

## Chemical Constituents of *Croton thurifer* Kunth as $\alpha$ -Glucosidase Inhibitors

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**Abstract:** Phytochemical investigation of *Croton thurifer*, collected in Loja-Ecuador, led to the isolation of seven known compounds identified as: (3R, 20S) -3-palmitate-20-hydroxydammar-24-ene (**1**); (3R, 20S)-3-acetoxy-20-hydroxydammar-24-ene (**2**); *trans*-phytol (**3**); vomifoliol (**4**);  $\beta$ -sitosterol (**5**); *trans*-tiliroside (**6**) and sparsifol (**7**). The structures of the isolated compounds were determined by NMR, MS, as well as by comparison with literature data. The hypoglycemic activity of the crude extracts and isolated compounds was assessed by their ability to inhibit  $\alpha$ -glucosidase activity. The hexane and methanol extracts did not show any inhibitory activity on  $\alpha$ -glucosidase, while the ethyl acetate extract (EtOAc) showed very low inhibitory activity with  $IC_{50}$ = 1.77 mg/mL. Two of the seven isolated compounds exhibited good inhibitory activity with  $IC_{50}$  values much higher than acarbose (377  $\mu$ g/mL). *trans*-tiliroside was the compound with the highest inhibitory activity ( $IC_{50}$  = 114.85  $\mu$ g / mL), while (3R, 20S)-3-Acetoxy-20-hydroxydammar-24-ene had moderate inhibitory activity ( $IC_{50}$ =292.87). *Trans*-tiliroside exerted a non-competitive inhibition with  $K_m$  values between 368 and 358  $\mu$ M and  $V_{max}$  value from 695 and 325 nM/min for uninhibited and inhibited reaction, respectively and a  $K_i$  of 163,6  $\mu$ M.

**Keywords:** *Croton thurifer*;  $\alpha$ -glucosidase; (3R, 20S)-3-Acetoxy-20-hydroxydammar-24-ene; *trans*-tiliroside. © 2019 ACG Publications. All rights reserved.

### 1. Introduction

The genus *Croton* comprises around 1200 species, which makes it the second largest genus in the family Euphorbiaceae [1,2] and one of the most diverse, with many species endowed with medicinal properties [3]. The continent of America is rich in plant species; in Brazil alone more than 350 species have been documented [4]. In Ecuador, 39 *Croton* species are recognized [5], and 13 of

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them are classified as native [6]; however several more species have been documented in recent years [7-11]. Ecuador is known to be one of the countries with the highest biodiversity in the world, and it has a long tradition in the use of medicinal plants. Many species under the *Croton* genus are used in the traditional medicine by the indigenous culture in the country [12,13]. Studies on the biological activities of species belonging to this genus from America, Asia and Africa, are shown to be antimicrobial [14], antioxidant [15], anti-inflammatory [16], anticancer [17,18] and wound healing [19,20].

The chemistry of *Croton* genus shows a high diversity of structures: diterpenes of different skeletons including crotofolanes [21,22], clerodanes [23-25], kaurenes [26-28], labdanes [29], terpenes [30,31]. Alkaloids and flavonoids are also common in the genus [32,33].

*Croton thurifer* Kunth is a native shrub widely distributed in the provinces of Azuay and Loja in the south of Ecuador, and in the northern region of Peru. Due to the taxonomic imbalance, this species has been identified as *C. wagneri* and *C. elegans*. It is known with the vernacular name of “mosquera”, the exudate latex of the stem bark is yellow, and it is used to eliminate warts and to treat wounds, sores, and ulcers [34]. The diabetes is one of the major health problems in Ecuador [35,36] where the people commonly use medicinal plants to control this metabolic disease. In addition, finding new antibacterial agents is a big issue in medicinal chemistry because increasing resistance to antibiotic agents that are currently in use. To take advantage of Ecuador's biodiversity, and its rich tradition of using medicinal plants, we used *C. thurifer* as material to conduct a phytochemical study and explore its potential as source of antidiabetic compounds.

## 2. Materials and Methods

### 2.1. General experimental procedures

LC-MS spectra were recorded with a Liquid chromatography Ultimate 3000 (Thermo Scientific LTQ XL, Germering, Germany) coupled to a Linear Ion Trap mass spectrometer equipped with an ESI source (Bruker, Bremen, Germany). Optical rotations were measured with an Automatic Polarimeter (Jinan Hanon Instruments Co. Ltd., Jinan, China) MRC P810.  $^1\text{H}$  and  $^{13}\text{C}$ , 1D and 2D nuclear magnetic resonance (NMR) spectra were obtained on a Varian Premium Shielded-400 spectrometer (400 MHz to  $^1\text{H}$  and 100 MHz to  $^{13}\text{C}$ ). Chemical shifts were reported in  $\delta$  (ppm), relative to the signal of tetramethylsilane (TMS) and coupling constants (J) in Hz. Thin Layer Chromatography (TLC) was performed with aluminium pre-coated Si plates with fluorescence indicator in the range of 254 nm (F254) (Merck, Darmstadt, Germany). Spraying with a vanillin/ $\text{H}_2\text{SO}_4$  solution revealed the substances. All the Chromatographic procedures were done over normal phase, Silica gel 60 (63–200  $\mu\text{m}$ ) from (Merck, KGaA, Darmstadt, Germany), and RP-18 (Merck, KGaA, Darmstadt, Germany, 40–63  $\mu\text{m}$ ). All organic solvents were bought from Brenntag (Brenntag, Guayaquil, Ecuador) and re-distilled before using.

