

## Isolation and Characterization of Chemical Constituents from the Crude Extracts of *Crossopteryx Febrifuga* (Afzel ex g. don) benth (Rubiaceae) and Evaluation of Their Phytotoxicity and Antioxidant Activity

Bealem Aristide<sup>1</sup>, Sakava Paul<sup>2\*</sup>, Lidia Favier<sup>3</sup>, Didier Hauchard<sup>3</sup>, Jean-Paul Guegan<sup>3</sup>, Nyemb Jean Noël<sup>4</sup>, Talla Emmanuel<sup>5</sup>, Celine Henoumont<sup>6</sup>, Sophie Laurent<sup>6</sup>, Mbafor Tanyi Joseph<sup>1</sup>

<sup>1</sup>Department of Organic Chemistry, Faculty of Science, University of Yaoundé I, P.O. Box 812 Yaoundé, Cameroon; <sup>2</sup>Department of Chemistry, Higher Teachers' Training College, The University of Bamenda, P.O. Box 39 Bambili, Cameroon; <sup>3</sup>Sciences chimiques Rennes, UMR CNRS 6226, Ecole Nationale Supérieure de Chimie de Rennes, Université de Rennes 1, Avenue du Général Leclerc, CS 50837, 35708 Rennes Cedex 7, France; <sup>4</sup>Department of Refining and Petrochemistry, Faculty of Mines and Petroleum Industries, University of Maroua, P.O. Box 08 Kaélé, Cameroon; <sup>5</sup>Department of Chemistry, Faculty of Science, University of Ngaoundéré, P.O. Box 454 Ngaoundéré, Cameroon; <sup>6</sup>Department of General, Organic and Biomedical Chemistry, Faculty of Medicine and Pharmacy, University of Mons, NMR and Molecular Imaging Laboratory, B-7000 Mons, Belgium

### ABSTRACT

The aim of this work is to isolate, characterize, and evaluate the antioxidant activity as well as the phytotoxicity of crude extracts and compounds obtained from *C. febrifuga*. Nine compounds were isolated from leaves, stem bark and roots (methylene chloride/methanol:1/1) crude extracts after repeated silica gel column chromatography corresponding to shanzhiside methyl ester (1), D-mannitol (2), a mixture of two isomeric pentacyclic triterpenes: oleanolic acid (3a) and ursolic acid (3b), 11-methylxoside (4), 3 $\beta$ -urs-12, 20(30)-diene-27,28-dioic acid (5), quinovin glycoside C (6), 3-O- $\beta$ -D-3-oxo-glucopyranosyl-ursa-12,20(30)-diene-27,28-dioic acid (7) and 3 $\beta$ -( $\alpha$ -L-rhamnopyranosyloxy)-ursa-12,20(30)-diene-27,28-dioic acid (8). The compound chemical structures were established by studying their <sup>1</sup>H and <sup>13</sup>C NMR (1D and 2D) and Mass Spectrometry (MS) spectra. The antioxidant capacity of extracts were evaluated using a recently developed electrochemical method consisting in interacting with the superoxide anion radical (O<sub>2</sub><sup>-</sup>) generated in situ by reduction of air molecular oxygen (O<sub>2</sub>) in aprotic medium (anhydrous DMF). The antioxidant index is expressed as the amount of extract needed to consume 30% (AI<sub>30</sub>) of electrogenerated O<sub>2</sub><sup>-</sup>. The lower is the antioxidant index, the more active the substrate is against O<sub>2</sub><sup>-</sup>. The stem bark crude extract is the most active with an AI<sub>30</sub> of 79.50mg/L while the leaves and root crude extracts showed a moderate activity against O<sub>2</sub><sup>-</sup> with AI<sub>30</sub> of 303.70mg/L and 450.00mg/L respectively. The phytotoxicity study were carried out using a germination test on *Lepidium sativum* seeds in absence (0mg/L) and in presence of increasing concentrations (10, 100, 500, 1000mg/L) of crude extracts and the isolated compound (1). The following physiological parameters of the seedlings were evaluated 72 hours after the experimentation: germination percentage (G %), germination index (GI), vigor index (VI), relative root growth (RRG %). Root extracts are the most phytotoxic on seed germination with G% ranging from 66.66 to 100% and RRG% from 8.20 to 88.45% while the isolated compound (1) stimulated growth between 10-500mg/L but showed a moderate phytotoxic effect on seedlings above 500mg/L.

**Keywords:** *Crossopteryx febrifuga*; crude extracts; isolation; superoxide radical anion; antioxidant activity; phytotoxicity

\*Correspondence to: Sakava Paul, Department of Chemistry, Higher Teachers' Training College, The University of Bamenda, P.O. Box 39 Bambili, Tel: +237674863715; E-mail: sakavapaul@yahoo.fr

Received Date: August 01, 2021; Accepted Date: October 05, 2021; Published Date: October 14, 2021

**Citation:** Aristide B, Paul S, Favier L, Hauchard D, Guegan JP, Noël NJ, et al. (2021) Isolation and characterization of chemical constituents from the crude extracts of *crossopteryx febrifuga* (afzel ex g. Don) benth (rubiaceae) and evaluation of their phytotoxicity and antioxidant activity. *Nat Prod Chem Res* 09:p320.

**Copyright:** © 2021 Aristide B, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

## INTRODUCTION

*Crossopteryx febrifuga*, etymologically fighting fever, is a monospecific genus of Rubiaceae family and belonging to the Ixoroideae subfamily [1]. The Rubiaceae have about 630 genera and more than 13,000 species divided into 3 subfamilies including Rubiodeae, Cinchinoideae and Ixoroideae, making this family one of the six largest families of Angiosperms [2]. *C. febrifuga* is a characteristic looking shrub with opposing branches that can reach 5 to 6m in height. It gives oval and opposite leaves often reddish at their base, very fragrant white flowers grouped in clusters at the top of the twigs, its round and green fruits turn blackish when ripe. The flowering period is from May to July. This shrub grows in dry forests on the sandy soils of southern Africa [3]. It is known under vernacular names of Ndeubeuh mann by indigenous peoples in southern Chad and Balembo by Bambara peoples in Mali [4]. Balembo is also the name of local antitussive syrup made with *C. febrifuga* roasted fruits. This plant is used in African folk medicine to treat various diseases such as intestinal worms, cough, gout, gonorrhea, fever, diabetes and diarrheal diseases [4-7]. In southern Chad, this species is known to have many therapeutic properties, thus a decoction of root powders is taken against stomachaches. In rural areas, the same decoction is recommended to women after childbirth for its antibiotic effects. The scraping of stem bark, brown in color, is sold in local markets in the form of herbal tea, commonly called Goub, for its taste and medicinal properties while a decoction of young leaves is taken as antipyretic against fever attacks. The leaves are also used in traditional veterinary medicine as a dewormer for livestock. This species is actually threatened with extinction in Chad due to its excessive use in folk medicine and overgrazing. Chadian Government recently raised the possibility of creating a botanical garden in collaboration with the National Association of Traditional Healers in order to safeguard interesting wild plant threatened with extinction. This measure could save *C. Febrifuga* in southern Chad.

