroids.

#### 2.4.7 Tannins

- **Ferric chloride test.** 0.5 g of extract was mixed with 10 mL of water and heated on water bath. The mixture was filtered and ferric chloride was added to the filtrate. A dark green solution indicates the presence of tannins.
- **Gelatin test.** To 5 mL of the extract dissolved in the appropriate solvent, few drops of a 1% gelatin solution containing sodium chloride (10%) were added. Formation of white precipitate indicates the presence of tannins.

#### 2.4.8 Terpenoids

- **Salkowski test.** 0.5 g of extract was treated with 10 mL of chloroform and filtered. Filtrates were treated with few drops of Concentrated Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

### 2.5 Quantitative Phytochemical Compositions of the Extracts

On the basis of positive results obtained for some of the phytochemicals and according to the antioxidant activity sought, the total phenolic and total flavonoids composition of the extracts were determined.

#### 2.5.1 Determination of total phenolic content (TPC)

Total phenolics were assessed by the Folin-Ciocalteu method previously describe by Talla et al. [34], using gallic acid (0.2 $g.L^{-1}$) as a standard.

The levels of total phenols in extract determined according to the Folin-Ciocalteu method are not absolute measurements of the amounts of phenolic materials but are in fact based on their chemical reducing capacity relative to an equivalent reducing capacity of gallic acid. The test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. A volume of 20 µL of extract solution (10 $g.mL^{-1}$) was added to a mixture of 200 µL of Folin-Ciocalteu reagent and 1.380 µL of distilled water followed by thorough mixing. After 3 min, 400 µL $Na_2CO_3$ (20%) were added. The mixture was allowed to stand for 20 min at 40°C with intermittent shaking. The absorbance was measured at 760 nm. The determination of the total phenolic compounds was carried out after standardization with Gallic acid using a straight line equation obtained from the standard Gallic acid calibration graph obtained by plotting optical densities against concentration of Gallic acid. The correlation equation constructed with Gallic acid (12.5 to 200 µg/mL) was $y = 2.593x + 0.014$ ($R^2 = 0.9878$).

The total phenolic content was measured as grams of Gallic acid equivalent per g of dry extract.

#### 2.5.2 Estimation of total flavonoid content (TFC)

The total content in flavonoids was determined by Aluminum chloride method described by Chang et al. [35] with slight modifications. Flavonoids are capable of forming complexes with metal ions and act as antioxidants. The plant extract (100 mg) was dissolved in 10 mL methanol at room temperature to give a concentration of 10 $mg.mL^{-1}$. The reaction mixture (2.0 mL) comprised of 1.0 mL of extract, 1.0 mL of aluminum chloride (2%) was incubated at room temperature for 60 min and absorbance measured at 430 nm. Quercetin was used as a positive control. The correlation equation constructed with quercetin (2 to 50 µg/mL) was $y = 0.2003x \pm 0.0096$ ($R^2 = 0.9993$). The flavonoid content was expressed in terms of standard equivalent ($mg.g^{-1}$ of extracted compound).

### 2.6 Measurement of *In vitro* Antioxidant Activity

#### 2.6.1 DPPH radical-scavenging activity assay

The DPPH radical scavenging activity was determined using the method of Talla et al. [34] with slight modifications. A series of 5 successive dilutions were prepared from sample stock solutions 1 $mg.mL^{-1}$ in methanol. For each concentration, 1 000 µL of $DPPH^{\bullet}$ (20 $mg.L^{-1}$ in methanol) was added to 500 µL of sample or extract. The mixtures were shaken vigorously and left to stand for 15 minutes of incubation in darkness at room temperature. After that, the absorbance of the mixtures were measured at 517 nm against a blank or control experiment (500 µL of extract or sample solution in 1 000 µL of methanol). The control experiment with a solution composed of 500 µL of pure methanol and 1000 µL of $DPPH^{\bullet}$ was used. Ascorbic acid (vitamine C) was used as reference. Varying concentrations of the standard were also prepared at similar concentrations and his absorbances were used in comparison with those of the extracts. Lower absorbance of the mixture indicated higher free radical scavenging activity. The percentage inhibition was calculated as

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] * 100$$

$A_0$ was the absorbance of the blank; $A_1$ was the absorbance in the presence of the extract or the standard, ascorbic acid.

#### 2.6.2 Evaluation of metal chelating activity

Ferrozine can quantitatively chelate with $Fe^{2+}$ and form a complex with a red color. This reaction is limited in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-$Fe^{2+}$ complexes. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions [36]. The chelation of ferrous ions is estimated using the method of Talla et al. [34].

To 200 µL of the extract at different concentration (ranging from 2 to 10 $mg.mL^{-1}$) is added a solution of 100 µL ferrous chloride (2 mM). The reaction is initiated by the addition of 400 µL of ferrozine (5 mM) and incubated at room temperature for 20 min and then the absorbance is measured at 700 nm. EDTA was used as a positive control. The assay was carried out at 20°C to prevent $Fe^{2+}$ oxidation. Lower absorbance indicated a higher iron chelating capacity. The negative control was without any chelating compound or test sample of extract. EDTA was prepared in same way as the test samples and treated with same reagent. Its values (absorbances) were used for comparison. The percent ferrous ion chelating capacity was

calculated accordingly by comparing the absorbance of the test samples with that of the negative control.

$$\text{Ferrous Ion Chelating capacity} = [(A_{control} - A_{extract})/A_{control}]*100$$

## 2.7 Statistical Analysis

All the experiments were carried out in triplicate, and the results were presented as mean ± SD (standard deviation). The comparisons between the dependent variables were determined using the analysis of variance (ANOVA) by XLSTAT 2007.8.04. The Duncan statistical test (LSD: least significant difference) were used in the comparison of means. Differences were considered statistically significant at $p < 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1 Qualitative Phytochemical Results

The CME of stem barks of *V. paradoxa* was screened for the presence of the following secondary metabolites: alkaloids, phenolic compounds, steroids, terpenoids, flavonoids, saponins, anthocyanins, coumarins, and tanins. The results of the phytochemical screening (Table 1) revealed the presence of all the organic compounds analyzed except steroids. The presence of these compounds in the extract is quite instructive as this lends credence of the use of the plant for medicinal purposes. Steroids are absents in the CME of *V. paradoxa* stem barks; this does not mean that this family of compounds is totally absent from the whole plant or from this part (stem barks) of the plant as the types of solvent and part of plants used may account for the variation in phytochemicals present [37]. Indeed, Oben et al. [38] reported the isolation of *β*-sistosterol and sigmasterol from the EtOAc extract of stem barks of *V. paradoxa*. The absence of steroids observed in this study could therefore suggested that they might be present in undetectable amount in the extract as we worked with crude extract which has a very complex composition and so some compounds may be masked [39], or this could be probably due to their low solubility in MeOH.