### 2.2. Plant material

The leaves of *C. thurifer* were collected in the flowering stage in the locality of Catamayo, province of Loja, Ecuador, in April 2016, at 1740 m a.s.l. (Coordinates 3°58'53.4''S; 79°20'29.0''W). The plant material was identified by botanist Nixon Cumbicus, and voucher specimens (HUTPL1050) were deposited at the Herbarium HUTPL at the Universidad Técnica Particular de Loja.

### 2.3 Extraction and isolation

The air-dried and powdered leaves of *C. thurifer* (500 g) were macerated with solvents of increasing polarity, starting with n-hexane, ethyl acetate and methanol (three times each x 4L) at room temperature for 24h. The solvents were evaporated under reduced pressure in a rotary evaporator, to yield the hexane (CtH) (12.5 g), ethyl acetate (CtEt) (5.15 g), and methanol (CtM) (166.3 g) extracts.

The hexane extract (2 g) was subjected to column chromatography on 200 g of Si-gel, and eluted with increasing gradient of polarity from Hex/EtOAc (95:0.5) to Hex/EtOAc (1:1). A total of 276 fractions (CtHI-01/CtHI-276), 50 mL each, were collected. Fractions with similar behavior on TLC were combined and evaporated, yielding 20 sub-fractions (CtHII-01/CtHII-20) for further separation and purification. The mixture of sub-fractions CtHII-03 to CtHII-05 (351.9 mg) was re-chromatographed on Si-gel column (30g), eluted with mixtures of n-hexane/ethyl acetate with increasing gradient of polarity, beginning with Hex/EtOAc (99:1) to (93:7). Twenty-four fractions (CtHIII-1/CtHIII-24) were collected, and the sub-fraction CtHIII-8 gave the compound (3R, 20S) -3-palmitate-20-hydroxydammar-24-ene (**1**) (33 mg) [37]. Fraction CtHIII-18 (60 mg) was subjected to chromatography on Si-gel and using a different elution system, starting with 7:3 CH<sub>2</sub>Cl<sub>2</sub>/cyclohexane and ending with 100% CH<sub>2</sub>Cl<sub>2</sub>. Four sub-fractions were obtained from this column, and the sub-fraction identified as CtHIV-3 yielded the triterpene (3R, 20S)-3-acetoxy-20-hydroxydammar-24-ene (**2**) (7 mg) [38].

Ethyl acetate extract (2 g) was subjected to column chromatography on 200 g of Si-gel and eluted with Hex/ETOAc, varying from 95:05 to a 65:35 proportion. Twenty-five fractions (CtEtI-01/CtEtI-25) were collected and the sub-fractions CtEtI-21 and CtEtI-22 were combined for RMN analysis, resulting in the identification of the compound *trans*-phytol (**3**) (24 mg) [39]. The sub-fraction CtEtI-23 was purified by column chromatography using 18g of silica gel and elution under isocratic conditions with (5:5) Hex/EtOAc. The pure substance obtained in this purification was identified as vomifoliol (**4**) (10 mg) [40]. The sub-fraction CtEtI-25 afforded the compound  $\beta$ -sitosterol (**5**) (14 mg) [41].

Finally, part of the methanol extract (CtM) (2g) was subjected to CC over reverse phase RP-18, using H<sub>2</sub>O/CH<sub>3</sub>OH (6:4) and nine fractions were collected. The fraction CtM-03 was purified on normal phase, and eluted with a mixture of (95:05) EtOAc/CH<sub>3</sub>OH to obtain the glycosylated flavonoid *trans*-tiliroside (**6**) (4.3 mg) [42].

The fraction CtM-01(170 mg) was rechromatographed on silica gel using isocratic conditions with a mixture of EtOAc/CH<sub>3</sub>OH/H<sub>2</sub>O (80:15:05) to obtain four fractions (CtMI-01/CtMI-04). The fraction CtMI-02 (105 mg) was chromatographed on silica gel with an increasing polarity elution system (EtOAc/CH<sub>3</sub>OH, from 8:2 to 6:4 ratio) resulting in the purification of sparsifol (**7**) (16.4 mg), which was isolated [43]and re-distilled before using.

