Oleanane- and ursane- type triterpenoids from *Eugenia grandis*

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Abstract

Arjunolic acid (an oleanane-type triterpenoid) and asiatic acid (an ursane-type triterpenoid) were obtained as an inseparable mixture from a chloroform extract of stem-bark of *Eugenia grandis* (Myrtaceae). They were characterised mainly by analysis of their spectral data. Arjunolic acid and asiatic acid are reported to have a variety of biological and pharmacological activities, which include antibacterial, anti-inflammatory, antioxidant, antidiabetic, antihyperlipidemic, cardiacprotective, antitumor, anticancer and hepatoprotective activities. Arjunolic acid has been used as a cardio-protective phytotonic in traditional Ayurvedic medicine for centuries.

Index Terms: Eugenia grandis, Eugenia, Myrtaceae, arjunolic acid, asiatic acid, pentacyclic triterpenoids, ^{13}C NMR, HMBC, HSQC-DEPT, MS

1. Introduction

The genus Eugenia consists of approximately 600 species in the tropics.[1] They are either trees or shrubs, which can yield edible fruits[1] and are often planted for ornaments in warm regions.[1] Triterpenoids have been reported as the main constituents from these species.[2-5] Examples of triterpenoids isolated from Eugenia plants include lupeol, betulinic acid, methyl arjunolate, α-amyrin , β-amyrin, methyl asiatate, methyl 2α-acetoxy-3β-hydroxyolean-12-en-28-oate, methyl 2α-acetoxy-3β-hydroxyurs-12-en-28-oate, arjunolic acid, 2α-hydroxyursolic acid, methyl maslinate (methyl 2α,3β-dihydroxyolean-12-en-28-oate), asiatic acid, methyl 2α,3β-dihydroxyurs-12-en-28-oate, oleanolic acid, epioleanolic acid, ursolic acid, epiursolic acid, crategolic acid, friedelin, erythrodiol and 3β-friedelinol.[2-5] The only steroid isolated has been β-sitosterol. *Eugenia caryophyllata*, *Eugenia mooniana*, *Eugenia aromatica*, *Eugenia biflora Jambosa caryophyllus*, *Eugenia jambolana Eugenia maire*, *Eugenia fructicosa*, *Eugenia wallichii* and *Eugenia javanica* are some examples of species belong to the Myrtaceae family.[6] Among the Eugenia species, *Eugenia caryophyllata* has been most extensively studied due to its medicinal properties and economic value.[6] Extracts from this plant have been used in treating toothache and gum diseases, dandruff control, scalp-treatment, transdermal and antitumour pharmaceuticals, food flavourings and as antioxidants for fats.[6]

Called locally as Sea apple or Jambu Laut, *Eugenia grandis* (synonym: *Syzygium grande*) is a common seashore tree but often planted along roadsides to give shade.[6] *E. grandis* grows to 30 m height and has an irregular crown.[6] The leaves are large, shiny, dark green, elliptic in shape and have a distinct down-turned tip.[6] The flowers are oblong, large, white and fluffy. Our literature search showed that this plant has not been explored well for phytochemical studies.[6] Castalagin, vescalagin and ellagitannin (1-*O*-

galloyl castalagin) have previously been isolated from the leaves of *E. grandis*.[7] Additionally, we reported the isolation and structural elucidation of a lupane- type triterpenoid *viz*. 2α,3β-dihydroxylup-12-en-28-oic acid **(1)** as a new compound from the chloroform extract of stem-bark of *E. grandis* together with six known compounds *viz*. 3β-hydroxylup-12-en-28-oic acid, fridelin, 3β-friedelinol, β-sitosterol, oleanolic acid and betulinic acid.[8] Herein, we report the isolation and identification of two pentacyclic terpenoids *viz*. arjunolic acid (**2**) (an oleanae- type terpenoid) and asiatic acid (**3**) (an ursane- type terpenoid) which were obtained as an inseparable mixture from the same chloroform extract of stem-bark of *E. grandis*. Compounds **2** and **3** are reported to have a variety of biological and pharmacological activities, which include antibacterial, anti-inflammatory, antioxidant, antidiabetic, antihyperlipidemic, cardiac-protective, antitumor, anticancer and hepato-protective activities.