Phytochemical components are responsible for both pharmacological and toxic activities in plants [40]. Phenolic compounds aid to plant defense to counteract reactive oxygen species (ROS) for surviving and prohibiting molecular damage and damage due to microorganisms, insects and herbivores [41]. Flavonoids are another important components, present in the part of plant examined, they have various pharmacological effects like antioxidant, free-radical scavenging, anti-cancer and anti-aging activities [42] etc. Tannins are anti-cancerous in nature [43]. Hence, the use of *V. paradoxa* for the treatment of cancer, epilepsy, diarrhea, dysentery, malaria and several other diseases by local herbalists or traditional healers is not surprising.

**Table 1. Results of the phytochemical screening of CME of *V. paradoxa* stem barks**

| Phytochemical constituents | Test | CME |
|---|---|---|
| Alkaloids | Mayer's test | + |
| | Dragendorff's test | + |
| Anthraquinone | Borntrager's test | + |
| Flavonoids | Alkaline reagent test | + |
| | Lead acetate test | + |
| Phenolic compounds | Ferric chloride test | + |
| Saponins | Froth test | + |
| | Foam test: | + |
| Steroids | Liberman-Burchard's test | - |
| Triterpenoids | Salkowski's test | + |
| Tanins | Ferric chloride test | + |
| | Gelatin test | + |

*+: positive reaction; -: negative reaction*

### 3.2 Total Phenolic Content (TPC) and Total Flavonoids Content (TFC)

Phytochemical composition of the CME was determined based on the results of phytochemical screening. TPC was determined by the Folin-Ciocalteu phenolic reagent method. This assay detects phenolic acids, flavonoids, tannins, anthocyanins, lignans and coumarins. TPC of the plant extracts was determined in mg of gallic acid equivalent per gram of dry extract (mgGAE.g$^{-1}$ dry extract) using the equation obtained from the standard gallic acid graph.

The results indicated that CME of stem barks of *V. paradoxa* contains significant amount of total phenolic compouds equivalent to 18.48 ± 1.43 mgGAE.g$^{-1}$ of dry weight of extract (mg of gallic acid equivalent per g of sample) and total flavonoids content was 3.98 ± 0.44 mgEQc.g$^{-1}$ dry weight of extract, *i.e.*, 1 g of the extract contains 18.48 mg of gallic acid equivalent and

3.98 mg of quercetin equivalent. From these results we can see that TPC is highly over than TFC. This would mean that flavonoids are not the major phenolic compounds of this extract, as phytochemical screening also revealed the presence of others phenolic compounds (tannins, anthraquinons…).

## 3.3 Antioxidant Test Results

According to Melo et al. [44], antioxidant activity can be classified based on the performance of the crude extract: I − good activity ($IC_{50}$ < 69 µg.mL$^{-1}$); II − moderate activity (69 µg.mL$^{-1}$ < $IC_{50}$ < 161 µg.mL$^{-1}$); III − low activity ($IC_{50}$ > 161 µg.mL$^{-1}$).

### 3.3.1 Free radical scavenging: DPPH assay

DPPH is a stable free radical and widely used to assess the radical scavenging activity of antioxidant compounds. The method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the non-radical form DPPH-H [45]. This transformation leads to a color change from purple to yellow, which is spectrophotometrically monitored at 517 nm. Ascorbic acid was chosen as the reference antioxidant for this test. The results showing the variation of percentage inhibition as a function of concentration of the extract and reference are shown in Fig. 1. All the $IC_{50}$ values (Table 2) were determined by graphical methods from the curves. These values are acceptable implying that they were in concordance with the plots. $IC_{50}$ is inversely proportional to AOA meaning that the greater the $IC_{50}$ value the lower the AOA. Scavenging of DPPH radical was found to rise with increasing concentration of the samples (Fig. 1). The highest scavenging was observed for CME with an $IC_{50}$ value of 12.28 ± 5.87 µg.mL$^{-1}$ followed by (-)-epicatechin with an $IC_{50}$ 22.18 ± 4.71 µg.mL$^{-1}$, against 9.32 ± 2.01 µg.mL$^{-1}$ for the standard ascorbic acid, which is a well-known antioxidant.

The high radical scavenging capacity of CME opposite to the one of (-)-epicatechin (**2**) suggest that CME could contain other compounds than (-)-epicatechin (**2**) that could have better radical scavenging capacity; or it could be the result of the synergic action of two or more antioxidant compounds present in the extract.

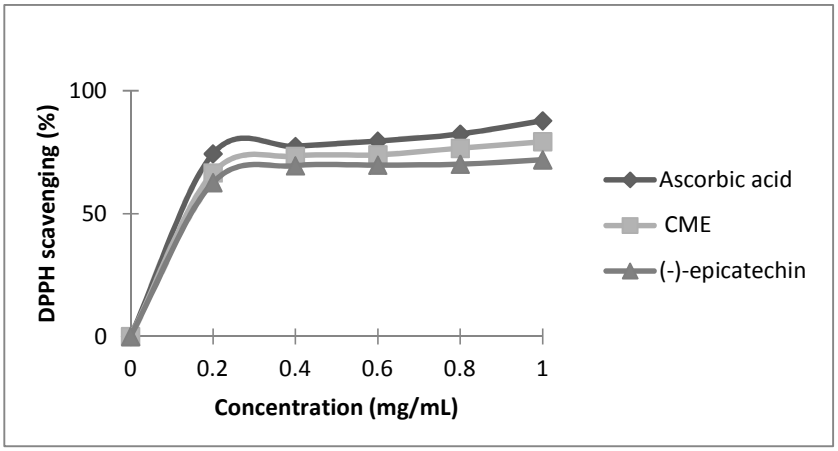


**Fig. 1. DPPH inhibition assay of CME and (-)-epicatechin (2)**

**Table 2. Radical scavenging activity (DPPH assay), and chelating activity (FIC assay) of CME of *V. paradoxa* stem bark**

| Sample | Antioxydant activity (AOA) | |
|---|---|---|
| | DPPH free radical scavenging $IC_{50}$ (µg.mL$^{-1}$) | FIC assay $IC_{50}$ (µg.mL$^{-1}$) |
| Ascorbic acid | 9.32 ± 2.01 [a] | - |
| EDTA | - | 0.73 ± 0.21 [a] |
| CME | 12.28 ± 5.87 [b] | 8946.32 ± 0.65 [b] |
| (-)-epicatechin (2) | 22.18 ± 4.71 [c] | - |

*Means followed by the same letter in a column do not differ significantly (n = 3, p <0.05)*

So, the high DPPH free radical scavenging of the CME could be attributed to the presence of (-)-epicatechin (**2**) and (+)-catechin (**1**), two well-knowns antioxidants isolated from this extract. Meanwhile, it could be possible that some more polar phenolic compounds such as tannins were present in the extract and were not isolated. However, our results showed that the CME of the barks of *V. paradoxa* has high radical scavenging capacity and can be used to inhibit the oxidation of vital substances by ROS, possibly by acting like a primary antioxidant as it has been suggested that extracts or compounds that exhibit activity against the DPPH free radical can be considered as primary antioxidants, since these compounds act as electron donors and interrupt the chain reactions [46,47].