#### 2.4. $\alpha$ -Glucosidase Inhibition Assay

The inhibitory effect over  $\alpha$ -Glucosidase enzyme was measured according to the method described by [44], with slight modifications, as final mix volume reaction and sample extract amount. The procedure was determined by using a 96-well microtiter plate with p-nitrophenyl- $\alpha$ -D-glucopyranoside (PNPG) as the substrate. The extracts were dissolved in methanol at a final concentration of 2mg/mL. The pure compounds were dissolved in a mixture of 1:1 MeOH:DMSO ratio due to the lack of complete solubility in MeOH. The maximum concentration tested for a given compound was 1mM. Several dilutions in PBS were made to find the concentration that yields complete enzyme inhibition. First, 75  $\mu$ L of PBS (SIGMA-P4417) was mixed with 5  $\mu$ L of the sample (extracts or compounds) and 20  $\mu$ L of the enzyme solution (0.15 U/mL in PBS pH 7.4), then the mixture was pre-incubated at 37 °C for 5 min prior to the initiation of the reaction by adding the substrate. After pre-incubation, 20  $\mu$ L of PNPG (5 mM in phosphate buffer, pH 7.4) was added and then incubated at 37 °C. The amount of p-nitrophenol (p-NP) released was measured in an EPOCH 2 (BIOTEK®) microplate reader at 405 nm at 5min intervals for 60 min. The results were expressed as inhibition percentage by means of the formula described by [45] as follows:

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

Where A<sub>0</sub> is the absorbance recorded for enzyme activity without inhibitor (control), and A<sub>s</sub> is the absorbance recorded for the enzymatic activity in the presence of the inhibitor (sample). IC<sub>50</sub> value was calculated by curve fitting of data (SPSS). Acarbose was used as positive control.

## 2.5 *trans*-Tiliroside kinetic analyses

K<sub>m</sub> and V<sub>max</sub> were determined for *trans*-tiliroside by measuring the change in enzyme velocity at different concentrations of PNPG and three concentrations of Inhibitor, including the one that yielded the IC<sub>50</sub> [46]. Progress curves for  $\alpha$ -glucosidase with 0.5, 1, 2.5, 5 and 7.5 mM of PNPG and for  $\alpha$ -glucosidase with a concentration of 115  $\mu$ M (IC<sub>50</sub>) were done. Once obtained the type of inhibition, K<sub>i</sub> was determined for three different concentrations (250, 125 and 62.5  $\mu$ M final concentrations) of inhibitor and three concentrations of PNPG (1, 2.5 and 5 mM). The amount of enzyme employed was the same as used in the enzyme inhibition assay above described, as well as, following the same procedure. All the results were analysed on GraphPad Prism 8.0.1 and expressed as the mean of three replicates  $\pm$  1SD.