2. Experimental Method

2.1 Plant materials

The plant material was collected in Singapore along Kent Ridge Road and identified by Associate Prof. Hugh Tan Tiang Wah, Dept. of Biological Sciences, NUS and Chua Keng Soon, Senior Laboratory Officer (RMBR), Herbarium, NUS. A voucher specimen (KM20041122) was deposited in the herbarium, Department of Biological Sciences, National University of Singapore, Singapore.

2.2 Extraction and Isolation

A whole plant weighing about 20 kg (wet weight) was cut. The leaves were removed using a knife and the stem-bark was chopped into small pieces. The air-dried stem-bark was then exhaustively extracted with chloroform under reflux conditions. The combined extract was chromatographed over silica gel column using hexane and eluted with the solvents of increasing polarity. Purification of eluted fractions by repeated column chromatography and/or preparative TLC afforded 2α,3β-dihydroxylup-12-en-28-oic acid (**1**), 3β-hydroxylup-12-en-28-oic acid, oleanolic acid, betulinic acid, β-sitosterol, friedelin and 3β-friedelinol.[8] All these seven compounds have previously been reported.[8] Additionally, we also obtained two pentacyclic terpenoids *viz*. arjunolic acid (**2**) and asiatic acid (**3**) as an inseparable mixture and the characterisation of this mixture is discussed in this article.

2.3 Instruments and chemicals used

Silica gel 60 (Merck, 0.063- 0.200 m) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60F 254, 0.25 mm or Baker Si250F, 0.25 mm) were used for preparative TLC and/or analytical TLC. Spots were detected using UV light or staining with iodine or by spraying with 50% H_2SO_4 followed by heating at 110˚C for 5 minutes. Lichroprep RP-18 (Merck, 40-63 μm) was used for separation and/or purification. HPLC was carried on a Waters associates, μ-Porasil (300 x 5 mm) column with a Shimadzu RID-10A, refractive index detector. ^{1}H, ^{13}C NMR and 2D NMR spectra were recorded on Bruker, 300 and/or 500 MHz spectrometers. Standard microprograms supplied by Bruker were used to run 1D and 2D NMR spectroscopy. Chemical shifts are reported in parts per million (ppm) with TMS as a reference standard or with reference to solvent peaks and coupling constants (J) expressed in hertz. LREIMS were measured on a Finnigan/MAT MAT 95 XL-T or VG Micromass 7035. HREIMS were measured on Finnigan/MAT MAT 95 XL-T mass spectrometers. IR spectra were recorded on a Bio Rad, Class II Laser product.

2.4 List of spectral data

Arjunolic acid and asiatic acid

Colourless crystals; IR (KBr) ν_{max} 3535, 3372, 2925, 2856, 1694, 1639, 1458, 1389, 1306, 1270, 1047 cm^{-1}; MS (EI, 70eV), *m/z* (rel.int. %): 488 $[M]^+$ (4), 470 (3), 452 (10), 248 (100), 203 (88), 189 (34), 133 (54), 119 (24), 69 (26), 41 (16); HREIMS m/z 488.3497 (calcd. for $C_{30}H_{48}O_5$, 488.3501); ^{1}H NMR (80% $CDCl_3$:20% CD_3OD, 300 MHz) δ 3.79, 3.79 (1H, ddd, J = 4.5, 10, 11.5 Hz, H-2; 1H, ddd, J = 4.5, 10, 11.5 Hz, H-2); 3.48, 3.48 (1H, d, J = 10 Hz, H-3; 1H, d, J = 10