In a previous study, Simo Tagne et al. [48] used the DPPH assay and found an $IC_{50}$ of 22.14 ± 0.39 µg.mL$^{-1}$ for the same CME extract of stem barks of *V. paradoxa*. This result implies that our sample is more efficient than they reported. Meanwhile, as the both sample of stem barks of *V. paradoxa* have been collected in the same region of Cameroon at the same period of the year, this significant difference in the activity may be due to some parameters like the solvent used for the preparation of sample and DPPH, the concentration of the DPPH working solution, the duration of the reaction of radical scavenging activity between DPPH solutions and sample, the wave length used for absorbance measurement of the discoloration of the reaction mixture, the standard solution and the sample to reagent ratio.

Some studies showed that the antioxidant effect of plant products is mainly due to radical-scavenging activity of phenolic compounds such as flavonoids, polyphenols, tannins, and phenolic terpenes [49]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [50].

#### 3.3.2 Metal chelating activity: Ferrous ion chelating capacity (FIC)

The main strategy to avoid reactive oxygen species generation that is associated with redox active metal catalysis involves chelating of the metal ions. The transition metal ion, $Fe^{2+}$ possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals [51].

The Fig. 2 below shows that the ferric ion chelating (FIC) capacity of extracts increased proportionally with the concentration. This can be explained by the fact that an increasing of the concentration of the extract lead to the formation of greater amount of $Fe^{2+}$ complexes by increasing the chelating agent concentration [34]. The positive control used (EDTA) has the highest percentage of ferrous ion chelating capacities of 99.45% at the concentration of 10 mg.mL$^{-1}$ than the CME at the same concentration (39.87%). Elsewhere, the result shows that, the CME has a FIC capacity assailable by the organism.

The transition metals in biological systems can catalyze the Haber-Weiss and Fenton reactions, resulting in the generation of hydroxyl radicals [52]. However, these transition metals can be chelated by antioxidants, resulting in the suppression of the generation of $HO^{\bullet}$ and an inhibition of peroxidation of biological molecules. Generally, Chelating capacity is attributed to flavonoids and phenolic compounds with free hydroxyl group which use their redox properties to chelate transition metals. Their oxygen atom and hydroxyl groups can chelate the $Fe^{2+}$ ions. Thus, the low FIC capacity of the CME of the stem bark of *V. paradoxa* suggests that it contains small amounts of ligands to compete with ferrozine and prevent the generation of hydroxyl radicals. According to Kostyuk et al. [53], flavonoids bind to metal ions and are much less prone to oxidation than free compounds in the presence of superoxide. This low chelator activity of the extract ($IC_{50}$ = 8946.32 ± 0.65 µg.mL$^{-1}$) could be due to the fact that flavonoids and phenolic compounds contained in the extract are mostly substituted, in other words the most high number of flavonoids and phenolics contained in the CME do not have enough free hydroxyl groups to chelate high amount of the $Fe^{2+}$ ion.

### 3.4 Identification and Structure Elucidation of Compounds

The CME of the stem bark of *V. paradoxa* was submitted to chromatographic techniques and yielded five compounds **1**–**4** (Fig. 3). Amoung these compounds, 4 were known and identified as (+)-catechin (**1**), (-)-epicatechin (**2**), betulinic acid (**3a)** and bassic acid (**3b**). The last two compounds **3a** and **3b** were obtained as a mixture.

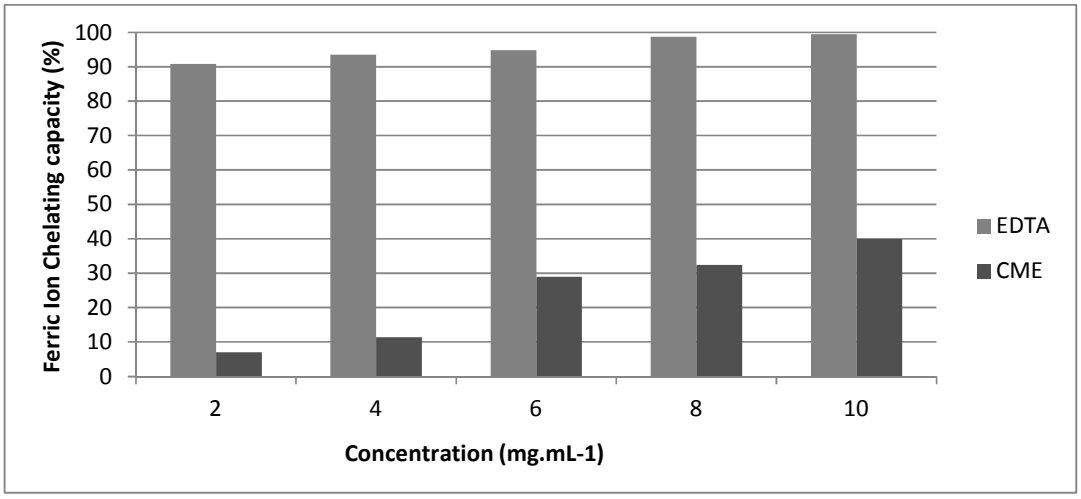


**Fig. 2. Chelating capacity of the CME of *V. paradoxa* stem barks**

The mixture of compounds **3a** and **3b** crystallizes as a white powder in Hex/EtOAc (5.5:4.5). It has not been further purified by silica gel chromatography because of the very small quantity isolated (5 mg). Comparison by co-TLC with the authentic sample of betulinic acid showed that this mixture is formed of betulinic acid and another more polar terpenoid (violet color of the spot). The structures of these compounds were described using only $H^1$ NMR, EI-MS, TOF-ESI-MS and comparative analysis with literature values.

The EIMS of the two compounds **3a** and **3b** from the mixture show similar fragmentation patterns which are analogous to the EIMS of terpenoids. Analysis of its EI mass spectrum didn't have any molecular ion peak. However, the mass spectrum displayed characteristic of a retro-Diels-Alder fragmentation of pentacyclic triterpenes at *m/z* 248, 219, 207, 203, 189 and 133. The identification of these compounds was therefore made possible by the different fragmentation mechanism that these compounds undergo and comparison with literature.

