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**Isolation of flavonoids and triterpenoids from the fruits of *Alphitonia neocaledonica* and evaluation of their antioxidant, antityrosinase and cytotoxic activities.**

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Phytochemical analysis of *Alphitonia neocaledonica* fruits

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## ABSTRACT :

**Introduction** – *Alphitonia neocaledonica* (Rhamnaceae) is an endemic tree of New Caledonia. Although three flavonoids were identified in the leaves, the secondary metabolite profile of the fruits has never been investigated.

**Objective** – Phytochemical investigation of *A. neocaledonica* fruits and evaluation of their antioxidant, antityrosinase and cytotoxic activities.

**Methodology** – A hydromethanolic extract was fractionated by liquid-liquid extraction to obtain ethyl acetate and *n*-butanolic fractions. The ethyl acetate-soluble part was purified by Silicagel column chromatography and high-performance liquid chromatography (HPLC). The *n*-butanol-soluble part was fractionated by centrifugal partition extraction (CPE) and the collected fractions were further purified by centrifugal partition chromatography (CPC) and HPLC. The chemical structures of the purified compounds were identified by nuclear magnetic resonance and mass spectrometry.

**Results** – Three triterpenoids and one flavonoid were isolated from the ethyl acetate-soluble part. Fractions enriched in triterpenoids, flavonoids and catechin derivatives were obtained from the *n*-butanol-soluble part. Gallocatechin and flavonoids were obtained as pure compounds by further CPC and HPLC purification. The *n*-butanolic-soluble part showed antioxidant and antityrosinase activities due to the presence of tannins and gallocatechin. The triterpenoid alphitolic acid showed a moderate cytotoxic activity against KB cells line (IC<sub>50</sub> = 8.5 μM).

**Conclusion** – Nine known compounds including three triterpenes, five flavonoids and (+) gallocatechin, as well as a new 3-*O*-(6-*E*-feruloyl)-β-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→2)-]α-L-rhamnopyranosyl-quercetin, were isolated from *A. neocaledonica* fruits. The hydromethanolic extract possess a potential cytotoxic activity due to the presence of triterpenes, and it can also be valuable as a cosmetic ingredient for its antioxidant and antityrosinase activities.

Keywords: Centrifugal partition extraction; Centrifugal partition chromatography; three-phase solvent system; triterpenoids, flavonoids; fruits ; *Alphitonia neocaledonica*

## INTRODUCTION

New Caledonia is well known for its high biodiversity with a global endemic area of 74.3%. This high endemism is partly due to the multiplicity of geological substrates with one-third of the total area covered by ultramafic rocks. Erosion and alteration of these rocks have led to nutrient depletion and soil enrichment with heavy metals resulting in the development of a unique flora. *Alphitonia neocaledonica* (Schltr.) Guillaumin (Rhamnaceae) is an endemic small forest tree of New Caledonia growing on this ultramafic soil rich in extractable nickel. This tree is found throughout the mainland, the Isle of Pines (Southern Province), Bélep and Yandé (Northern Province). The fruits are black, round, about the size of a small cherry, not edible, and contain 2-3 very hard nuclei with shiny, black and very hard seeds (Suprin 1992). The fresh bark, bitter and astringent, exudes a clear balsamic odor of methyl salicylate and is traditionally used to treat skin diseases (eczema, ecthyma, pityriasis) and rheumatic pains (Suprin 1992). The leaves are used locally to facilitate childbirth (Suprin 1992). The fatty acids profile of the seeds confers a potential nutritional value to the oil (Bombarda et al. 2010).

A range of phenolic compounds have already been identified in various *Alphitonia* species (Guise et al. 1962, Branch et al. 1972, Jou et al. 2004). More precisely, three isorhamnetin-derived flavonoids have been identified in the leaves of *A. neocaledonica* (Lin et al, 1995). Pentacyclic triterpenoids including ceanothic acid, betulin, betulinic acid, alphitolic acid with *in vitro* cytotoxic, anti-inflammatory, and antimicrobial activities (Dzuback et al. 2006) have also been detected in several *Alphitonia* species (Branch et al. 1972, Jou et al. 2004, Setzer et al. 2004).

The objective of this work was to investigate the secondary metabolite profile of a crude hydromethanolic extract of *Alphitonia neocaledonica* fruits, with a particular focus on flavonoids and triterpenoids which have been reported as potent antioxidant or whitening agents due to their radical scavenging (Ko et al. 2011), or tyrosinase inhibitory activities (Parvez et al. 2007; Ullah et al. 2007). In this study, the radical scavenging ability of the extracts was investigated, as well as the tyrosinase inhibitory activity and cytotoxic activity against KB cells of some isolated compounds.

## EXPERIMENTAL

## Chemicals and reagents

Acetone, acetonitrile of HPLC grade, *n*-butanol, chloroforme, cyclohexane, dimethylsulfoxide (DMSO), ethanol, ethyl acetate, *n*-heptane, *n*-hexane, isopronalol, methanol, methyl-*tert*-butyl ether, methyl ethyl ketone, potassium hydroxide, pyridine, sulphuric acid, toluene, and trifluoroacetic acid (TFA) were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). Acetic acid was purchased from Acros Organics (New Jersey, USA) and formic acid was purchased from Prolabo (Paris, France). Deuterated methanol-*d*<sub>4</sub>,  $\alpha$ -naphthol, DPPH, mushroom tyrosinase (EC 1.14.18.1), L-DOPA, kojic acid (purity 99%), ascorbic acid, and  $\alpha$ -hederin were purchased from Sigma-Aldrich (Saint-Quentin, France). WST-1 was obtained from Roche (Meylan, France) and DMEM F12 was purchased from Gibco-Invitrogen (Cergy Pontoise, France). The KB cell line DSMZ ACC136 was purchased from Interchim<sup>®</sup> (Montluçon, France). Deionised water was used to prepare all aqueous solutions.

## Plant material

*Alphitonia neocaledonica* (Schltr.) Guillaumin fruits were collected by Pr. Mohammed Nour in September 2009 at the end of the cool season in southern province. The botanical identification was made at the Laboratoire Insulaire du Vivant et de l'Environnement of the New Caledonia University. A voucher specimen (09NM001) was deposited in the Herbarium of Noumea (New Caledonia).