Hz, H-3); 5.24, 5.28 (1H, t, J = 4 Hz, H-12; 1H, t, 4 Hz, H-12); 2.20, 2.88 (1H, d, J = 12 Hz, H-18; 1H, dd, J = 13.5, 4.0 Hz, H-18); 3.44, 3.43, 3.71, 3.70 (1H, d, J = 10.5 Hz, H_α-23; 1H, d, J = 10.5 Hz, H_β-23; 1H, d, J = 10.5 Hz, H_α-23; 1H, d, J = 10.5 Hz, H_β-23); 0.68, 0.71 (3H, s, H-24; 3H, s, H-24); 0.99, 1.00 (3H, s, H-25; 3H, s, H-25); 0.83, 0.86 (3H, s, H-26; 3H, s, H-26); 1.04, 1.09 (3H, s, H-27; 3H, s, H-27); 0.89, 0.90 (3H, s, H-29; 3H, d, H-29); 0.90, 0.91 (3H, s, H-30; 3H, d, H-30); ^{13}C NMR (80% $CDCl_3$: 20% CD_3OD, 125.7 MHz) δ 46.1, 47.1 (C-1), 66.7, 66.8 (C-2), 75.1, 75.2 (C-3), 42.7, 43.5 (C-4), 48.4, 48.4 (C-5), 18.6, 18.6 (C-6), 32.4, 33.1 (C-7), 39.4, 40.1 (C-8), 47.3, 48.5 (C-9), 38.5, 38.5 (C-10), 23.3. 23.8 (C-11), 122.6, 125.9 (C-12), 139.0, 144.6 (C-13), 42.0, 42.4 (C-14), 27.9, 28.3 (C-15), 23.9, 24.1 (C-16), 47.9, 47.9 (C-17), 43.5, 52.8 (C-18), 39.2, 46.3 (C-19), 30.8, 39.0 (C-20), 33.8, 30.6 (C-21), 30.5, 34.2 (C-22), 63.3, 63.4 (C-23), 180.1, 181.1 (C-28), 13.1, 13.2, 16.8, 17.0, 17.2, 17.3, 17.4, 23.6, 23.7, 23.7, 26.1, 33.2 (C-24, C,25, C-26, C-27, C-29, C-30).

3. Results and Discussion

Arjunolic acid **(2)** and asiatic acid **(3)** were obtained as colourless crystals. The IR spectrum gave absorption peaks at ν_{max} 3535 & 3372, 1694 and 1639 cm^{-1}, which are characteristics of hydroxyl, carboxyl and double bond respectively.[8] Both compounds have the same molecular formula, $C_{30}H_{48}O_5$, deduced from HREIMS *m/z* 488.3497. The EIMS gave a single molecular ion peak at *m/z* 488. We have confirmed this molecular ion peak with ESI and FAB mass spectra and gave $[M-1]^+$ ion at *m/z* 487. However, there was an inconsistency in the observed molecular weight and the number of ^{13}C NMR signals. In the ^{13}C NMR spectrum, we observed more than forty peaks. Some peaks displayed doublets with unequal intensities, which were not due to incomplete ^{1}H-decoupling. For example, a peak at about δ 181, which indicated the carboxyl carbon, showed two very close signals at δ 180.9 and 181.1. Further, both HMBC and HSQC-DEPT gave peaks with stacking one over another or with very close chemical shift values. Based on these observations, we understood that it was a mixture of two closely related compounds. Additionally, we also confirmed that these two compounds were pentacyclic triterpenoids since a fragment ion was observed at *m/z* 248 in the MS, which was also a base peak. A fragment ion at *m/z* 248 in the MS was a clear and unambiguous indication of the presence of pentacyclic triterpenoids (refer to ***Figures 2*** and ***3***). Other fragmentation peaks were observed at *m/z* 203, 189 and 133.[8] These are characteristics of a oleanane- or ursane- or lupane-type triterpenoids.[8, 9-11] Furthermore, the major MS fragmentation pattern of the previously reported new compound **1** was found to be *m/z* = 472, 454, 243 (base peak), 203, 189 and 133.[8] The major MS fragmentation pattern of **2** and **3** were also very similar to **1** (refer to ***Figures 2*** and ***3*****)** except a mass difference of 16 *amu*. This observation allowed us to place an extra hydroxyl group in the mixture of pentacyclic terpenoids relative to compound **1**. As expected, on inspection of its chemical shift values in ^{13}C and DEPT spectra at the hydroxyl region, three oxygenated carbons were observed, one at δ 75.2 another at δ 66.8, both attached to methine carbons with the third one at δ 63.3 attached to a methylene carbon. However, each of the three peaks displayed two peaks with a negligibly small chemical shift difference. We have placed one hydroxyl group at C-2 and another group at C-3 based on the spectral correlations obtained in the previously reported compound **1**. According to the number of different types of carbons, the third hydroxyl group, as it attached to methylene carbon, should replace any one of methyl hydrogens, so that it will form $–CH_2OH$ group. It is impossible to use all other positions which will make the carbon either methine or quaternary.