The ions at *m/z* 189.0, and 207.0 [$M^+$-$C_{16}H_{27}$], are generally characteristics of a pentacyclic triterpenes (Fig. 5) with an isopropenyl group [54], while those at *m/z* 203.0, 189.0, and 133.1 are characteristic skeletal fragments produced by oleanane-type triterpènes [55]. These last fragments in addition of the one at *m/z* 248.0 resulted from a retro-Diels-Alder cleavage of ring C, typical fragmentation mechanism of pentacyclic oleanane-type triterpenes with a double bond at C-12 (13) and carboxylic group at C-17 (figure). So, we have a mixture of an oleanane-type and lupane-type triterpenes. $^1$H NMR spectral data of the mixture of compounds **3a** and **3b** showed thirteen methyls signals at $\delta_H$ 0.73 (6H, *s*), 0.80 (6H, *s*), 0.91 (3H, *s*), 0.92 (3H, *s*), 0.93 (3H, *s*), 0.94 (6H, *s*), 0.95 (3H, *s*), 0.97 (3H, *s*) and 1.67 (6H, *s*). Among which 7 (0.73, 0.80, 0.91, 0.94, 0.95, 1.67) were attributed to compounds **3a** (lupeol) for the tertiary methyl protons H-23 to 27 and 30, respectively, and six (0.73, 0.80, 0.92, 0.93, 0.94, 0.97 and 1.67) to compounds **3b**, for angular methyl protons H-23 to H-30 except H-28 due to the presence of carboxylic group at this position. The presence of a double doublet of two protons at $\delta_H$ 3.17 (*dd*, *J* = 12.0, 4.8 *Hz*) is due to the methane proton germinal to an OH group; this signal have been assigned to the proton H-3 of compounds **3a**. The coupling constants of H-3 (3.17, *dd*, *J*= 12.0, 4.8 Hz) observed in the $^1$HNMR spectrum indicated *β*-orientation of the OH group at C-3. The proton H-3 of compound **3b** appears at 5.02 (1H, *ov*). The proton at $\delta_H$ 2.95 (*J* = 15.6 *Hz*, *J* = 10.8 *Hz*, *J* = 4.8 *Hz*) was assigned to H-19 of **3a** and H-18 of **3b** was detected at $\delta_H$ 3.01 (*d*, *J* = 4.8 *Hz*). The spectrum also reveals typical signals of pentacyclic lupane-type triterpenes with a pair of olefenic protons at $\delta_H$ 4.58 and 4.71 (2H-29 of compound **3a**) that was indicative of a terminal isopropenyl group.

The $^1$H NMR spectrum of this mixture exhibits furthermore two overlapping of one proton each at $\delta_H$ 5.26 and 5.65, which reveals the presence of two olefinic protons (1H, *m*, H-12 and 1H, *m*, H-6) in the B and C rings of the triterpenoid **3b**. The overlapped signals at 5.09 and 5.02 were

attributed to two oxymethine protons H-2 and H-3 respectively in the ring A of **3b**. While the ones at 4.05 and 3.47 (1H, *s* each) was assigned to the hydroxymethylene protons at C-23 of compound **3b**. Comparison of the $^{1}$H NMR and EIMS data of the mixture of compound **3a** and **3b** with reference literatures indicated that these were in accord with those of betulinic acid for compound **3a** [56] and bassic acid for **3b** [57,58]. These two structures are supported by the fragmentation shown below (Figs. 4 and 5).

Compound **4** was obtained as a white powder and gave a positive Liebermann–Burchard reaction. It was assigned the molecular formula $C_{39}H_{52}O_9$ from the TOF-MS-ESI+ analysis, which showed the quasi-molecular ion $[M-H]^+$ at *m/z* 663.3, as well as from its NMR spectroscopic data. The $^{1}$H NMR (300 MHz, acetone-$d_6$) of compound **4** exhibited six tertiary methyl groups detected at $\delta_H$ 0.98 (*s*, 3H-24), $\delta_H$ 0.77 (*s*, 3H-25) $\delta_H$ 0.84 (*s*, 3H-26), $\delta_H$ 1.35 (*s*, 3H-27), $\delta_H$ 1.17 (*s*, 3H-29), and a secondary methyl at $\delta_H$ 0.94 (*br*, *d*, 5.1 Hz, 3H-30) (Table 2) ; a primary hydroxyl methylene at $\delta_H$ 3.55 (*m*, H-23*ax*), 3.37 (*m*, H-23*eq*), three secondary oxymethines at $\delta_H$ 4.05 (*m*, H-3) and 3.89 (*m*, H-2) 3.29 (m, H-3) and $\delta_H$ 3.29 (*m*, H-3).