## Apparatus

**Centrifugal partition extraction.** A laboratory-scale Fast Centrifugal Partition Extractor FCPE300<sup>®</sup> (Kromaton Technology, Angers, France) of 303.5 mL capacity and containing a total of 231 partition twin-cells (~1 mL each) was used to fractionate the *n*-butanol extract of *A. neocaledonica* fruits. The mobile phase was pumped by a Preparative Pump 1800W V7115 (Knauer, Berlin, Germany). The system was coupled to a UVD 170S detector (Dionex, Sunnivale, CA, USA) monitored at 210 and 254 nm. Fractions of 20 mL were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). All experiments were carried out at room temperature ( $22 \pm 2$  °C).

**Centrifugal partition chromatography.** CPC was performed on an ASCPC250<sup>®</sup> apparatus (Armen Instrument, Vannes, France) of 250 mL capacity and containing 1890 partition twin-

cells (~0.1 mL). The solvents were pumped by a binary gradient Armen AP-50 pump. The system was coupled to an AR-600 UV/Vis detector (Armen) monitored at 210 and 254 nm. Fractions of 4 mL were collected by an Armen LS-5600 collector. Data were acquired using the Armen Glider CPC control software version 2.9. Experiments were conducted at room temperature ( $22 \pm 2$  °C).

**Preparative HPLC.** Preparative HPLC was performed on a NW 50 preparative HPLC system equipped with a Merck column of 180 x 50 mm packed with a Lichroprep C<sub>18</sub> reversed phase (40-63 μm) (Merck, Darmstadt, Germany). The mobile phase was pumped by an Armen quaternary gradient pump AP 250/500 pump and the sample were introduced into the HPLC column via an Armen ACC 250/500 injector equipped with a 10 mL sample loop. The effluent was monitored at 254 nm by a Knauer K-2501 UV/Vis detector and plotted with a Kipp & Zonen recorder (Emerainville, France). The fractions were collected with a Büchi C-660 collector (Rungis, France).

**Analytical and semi-preparative HPLC.** Analytical and semi-preparative HPLC were performed on a Dionex apparatus equipped with an ASI-100 automated sample injector, a STH 585 column oven, a P580 pump, a diode array detector UVD 340S and the Chromeleon<sup>®</sup> software version 6.8. Semi-preparative HPLC separations were performed on a prepacked C<sub>18</sub> reversed phase column Luna (250 x 10mm, 5μm, Phenomenex, France). Analytical HPLC separations were performed on a prepacked C<sub>18</sub> reversed phase column Luna (250 x 4.6 mm, 5μm, Phenomenex, France). The chromatograms were monitored at 205, 210, 254 and 312 nm.

**Open column liquid chromatography (CC) and thin layer chromatography (TLC).** CC was performed over Kieselgel 60 (63-200 mesh, Merck, Darmstadt, Germany). Preparative and analytical TLC were carried out on precoated silica gel 60 F<sub>254</sub> plates (Merck, Darmstadt, Germany). Spots were visualized after spraying with 50% sulfuric acid and heating at 100 °C for 1 min.

**Optical rotation.** Optical rotations were measured in methanol or chloroform with a Perkin-Elmer 241 polarimeter (Perkin Elmer, Courtaboeuf, France).

**NMR analyses.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker Avance III 500 spectrometer ( $^1\text{H}$  at 500 MHz and  $^{13}\text{C}$  at 125 MHz, Bruker, Wissembourg, France) or on a Bruker Avance III 600 spectrometer equipped with a cryoprobe ( $^1\text{H}$  at 600 MHz and  $^{13}\text{C}$  at 150 MHz). 2D-NMR experiments were performed using standard Bruker microprograms. Chemical shift ( $\delta$ ) were reported in ppm using the internal solvent resonances at  $\delta_{\text{H}}$  3.33 and  $\delta_{\text{C}}$  47.6 ( $\text{CD}_3\text{OD}$ ).

**ESI-MS analyses.** High resolution ESI-MS experiments were performed using a hybrid quadrupole/time-of-flight (Q-TOF) instrument, equipped with a pneumatically assisted electrospray ion source operated in positive ionization mode (Micromass, Manchester, UK). The samples were introduced by direct infusion in a solution of **methanol** at a flow rate of 5  $\mu\text{L}/\text{min}$ . The spray capillary voltage was set at 3500V, and the extraction cone voltage between 30-60V. The source temperature was 80  $^{\circ}\text{C}$  and the desolvation temperature was 100  $^{\circ}\text{C}$ .

**Biological activity measurements.** 96-well microplates Greiner<sup>®</sup> F Bottom (BMG-LABTECH, Champigny sur Marne, France) and a BMG-LABTECH UV-Vis Spectrophotometer Micro-plate reader FLUOstar Omega were used for absorbance measurements in biological assays.

### **Preparation of crude extracts**

Powdered air-dried fruits of *A. neocaledonica* (475 g) were macerated in 5 L **methanol:water** (8:2, v/v) over night and then lixiviated with 15 L **methanol:water** (8:2, v/v). After **methanol** evaporation under reduced pressure, the resulting aqueous solution (1 L) was extracted successively with **cyclohexane**, **ethyl acetate** and *n*-**butanol** (3 x 1 L each), then dried under reduced pressure to yield a **cyclohexane**-soluble part of 0.75 g, a **ethyl acetate**-soluble part of 3.6 g, a *n*-**butanol**-soluble part of 41.2 g and a **water**-soluble part of 140.3 g (Fig. 1).

### **Separation procedure of the **ethyl acetate**-soluble part**

The **ethyl acetate**-soluble part (3.6 g) was purified by **Silicagel CC** using a gradient of **cyclohexane:chloroform:methanol** (3:7:0 to 0:0:1, v/v), to afford 60 fractions (200 mL each). Fractions [8-9] (443.8 mg) and [42-44] (859.3 mg) were further purified by **Silicagel CC** and subsequently by preparative TLC (Fig. 1). Fractions [57-58] (597.2 mg) were purified by preparative HPLC using a binary gradient of **methanol:water** (2:8 to 1:0, v/v) in 210 min at 35

mL/min to yield 120 fractions. Fractions [111-113] (29.1 mg) were further purified by semi-preparative HPLC by using a gradient of **acetonitrile:water** (3:7 to 6:4, v/v) (see Supporting information for more details).