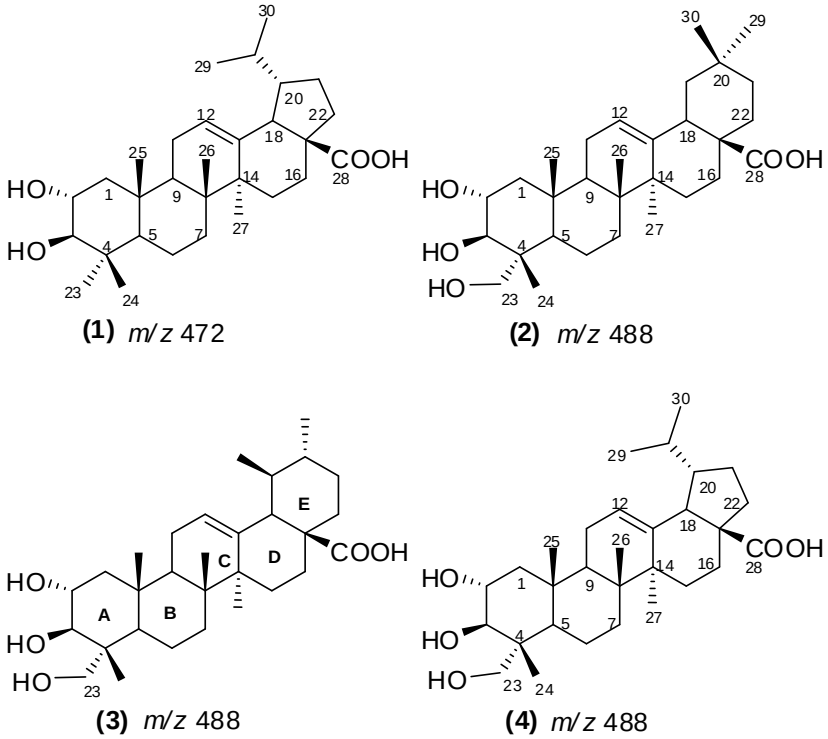

Figure 1. Structures of compounds **1**, **2**, **3** and **4**.

As mentioned previously, EIMS gave a base peak at *m/z* 248 and other fragment peaks at *m/z* 203, 189 and 133. These are indicative of lupene or oleanene or ursene type triterpenoids with a double bond between C-12 and C-13 and a carboxyl group at C-17 position.[8] This observation allowed us to exclude the presence of a hydroxyl group in rings C, D and E. Any changes in any one of the position in these rings will not follow this fragmentation pattern. In other words, it is impossible to utilise the methyl group present in these rings for the formation of $–CH_2OH$. The only possibility is to utilise one of the methyl groups present in ring A or B i.e. C-23, C-24, C-25 and C-26. Inspection of its HMBC revealed that the single proton at C-3 position correlates with an oxygenated methylene carbon at C-23 position. This allowed us to exclude the possibility of C-24, C-25 and C-26 positions. This is consistent with previous reports that NMR signals of methyl groups of pentacyclic triterpenoids will have oxygen functions at 2, 3 and 23 positions.[12] For this interpretation, three structures are possible viz. 2α,3β,23-trihydroxyolean-12-en-28-oic acid (arjunolic acid, **2**, an oleanane-type triterpenoid), 2α,3β,23-trihydroxyurs-12-en-28-oic acid (asiatic acid, **3**, an ursane-type triterpenoid) and 2α,3β,23-trihydroxylup-12-en-28-oic acid **(4,** a lupane type- triterpenoid**)** (refer to ***Figure 1***).