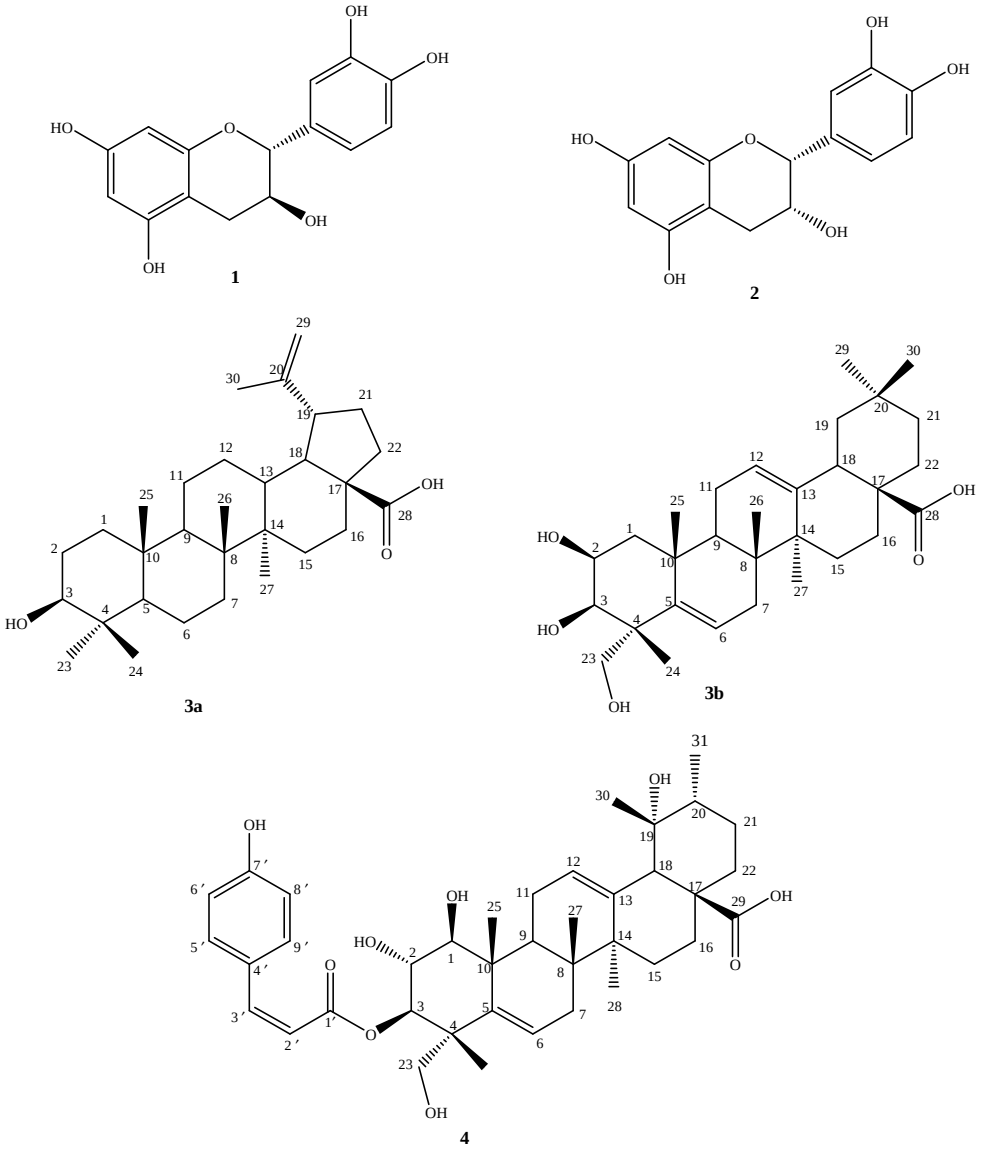


**Fig. 3. Chemical structures of compounds isolated from the stem barks of *V. paradoxa***

The proton spectrum exhibits furthermore a triplet of two protons at $\delta_H$ 5.28 (*br, t*, *J* = 3.5 Hz), which reveals the presence of two olefinic protons (H-6 and H-12) in the B and C rings of the triterpenoid. A singlet at $\delta_H$ 2.54 is detected for another methine proton (H-18). The signal at $\delta_H$ 2.63 (*ddd*, 3.6, 9.0, 12.6 Hz), integrating for one methine proton is attributed to H-11a, while the one at $\delta_H$ 1.95 (2H, *d*, 3.6 Hz) is assigned to H-7a and H-11b. Furthermore, $^1$H NMR spectrum of **4** (Table 2) showed an AB system of two olefinic protons at $\delta_H$ 6.90 (*d*, *J* = 8.7 Hz, 1H, H-3′) and 7.90 (*d*, *J* = 8.7 Hz, 1H, H-2′), an AA′BB′ system of four aromatic protons at $\delta_H$ 7.42 (*m*, H-5′/9′) 7.69 (*m*, H-6′/8′). The small coupling constant (*J* = 8.7 Hz) between H-2′ and H-3′ indicated a *cis* configuration at the double bond [59]. All these signals suggested the presence of a 4-hydroxy-cinnamoyl moiety. This is supported in EIMS by the fragments at *m/z* 163.1, 147.1, 119.2, 93.1 and 77.1 (Fig. 7). The $^{13}$C-NMR and $^{13}$C DEPT 135 spectra displayed three oxymethines signal at $\delta_C$ 84.4 (C-1), 69.3 (C-2) and 77.9 (C-3), one oxygenated methylene signals at $\delta_C$ 65.3 (C-23). The signal at $\delta_C$ 53.2 was assigned to C-18, while the one of a quaternary carbon at $\delta_C$ 71.7 is attributed to the oxygenated quaternary carbon C-19. The confirmation of its position came from the EI-MS analysis which showed a pic at m/z 219.1 ($C_{15}H_{23}O$) resulting from the retro Diels-Alder fragmentation of ring C followed by the loss of a carboxylic group –COOH. The further loss of $H_2O$ yields to m/z 201. Based on biogenetic considerations and spectral data in comparison with those of known compounds, the cynnamoyl group is fixed at C-3, the orientation being *β*-equatorial [29, 57,58]. The $^1$H and $^{13}$C NMR data of **4** indicated that it had the same hexacyclic triterpene skeleton than 2,3-O-isopropy-lidene-1*β*,2*β*,3*β*,19*α*-tetrahydroxyurs-12-en-28-oic acid [60]. The major difference between **4** and the last one was the presence of the double bond at C-5(6), the oxymethylene at C-23, and the substituents at C-2 and C-3 in **4**. Thus, the planar structure of **4** was established as shown. According to Mahato and Kundu [61], configurational determination of hydroxyl groups can be deduced from the inspection of the $^{13}$C NMR data of various mono- and poly- hydroxy triterpenes; the hydroxyl bearing carbon is less deshielded by the adjacent axial hydroxyl than by the equatorial one. The relative configuration of **4** was therefore elucidated from the $^{13}$C NMR (Table 3) and comparison with those of 2,3-O-isopropylidene-1*β*,2*β*,3*β*,19*α*-tetrahydroxyurs-12-en-28-oic acid [60]. The same configuration was observed for compound **4** except for C-2. Indeed, the less deshielded C-2 of **4** suggested the *α*-orientation of hydroxyl group at this position. Thus, the structure of **4** was established as 3-O-*β*-(*p*-hydroxy-*cis*-coumaroyl)-1*β*-2*α*-19α-23-tetrahydroxy-urs-5,12-dien-28-oic acid, and it was named vitellaric acid. This is to the best of our knowledge, the first report of this compound in the plant kingdom.