## 3. Results and Discussion

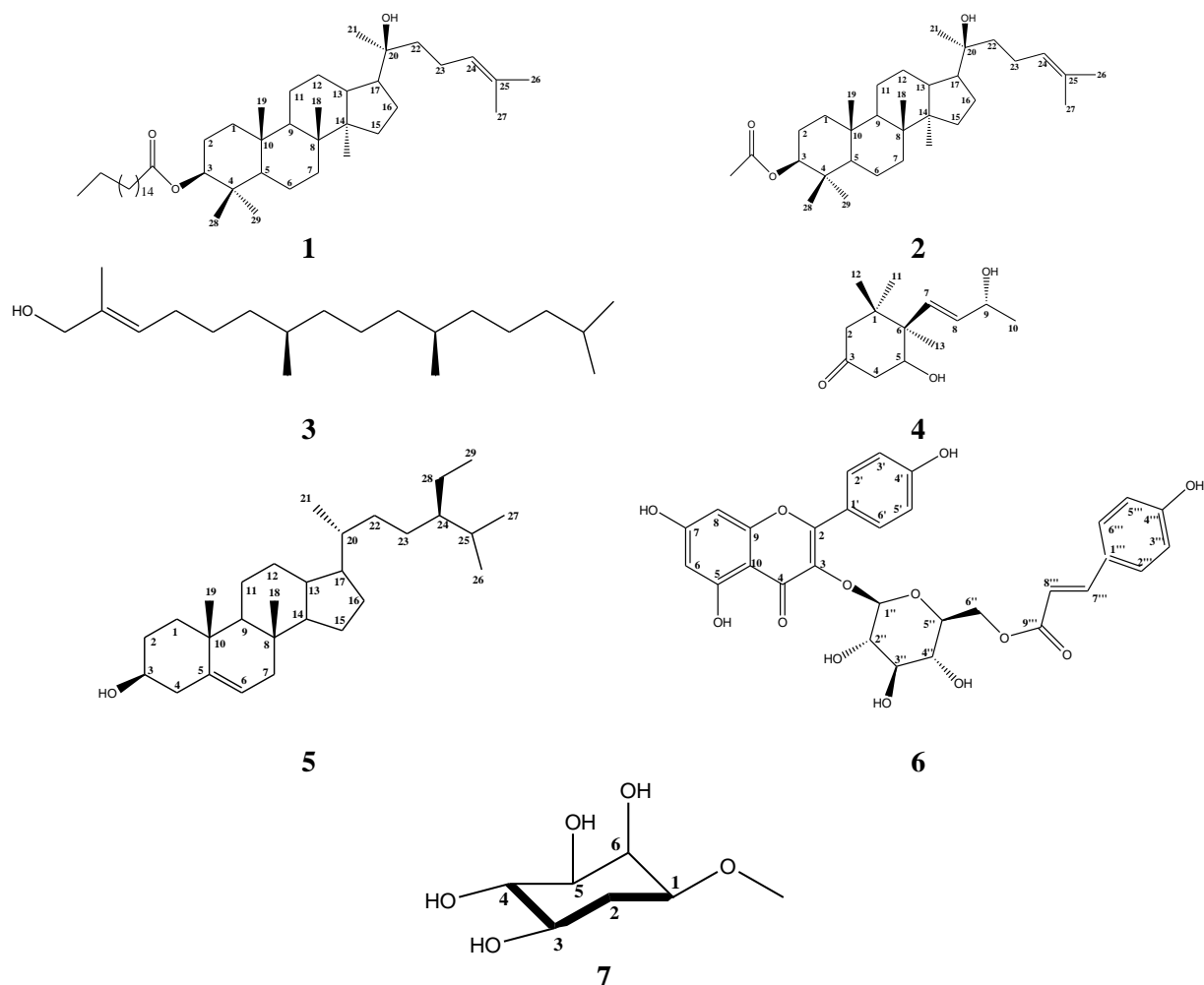
### 3.1. Structure Elucidation

Seven compounds were identified from organic extracts (Figure 1). Of these, two compounds, dammarane-type triterpenoids (**1**, **2**), were identified from n-hexane and ethyl acetate (EtOAc) extracts, three compounds, *trans*-phytol (**3**), vomifoliol (**4**) and  $\beta$ -sitosterol (**5**) [47], were isolated from methanol extract, glycosidic flavonoid *trans*-tiliroside (**6**) [48] and sparsifol (**7**), were isolated from methanol extract of species.

(3*R*, 20*S*)-3-Palmitate-20-hydroxydammar-24-ene (**1**). Colorless crystals; EIMS *m/z* 682 [M<sup>+</sup>]; [ $\alpha$ ]<sub>D</sub> = +45.75 (c, 0.115, CHCl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$ : 5.11 (1H, dt, *J* = 7.2, 0.8 Hz, H-24), 4.47 (1H, dd, *J* = 5.6 Hz, 10.4 Hz, H-3), 1.69, 1.68, 1.62, 1.13, 0.95, 0.87, 0.86, 0.85, 0.84 (3H each, s, Me-18, 19, 21, 26, 27, 28, 29, 30, 16'); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$ : 38.8 (C-1), 23.8 (C-2), 80.7 (C-3), 40.5 (C-4), 56.0 (C-5), 18.3 (C-6), 35.3 (C-7), 40.4 (C-8), 50.6 (C-9), 37.1 (C-10), 21.6 (C-11), 27.6 (C-12), 42.4 (C-13), 50.4 (C-14), 31.3 (C-15), 24.9 (C-16), 49.9 (C-17), 15.6 (C-18), 16.4 (C-19), 75.5 (C-20), 25.5 (C-21), 40.6 (C-22), 22.6 (C-23), 124.8 (C-24), 131.7 (C-25), 25.8 (C-26), 17.8 (C-27), 28.1 (C-28), 16.6 (C-29), 16.5 (C-30), 173.8 (COO<sup>-</sup>), 35.0 (C-2'), 29.3-29.7 (C-4'-13'), 32.0 (C-14'), 22.8 (C-15'), 14.2 (C-16').

(3*R*, 20*S*)-3-Acetoxy-20-hydroxydammar-24-ene (**2**). Colorless oil, C<sub>32</sub>H<sub>54</sub>O<sub>3</sub>; EIMS *m/z* = 486.6 [M<sup>+</sup>]. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$ : 5.11 (1H, dt, *J* = 7.2, 0.8 Hz, H-24), 4.81 (1H, t, *J* = 5.6 Hz, H-3), 2.04 (s, CH<sub>3</sub>-COO), 1.68, 1.62, 1.25, 1.13, 0.95, 0.87, 0.86, 0.84 (3H each, s, Me-18, 19, 21, 26, 27, 28, 29, 30); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$ : 35.3 (C-1), 23.8 (C-2), 81.7 (C-3), 38.1 (C-4), 50.4 (C-5), 18.3 (C-6), 37.2 (C-7), 40.7 (C-8), 50.7 (C-9), 37.2 (C-10), 21.7 (C-11), 25.6 (C-12), 42.4 (C-13), 50.7 (C-14), 31.5 (C-15), 27.6 (C-16), 49.9 (C-17), 15.6 (C-18), 16.4 (C-19), 75.6 (C-20), 25.9 (C-21), 40.6 (C-22), 22.7 (C-23), 124.8 (C-24), 131.8 (C-25), 25.9 (C-26), 17.8 (C-27), 28.1 (C-28), 21.5 (C-29), 16.5 (C-30), 171.2 (COO<sup>-</sup>).