We were unable to get the two possible structures based on their 2D NMR spectral correlations, due to their extreme complexity, particularly at the high-field region. Fortunately, analysis of its ^{13}C NMR or DEPT spectrum in the olefinic region showed that peaks were not stacked one over another; we observed four independent peaks with reasonably good chemical shift difference. Additionally, it was also observed that two of them were methine and the other two were quaternary carbons. These peaks were due to C-12 and C-13 positions. The chemical shift values of one of the methine and a quaternary carbon were observed at δ 122.6 and 144.6, respectively. These values were in very good agreement with chemical shift values of arjunolic acid (**2**) at its C-12 and C-13 positions.[13-17] Similarly the chemical shift values of another methine and a quaternary carbon were observed at δ 125.9 and δ 139.0, respectively. These values were in very good agreement with chemical shift values of

asiatic acid (**3**) at its C-12 and C-13 positions.[13-17] As stated previously that the ^{13}C NMR and HMBC spectra were very complex at the high field region; we were unable to get the chemical shift values for the individual compounds. Thus, we tentatively assigned that the mixture of two compounds were arjunolic acid (**2**) and asiatic acid (**3**). Our literature search indicated that reports of a mixture containing two compounds are very common, particularly in the case of the pentacyclic triterpenoids.[18,19] Our literature search also revealed that the existence of the compound, 2α,3β,23-trihydroxylup-12-en-28-oic acid (**4**) has not been reported so far. Further, oleanane- and ursane-type compounds have previously been reported as a mixture[18,19] rather than lupane & oleanane or lupane & ursane types. The NMR chemical shift values for the individual compounds and/or their methyl esters are available in the literature.[13-17]