Some recent phytochemical studies revealed the presence of iridoids, phenols, flavonoids, tannins, triterpenes, saponins and steroids in *C. febrifuga* extracts [8-10]. Secondary metabolites with high pharmacological potentials isolated from Rubiaceae justify the growing interest in the scientific investigation of the species in this family [11]. Despite the plant's many therapeutic properties recognized in ethnopharmacology, relatively few studies have been carried out in order to validate them scientifically. In this study, we evaluated for first time the phytotoxicity of crude extracts and the isolated compound (1) on the germination and growth of *Lepidium sativum* seeds. The antioxidant capacity was measured according to a relatively new electrochemical method consisting in evaluating the capacity of the substrate to trap the superoxide anion radical ( $O_2^{\cdot-}$ ) generated in situ by reduction of molecular oxygen ( $O_2$ ) [12]. The superoxide anion radical is one of the reactive oxygen species called "ROS" whose unregulated accumulation in the human body can cause various disorders such as cancers,

neurodegenerative diseases [13-14]. We also report a direct isolation of a saponin ( $3\beta$ - $(\alpha$ -L-rhamnopyranosyl)-ursa-12,20(30)-diene-27,28-dioic acid) (8) previously obtained from *C. febrifuga* extracts by acid hydrolysis of a bisdesmosidic saponin [15] as well as eight other known compounds.

## MATERIALS AND METHODS

### General experimental procedures

The masses were measured on a 1/1000 Sartorius-type electronic balance. Column chromatography (CC) was carried out on a high purity grade silica gel adsorbent, pore size 60Å, 70-230 mesh, 63-200µm, for column chromatography. The extracts were concentrated on a Büchi R-110 brand rotavapor. Thin Layer Chromatography (TLC) was carried out on square aluminum plates (20x20cm<sup>2</sup>) covered with silica (0.2mm thick) of the SIL G / UV254 Merck type, the spots were deposited with capillaries (Blaubrand® intraMark) of 5 to 20µL and visualized under an UV lamp at 254 or 236nm or revealed by spraying a H<sub>2</sub>SO<sub>4</sub> (10%) solution followed by heating in an oven. NMR spectra were recorded on a bruker Avance III 400 spectrometer operating at 400.13MHz for <sup>1</sup>H, equipped with a BBFO probe with a z-gradient coil and a GREAT 1/10 gradient unit. All experiments (<sup>1</sup>H NMR, <sup>13</sup>C NMR and 2D as COSY, HSQC and HMBG) were carried out at 25°C. <sup>1</sup>H chemical shifts ( $\delta$ ) are given in ppm relative to the solvent residual peak and <sup>13</sup>C chemical shifts are relative to the central peak of the solvent signal. The products to be analyzed were solubilized in deuterated solvents (MeOH-d<sub>4</sub> or DMSO-d<sub>6</sub>). The chemical shifts  $\delta$ (ppm) were recorded with reference to tetramethylsilane (TMS,  $\delta = 0$ ). The MestReNova software was used to process the NMR spectra. The Mass Spectra (MS) are recorded on an Orbitrap Q-Extractive type apparatus (Thermo Fisher Scientific, Waltham (MA), USA) in direct infusion, in Electrospray ionization (+)/(-) mode(s). A JASCO 320-A type IR spectrophotometer using KBr pellets recorded the IR spectra. The cyclovoltammogram was performed on a Potentiostat-Galvanostat (Autolab) Echo chemie type measuring device coupled to a computer. The data were recorded and processed by GPES Manager Software (General purpose electrochemical system, version 4.9, Echo chemie B.V). A thermostatically controlled cell containing 10mL of 0.1M solution (Bu<sub>4</sub>NPF<sub>6</sub>), maintained at 21±0.3°C by a Fisher scientific-polystat 5L/8662H type thermo regulator, in which were bathed 3 electrodes playing specific roles, namely a working electrode at glassy carbon disc (polished using a struers Dap-7 polisher fitted with a green Sci-paper, grit 1200, size: 200mm dia, and rinsed with distilled water then dried after each use), an electrode reference (Ag/AgCl) in ethanol saturated with LiCl and a counter-electrode, in platinum wire which completes the circuit by leading the electrons through the solution to the working electrode. Germination bioassay was carried out in sterile Pétri boxes ( $\varnothing$  90 mm) containing a layer of filter paper.

## Chemicals and instruments

Commercial chemicals were used in the phytochemical study as well as in the anti-oxidant assay. The solvent systems (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 1:1) were used for all the extraction phase. N hexane, ethylacetate and methanol were used as eluting solvents for CC. Tetrabutylammonium hexafluorophosphate (Bu<sub>4</sub>NPF<sub>6</sub>, M = 387.43g.mol<sup>-1</sup>), puriss electrochemical grade, anhydrous N, N Dimethylformamide (DMF) used for electrochemical study were obtained from sigma-Aldrich Switzerland. The reagents are of analytical purity and were purchased from Sigma-Aldrich. The CC tubes are of different sizes depending of the quantity of the treated sample. The extracting tanks are gloomy and hermetic to avoid any photochemical reaction as well as solvent evaporation during the extraction phase.

## Plant material

Leaves, stem bark and root scraping of *C. febrifuga* were freshly harvested, in March 2014, in Béti, Department of Pendé/Logone Oriental Region, Republic of Chad, after identification by Mr Dodorom Téblé Wolwaï, botanist at the Faculty of Science and Technics, University of Doba (Chad) and confirmed by the Yaoundé National Herbarium Voucher specimen number 41791/HNC. These samples were transported to the laboratory and gently cleaned with distilled water to remove dust and then dried for two weeks at room temperature out of direct sunlight.

## Extracts preparation

After preliminary stages of drying and manual powdering the plant material, 1.0kg of powder from the leaves, 1.0 kg of powder from the stem bark and 1.0kg of powder from the root were separately extracted by maceration, at room temperature, for 48 hours with 6L of methylene chloride/methanol (1/1, v/v) system. Maceration is a liquid-solid extraction method that takes place at room temperature and has the advantage of preserving heat-sensitive molecules before any intentional modification of their structures in organic synthesis. This operation was repeated twice in order to optimize the extraction. The different resulting solutions were filtered using whatmann paper and then concentrated under reduced pressure at 55°C on a rotavapor to give successively 80g of a soft green-brown paste for the leaves, 80g of an amorphous solid of chocolate color for the root and 100g of raw extract of creamy consistency and red-brown in color for the stem bark. These crude extracts were stored at 4°C until next stage of the study.

## Column chromatography and isolation

Samples of 50g of crude extracts obtained were subjected separately to liquid silica gel column chromatography (pore size 60Å, 70-230 mesh, 63-200 µm, 300 g) and eluted with n-hexane-ethyl acetate and ethyl acetate-methanol systems in increasing polarity (100:0→0:100). Fractions of 200 mL each collected at the outlet of the chromatographic column were concentrated using a rotavapor. This operation yielded a total of 218 fractions for stem bark crude extracts sample, 340 fractions for leaves crude extracts and 400 fractions for root crude extracts and then

gathered into major sub-fractions according to their chromatographic profiles after an analytical TLC. Successive purifications of some UV absorbing sub-fractions afford to nine compounds identified using spectroscopic NMR, Mass Spectrometry methods and corresponding to the following known compounds:

### Leaves crude extracts

Four compounds were isolated from this extract: Shanzhiside methyl ester (1), a white powder (250.20 mg) precipitated in the EtOAc/CH<sub>2</sub>Cl<sub>2</sub> 1:1 system, D-mannitol (2), white powder (19.7mg, EtOAc/CH<sub>2</sub>Cl<sub>2</sub> 1:1), a mixture of oleanolic acid (3a) and ursolic acid (3b), white powder (31.71mg, n-C<sub>6</sub>H<sub>14</sub>/EtOAc 7.5:2.5)

### Root crude extracts

Three compounds including 11-methylxoside (4), yellowish amorphous powder (7.52 mg, EtOAc/MeOH 9:1), 3β-urs-12,20(30)-diene-27,28-dioic acid (5) white powder (5.23mg, n-C<sub>6</sub>H<sub>14</sub>/EtOAc 3:2) and quinovinglycoside C (6), white amorphous powder (8.63mg, n-C<sub>6</sub>H<sub>14</sub>/EtOAc 1:9)

### Stem bark crude extracts

Two compounds including 3-O-β-D-3-oxo-glucopyranosyl-ursa-12,20(30)-diene-27, 28-dioic acid (7), brownish powder (11.80mg, n-C<sub>6</sub>H<sub>14</sub>/EtOAc 1:1) and 3β-(α-L-rhamno- pyranosyl)-ursa-12,20(30)-diene-27,28-dioic acid (8), beige powder (18.0mg, EtOAc/MeOH 9:1) were isolated.