### **CPE fractionation of the *n*-butanol-soluble part**

The solvent system was prepared by mixing *n*-heptane (500 mL), **methyl-*tert*-butyl-ether** (500 mL), **acetonitrile** (500 mL) and **water** (500 mL) in a separatory funnel. After separation of the *n*-heptane-rich upper phase (UP), one equivalent volume of **methyl-*tert*-butyl-ether** (500 mL) was added to the mixture of middle and lower phases in order to slightly reduce the polarity of the remaining two-phase solvent system (Hamzaoui et al. 2013). After decantation, the final middle and lower phases were separated. The fractionation was performed at 20 mL/min with a column rotation speed of 1000 rpm. **Four** g of the *n*-butanol-soluble part of A.

*neocaledonica* fruits were solubilized in 30 mL of an LP/UP mixture (8:2, v/v) and loaded into the column. The upper liquid phase was pumped for 27 min to elute all hydrophobic compounds. The moderately polar middle phase was then pumped for 30 min to elute the moderately polar compounds, and finally the aqueous lower phase was pumped in the descending mode for 15 min in order to recover the most hydrophilic compounds retained inside the column.

All fractions were checked by TLC developed with toluene:**acetone:formic acid** (6:6:1, v/v) and those showing the same profile were pooled to yield eight sub-fractions noted from F<sub>1</sub> to F<sub>8</sub> [F<sub>1</sub>: 597 mg, F<sub>2</sub>: 35.6 mg, F<sub>3</sub>: 137.8 mg, F<sub>4</sub>: 402.6 mg, F<sub>5</sub>: 92.6 mg, F<sub>6</sub>: 293.0 mg, F<sub>7</sub>: 120.5 mg, F<sub>8</sub>: 1590.3 mg]. These sub-fractions were analyzed by analytical HPLC using a gradient of **acetonitrile:0.025% TFA in water** (1:9 to 4:6, v/v) for 35 min.

### **Purification of the CPE-fractions F<sub>3</sub> to F<sub>6</sub>**

Fractions F<sub>3</sub>, a part of fraction F<sub>4</sub> (90.0 mg), fraction F<sub>5</sub> and a part of fraction F<sub>6</sub> (90.0 mg) were purified by semi-preparative HPLC (Fig. 1) by using a gradient of **acetonitrile:0.025% TFA in water** (see Supporting information for more details).

A part of fraction F<sub>4</sub> (290 mg) was solubilized in 20 mL of aqueous phase/organic phase (8:2, v/v) of the biphasic solvent system *n*-hexane:**ethyl acetate:methanol:water** (1:9:1:9, v/v). The CPC column was filled by the aqueous stationary phase. The organic mobile phase was pumped at 4 mL/min with a rotation speed of 1200 rpm in the ascending mode in order to equilibrate the CPC column. The sample was then injected and the organic mobile phase was

pumped at 4 mL/min for 180 min. Finally, the aqueous phase was pumped in the descending mode at 20 mL/min for 50 min. The CPC-fractions [186-192] (27.5 mg) were purified by semi-preparative HPLC using a binary gradient of **acetonitrile:0.025% TFA in water** (1:9, v/v) for 10 min, from 1:9 to 23:77 (v/v) in 3 min, and to 25:75 (v/v) in 23 min at 4 mL/min.

### **Acid hydrolysis and identification of sugar units**

One g of the crude **methanol:water** extract was refluxed with 25 mL of **2N trifluoroacetic acid** for 4 h. After extraction with **ethyl acetate** (3 × 25 mL), the aqueous layer was neutralized to pH 6 with 50 mM **potassium hydroxide** and freeze-dried to provide the monosaccharide residue. The sugars profile was determined by comparison with authentic samples on TLC in **methyl ethyl ketone:iso-propanol:acetone:water** (20:10:7:6, v/v). Detection was performed with  $\alpha$ -naphthol. The monosaccharide residue (40 mg) was solubilized in **water** (1 mL) and purified by semi-preparative HPLC, on a specific column ROA (250 x 15 mm, T = 35 °C) eluted isocratically with a solution of **0.25  $\mu$ M sulphuric acid** at a flow rate of 3.5 mL/min, to give the four sugars. The chromatogram was monitored by a refractive index detector RI-410 (T = 35 °C). Each fraction was neutralized with a 50 mM solution of **potassium hydroxide**, dried, dissolved in 1 mL pyridine, and filtered to remove potassium sulfate salts. After pyridine evaporation, the sugars were dissolved (0.1-0.5 mg/mL) in a mixture *n*-hexane:**ethanol:trifluoroacetic acid** (70:30:1, v/v) and analyzed by HPLC on an analytical chiral column Chiralpak<sup>®</sup> ICA, using the mobile phase *n*-hexane:ethanol:trifluoroacetic acid (80:20:0.1, v/v) isocratically at a flow rate of 0.5 mL/min. Chromatograms were monitored by a refractive index detector RI-410 and identification of the sugars was carried out by comparing the retention times of standard sugars (Table 1).

### **Structure identification of compounds**

All compounds were identified by extensive spectroscopic methods including 1D- (<sup>1</sup>H and <sup>13</sup>C) and 2D-NMR (COSY, HSQC, HMBC and NOESY) experiments as well as HR-ESI-MS analysis and by comparison with spectral data from the literature values for the known compounds.