*trans*-phytol (**3**): C<sub>20</sub>H<sub>40</sub>O, EIMS *m/z* = 296 [M<sup>+</sup>]; <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  0.87 (15H, 2d, *J* = 6.8 Hz, H-16, H-17, H-18, H-19), 1.48 - 2.04 (20 H, m), 1.66 (3H, s, H-20), 2.06 (2H, m), 4.14 (2H, dd, *J* = 6.4, 0.4 Hz, H-1), 5.78 (1H, t, *J* = 6.4 Hz, H-2). <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz):  $\delta$  16.3 (C-20), 19.8 (C-19), 19.9 (C-18), 22.7 (C-16), 22.8 (C-17), 24.6 (C-9), 24.9 (C-13), 25.3 (C-5), 28.1 (C-15), 32.9 (C-11), 36.8 (C-12), 37.4 (C-6), 37.5 (C-8), 37.6 (C-10), 38.2 (C-8), 39.5 (C-14), 40.7 (C-4), 59.6 (C-1), 123.2 (C-2), 140.4 (C-3).



**Figure 1.** Compounds isolated from the leaves of *Croton thurifer*. (3R, 20S) -3-Palmitate-20-hydroxydammar-24-ene (1), (3R, 20S)-3-Acetoxy-20-hydroxydammar-24-ene (2), *Trans*-phytol (3), Vomifoliol (4),  $\beta$ -Sitosterol (5), *Trans*-tiliroside (6) and Sparsifol (7)

*Vomifoliol* (4):  $C_{13}H_{20}O_3$ ; EIMS  $m/z = 224$  [M<sup>+</sup>];  $[\alpha]_D = +216.04$  (c, 0.107,  $CHCl_3$ );  $^1H$  NMR ( $CDCl_3$ , 400 MHz):  $\delta$  5.90 (1H, m, H-5), 5.85 (1H, d,  $J = 10.8$  Hz, H-8), 5.83 (1H, m, H-7), 4.40 (1H, q,  $J = 5.6$  Hz, H-9), 2.43 (1H, d,  $J = 16.8$  Hz, H-3a), 2.23 (1H, d,  $J = 16.8$  Hz, H-3b), 1.89 (3H, s, H-13); 1.28 (3H, d,  $J = 6.8$  Hz, H-10), 1.07 (3H, s, H-12), 1.00 (3H, s, H-11);  $^{13}C$  NMR ( $CDCl_3$ , 100 MHz): 198.2 (C-4), 162.9 (C-6), 135.9 (C-8), 129.8 (C-7), 127.0 (C-5), 79.2 (C-1), 68.2 (C-9), 49.8 (C-3), 41.3 (C-2), 23.9 (C-10), 24.1 (C-11), 23.0 (C-12), 19.0 (C-13).

$\beta$ -*Sitosterol* (5).  $C_{29}H_{50}O$ ; crystals;  $^1H$ -NMR ( $CDCl_3$ , 400 MHz):  $\delta$  (ppm): 3.50 (1H, m, H-3), 5.34 (1H, d,  $J = 4.6$  Hz, H-6), 0.92 (3H, d,  $J = 6.5$  Hz, H-21), 0.84 (3H, t,  $J = 7.3$  Hz, H-21), 0.82 (3H, d,  $J = 7.5$  Hz, H-29), 0.80 (3H, s, H-26), 0.67 (3H, s, H-18), 1.00 (3H, s, H-19);  $^{13}C$ -NMR ( $CDCl_3$ , 100 MHz)  $\delta$  (ppm): 37.4 (C-1), 31.8 (C-2), 71.9 (C-3), 42.5 (C-4), 140.9 (C-5), 121.9 (C-6), 32.1 (C-7), 32.1 (C-8), 50.3 (C-9), 36.7 (C-10), 21.2 (C-11), 39.9 (C-12), 42.5 (C-13), 56.9 (C-14), 26.2 (C-15), 28.4 (C-16), 56.2 (C-17), 36.3 (C-18), 19.2 (C-19), 34.1 (C-20), 26.2 (C-21), 45.9 (C-22), 23.2 (C-23), 12.1 (C-24), 29.3 (C-25), 19.9 (C-26), 19.5 (C-27), 18.9 (C-28), 12.0 (C-29).

*trans*-tiliroside (6):  $C_{30}H_{26}O_{13}$ ; amorphous yellow solid; mp 268-270 °C; EIMS  $m/z = 594$  [M<sup>+</sup>];  $^1H$ -NMR ( $CD_3OD$ , 400 MHz)  $\delta$  (ppm):  $\delta$  7.98 (2H, d,  $J = 8.8$  Hz, H-2' and H-6'),  $\delta$  7.39 (1H, d,  $J = 16$  Hz, H-7'''),  $\delta$  7.30 (2H, d,  $J = 8.5$  Hz, H-2''' and H-6'''),  $\delta$  6.81 (2H, d,  $J = 8.8$  Hz, H-3' and H-5'),  $\delta$  6.79 (2H, d,  $J = 8.5$  Hz, H-3''' and H-5'''),  $\delta$  6.30 (1H, brs, H-8),  $\delta$  6.13 (1H, brs, H-6),  $\delta$  6.03 (1H, d,  $J = 16$  Hz, H-8'''),  $\delta$  5.23 (1H, d,  $J = 7.6$  Hz, H-1''),  $\delta$  4.30 (1H, dd,  $J = 11.8, 1.9$  Hz, H-6 $\beta$ '''),  $\delta$  4.18 (1H, dd,  $J =$