## Phytotoxicity test

We evaluated the phytotoxicity of crude extracts and the isolated compound (1) on the germination and growth of *Lepidium sativum* seeds, a vegetable plant of Brassicaceae family. The study was performed according to the protocol described by Mihaela [16]. with a slight modification. For each sample, series of concentrations of 10, 100, 500 and 1000mg/L were prepared using distilled water. The solutions obtained are further homogenized in an ultrasonic bath. A negative control was made with distilled water containing no substrate (0mg/L). 10 seeds of regular contours and similar sizes, selected after a visual examination, are inoculated with 5ml of each solution previously prepared in sterile Petri dishes where filter papers are previously placed serving as germination surface. The seeds are distributed over the entire surface of the box to prevent any hindrance during germination period. This operation was repeated 3 times for each concentration. Before the experimentation, physico-chemical parameters of solutions such as pH and conductivity (µS/cm) were measured. After 3 days of incubation at room temperature and protected from light, the following physiological parameters of the seedling were measured: germination percentage (G %), germination index (GI), relative root growth (RRG %) and vigor index (VI) expressed as follows [16-17].

$$G\% = \frac{\text{Nbr of germinated seeds in the sample}}{\text{Nbr of germinated seeds in the control}} \times 100$$

$$RRG\% = \frac{\text{Mean of root length in the sample}}{\text{Mean of root length in the control}} \times 100 \quad ; \quad GI = \frac{G\%}{RRG\%} \times 100$$

$$VI = \frac{\text{Mean of root length}}{\text{Mean of shoot length}} \times G\%$$

In the germination process, we considered as germinated seeds with root lengths  $\geq 1$ mm. Comparisons with the control (0mg/L) allowed us to know the allelopathic response of the seedling to the external stress. Allelopathy is any direct or indirect effect, positive or negative, of a plant on another through the production of chemical compounds released into the environment [18]. The allelopathic activity of Brassicaceae family is attributed to glucosinolates, a group of sulfur-containing carbohydrate compounds common in this family [19-20].

### Determination of the antioxidant activity of the crude extracts

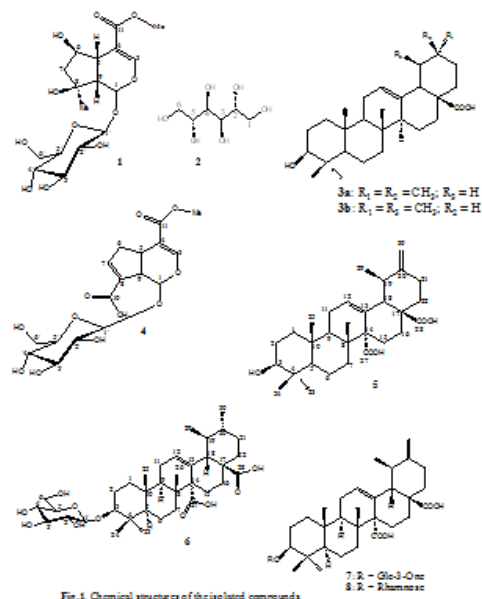
0.1M solution (Bu4NPF6) were prepared by dissolving 0.387g of Bu4NPF6 (support electrolyte to conduct the current) in 10 mL of anhydrous DMF (99.8%) and saturated with dry air for 10min. Under the operating conditions, the solubility of oxygen in air is approximately  $S(O_2) = 0.94 \cdot 10^{-3}M$ . First, the molecular oxygen ( $O_2$ ) reduction in cyclic voltammogram was recorded, in the absence of any antioxidant substrate, at a scan rate of 0.1V.s<sup>-1</sup>. The initial potential was set at 0V vs Ag/AgCl. The sweep potential was reversed at -0.9 V relative to Ag/AgCl. The presence of the radical ( $O_2\cdot^-$ ) in the medium is easily detected by measuring its anodic oxidation current ( $I_{pa}^0$ ) during reverse scanning [12]. We then prepared 5 mL of stock solutions (10g.L<sup>-1</sup>) each by dissolving crude extracts (root, stem bark, leaves) in anhydrous DMF. Gradual volumes of these freshly prepared stock solutions were added to the 10mL of 0.1M solution (Bu4NPF6) in the thermostatically controlled cell to obtain a range of antioxidant substrate concentrations from 0-700mg.L<sup>-1</sup>. After each addition, the cyclic voltammogram of the progressive reduction of the superoxide radical was recorded. The consumption of  $O_2\cdot^-$  is correlated with the concentration of crude extracts gradually added in the electrochemical cell, thus reflecting their antioxidant capacity. This antioxidant capacity is estimated by the Antioxidant Index (IA30) defined as the concentration of extract required to consume 30% of electrogenerated superoxide anion radical. The smaller the IA30, the more active the extract is against the superoxide anion radical [21].

## RESULTS AND DISCUSSION

### Structures of the isolated compounds

After a column chromatographic fractionation of the crude extracts followed by successive purifications on sephadex LH20, the following nine known compounds, belonging to four organic compounds groups (iridoids, triterpenes, saponins, carbohydrates) were isolated and identified by 1H, 13C NMR

1D and 2D (COSY, HSQC, HMBC) and by comparison with literature data: Shanzhiside methyl ester (1) [22]. D-mannitol (2), a primary metabolite common to several species [23], a mixture of two isomeric pentacyclic triterpenes: oleanolic acid (3a) and ursolic acid (3b) [24-26]. 11-methylxoside (4) [27]. 3- $\beta$ -urs-12,20 (30)-diene-27,28-dioic acid (5) [28], quinovin glycoside C (6), an antivenom saponoside previously isolated from extracts of *Mitragyna Stipulosa* [29]. 3-O- $\beta$ -D-3-oxo-glucopyranosyl-ursa-12,20 (30)-diene-27,28-dioic acid (7) [8]. and 3- $\beta$ -( $\alpha$ -L-rhamnopyranosyloxy)-ursa-12,20(30)-diene-27,28-dioic acid (8) isolated for the first time from extracts of *C. febrifuga* (see Fig.1).