### **WST cytotoxicity assay**

The cytotoxic activities of compounds **1-3** on KB cell lines (ATTC<sup>®</sup> CCL<sup>TM</sup>-17) were determined by colorimetry based on the cleavage of the WST-1 tetrazolium salt. Tetrazolium

salts were cleaved to formazan dye by cellular enzymes in the viable cells, and the level of absorbance was directly correlated to the metabolically active cells. The stock solutions of triterpenoids (1 mg/mL) were prepared in **dimethylsulfoxide**. Sample dilutions were then performed in medium DMEM F12 (1, 2.5, 5, 7.5 or 10 µg/mL). The cells were prepared as previously published, using DMEM F12 medium (Chwalek et al. 2006). After removal of pre-incubated culture medium, 200 µL of DMEM F12 containing various concentrations of samples were added and further incubated for 48 h at 37 °C. Cell viability was determined by adding WST-1 tetrazolium salt and by measuring the absorbance at 450 nm after ≈1 h. Each assay was realized in triplicate in 96-well microplates. A dose–response curve was plotted for each compound, and the concentration giving 50% inhibition (IC<sub>50</sub>) was calculated by using MSEXcel based program. α-hederin was employed as a positive control, which exhibited an IC<sub>50</sub> value of 5.5 µM under the above conditions (Chwalek et al. 2006).

#### **DPPH radical scavenging assay**

The free radical scavenging capacity of the **ethyl acetate**- and *n*-**butanol**-soluble parts of *A. neocaledonica* fruits was determined by using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Cheng et al. 2006). Briefly, a stock solution of DPPH was prepared at 158 µM in **ethanol:water** (1:1, v/v). Each sample was dissolved in **dimethylsulfoxide** (200 µg/mL) and 5 µL were added to the DPPH stock solution (95 µL), in triplicate in 96-well plates. The DPPH<sup>•</sup> absorbance in each reaction mixture was monitored at 515 nm. Absorbance measurements were performed at regular interval of 1.5 min for 30 min at 37 °C for all samples. The *n*-**butanol**-soluble part was then tested at 200, 100, 50 and 10 µg/mL to calculate the concentration able to quench 50% of the reaction system (EC<sub>50</sub>) at 30 min. The **ethanol:water** (1:1, v/v) solution was used as a blank, the free DPPH solution was used as a negative control and ascorbic acid (5 µg/mL) was used as a positive control. Results were expressed as percentage decrease with respect to control values.

#### **Tyrosinase inhibitory activity assay**

The tyrosinase inhibitory activity of the *n*-**butanol**-soluble part, of the CPE-fractions F<sub>1</sub>, F<sub>3</sub>-F<sub>6</sub> and of the isolated pure compounds **1**, **2**, **5-10** was determined against mushroom tyrosinase. The assay was performed according to a slight modification of a previously described method using L-DOPA as substrate (Alabdul Magid et al. 2008). Briefly, the tested compounds were dissolved in **10% DMSO** and mixed (1:1) with **sodium**-phosphate buffer (PBS, pH 6.8) to

obtain a concentration of 4 mg/mL for the extracts or 1 mg/mL for the compounds. Tyrosinase (100  $\mu$ L; 135 U/mL) was first pre-incubated with the tested compounds (100  $\mu$ L) at 25 °C for 10 min, and then 100  $\mu$ L of L-DOPA (0.5 mM, PBS pH 6.8) was added. The enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 25 °C) after 10 min incubation. These solutions were prepared in triplicate in 96-well plates. Kojic acid (1 mM) was used as positive control. The inhibitory percentage of tyrosinase was calculated as follows: % inhibition =  $\{[(A - B) - (C - D)]/(A - B)\} \times 100$  ( $A$ : OD at 475 nm without test substance;  $B$ : OD at 475 nm without test substance and tyrosinase;  $C$ : OD at 475 nm with test substance;  $D$ : OD at 475 nm with test substance, but without tyrosinase).

## RESULTS AND DISCUSSION

The presence of triterpenes, polyphenols, and a high amount of condensed tannins was firstly highlighted by screening the hydromethanolic extract of *A. neocaledonica* fruits by analytical TLC and HPLC. The hydromethanolic extract was preliminarily partitioned by liquid-liquid extraction to give an **ethyl acetate**- and a *n*-**butanol**-soluble parts. Extraction yield, antioxidant activity and anti-tyrosinase activity of these **ethyl acetate**- and a *n*-**butanol**-soluble-parts are given in Table 2. The *n*-**butanol** soluble part was the most active with an  $EC_{50} = 20 \mu\text{g/mL}$  for the DPPH radical scavenging activity and 92.1% inhibition of the tyrosinase activity. The *n*-**butanol**-soluble part was thus investigated for identification of phenolic compounds. Although less active as compared to the *n*-**butanol**-soluble part, the secondary metabolite profile of the **ethyl acetate**-soluble part was also investigated in order to screen *A. neocaledonica* fruits in the broadest possible sense.

### Characterization of the **ethyl acetate** -soluble part

The **ethyl acetate**-soluble part was fractionated by silicagel CC. Compound **1** (141.3 mg) was detected in fractions [22-35], and **2** was detected in fractions [41] (325.3 mg) and [22-27] (134.9 mg). Compounds **2** (8.9 mg) and **3** (6.5 mg) were also obtained by preparative TLC of fractions [62-65] (Fig. 1). These compounds were identified as three known triterpenes including two major products derived from lupane: betulinic acid (**1**) (Ikuta and Itokawa 1988, Jou et al. 2004), and alphitolic acid (**2**) (Aguirre et al. 2006), as well as an ursane derivative: corosolic acid (**3**) (Aguirre et al. 2006) (Fig. 2). The known flavonoid 3-*O*-[6"-*E*-feruloyl]- $\beta$ -D-glucopyranosylisorhamnetin (**4**) (Wang et al. 2008) (Fig. 2) was also purified by combination of preparative and semi-preparative HPLC of fractions [57-58] ( $R_t = 9.8$ , 3.4 mg). Even if minor, this

flavonoid contributed probably to the slight but significant DPPH radical scavenging activity of the **ethyl acetate**-soluble part (Table 2). The slight tyrosinase inhibitory activity (10%) of the **ethyl acetate**-soluble part (Table 2) might be attributed to the presence of betulinic acid (**1**) and alphitolic acid (**2**) which display 45.5 and 36.5 % inhibition, respectively (Table 3). The very small recovered quantity of corosolic acid (**3**) did not enable the evaluation of its tyrosinase inhibitory activity.