11.6, 6.6 Hz, H-6 $\alpha$ "),  $\delta$  3.55 (1H, m, H-5"),  $\delta$  3.46 (1H, m, H-3"),  $\delta$  3.45(1H, m, H-2"),  $\delta$  3.44 (1H, m, H-4");  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ , 100 MHz)  $\delta$  (ppm): 159.4 (C-2), 135.2 (C-3), 179.4 (C-4), 158.5 (C-5), 99.9 (C-6), 165.9 (C-7), 94.8 (C-8), 162.9 (C-9), 105.6 (C-10), 122.7 (C-1'), 132.2 (C-2', C-6'), 116.1 (C-3', C-5'), 116.8 (C-3', 6'), 103.9 (C-1"), 75.8 (C-2"), 75.7 (C-3"), 71.7 (C-4"), 77.9 (C-5"), 63.9 (C-6"), 127.2 (C-1""), 133.2 (C-2""), 114.7 (C-3""), 131.2 (C-4""), 116.0 (C-5""), 133.7 (C-6""), 146.6 (C-7""), 116.8 (C-8""), 168.8 (C-9").

*Sparsifol* (7): colorless solid; mp 177-180°C: EIMS  $m/z$ = 194 [M+],  $\text{C}_7\text{H}_{14}\text{O}_6$ ,  $[\alpha]_D = -50.60$  (c, 0.164,  $\text{H}_2\text{O}$ );  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz)  $\delta$  (ppm): 4.27 (1H, t,  $J = 3.2$  Hz, H-1), 3.40 (1H, t,  $J = 3.6$  Hz, H-2), 3.62 (1H, m, H-3), 3.60 (1H, d,  $J = 3.6$  Hz, H-4), 3.75 (1H, dd,  $J = 3.6, 6.4$  Hz, H-5), 4.06 (1H, t,  $J = 3.6$  Hz, H-6), 3.47 (s, OCH<sub>3</sub>);  $^{13}\text{C}$  NMR ( $\text{D}_2\text{O}$ , 125 MHz)  $\delta$  (ppm): 80.0 (C-1), 71.2 (C-2), 72.7 (C-3), 70.5 (C-4), 71.8 (C-5), 67.0 (C-6), 56.8 (OCH<sub>3</sub>).

### 3.2 $\alpha$ -Glucosidase Inhibition Activity

The inhibitory activity of crude extracts was null or very weak as shown in Table 1. Only the ethyl acetate extract yielded an inhibition of  $\text{IC}_{50}$  of 1.77 mg/mL, while the other extracts exhibited a maximum of 30% of inhibition at their maximum dose tested (2mg/mL). On the other hand, only two of the six pure compounds assessed yielded an  $\text{IC}_{50}$  below 300  $\mu\text{M}$ . *Trans*-tiliroside yielded an  $\text{IC}_{50}$  of 114.85  $\mu\text{M}$ , and the triterpene (3R, 20S)-3-acetoxy-20-hydroxydammar-24-ene yielded an  $\text{IC}_{50}$  of 292.87  $\mu\text{M}$ . Both compounds showed a strong inhibitory effect, even at concentrations lower than Acarbose (used as a positive control). In contrast, (3R, 20S) -3-palmitate-20-hydroxydammar-24-ene, *trans*-phytol,  $\beta$ -sitosterol, sparsifol, each with an  $\text{IC}_{50}$  below than 1000  $\mu\text{g/mL}$ , had poor or no inhibitory effect on the enzyme

**Table 2.** In vitro  $\alpha$ -glucosidase inhibitory activities of extracts and compounds isolated from *Croton thurifer*

No	Extract/Compound	$\alpha$ -Glucosidase inhibition (%)	$\alpha$ -Glucosidase $\text{IC}_{50}$ (mg/mL, $\mu\text{M}$ †)
1	Hexane extract	21.67 $\pm$ 0,24	> 2000
2	MeOH extract	30.55 $\pm$ 0,51	> 2000
3	EtOAc extract	50	1.77 $\pm$ 0.03
4	<i>trans</i> -tiliroside	50	114.85 $\pm$ 0,05†
5	(3R, 20S) -3-palmitate-20-hydroxydammar-24-ene	40.03 $\pm$ 0,09	> 1000
6	(3R, 20S)-3-Acetoxy-20-hydroxydammar-24-ene	50	292.87 $\pm$ 0.03†
7	<i>trans</i> - phytol	2.97 $\pm$ 0.28	> 1000
8	Vomifoliol	*ND	*ND
9	$\beta$ -sitosterol	10.39 $\pm$ 0,49	> 1000
10	Sparsifol	6.11 $\pm$ 0.08	> 1000
11	**Acarbose	50	377.0 $\pm$ 1.9†