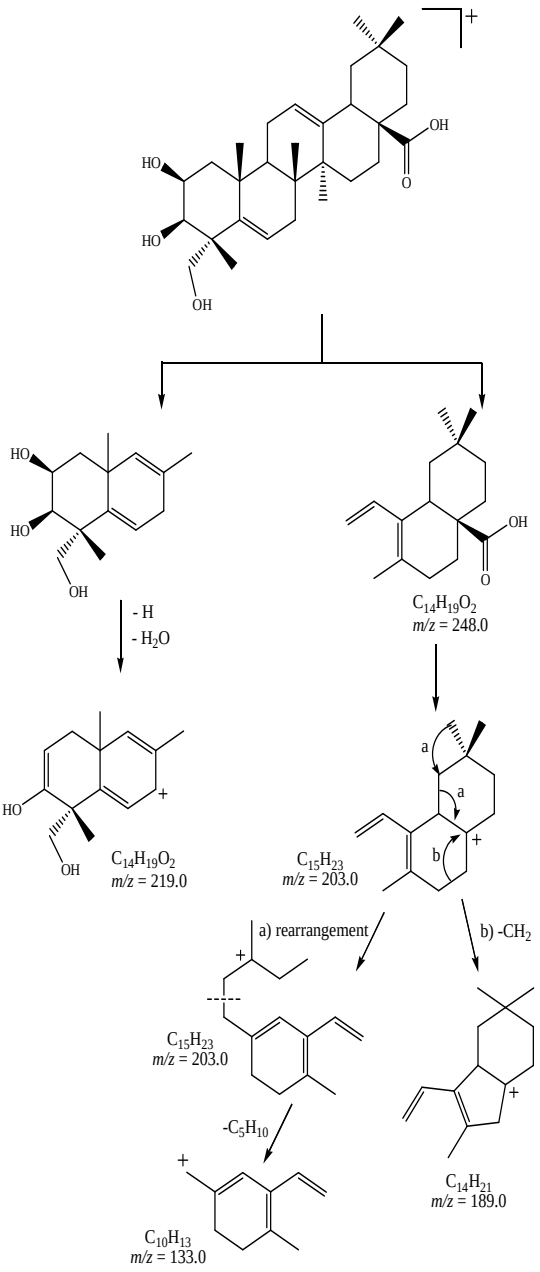


**Fig. 4. Proposed fragmentation mechanism of bassic acid (compound 3a)**

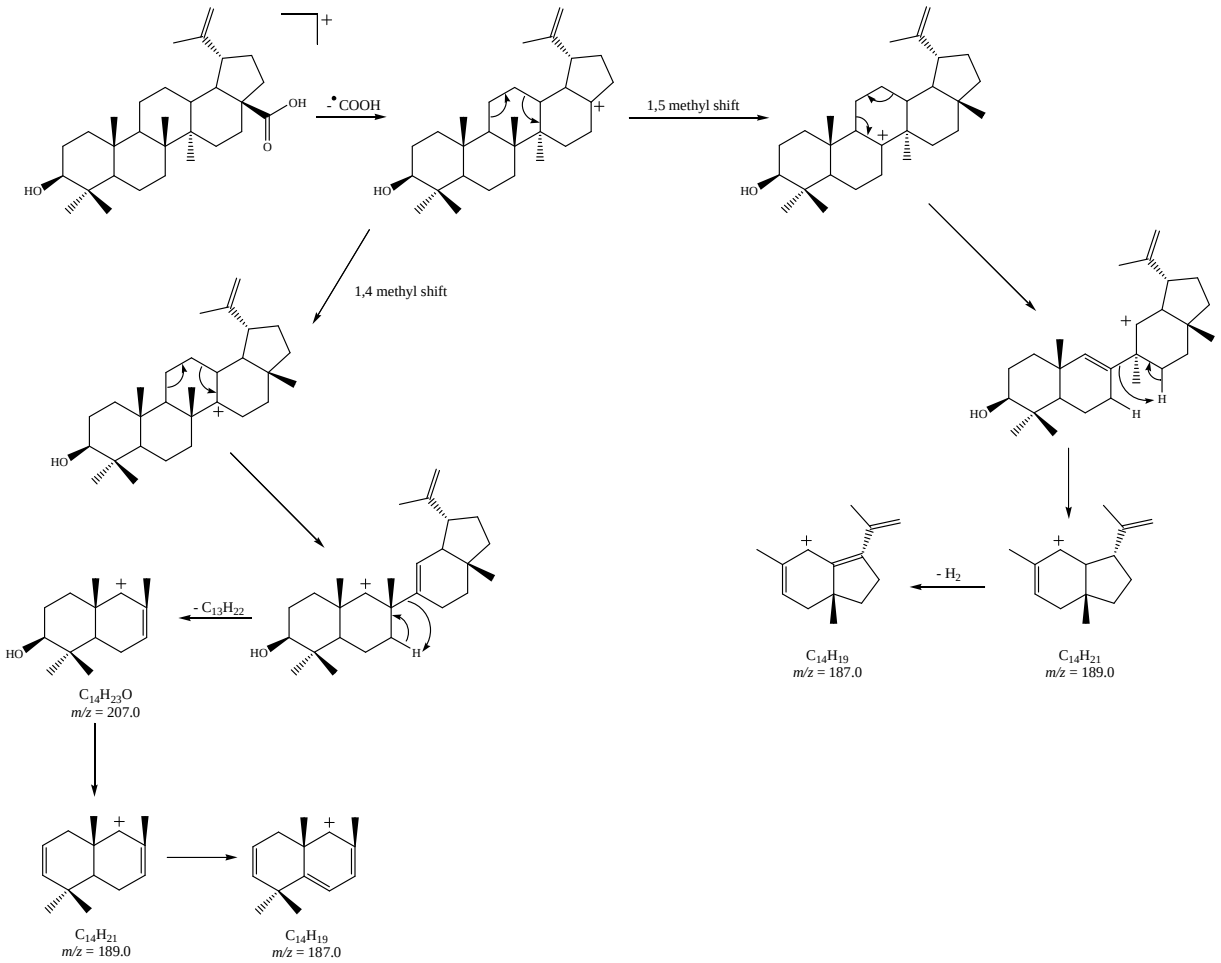


**Fig. 5. Fragmentation by EI of betulinic acid (compound 3b)**

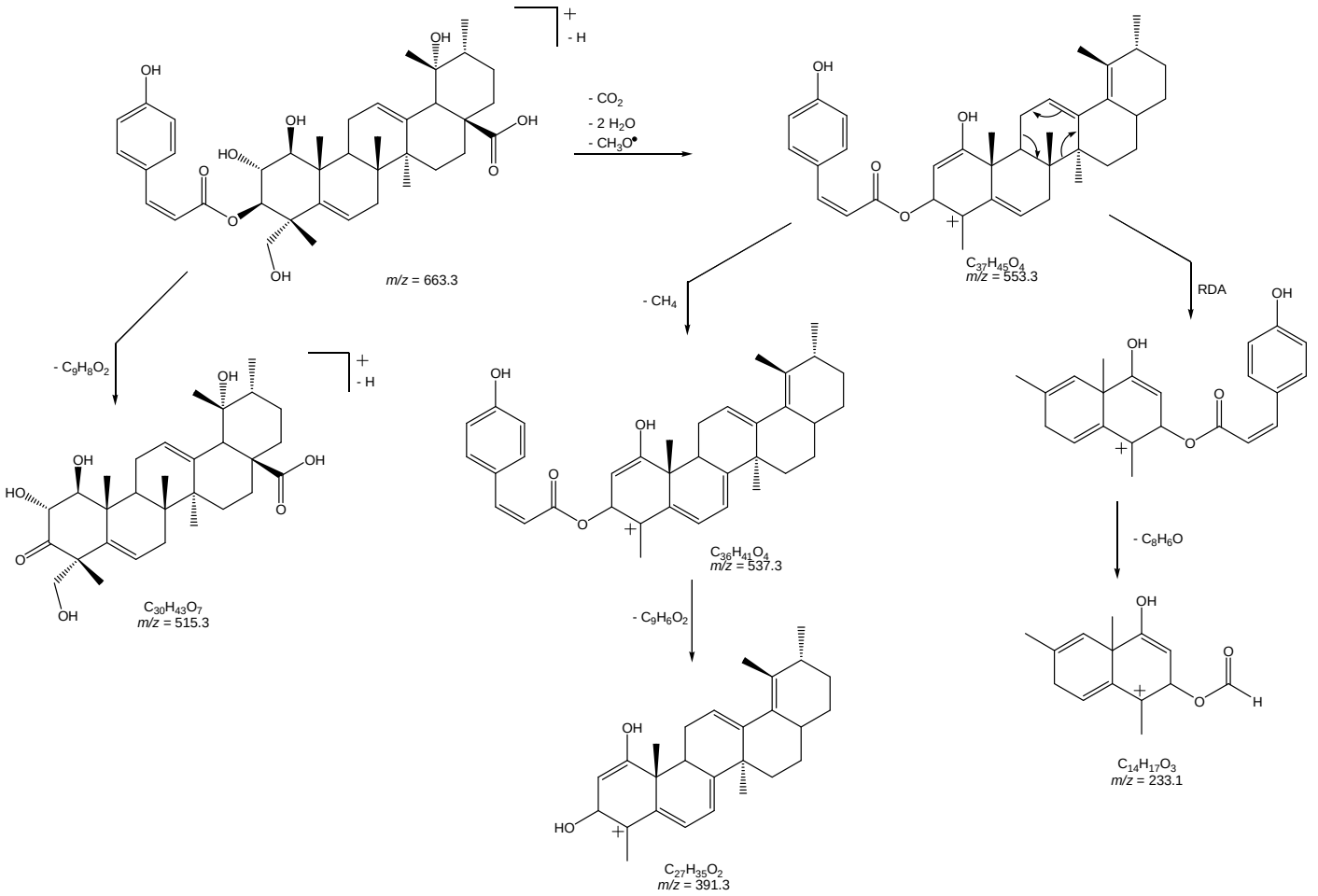


**Fig. 6. Proposed fragmentation of compound A from TOF MS ESI+ analysis**

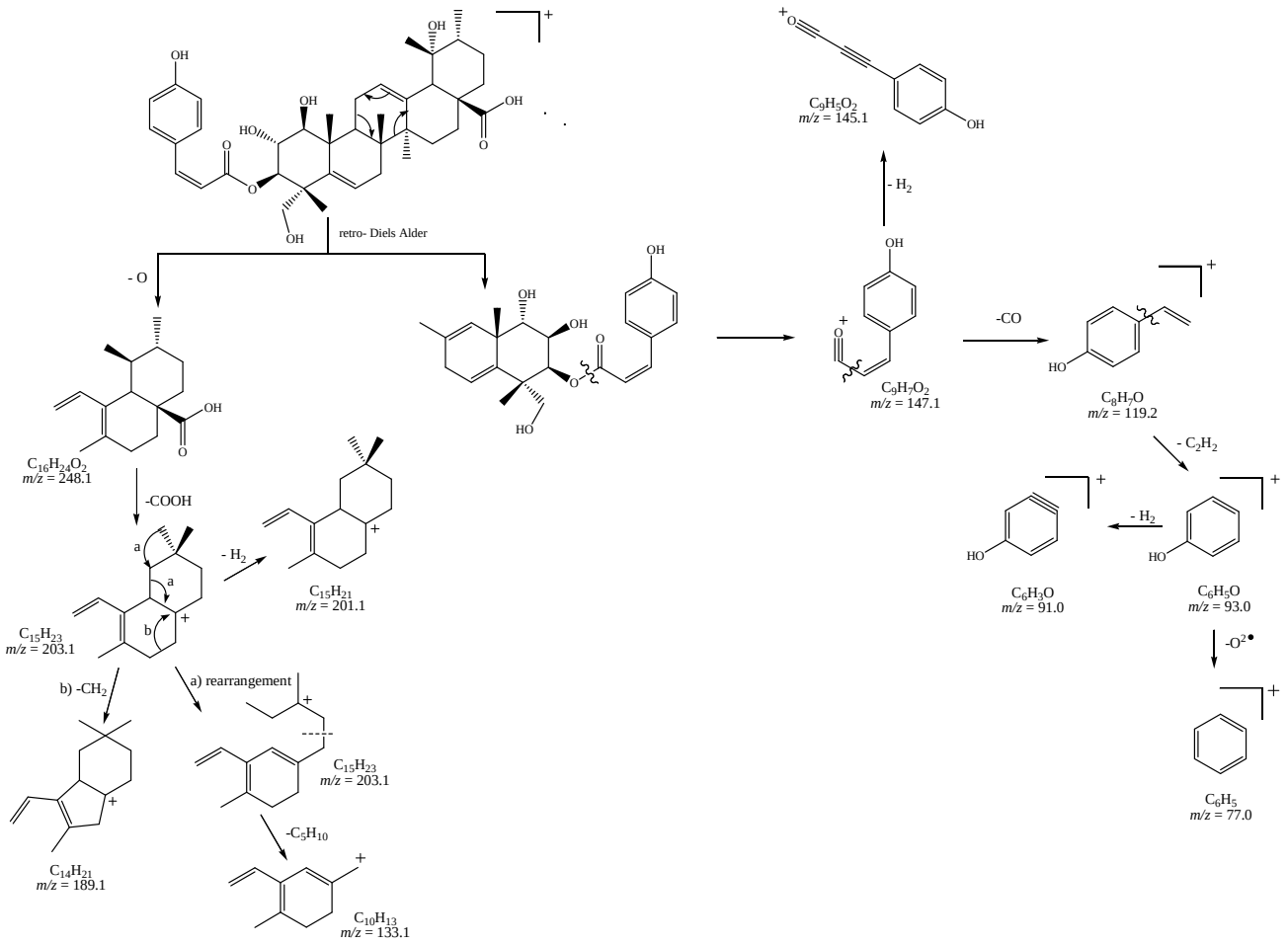


**Fig. 7. Proposed fragmentation of compound 4 from EIMS analysis**

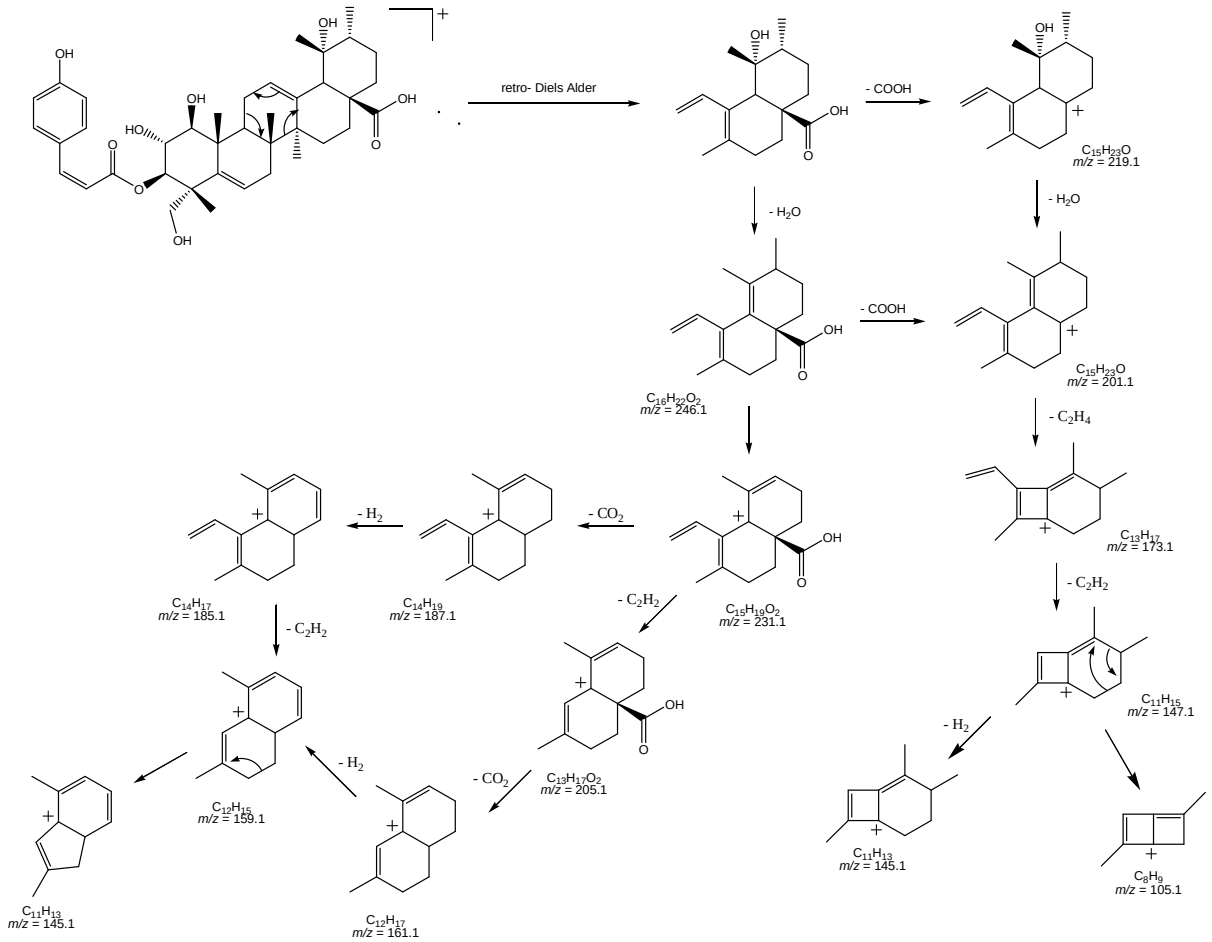


**Fig. 7. (Continuation) Proposed fragmentation of compound 4 from EIMS analysis**

**Table 3. $^{1}$H (acetone-$d_6$, 300 MHz) and $^{13}$C-NMR (DMSO-$d_6$, 125 MHz) spectral data (chemical shifts and coupling constants) for compound 4 in acetone-$d_6$**

| Position | $\delta_H$ ppm (Mult.) | $\delta_c$ ppm |
|---|---|---|
| **1** | 4.05 (*m*) | 84.40 |
| **2** | 3.89 (*m*) | 69.3 |
| **3** | 3.29 (*m*) | 77.9 |
| **4** | / | 44.9 |
| **5** | / | 152.8 |
| **6** | 5.29 (*br*, *t,* 3.6 Hz) | 126.8 |
| **7** | 1.95 (*br, d,* 3.6 Hz); 1.75 (*br, d,* 3.6 Hz) | 35.5 |
| **8** | / | 37.4 |
| **9** | 1.69 (*m*) | 41.2 |
| **10** | / | 37.9 |
| **11** | 2.63 (*ddd, 3.6, 9.0, 12.6* Hz); 1.95 (*br, d, 3.6* Hz) | 25.6 |
| **12** | 5.28 (br, *t,* 3.6 Hz) | 121.7 |
| **13** | / | 141.6 |
| **14** | / | 42.7 |
| **15** | 1.29 (*m*); 1.00 (*m*) | 28.6 |
| **16** | 1.78 (*m*); 1.62 (*m*) | 23.6 |
| **17** | / | 48.3 |
| **18** | 2.54 (*s*) | 53.2 |
| **19** | / | 71.7 |
| **20** | 1.83 (*m*) | 41.7 |