### **WST cytotoxic activity of triterpenes**

The cytotoxic activity of betulinic acid (**1**) has been previously reported against more than 40 cell lines derived from the most common human cancers (breast, colon, brain, lung and prostate) with an IC<sub>50</sub> ranging from 1.0 to 11.6 µg/mL by MTT assay, with a good selectivity on cancer cell lines with respect to healthy human cells, a low toxicity and a wide therapeutic index (Dzuback et al., 2006, Shanmugan et al. 2012). It has been also tested in clinical phase II trials (Dzuback et al., 2006). Alphitolic acid (**2**) (Kim et al. 2012) and corosolic acid (**3**) (Salvador et al. 2012) have also shown a cytotoxic activity against various cell lines but not KB cells for corosolic acid. In order to compare the cytotoxic activities of these compounds (**1-3**) against KB cell line, using the same method, a WST-1 proliferation test, we determined their IC<sub>50</sub> values after 48 h of contact of the compounds with the cells. Betulinic acid (**1**) and corosolic acid (**3**) showed a low cytotoxic activity with inhibitions of 45.7% and 49.3%, respectively at 10 µg/mL (IC<sub>50</sub> > 10 µg/mL) and alphitolic acid (**2**) showed a moderate cytotoxic activity with an IC<sub>50</sub> of 4 ± 0.3 µg/mL (8.5 µM). This is the first comparison of these compounds against KB cells line.

### **Characterisation of the *n*-butanol-soluble part**

Centrifugal Partition Extraction was used to prefractionate the *n*-butanol-soluble part and to separate the abundant mixture of tannins from the other potentially interesting constituents. This solid support-free liquid-liquid fractionation technique was particularly suited for this purpose due to the liquid nature of the stationary phases which enables a total recovery of the injected mass while avoiding irreversible adsorption of polar compounds as observed on polymeric resins or silica gel (Hamzaoui et al. 2013). As a result, eight fractions of simplified chemical composition were obtained from 4 g of the *n*-butanol-soluble part, with the last fractions F<sub>7</sub> and F<sub>8</sub> containing all the tannins (75.7%) retained inside the column over the whole separation process. According to UV spectra obtained from HPLC-DAD chromatogram and

TLC analyses, fractions F<sub>1</sub>-F<sub>2</sub>, eluted with the *n*-heptane-rich upper phase, comprised a diversity of triterpenoids, while fractions F<sub>3</sub>-F<sub>6</sub>, eluted with the methyl-*tert*-butyl ether/acetonitrile-rich intermediate phase, mostly contained flavonoids and phenolic compounds.

CPC and semi-preparative HPLC were then used as orthogonal techniques to further purify fractions F<sub>3</sub> to F<sub>6</sub> (Fig. 1). Fraction F<sub>3</sub> resulted in the purification of compounds **6** (Rt = 28.2, 4.4 mg), **5** (Rt = 30.2, 6 mg), **9** (Rt = 33.7, 2.6 mg) and **8** (Rt = 34.3, 2.0 mg). Fraction F<sub>4</sub> yielded compounds **6** again (Rt = 29.3, 5.6 mg) and **5** (Rt = 33.5, 8.1 mg). Fraction F<sub>5</sub> give compounds **7** (Rt = 15.5, 2.4 mg) and **9** (Rt = 21.4, 3.1 mg) and **8** (Rt = 11.1, 2.2 mg), **7** (Rt = 15.2, 3.1 mg) and **9** (Rt = 21.1, 2.8 mg) were isolated from fraction F<sub>6</sub> (Fig. 1). During the purification of fraction F<sub>4</sub>, compounds **5** and **6** were eluted separately but **10** was eluted with a mixture of derivatives. Fraction F<sub>4</sub> was thus purify by CPC and compounds **5** (15 mg), **6** (5.0 mg) and **10** (10.9 mg) were obtained (Fig. 1).

Compounds **5-8** were identified as four known flavonoids 3-*O*- $\beta$ -D-galactopyranosyl-isorhamnetin (**5**) (Bierner 1979), 3-*O*- $\beta$ -D-galactopyranosyl-quercetin (**6**) (Bierner 1979), 3-*O*-[ $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-galactopyranosyl]-isorhamnetin (**7**) (Buschi and Pomilio 1982), and 3-*O*-[ $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\alpha$ -L-rhamnopyranosyl]-quercetin (**8**) (Hasler et al. 1992) (Fig. 2). Compound **10** was identified as (+) gallocatechin (Foo et al. 1997).

Compound **9** was a yellow powder with an  $[\alpha]_D$  of -44.0 (*c* 0.1, methanol). The molecular formula C<sub>42</sub>H<sub>46</sub>O<sub>23</sub> was obtained from the HR-ESI-MS analysis in the positive ionization mode. A pseudo-molecular ion at *m/z* 941.2324 [M + Na]<sup>+</sup> (calcd 941.2328) was also detected. <sup>1</sup>H NMR signals of quercetin were observed at  $\delta_H$  6.14 (s, 2H), 7.36 (*d*, *J* = 2.2 Hz, H-2'), 6.92 (*d*, *J* = 8.4 Hz, H-5') and 7.27 (*dd*, *J* = 8.4, 2.2 Hz, H-6') as for **6** and **8**. The saccharide part was characterized by the presence of three anomeric protons detected at  $\delta_H$  4.50 (*d*, *J* = 7.7 Hz), 4.53 (*d*, *J* = 8.1 Hz) and 4.72 ppm (*d*, *J* = 2.0 Hz) with their corresponding anomeric carbons at  $\delta_C$  106.2, 104.0 and 98.9, respectively (Fig. S1 and S2 in supporting information), attributed after analysis of the HSQC-*J*modulate spectrum. Combined analysis of the COSY, HSQC-*J*modulate spectra and coupling constants from the <sup>1</sup>H NMR spectrum allowed us to identify three sugars, a  $\alpha$ -L-rhamnopyranose substituted in position 2 ( $\delta_C$  82.6), a  $\beta$ -D-glucopyranose esterified in position 6 with its deshielded signals at  $\delta_H$  4.16 (*dd*, *J* = 11.8, 6.0 Hz, H-6a'') and 4.53 (*dd*, *J* = 11.8, 2.9 Hz, H-6b'') and a terminal  $\beta$ -D-xylopyranose (Table 4). D-Glucose, D-galactose, L-rhamnose, and D-xylose were identified after acid hydrolysis of the hydromethanolic extract (Table 1). To confirm the presence of L-rhamnose in our samples the optical rotation of the