\*NR: Not determined due to the low yield obtained

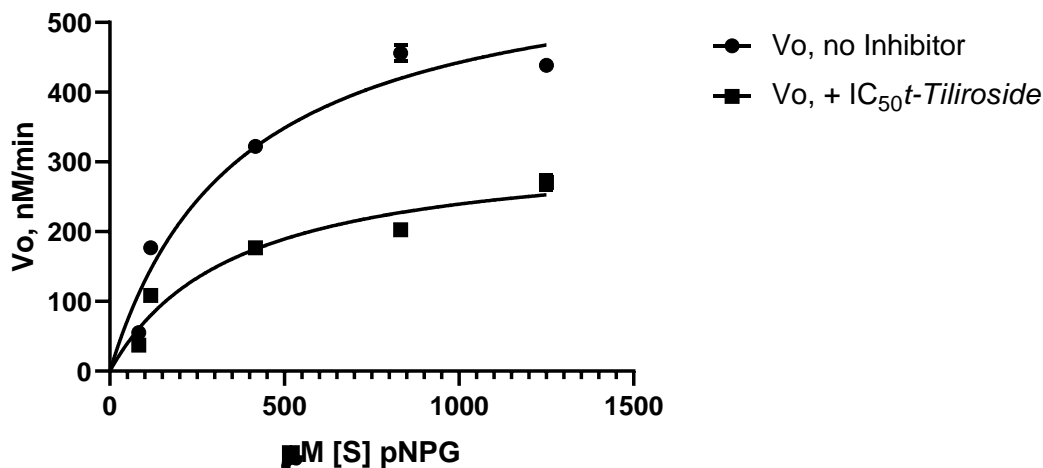
\*\*Acarbose was used as positive control

†  $\alpha$ -glucosidase inhibitory activity for pure compounds and positive control (Acarbose) are expressed in  $\mu\text{M}$ .

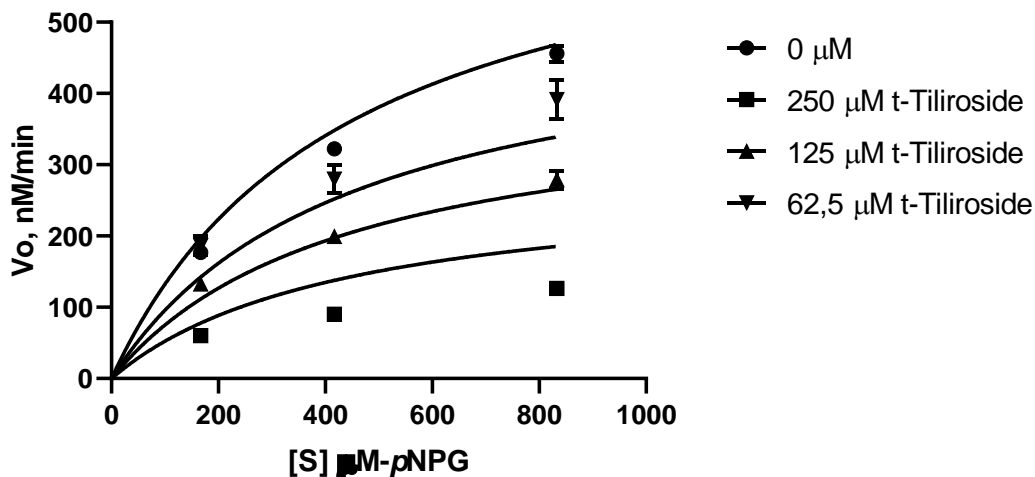
Following their identification, compounds 1-7 were assessed together with the different extracts for  $\alpha$ -glucosidase inhibition. The results indicated that the ethyl acetate extracts and two of the pure

compounds, the (3R, 20S)-3-acetoxy-20-hydroxydammar-24-ene (**2**) and the glycoside flavonoid tiliroside (**6**) showed some inhibition.

*Trans*-tiliroside exerted a non-competitive inhibition mode due to the variation in  $V_{max}$  instead of  $K_m$  values obtained from the non-linear regression analysis performed ( $Y = V_{max} * X / (K_m + X)$  model).  $V_{max}$  presented a value of 605.7 nM/min while as for the reaction inhibited by *trans*-tiliroside a decreasing value of 325.4 nM/min was obtained.  $K_m$  varied from 368.8 to 358.9 for the non-inhibited and inhibited reaction, respectively.  $V_{max}$  decreasing is presented in Figures 2 and 3 and also Lineweaver-Burk plot is illustrated to demonstrate the non-competitive mode of inhibition (Figure 4).  $K_i$  as obtained from non linear regression analysis ( $(V_{maxinh} = V_{max} / (1 + I/K_i))$ ;  $Y = V_{maxinh} * X / (K_m + X)$  model, least square fit), presented a value of  $163.6 \pm 2.65 \mu\text{M}$ .