| **21** | 1.58 (*br, d, 4.5* Hz); 1.49 (*m*) | 36.7 |
| **22** | 1.72 (*m*); 1.54 (*m*) | 32.5 |
| **23** | 3.55 (*m*); 3.38 (*m*) | 65.3 |
| **24** | 0.98 (*s*) | 16.1 |
| **25** | 0.77 (*s*) | 17.6 |
| **26** | 0.84 (*s*) | 21.8 |
| **27** | 1.35 (*s*) | 24.0 |
| **28** | / | 179.8 |
| **29** | 1.17 (*s*) | 27.2 |
| **30** | 0.96 (*br, d*, 5.1 Hz) | 16.8 |
| **1'** | - | 166.8 |
| **2'** | 7.90 (*d*, *J* = 8.7 Hz) | 116.8 |
| **3'** | 6.90 (*d*, *J* = 8.7 Hz) | 143.6 |
| **4'** | - | 134. 5 |
| **5'; 9'** | 7.69 (*m*) | 129.7 |
| **6'; 8'** | 7.42 (*m*) | 115.9 |
| **7'** | - | 151.4 |

*δ in ppm from TMS*

The EI mass spectrum of **4** did not yield the molecular ion but revealed a characteristic pic at *m/z* 248.1 ($C_{16}H_{24}O_2$), which is assigned to a fragment obtained from the retro-Diels-Alder cleavage (ring C) of the parent ion followed by the loss of an oxygen atom (Fig. 7). The loss of –COOH group from the secondary fragment *m/z* 248.1, yields *m/z* 203.0 ($C_{15}H_{23}$) and *m/z* 189.0 ($C_{14}H_{21}$). Rearrangement of the m/z 203.0 ion, and loss of a neutral 5-carbon fragment relatively gave rise to another stable ion at *m/z* 133.0 ($C_{10}H_{13}$). These data provide a worthwhile evidence of the structural arrangement of the compound, suggesting that the compound **4** is composed of rings and has a carboxylic acid group with an additional hydroxyl group in ring D/E portion of the molecule. The ready loss in the TOF-MS-ESI+ of the *p*-hydroxylcynnamoyl group at C-3 gives rise to a fragment at *m/z* 515.3 (Fig. 6).

## 4. CONCLUSION

Qualitative phytochemical analysis performed on the CME of *V. paradoxa* stem barks revealed the presence of important classes of compounds like alkaloids, flavonoids, saponins, phenolic compounds, coumarins and triterpenes. Qualitative phytochemical screening has not yet been reported for *V. paradoxa*. Our results

showed that the extract is rich in phenolic constituents and demonstrated good antioxidant activity measured by different methods. The study of MeOH extract from the stem barks of *V. paradoxa* resulted in the isolation and structural elucidation of a new ursane-type triterpenoid named vitellaric acid, along with four known compounds belonging to the family of pentacyclic triterpenes and flavonoids. Among these known compounds, a mixture of betulinic acid (**3a**) and bassic acid (**3b**) was also obtained and its composition was identified without the need for further separation. This work demonstrates the effective use of mass spectrometry for the structural determination of small amounts of compounds. The study provides scientific evidence for the use of *V. paradoxa* stem barks for the treatment of diseases associated with oxidative stress due to ROS. This plant rich in flavonoids and phenolic acids could be a good source of natural free radical scavengers.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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