### General information on the isolated compounds

Compound 1 (Shanzhiside méthyl ester) white powder (ethyl acetate/methanol 1:1), (HRESI-MS) (+): m/z 429.13673 [M+Na]<sup>+</sup> (calculated for C<sub>17</sub>H<sub>26</sub>O<sub>11</sub>Na: 429.1367) corresponding to the formula C<sub>17</sub>H<sub>26</sub>O<sub>11</sub>, DI=5,1H NMR ( $\delta$ ppm, DMSO-d<sub>6</sub>, 400MHz): 7.33 (d, J=1.25Hz, H-3), 5.50 (d, J= 2.3Hz, H-1), 4.45 (d, J=7.9Hz, H-1'), 3.92 (m, H-6), 3.70 (ddd, J=11.9Hz, J=6.4Hz, J=1.9Hz, Ha-6'), 3.65 (s, OMe-12), 3.44 (dt, J=11.9Hz, J=6.0Hz, Hb-6'), 3.15 (o) m, H-5', 3.12 (o) m, H-3', 3.04 (ddd, J=9.4Hz, J=8.7Hz, 5.3Hz, H-4'), 2.93 (ddd, J=8.7Hz, J=8.0Hz, J=5.4Hz, C-2'), 2.81 (dd, J=9.9Hz, J=2.5Hz, H-5), 2.45 (dd, J=9.8Hz, J=2.3Hz, H-9), 1.83 (dd, J=13.1Hz, J=6.6Hz, Ha-7), 1.63 (dd, J=13.1Hz, J=6.4Hz, Hb-7), 1.11 (s, H-10), 13C NMR (100MHz) : 167.19 (C-11), 151.01(C-3), 109.57(C-4), 97.98(C-1'), 92.78(C-1), 77.31(C-5'), 76.92(C-8), 76.73(C-3'), 74.93(C-6), 73.07(C-2'), 70.02(C-4'), 61.09(C-6'), 51.15(OMe-12), 49.89(C-9), 49.08(C-7), 40.19(C-5), 24.70(C-10).

Compound 2 (D-mannitol) white Powder (ethyl acetate/methanol 1:1), 1H NMR ( $\delta$ ppm, DMSO-d<sub>6</sub>, 400MHz): 4.41(d, J=5.6Hz, OH-1/6), 4.34(t, J=5.6Hz,OH-2/5), 4.13 (d, J=7.0Hz, OH-3/4), 3.61(ddd, J=10.7Hz, J=5.6Hz, J=3.4Hz, Ha-1/6), 3.53 (t, J=7.6Hz, H-3/4), 3.45(ddd, J=10.7Hz, J=5.6Hz, J=3.5Hz, H-2/5), 3.39(dd, J=10.7Hz, J=5.7Hz, Hb-1/6), 13C NMR (100MHz) : 71.35(C-2/5), 69.71(C-3/4), 63.90(C-1/6).

Compounds 3a (oleanolic acid): white powder (hexane/ethyl acetate 7.5:2.5), ESI- m/z 455 [M-H]<sup>-</sup> compatible with C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>, DI= 7, IR (KBr): ν<sub>max</sub>=3,300cm<sup>-1</sup>(-OH), 3,000cm<sup>-1</sup>(Csp<sup>3</sup>-H), 1,700cm<sup>-1</sup>(C=O), <sup>13</sup>C NMR (125MHz, DMSO-d<sub>6</sub>): 178.2 (C-28), 143.8 (C-13), 121.5 (C-12), 76.8 (C-3), 55.9 (C-5), 48.2 (C-9), 46.7 (C-17), 46.6 (C-19), 42.2 (C-14), 42.1 (C-18), 39.4 (C-4), 39.8 (C-8), 39.1(C-1), 37.4 (C-10), 34.3 (C-21), 33.4 (C-7/29), 33.2 (C-22), 31.0 (C-20), 28.8 (C-23), 28.4 (C-15), 28.0 (C-2), 26.22 (C-27), 23.8 (C-11/16/30), 18.8 (C-6), 17.6 (C-26), 16.5 (C-24), 15.6 (C-25).

Compounds 3b (ursolic acid): white powder (hexane/ethyl acetate 7.5:2.5), ESI- m/z 455 [M-H]<sup>-</sup> compatible with C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>, DI= 7, IR (KBr): ν<sub>max</sub>=3,300cm<sup>-1</sup>(-OH), 3,000cm<sup>-1</sup>(Csp<sup>3</sup>-H), 1,700cm<sup>-1</sup>(C=O), <sup>13</sup>C NMR (125MHz, DMSO-d<sub>6</sub>): 178.6 (C-28), 138.2 (C-13), 124.5 (C-12), 76.8 (C-3), 55.9 (C-5), 53.6 (C-18), 48.2 (C-9), 48.1 (C-17), 42.7 (C-14), 40.1 (C-8), 39.7 (C-4), 39.3 (C-1), 39.5 (C-19), 39.4 (C-20), 37.6 (C-10), 37.4 (C-22), 33.8 (C-7), 31.1 (C-21), 28.9 (C-15), 28.8 (C-23), 28.3 (C-2), 25.0 (C-16), 24.0 (C-27), 23.8 (C-11), 21.4 (C-30), 18.9(C-6), 17.5 (C-26/29), 16.5 (C-24), 15.7 (C-25).

Compound 4 (11-Methylxoside) yellowish amorphous powder (ethylacetate/methanol 9:1), High Resolution ESI Mass spectrum (HRESI-MS) (+)/(-), ESI<sup>+</sup> : m/z 425 [M+Na<sup>+</sup>] and ESI- m/z 401 [M-H]<sup>-</sup> compatible with the formula C<sub>17</sub>H<sub>22</sub>O<sub>11</sub>, <sup>1</sup>H NMR (δppm CH<sub>3</sub>OH-d<sub>4</sub>, 400MHz): 7.49(d, J=1.1Hz, H-3), 6.90 (dt, J=2.5Hz, J=1.6Hz, H-7), 5.69(d, J=5.0Hz, H-1), 4.62(d, J=7.9Hz, H-1'), 3.86(m, Ha-6'), 3.71(s, OMe-12), 3.68(m, Hb-6'), 3.36(m, H-3'), 3.33(m, H-5), 3.30(m, H-5'), 3.29(m, H-4'), 3.22(m, H-9), 3.19(dd, J=8.9Hz, 7.9Hz, H-2'), 2.93(dt, J=18.7Hz, 8.0Hz, 2.3Hz, Ha-6) and 2.43(m, Hb-6), <sup>13</sup>C NMR(100MHz): 169.17 (C-11), 168.01(C-10), 153.54(C-3), 147.53(C-7), 136.22(C-8), 112.43(C-4), 100.38 (C-1'), 96.29(C-1), 78.22(C-5'), 77.80(C-3'), 74.60(C-2'), 71.40(C-4'), 62.64(C-6'), 51.73 (OMe-12), 47.42(C-9), 40.15(C-6) and 35.0(C-5).

Compound 5 (3β-urs-12,20 (30) -diene-27,28-dioic acid) white powder (hexane/ethyl acetate 6 :4), (HRESI-MS) (+)/(-), ESI<sup>+</sup> m/z [M+Na]<sup>+</sup> 507.30809 (calculated for C<sub>30</sub>H<sub>44</sub>O<sub>5</sub>Na: 507.3082) and ESI<sup>-</sup> , m/z [M-H]<sup>-</sup> 483.3116 (calculated for C<sub>30</sub>H<sub>43</sub>O<sub>5</sub>) compatible with the formula C<sub>30</sub>H<sub>44</sub>O<sub>5</sub>(DI=9), <sup>1</sup>H NMR (δppm, DMSO-d<sub>6</sub>, 400MHz): 5.50 (m, H-12), 4.67 (brs, Hb-30), 4.59 (brs, Ha-30), 4.32 (brs, OH-3), 2.95 (brs t, J=7.2Hz, H-3), 2.22 (d, j=11.7Hz, H-18), 2.18 (o, H-9), 2.12 (o, 2H-21), 2.05 (o, Hb-16), 1.98 (o, Ha-15), 1.9 (o, Hb-11), 1.84 (o, H-19), 1.8 (o, Ha-11), 1.70 (o, Hb-15), 1.69 (o, Ha-22), 1.63 (o, Ha-16), 1.6 (o, 2H-1), 1.6 (o, Hb-7), 1.55 (o, Ha-7), 1.46 (o, Hb-22), 1.45 (m, Ha-6), 1.45 (o, 2H-2), 1.25 (o, Hb-6), 0.95 (d, J=6.3Hz, H-29), 0.87 (s, 3H-25), 0.86 (s, 3H-23), 0.79 (s, 3H-26), 0.66 (s, 3H-24), 0.58 (d, J=10.9Hz, H-5), <sup>13</sup>C NMR (100MHz): 177.7(C-28), 176.0(C-27), 152.5(C-20), 132.5(C-13), 128.3(C-12), 105.8(C-30), 77.0(C-3), 55.7(C-18), 55.4(C-14), 55.0(C-5), 47.4(C-17), 46.12(C-9), 39.0(C-8), 38.4(C-1), 38.4(C-4), 38.1(C-22), 36.5(C-10), 36.3(C-7), 35.0(C-19), 31.5(C-21), 28.34(C-23), 27.0(C-2), 25.15(C-16), 24.1(C-15), 22.5(C-11), 18.2(C-26), 18.0(C-6), 16.1(C-24), 16.0(C-25), 15.99(C-29).