isolated L-rhamnose was measured.  $^1\text{H}$  NMR spectrum also revealed signals of a methoxy group ( $\delta_{\text{H}}$  3.79), a second ABX system at  $\delta_{\text{H}}$  6.93 (*d*,  $J = 1.9$  Hz, H-2'''),  $\delta_{\text{H}}$  6.69 (*d*,  $J = 8.2$  Hz, H-5''') and  $\delta_{\text{H}}$  6.87 (*dd*,  $J = 8.2, 1.9$  Hz, H-6'''), indicating the presence of a second trisubstituted aromatic ring, and signals of two olefinic doublets at  $\delta_{\text{H}}$  7.45 and 6.13 (*d*,  $J = 15.9$  Hz), suggesting a substitution by an organic acid derivative of cinnamic acid. These two olefinic protons were correlated in the HMBC spectrum with the signal of a carbonyl group at  $\delta_{\text{C}}$  167.4, indicating the presence of an  $\alpha,\beta$ -unsaturated ketone. Analysis of the NOESY spectrum showed a correlation between the methoxy protons ( $\delta_{\text{H}}$  3.79) and proton H-2'''' ( $\delta_{\text{H}}$  6.93, *d*,  $J = 1.9$  Hz), and allowed us to locate the methoxy group at C-3'''. These data resulted in the identification of the organic acid as ferulic acid as in compound **5** (Wang et al. 2008). The sequence Xyl-(1 $\rightarrow$ 2)-Glc-(1 $\rightarrow$ 2)-Rha of the three sugars was established from the HMBC correlations observed between H-1''' Glc/C-2'' Rha and H-1'''' Xyl/C-2''' Glc and inversely H-2'' Rha/Glc C-1'''' and H-2''' Glc/C-1'''' Xyl. These results are in agreement with those obtained by analysis of the NOESY spectrum showing correlations between H-2'' Rha/H-1'''' Glc and H-2''' Glc/H-1'''' Xyl. The quercetin was glycosylated at position 3 as observed by the HMBC correlations between H-1'' Rha/C-3 and the esterification of the glucose unit was confirmed by the two HMBC correlations observed between the carbonyl of ferulic acid and both geminal protons Ha, b-6''' Glc. On the basis of these data, the structure of compound **9** was established as 3-*O*-(6-*E*-feruloyl)- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)-[ $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 2)-] $\alpha$ -L-rhamnopyranosyl-quercetin (Fig. 3).

All flavonoids (**4-9**) of the fruits were detected for the first time in *Alphitonia* species and were different from the flavonoids previously identified in the leaves (Lin et al. 1995). Flavonoids **5**, **6**, and **8** have already demonstrated a DPPH radical scavenging activity with an  $\text{IC}_{50} = 67.92$   $\mu\text{M}$ , 4.29  $\mu\text{M}$  (Demirkiran et al. 2013) and 26.4  $\mu\text{M}$  (Tang et al. 2001), respectively. Similarly, the DPPH radical scavenging activity of galocatechin **10** has already been demonstrated with an  $\text{IC}_{50} = 6.7$   $\mu\text{g/mL}$  (Ko et al. 2011). These compounds are thus probably significantly involved in the DPPH radical scavenging activity of the *n*-butanol-soluble part ( $\text{EC}_{50} = 20$   $\mu\text{g/mL}$ ). This extract was however less active than the positive control ascorbic acid ( $\text{EC}_{50} = 5$   $\mu\text{g/mL}$ ).

### Tyrosinase inhibitory activity of the *n*-butanol-soluble part

The CPE-fractions F<sub>1</sub>, F<sub>3</sub>-F<sub>6</sub> (Table 3) and the isolated pure compounds **5-10** (Fig. 2) were evaluated for their anti-tyrosinase activity. Fraction F<sub>1</sub> with triterpenes showed a slight

inhibitory activity (25.6%). Fractions F<sub>3</sub> and F<sub>4</sub> with a similar profile (flavonoids and catechins derivatives), exhibited similar inhibition activities (67.2 and 68.0%), and both fractions F<sub>5</sub> and F<sub>6</sub> rich in flavonoids and a part of tannins had both a significant activity (73.4 and 58.3%). All compounds (**5-10**) isolated from fractions F<sub>3</sub> to F<sub>6</sub> were tested at 1 mg/mL and no interesting activity was observed with the five flavonoids (**5-9**) (Table 3), probably due to the glycosylation at C-3, as observed previously with various flavonoids (Parvez et al 2007). Gallocatechin **10** inhibits 87.4% of the enzymatic activity, this activity has already been demonstrated with an IC<sub>50</sub> = 4.8 µg/mL (Ko et al. 2011). Thus, the high antityrosinase activity of the *n*-butanol-soluble part could be explained by the presence of tannins and/or catechine derivatives. This activity may prove to have practical values as future cosmetic ingredients.

In conclusion, *Alphitonia neocaledonica* fruits are readily available because this species is used in the revegetation of New-Caledonian serpentine nickel-mining soils. These fruits can be valuable, as topic utilization, for their antioxidant and antityrosinase activities due to their phenolic compounds. However attention must be given because these fruits possess two abundant triterpenes, betulinic acid and alphitolic acid, with cytotoxic activity against various cell lines. For this reason a cytotoxic evaluation of the fruits could be made previous utilization. It should also be noted that these fruits could also be used as a new source of betulinic acid.

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### Supporting information

Supporting information can be found in the online version of this article.