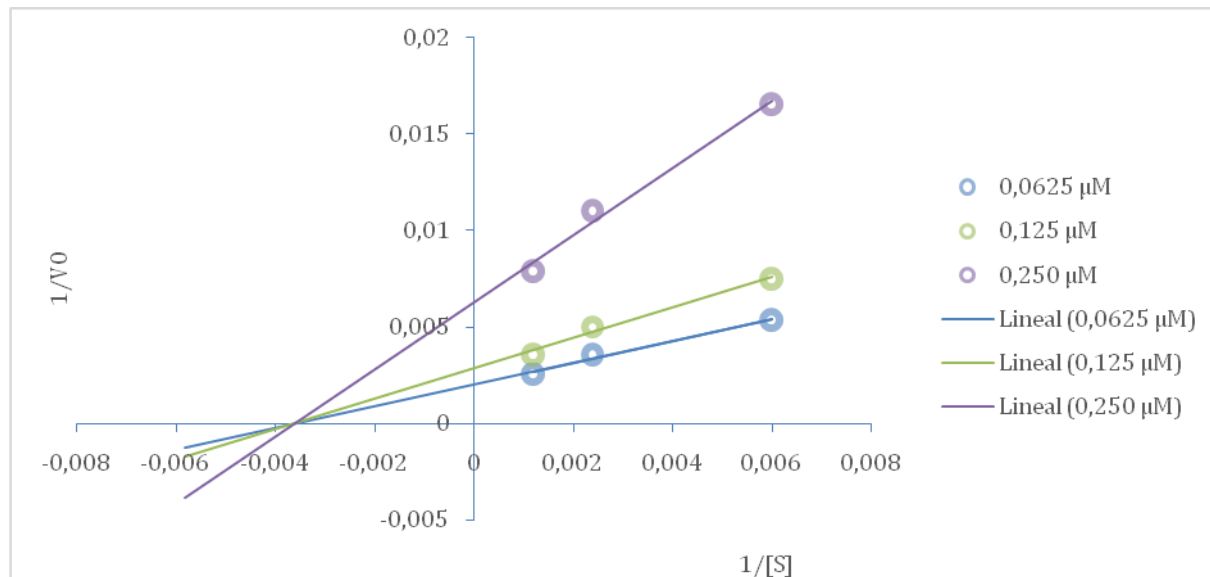
**Figure 2.** M-M plot showing variation in  $V_{max}$  for  $\alpha$ -glucosidase with a concentration equivalent to  $IC_{50}$  of *trans*-tiliroside



**Figure 3.** M-M plot showing variation in  $V_{max}$  for  $\alpha$ -glucosidase with three different concentrations of *trans*-tiliroside

The dammaranes are a kind of compounds belonging to the tetracyclic triterpenes. Steroids, saponins and triterpenes in general show a great variety of structures as well as a wide spectrum of biological activities [50]. They are found frequently in many species of different plant families, including Euphorbiaceae, to which belongs the *Croton* genus. Some of the dammarane-type triterpenes are endowed with cytotoxic[51], antiviral [52], and antifungal [53] activities. A new dammarane type

triterpene was isolated from *Castenea mollisima* showing potent  $\alpha$ -glucosidase inhibition with an  $IC_{50}$  value of  $0,1\mu M$  [54]. Dammarane-type compounds are naturally occurring triterpene saponins isolated from some species of Ginseng, and they have a variety of biological activities including anticancer and hepatoprotective effects [38,52]. To our knowledge, this is the first report for the in vitro inhibition of  $\alpha$ -glucosidase by a known dammaran triterpene type (**2**) [55], isolated from *Croton thurifer*



**Figure 4.** Lineweaver-Burk plot for three different concentrations of *trans*-tiliroside and three different concentrations of PNPG, showing the variation in  $V_{max}$  instead of  $K_m$ . Initial velocity is expressed in  $nM/min$  and substrate concentration in  $\mu M$

Additionally from the antidiabetic potential, tiliroside has been considered a dietary flavonoid because is contained in several edible plants and has demonstrated a variety of biological properties, including anticholinesterase, antiprotozoal, antioxidant, anti-inflammatory, hepatoprotective and moderate neuroprotective effect [49].

The results obtained with the glycosidic flavonoid tiliroside (**6**) are not surprising because this compound have been reported to have a wide range of pharmacological properties such as antidiabetic and antihyperlipidemic, also point out that this compound has exhibited anti-inflammatory, antirheumatic, antimicrobial, antioxidants, antiviral, cytotoxic, and anticholinesterase. Previous studies reported a good antidiabetic activity for tiliroside, via inhibition of  $\alpha$ -glucosidase, with  $IC_{50}$  values of  $28.36\mu M$  [56] and  $14.5\mu M$  [57]. The difference observed in our results could be due to the lower amount of enzyme used in assays of the previously mentioned reports. Moreover, the results reported by Silva et al. [58] show the  $IC_{50}$  value in  $\alpha$ -glucosidase for *trans*-tiliroside is above  $210\mu M$ [59], which is in agreement with our results.

The inhibitory effect of (3R, 20S)-3-acetoxy-20-hydroxydammar-24-ene (**2**) and *trans*-tiliroside (**6**) on  $\alpha$ -glucosidase is shown in Table 1, and it is comparable to and even higher than the commercial inhibitor Acarbose. Thus further studies of toxicity that employ the above-mentioned compounds as novel  $\alpha$ -glucosidase inhibitors are needed.

These results support the many uses claimed for plants belonging to this genus. It is well known that the red latex that many *Croton* species exudes is endowed with medicinal properties, including burn and wound healing, cancer, inflammation, pain, and diabetes among others. In Ecuador like in others regions of America and Africa, these plants are used frequently by people for their medicinal properties [13].



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