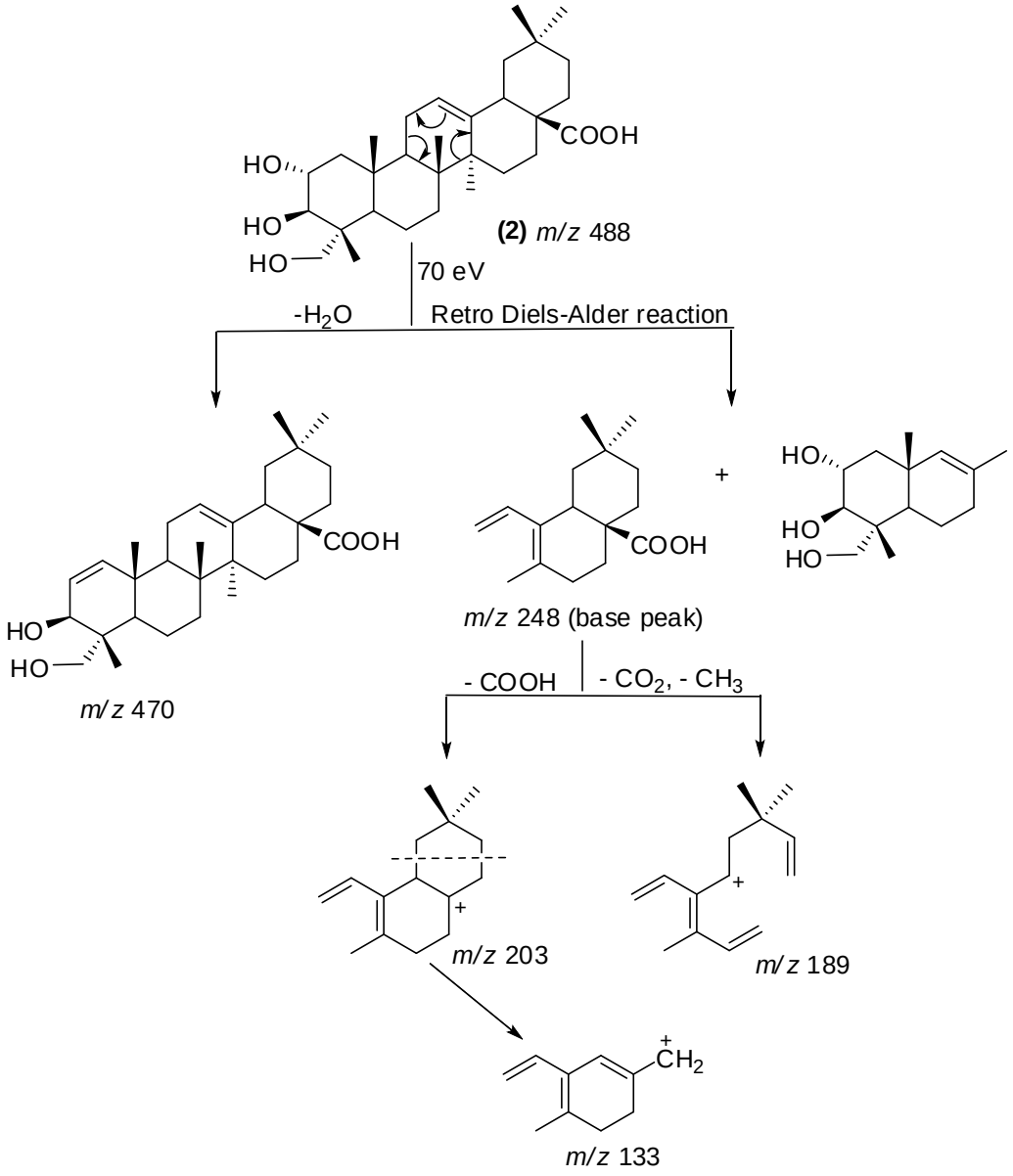

Figure 2. Proposed major MS fragmentation pattern of arjunolic acid (**2**).

In general, pentacyclic triterpenoids are reported to have a wide range of biological activities.[20] Arjunolic acid (**2**) has been used as a cardio-protective tonic in traditional Indian medicine for

centuries.[21] Compound **2** is reported to have many beneficial effects to humans such as lowering of blood pressure, cholesterol levels and heart rate.[21] Compound **2** protects against myocardial necrosis, platelet coagulation and aggregation.[21] Compound **2** protects the cells from metal induced toxicity.[21] Compound **2** possesses antidiabetic, anti-inflammatory, antitumor and antimicrobial activity.[21] Compound **2** also serves as a potent free radical scavenger and antioxidant.[22] Compound **2** and its semisynthetic derivatives were shown to exhibit inhibitory effects on Epstein-Barr virus (EBV) activation in Raji cells.[6] Arjunolic acid derivatives could be valuable compounds as antitumour-promoters.[6] Their inhibitory effects on skin tumour promoters were greater than those of previously studied natural products.[23,24] Compound **2** decrease Ehrlich Ascites Carcinoma cell viability and increases cell toxicity in experimental animals. Compound **2** reduced cell count and tumour volume.[25] Overall, **2** is regarded as a phytochemical with multifunctional therapeutic applications.[21,26]