Compound 6 (quinovin glycoside C): white amorphous powder (hexane/ethyl acetate 1:9), (HRESI-MS)(+)(-), ESI<sup>+</sup> m/z [M+Na]<sup>+</sup> + 671, ESI- m/z [M-CO<sub>2</sub>-H]<sup>-</sup> 603 compatible with the formula

C<sub>36</sub>H<sub>56</sub>O<sub>11</sub>, DI=9, <sup>1</sup>H NMR (δppm, DMSO-d<sub>6</sub>, 400MHz): 5.49 (m, H-12), 4.14 (d, J=7.8Hz, H-1'), 4.02 (m, H-4'), 3.64 (dd, J=11.0Hz, J=3.4Hz, Ha-6'), 3.41 (o, Hb-6'), 3.1 (t, J=8.6Hz, H-3'), 3.06 (m, H-5'), 2.99 (dd, J=11.8Hz, J=4.6Hz, H-3), 2.94 (dt, J=7.8Hz, J=4.6Hz, H-2'), 2.14 (o, H-9/18), 1.92 (o, Ha-16), 1.90 (o, Ha-15), 1.87 (o, Ha-11), 1.80 (o, Ha-2), 1.78 (o, Hb-11), 1.58 (o, Ha-1), 1.57 (o, Ha-7), 1.56 (o, Hb-16), 1.54 (o, Hb-2), 1.53 (o, Hb-15), 1.50 (o, 2H-22), 1.44 (o, Ha-6), 1.39 (o, 2H-21), 1.23 (o, Hb-6), 0.94 (s, 3H-23), 0.89 (o, H-19/20), 0.87 (d, J=4.6Hz, 3H-30), 0.88 (s, 3H-26), 0.86 (o, Hb-1/7), 0.81 (d, J=5.3Hz, 3H-29), 0.78 (s, 3H-25), 0.73 (s, 3H-24), 0.6 (d, J=11.4Hz, H-5), <sup>13</sup>C NMR (100MHz): 178.34 (C-28), 176.6 (C-27), 132.62 (C-13), 127.93 (C-12), 105.3 (C-1'), 87.89 (C-3), 76.9(C-3'), 76.61 (C-5'), 74.0(C-2'), 70.23(C-4'), 61.26 (C-6'), 55.34 (C-14), 55.26 (C-5), 53.67 (C-18), 47.3 (C-17), 46.19 (C-9), 39.0 (C-10), 38.68 (C-4), 38.59 (C-1), 38.46 (C-20), 36.73 (C-7), 36.53 (C-19), 36.22 (C-8), 35.87 (C-22), 29.83 (C-21), 27.74 (C-23), 25.61 (C-2), 24.99 (C-15), 24.05 (C-16), 22.41 (C-11), 21.2 (C-30), 18.17 (C-25), 17.78 (C-6), 17.58 (C-29), 16.55 (C-24), 16.12 (C-26).

Compound 7 (3-O-β-D-3-oxo-glucopyranosyl-ursa-12,20(30)-diene-27,28-dioic acid): brownish powder (hexane/ethyl acetate 1:1), (HRESI-MS)(-): m/z 643.3492 [M-H]<sup>-</sup> (calculated for C<sub>36</sub>H<sub>51</sub>O<sub>10</sub>) compatible with C<sub>36</sub>H<sub>52</sub>O<sub>10</sub>, <sup>1</sup>H NMR (δppm, CH<sub>3</sub>OH-d<sub>4</sub>, 400MHz): 5.63 (m, H-12), 4.68 (s, Hb-30), 4.64 (s, Ha-30), 4.40 (d, J = 7.8Hz, H-1'), 4.22 (dd, J = 10.1Hz, J = 1.7Hz, H-4'), 4.11 (dd, J = 7.8Hz, J=1.7Hz, H-2'), 3.92 (dd, J = 12.0Hz, J = 2.0Hz, Hb-6'), 3.79 (dd, J = 12.0Hz, J = 4.6Hz, Ha-6'), 3.29 (ddd, J = 10.1Hz, J = 4.6Hz, J = 2.0Hz, H-5'), 3.21 (dd, J = 11.5Hz, J = 4.5Hz, H-3), 2.32 (d, J = 11.8Hz, H-18), 2.26 (o, H-9), 2.25 (o, Ha -16), 2.25 (o, Hb-21), 2.19 (o, Ha-21), 2.1 (o, Ha-15), 1.98 (o, H-19), 1.97 (o, Hb -11), 1.91 (o, Ha -11), 1.90 (o, Hb-2), 1.80 (o, Ha-7), 1.78 (o, Ha-2), 1.77 (o, Hb-16), 1.75 (o, Hb-15), 1.70 (o, 2H-1), 1.67 (o, 2H-22), 1.55 (o, Ha-6), 1.55 (o, Hb-7), 1.37 (o, Hb-6), 1.04 (d, J = 6.4Hz, 3H-29), 1.02 (s, 3H-23), 0.99 (s, 3H-25), 0.90 (s, 3H-26), 0.85 (s, 3H-24), 0.77 (m, H-5), <sup>13</sup>C NMR(100MHz): 207.55 (C-3'), 180.8 (C-28), 178.8 (C-27), 154.1 (C-20), 133.6 (C-13), 131.08 (C-12), 107.9 (C-1'), 106.2 (C-30), 91.09 (C-3), 78.92 (C-2'), 78.03 (C-5'), 73.75 (C-4'), 62.58 (C-6'), 57.69 (C-18), 57.3 (C-14), 56.91 (C-5), 49.55 (C-17), 47.96 (C-9), 40.74 (C-8), 40.1 (C-4), 39.98 (C-7), 39.87 (C-1), 37.97 (C-22), 37.86 (C-10), 36.71 (C-19), 32.99 (C-21), 28.4 (C-23), 26.98 (C-2), 26.58 (C-16), 25.75 (C-15), 23.89 (C-11), 19.28 (C-6), 19.02 (C-26), 17.04 (C-24), 16.88 (C-25), 16.58 (C-29).