**Table 1:** Time retention of sugars

Sucre	t <sub>r</sub> (min)
D-glucose ( $\alpha$ & $\beta$ )	18.3–23.2
D-galactose ( $\alpha$ & $\beta$ )	24.8-26.8
L-rhamnose ( $\alpha$ & $\beta$ )	11.7
L-arabinose ( $\alpha$ & $\beta$ )	14.6-15.5
D-xylose ( $\alpha$ & $\beta$ )	16.6-18.5

**Table 2:** Extraction yield, antioxidant and anti-tyrosinase activities of *A. neocaledonica* fruits

	extraction yield (%)	antioxidant activity (%) at 200 $\mu$ g/mL	Tyrosinase inhibitory activity (%) at 4 mg/mL
Ethyl acetate	0.76	44.7	10
<i>n</i> -Butanol	8.76	93.2	92.1
Water	29.54	nt	nt
Ascorbic acid		100	
Kojic acid (1mM)			69.3 $\pm$ 1.6

nt : not tested

**Table 3:** Effect of CPE fractions and compounds **1, 2, 5-10** on anti-tyrosinase activity

	Percent inhibition at 4 mg/mL (mean $\pm$ SD)	Percent inhibition at 1mg/mL (mean $\pm$ SD)
F <sub>1</sub>	25.6 $\pm$ 0.7	
F <sub>3</sub>	67.2 $\pm$ 1.6	
F <sub>4</sub>	68.0 $\pm$ 1.7	
F <sub>5</sub>	73.4 $\pm$ 1.5	
F <sub>6</sub>	58.3 $\pm$ 0.2	
<b>1</b>		45.5 $\pm$ 1.2
<b>2</b>		36.5 $\pm$ 0.6
<b>5</b>		6.4 $\pm$ 1.6
<b>6</b>		2.5 $\pm$ 1.7
<b>7</b>		14.0 $\pm$ 0.3
<b>8</b>		9.2 $\pm$ 1.3
<b>9</b>		14.4 $\pm$ 1.2
<b>10</b>		87.4 $\pm$ 2.2

**Table 4:** <sup>1</sup>H (600 MHz) and <sup>13</sup>C (150 MHz) NMR data of compound **9**.

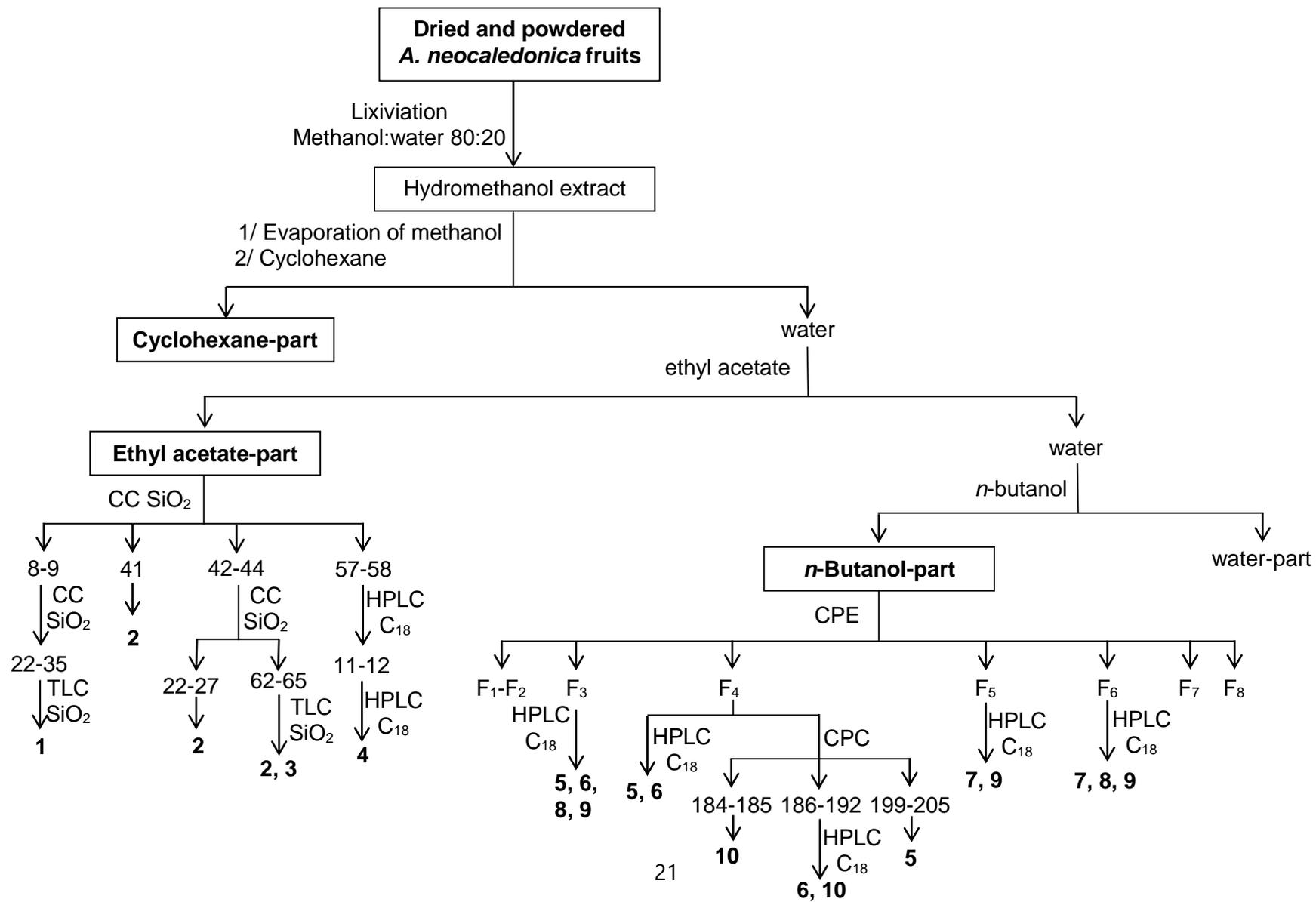
Position	$\delta_C$	$\delta_H$ (J in Hz)	Position	$\delta_C$	$\delta_H$ (J in Hz)
-			<b>Glc</b>		
2	156.7	-	1'''	104.0	4.53, <i>d</i> (8.1)
3	135.2	-	2'''	83.7	3.42, <i>dd</i> (9.2, 8.1)
4	178.0	-	3'''	76.0	3.62, <i>t</i> (9.2)
5	161.6	-	4'''	70.2	3.38, <i>t</i> (9.5)
6	98.9	6.14, <i>sl</i>	5'''	73.9	3.47, <i>ddd</i> (9.7, 6.0, 2.9)
7	165.9	-	6'''	62.7	4.16, <i>dd</i> (11.8, 6.0)
8	93.7	6.14, <i>sl</i>			4.53, <i>dd</i> (11.8, 2.9)
9	157.0	-	<b>Xyl</b>		
10	104.1	-	1''''	106.2	4.50, <i>d</i> (7.7)
1'	121.5	-	2''''	74.5	3.28, <i>dd</i> (9.7, 7.6)
2'	115.5	7.36, <i>d</i> (2.2)	3''''	76.4	3.31, <i>t</i> (9.9)
3'	145.0	-	4''''	69.4	3.47, <i>dd</i> (10.0, 5.3)
4'	148.6	-	5''''	65.7	3.25, <i>ddd</i> (11.3, 10.0, 5.3)
5'	115.0	6.92, <i>d</i> (8.4)			3.99, <i>dd</i> (11.3, 5.3)
6'	121.4	7.27, <i>dd</i> (8.4-2.2)	<i>E</i> -feruloyl		
			1'''''	126.0	-
			2'''''	109.8	6.93, <i>d</i> (1.9)
			3'''''	147.8	-
<b>Rha</b>			4'''''	149.1	-
1''	98.9	4.72, <i>d</i> (2.0)	5'''''	114.8	6.69, <i>d</i> (8.2)
2''	82.6	4.43, <i>dd</i> (2.8, 2.0)	6'''''	122.7	6.87, <i>dd</i> (8.2, 1.9)
3''	70.6	3.78, <i>dd</i> (9.4, 2.8)	7'''''	145.5	7.45, <i>d</i> (15.9)
4''	72.8	3.28, <i>t</i> (9.4)	8'''''	113.7	6.13, <i>d</i> (15.9)
5''	70.4	3.73, <i>dq</i> (9.4, 6.2)	9'''''	167.4	-
6''	16.3	1.09, <i>d</i> (6.2)	3'''''-OCH <sub>3</sub>	54.8	3.79 ( <i>s</i> )