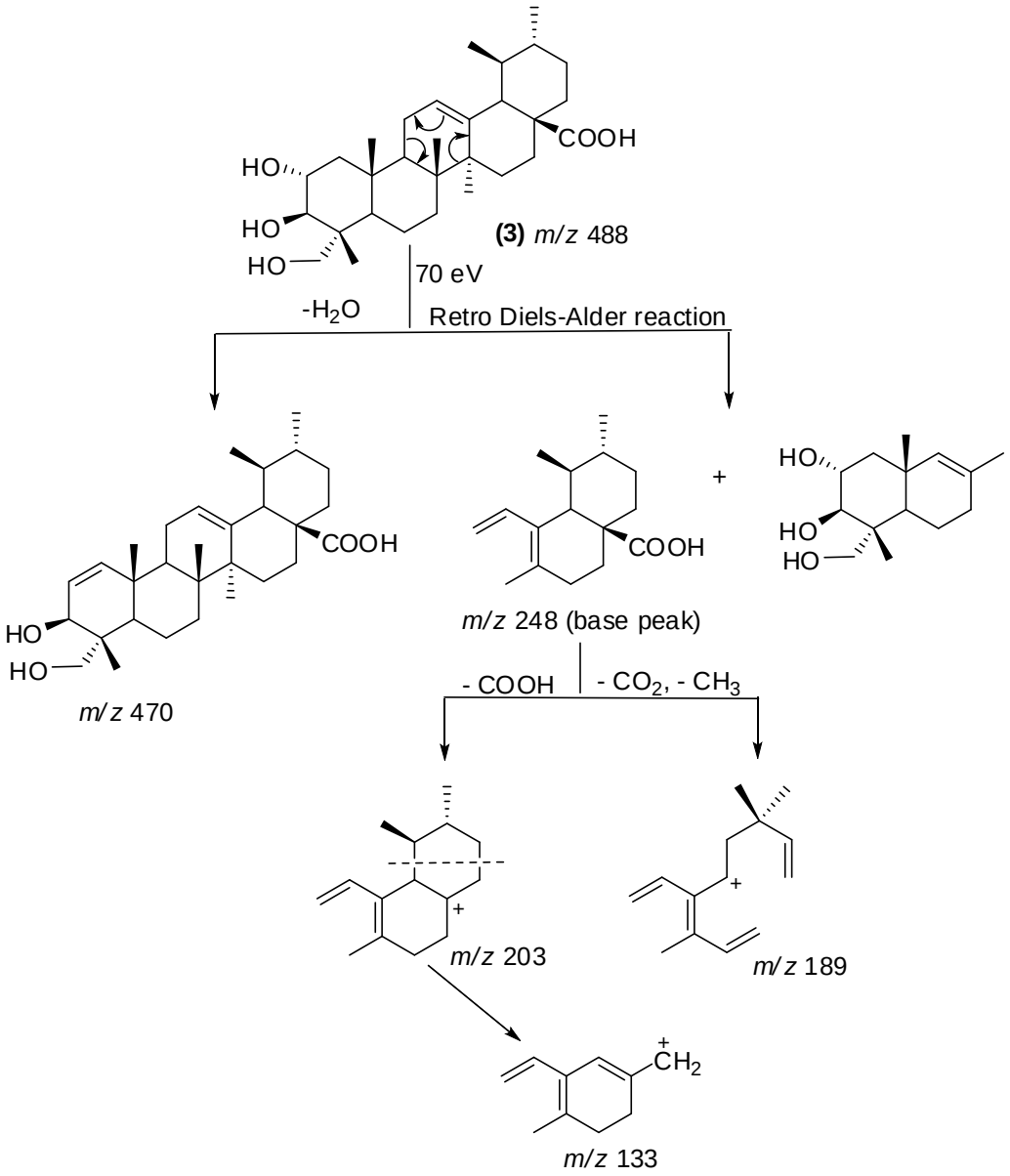

Figure 3. Proposed major MS fragmentation pattern of asiatic acid (**3**).

Asiatic acid (**3**) also exhibits a wide variety of biological and pharmacological activities, which include antioxidant, anti-inflammatory and hepatoprotective activities.[27-29] Compound **3** showed antidiabetic and antihyperlipidemic activities in experimental animals.[30-32] The antihyperlipidemic activity of **3** was found to be comparable to glibenclamide, a well-known antihyperglycemic prescription drug. The anticancer effect of asiatic acid in two human breast cancer cell lines, MCF7 and MDA-MB-231 has previously been reported.[6] Compound **3** exhibited effective cell growth inhibition by inducing cancer cells to undergo S-G2/M phase arrest and apoptosis.[33] Compound **3** decreased viability and induced apoptosis in SK-MEL-2 (Human melanoma cells) and HepG2 (Human hepatoma cells) in a time- and dose dependent manner.[34,35] Compound **3** dose-dependently showed cytotoxicity in HT29 (Human colon adenocarcinoma cell lines). The structural relationships of **3** and its derivatives to cytotoxicity and antihepatofibrotic activity in HSC-T6 cells have been reported. Modification of the carboxylic acid group at C28 also reduced the cytotoxicity in HSC-T6 cancer cell lines.[36]

4. Conclusion

Arjunolic acid (**2)** and asiatic acid (**3)** were obtained as an inseparable mixture from the chloroform extract of stem-bark of *Eugenia grandis*. They were characterised mainly by analysis of their IR, NMR and MS spectral data. Compounds **2** and **3** exhibit a variety biological and pharmacological activities. Additionally, we proposed the possibility of the existence of compound **4** in the mixture based on rational analysis. Interestingly, our literature search showed that **4** has never been reported not only from the natural product kingdom but also by synthesis. Therefore, **4** could be a potential synthetic target molecule. Due to its structural similarity to **2** and **3**, it is expected that **4** could potentially exhibit a variety of biological and pharmacological activities in line with **2** and **3**. Species from the genus, Eugenia reported to have many therapeutic applications. Further studies on *E. grandis* is required to explore this plant for its therapeutic applications.

Conflict of interest

The authors have no conflicts of interest to declare.

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