Compound 8 (3-β-(α-L-rhamnopyranosyloxy)-ursa-12,20(30)-diene-27,28-dioic acid): beige powder (ethyl acetate/methanol 9:1), (HRESI-MS)(+): m/z 653.82 [M+Na]<sup>+</sup> calculated for C<sub>36</sub>H<sub>54</sub>O<sub>9</sub>, DI=10, <sup>1</sup>H NMR (δppm, DMSO-d<sub>6</sub>, 400MHz): 5.53 (m, H-12), 4.59 (brs, Ha-30); 4.68 (brs, Hb-30), 4.15 (d, J = 7.7Hz, H-1'), 3.15 (m, H-5'), 3.07 (dt, J = 8.9Hz, J = 4.5Hz, H-3'), 2.98 (d, o, H-3), 2.97 (m, H-2'), 2.78 (dt, J = 8.9Hz, J = 5.4Hz, H-4'), 2.23 (d, J = 11.8Hz, H-18), 2.16 (o, H-9), 2.14 (o, 2H-21), 1.99 (o, Ha-15), 1.90 (o, Ha-11), 1.84 (o, H-19), 1.81 (o, Hb-11), 1.75 (o, Hb-2), 1.71 (o, Ha-22), 1.66 (o, 2H-16), 1.61 (o, Hb-15), 1.61 (o, 2H-1), 1.55 (o, Ha-2), 1.55 (o, 2H-7), 1.48 (o, Hb-22), 1.46 (o, Ha-6), 1.25 (o, Hb-6), 1.13 (d, J = 6.1Hz, 3H-6'), 0.96 (d, J = 6.5Hz, 3H-29), 0.94 (s, 3H-23), 0.89 (s, 3H-25), 0.79 (s, 3H-26), 0.74 (s, 3H-24), 0.61 (o, H-5), <sup>13</sup>C NMR(100MHz): 177.7 (C-28), 176.09 (C-27), 152.34 (C-20), 132.27 (C-13),

128.53 (C-12), 105.91 (C-30), 105.23 (C-1'), 87.92 (C-3), 76.6 (C-3'), 75.36 (C-4'), 74.18 (C-2'), 71.19 (C-5'), 55.66 (C-18), 55.32 (C-14), 55.26 (C-5), 47.42 (C-17), 46.16 (C-9), 39.06 (C-8), 38.7 (C-4), 38.55 (C-1), 38.1 (C-22), 36.23 (C-10), 36.22 (C-7), 35.05 (C-19), 31.45 (C-21), 27.71 (C-23), 25.75 (C-2), 25.1 (C-16), 24.06 (C-15), 22.42 (C-11), 18.12 (C-6'), 18.09 (C-26), 17.84 (C-6), 16.51 (C-24), 16.06 (C-25), 15.95 (C-29).

Antioxidant activity

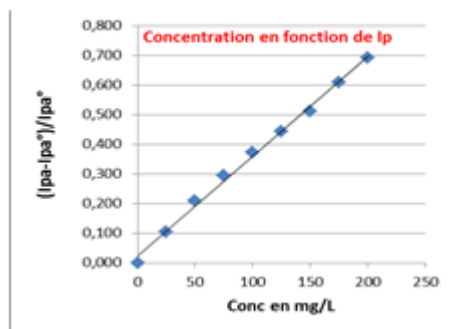


Figure2: Linear response of O2. - Consumption depending on the stem bark extracts concentration.

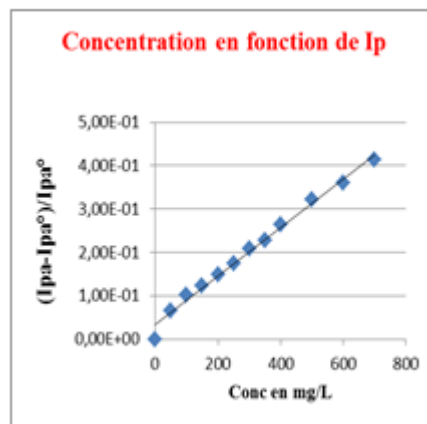


Figure4: Linear response of O2 consumption as a function of root extracts concentration.

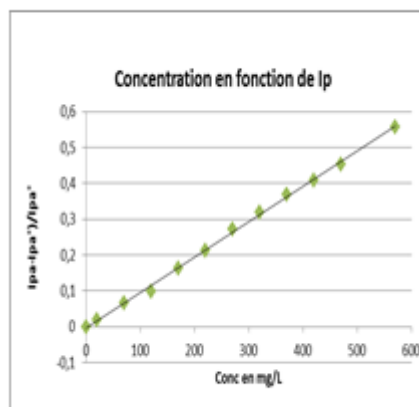


Figure5: Linear response of O2 consumption as a function of leaves extract concentration.

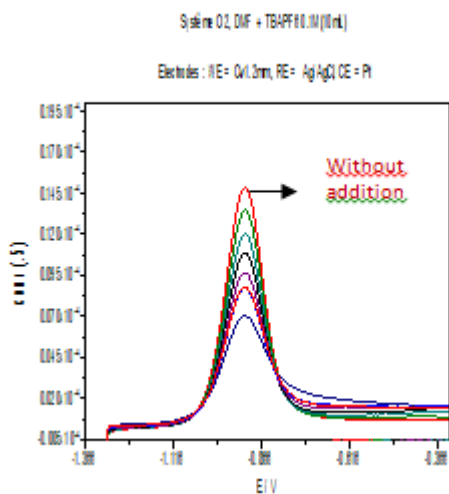


Figure3: Cyclic voltammograms of O2 reduction in absence and presence of increasing concentrations of stem bark extract at a stable glassy carbon disc electrode in 0.1M (Bu4NPF6) solution. 0.1 V / s scan rate.

This method of measuring antioxidant capacity of a substrate has been validated with Carine’s work that used it to measure flavonoids antioxidant capacity [12]. Our results clearly showed that the extracts tested, using this method, showed an antioxidant activity which is quantified for a better assessment by calculating the Antioxidant Index AI30  $((Ipa-Ipa^{\circ})/Ipa^{\circ} = 0.30)$ . The stem bark extract is the most active with a lower AI30 of 75.90 mg/L, meaning that a substrate concentration of 75.9mg/L is required to consume 30% of electrogenerated O2- while leaves and root crude extracts showed moderate activities with AI30 of 307.70 mg/L and 450.00mg/L respectively. Generally in Chadian folk medicine, the stem bark is the most used part of the plant, then powders of stem bark is used as an herbal tea locally called Goub, as well as a hemostatic agent for bloody wounds. From the stem bark extracts, we isolated two saponins including 3-O-β-D-3-oxo-glucopyranosyl-ursa-12,20(30)-diene-27,28-dioic acid known for its moderate antibacterial activity [8] and 3β-(α-L-rhamnopyranosyloxy)-ursa-12,20(30)-diene-27,28-dioic acid isolated for the first time from extracts of *C. febrifuga*.

### Phytotoxicity

Roots crude extracts

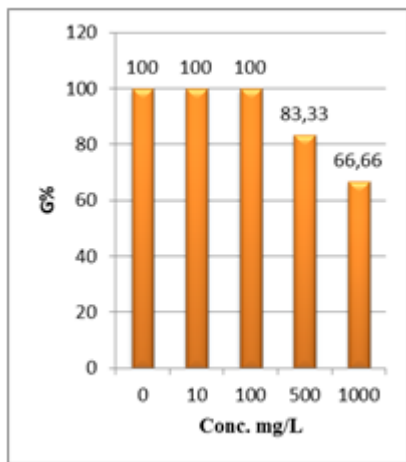


Figure6: Percentage of germination (G %) of *Lepidium sativum* seeds exposed to different concentrations of root crude extracts.

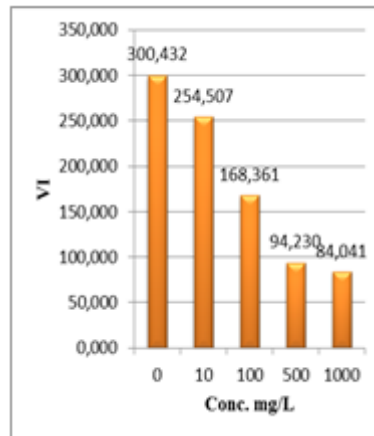


Figure9: Vigor index (VI) of *Lepidium sativum* exposed to different concentrations of root crude extracts.