Figure 1. Purification of the fruits of *Alphitonia neocaledonica*

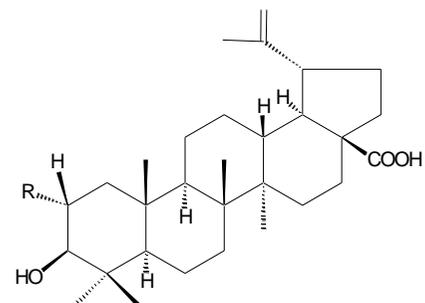
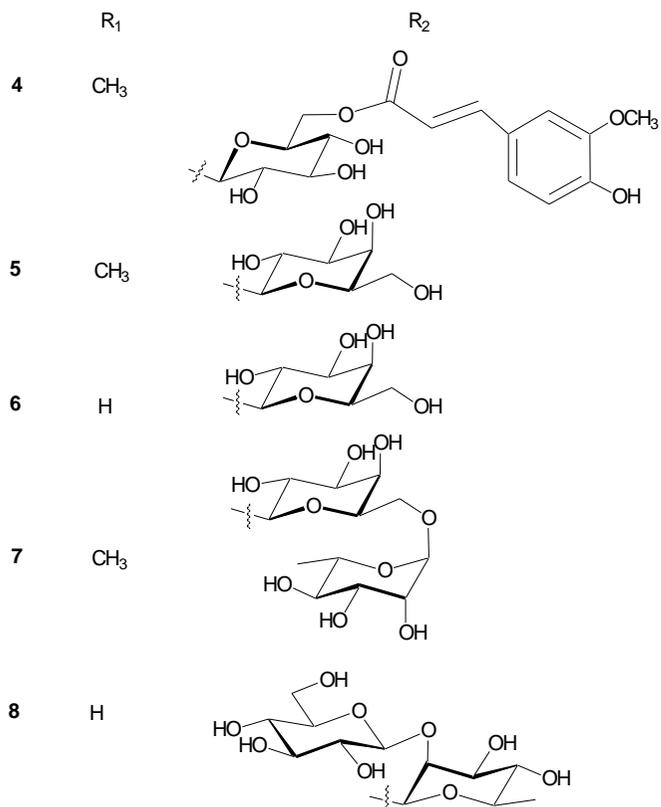
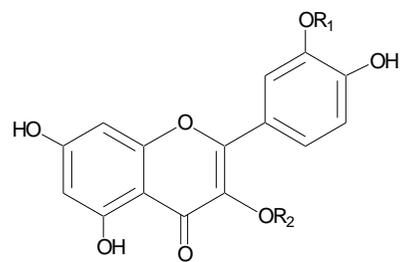
Figure 2: Structures of the known compounds (**1-8, 10**) isolated from *A. neocaledonica* fruits

Figure 3: Structure of compound **9**

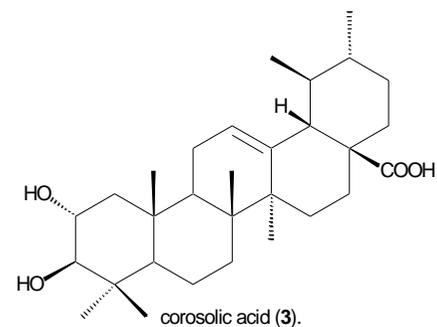
Figure 1



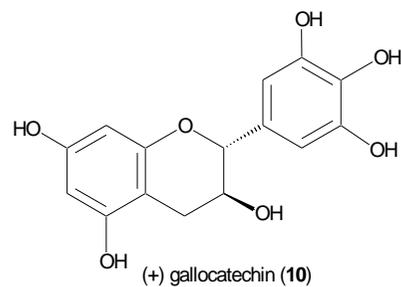
*Figure 2*



betulinic acid (1) : R = H  
 alphitolic acid (2) : R = OH



corosolic acid (3).



(+) gallocatechin (10)

Figure 3