Shanzhiside methyl ester (1)

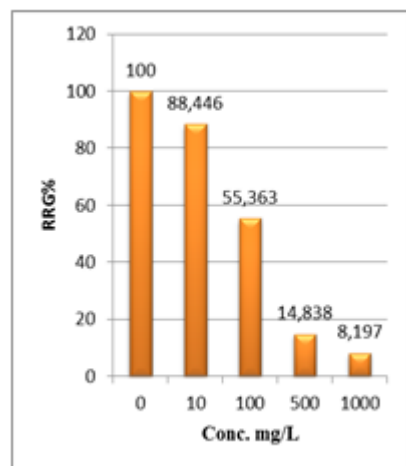


Figure7: Relative root growth (RRG %) of *Lepidium sativum* exposed to different concentrations of root crude extracts.

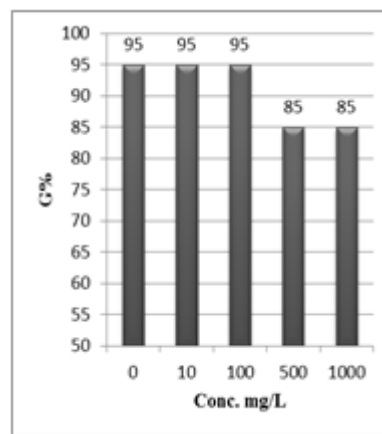


Figure10: Percentage of germination (G %) of *Lepidium sativum* seeds exposed to different concentrations of compound (1).

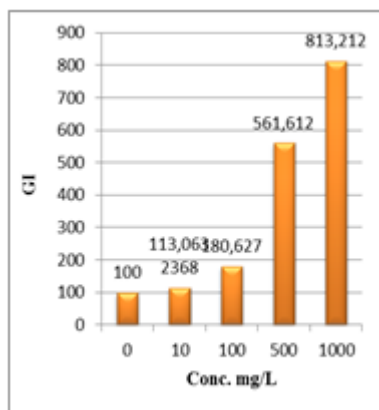


Figure8: Germination Index (GI %) of *Lepidium sativum* exposed to different concentrations of root crude extracts.

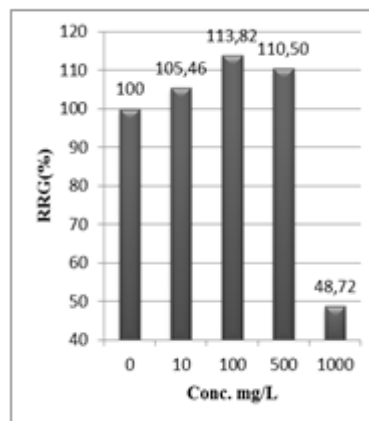
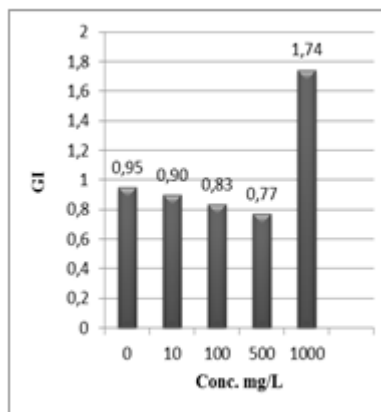
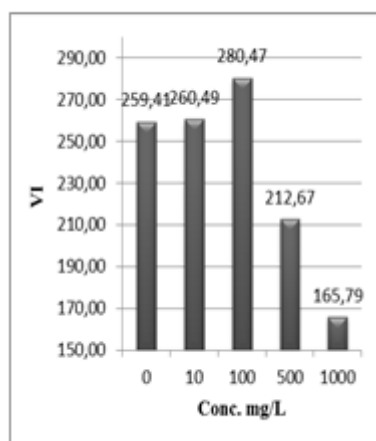


Figure11: Relative root growth (RRG %) of *Lepidium sativum* exposed to different concentrations of compound (1).



**Figure12:** Germination index (GI%) of *Lepidium sativum* exposed to different concentrations of the compound (1).



**Figure13:** Vigor index (VI %) of *Lepidium sativum* exposed to different concentrations of the compound (1).

Our results revealed that all the tested crude extracts as well as the compound (1) exhibited phytotoxicity on the germination and growth of seeds at varying degrees. Most plants contain inhibiting substances on seed germination and the degree inhibition is proportional to the concentration [30]. We found that at a certain concentration, mostly relatively low (10 and 100mg/L), the extracts have a stimulating effect on growth but become toxic or phytotoxic at high concentrations. Some compounds inhibit seed germination at 50ppm but stimulate growth below 1ppm [30]. Root extracts are more toxic against seed germination with the germination percentage (G %) ranging from 66.66 to 100% and the relative root growth (RRG %) from 8.20 to 88.45%. From 500mg/L, the seedlings are stunted with average root and stem lengths of 3.36 and 4.01mm respectively, whereas these values are respectively 41.05 and 13.66mm for the control (in the absence of extract). In addition to the physiological parameters reduced between 500 and 1000mg/L, yellowing of the foliage is observed, reflecting the external stress undergone by the seedling. Compound (1), belonging to the iridoids class, stimulated growth between 10-500mg/L but showed a toxic effect above 500mg/L. The (G

%) values is 95% for the control (0mg/L), 95% (10mg/L), 95 (100mg/L), 85% (500mg/L), 85% (1000mg/L) showed that the compound (1) have an influence on seed germination while the (RRG %) ranging from 48.72 to 113.82 showed also a negative allelopathic effect on seed germination at high concentrations ( $\geq 500$ mg/L). Iridoids are compounds originally isolated from *Iridomirmex* genus ants in Australia and are involved in the defense mechanism of these ants. Iridoids, are known to have a wide range of biological activities including antibacterial, antispasmodic, and antioxidant properties [31]. We evaluated in this study for first time the phytotoxicity of an iridoid compound.

## CONCLUSION

Phytochemical study of crude extracts from *C. febrifuga* led to the isolation and structural identification of nine compounds belonging to four classes of organic compounds (iridoids, saponins, triterpenes, and sugars). We report the direct isolation of a saponin ( $3\beta$ - $\alpha$ -L-rhamnopyranosyloxy)-ursa-12,20(30)-diene-27,28-dioic acid) previously obtained by acid hydrolysis of  $3\beta$ - $\alpha$ -L-rhamnopyranosyloxy)-28-O-( $\beta$ -D-glucopyranosyl)urs-12,20(30)-diene-27,28-dioic acid isolated from root extracts of *C. febrifuga* [15]. The tested compound (1), an iridoid, stimulated seedling growth at low doses (10-100mg/L) and exhibited moderate phytotoxicity at 500mg/L. The stem bark extract was the most effective against the superoxide anion in electrochemical measurements with an AI30 of 75.9mg/L. This study completes the wide range of biological activities of *C. febrifuga* extracts as well as an iridoid compound.

## ACKNOWLEDGEMENTS

We are grateful to Mrs. Geneviève Pillet (Switzerland), Mrs. Arlette Mermier (France), Mr Michel Blanc (France), Mrs. Ginette Blanc (France), Mrs. Danielle Blanc (France), and Mr Bealem Ferdinand (Chad) for supporting this work through a financial help to Bealem Aristide. We also thank Mr. Philippe Jehan (CREMPO, University of Rennes 1) for having carried out mass spectra experiments and for his advices.

## REFERENCES

1. Brigitta B and Torsten E. Time Tree of Rubiaceae: Phylogeny and dating the family, subfamilies, and tribes. *Int J Plant Sci* 2009; 170(6): 766-793.
2. Simplicie DK, Tchadjobo T, Denise P, Ilboudo JS. Sub-saharian Rubiaceae: a review of their traditional uses. *Phytochemistry and biological activities*. *Pak J Biol Sci*. 2011; 14(3):149-169.
3. Pierluigi G, Luisella V, Bruno G. Saponins from *Crossopteryx febrifuga*. *Phytochemistry* 1990; 29(8):2629-2635.
4. Ababacar M, Karl EM, Drissa D, Berit SP. Antioxidant and 15-lipoxygenase inhibitory activities of the Malian medicinal plants *Diospyros abyssinica* (Hiern) F. White (Ebenaceae), *Lannea velutina* A. Rich (Anacardiaceae) and *Crossopteryx febrifuga* (Afzel) Benth. (Rubiaceae). *J Ethnopharmacol*. 2006;104:132-137
5. Elisee KK, Thierry AD, Sarah KA, Jean David N, Gisele K-S. Antipyretic activity of aqueous extract of *Crossopteryx febrifuga*



- stem barks in wistar rats. World journal of pharmacy and pharmaceutical sciences. 2018;7(9):190-200.
- Muluh EK, Adebayo SA, Terumon AT-A, Abel A. Isolation and characterization of spinasterol from *Crossopteryx febrifuga* stem bark. Progress in Chemical and Biochemical Research. 2019; 2:68-73.
  - Elufioye TO and Agbedahunsi JM. Antimalarial activities of *Tithonia diversifolia* (Asteraceae) and *Crossopteryx febrifuga* (Rubiaceae) on mice in vivo. J Ethnopharmacol. 2004;93:167-171.
  - Modjinar K, Raymond NN, Jonas K, Roland T, Beaudelaire KP, Kristina JS, Rémy BT, Jean PD, Till O, Matthias FM, Léon A T. A new ursane-type triterpene oxoglucopyranoside from *Crossopteryx febrifuga*. Z. Naturforsch. 2019; aop.
  - Francisco AT-B and Kurt H. A cytotoxic triterpenoid and flavonoids from *Crossopteryx febrifuga*. Planta medica 1988; 266-267.
  - Nma N, Mann A, Muammad BM. GC-MS analysis of bioactive compounds in the ethyl acetate fraction of *Crossopteryx febrifuga* leaves. J Chem Pharm Res. 2018; 10(3):75-79.
  - Daiane M and Cecilia VN. Secondary metabolites from Rubiaceae species. Molecules 2015; 20:13422-13495.
  - Carine LB, Didier H, André D, Jean-Louis B, Marie-Laurence A. Validation of a new method using the reactivity of electrogenerated superoxide radical in the antioxidant capacity determination of flavonoids. Talanta. 2008; 75:1098-1103.
  - Camille M and Mirelle S. Espèces réactives de l'oxygène et stress oxydant. Médecine/sciences 2011; 27:405-412.
  - Irwin F. Superoxide anion radical (O<sub>2</sub><sup>-</sup>), superoxide dismutases, and related matters. J Biol Chem. 1997; 272(30):18515-18517.
  - Babady-Bila, Tshiamuene N, Amuri K, Suzanne T, Frans C, Georges H. An ursadienedioic acid glycoside from *Crossopteryx febrifuga*. Phytochemistry 1991;30(9):3069-3072.
  - Mihaela C, Bianca F, Geta C, Vasilia B, Alina VI, Fernanda M, Jorge HL, Silvia AS, Rodica MD. Antifungal, Antitumoral and Antioxydant potential of the Danube Delta *Nymphaea alba* extracts. Antibiotics 2020; 9,7.
  - Dianlei L, Beizhen X, Chen D, Ganghui L, Dawei H, Youcai Q, Hongyan L, Hong L. Effect of fertilizer prepared from human feces and straw on germination, growth and development of wheat. Acta Astronica 2018; 145:76-82.
  - Rice EL. 1984. Allelopathy. 2nd ed. Florida: Academic Press, Inc. Orlando.
  - Couëdel A, Seassau C, Wirth J, Alletto L. Potentiels de régulation biotique par allélopathie et biofumigation ; services et dis-services produits par les cultures intermédiaires multiservices de crucifères. Innovations Agronomiques. 2017; 62:71-85.
  - Raymond R, Jean-Marie B, Jean-Paul B, Thierry D, Sabah E, Anne M, Bernard N, Sylvain P, Christian P, Alain Q, Christophe S, Bernard S, Bernard T. 2005.OCL 12(3) Mai-Juin.
  - Naïma G-B, Khodir M, Mohamed C, Lila B-M, Didier H, Martin K, Caroline S, Philippe NO, Pierre D. Phenolic compounds, antioxydant and antibacterial activities of three Ericaceae from Algeria. Ind Crops Prod. 2015; 70:459-466.
  - Hui-Lan Y, Xiao-Hui Z, Qi-Lan W, Yan-Duo T. Separation and purification of water-soluble iridoid glucosides by high speed counter-current chromatography combined with macroporous resin column separation. J Chromatogr B Biomed Appl. 2013; 936:57- 62.
  - Alessandro B, Jener DGS, Monalisa MAMP, Juan TAO, Luciano SL, Jorge MD. D-mannitol from *Agave sisalana* biomass waste. Ind Crops Prod. 2010; 32:507-510.
  - Werner S, Nebojsa S, Robert W, Robert S, Olaf K. Complete assignments of <sup>1</sup>H and <sup>13</sup>C NMR resonances of oleanolic acid, 18 $\alpha$ -oleanolic acid, ursolic acid and their 11-oxoderivatives. Magn Reson Chem. 2003; 41:636 - 638.
  - Yong Z, Kungpeng X, Eugene YZ, Yin L, Lixin Y, Xiaoyan Y, Xiaomei X. Determination of oleanolic acid and ursolic acid in Chinese medicinal plants using HPLC with PAH polymeric C18. Pharmacogn Mag. 2013; 9(1):19-24.
  - Mahato SB and Kundu A P. Review article number 98 "13C NMR spectra of pentacyclic triterpenoids - A compilation and some salient features". Phytochemistry. 1994; 37(6):1517-1575.
  - Jeet SJ, Rita PA, Teresa M, Raghbir S. Isolation and in vitro cytotoxic activity of 11-methylxoside isolated from bark of *Randia dumetorum* Lamk. Herba polonica. 2013; 59(1).
  - Jean RC, Jean-de-Dieu T, Pépin N-E-A, Bruno NL, Norbert S. Antimicrobial triterpenes from the stem bark of *Crossopteryx febrifuga*. Z. Naturforsch. 2015; 70(7-8)c:169-173
  - Naheed F, Leon AT, David L, Sondengam BL, Atta-Ur-Rahman, Iqbal CM. Quinovic Acid Glycosides From *Mitragyna stipulosa* - first examples of natural inhibitors of snake venom phosphodiesterase I. Nat Prod Lett. 2002;16(6):389-393.
  - Edwin HC and Watson RD. Effect of various aqueous plant extracts upon seed germination. Bot Gaz. 1968; 129(1):57-62.
  - Rosa T, Monica RL, Federica M, Giancarlo AS, Francesco M. Biological and pharmacological activities of iridoids: Recent Developments. Mini-reviews in Medicinal Chemistry. 2008; 8:399-420.