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Identification of Daphnane Diterpenoids from *Wikstroemia indica* Using Liquid Chromatography with Tandem Mass Spectrometry

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Abstract: Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has emerged as a powerful tool for the rapid identification of compounds within natural resources. Daphnane diterpenoids, a class of natural compounds predominantly found in plants belonging to the Thymelaeaceae and Euphorbiaceae families, have attracted much attention due to their remarkable anticancer and anti-HIV activities. In the present study, the presence of daphnane diterpenoids in *Wikstroemia indica*, a plant belonging to the Thymelaeaceae family, was investigated by LC-MS/MS analysis. As a result, 21 daphnane diterpenoids (**1**–**21**) in the stems of *W. indica* were detected. Among these, six major compounds (**12**, **15**, **17**, **18**, **20**, and **21**) were isolated and their structures were unequivocally identified through a comprehensive analysis of the MS and NMR data. For the minor compounds (**1**–**11**, **13**, **14**, **16**, and **19**), their structures were elucidated by in-depth MS/MS fragmentation analysis. This study represents the first disclosure of structurally diverse daphnane diterpenoids in *W. indica*, significantly contributing to our understanding of bioactive diterpenoids in plants within the Thymelaeaceae family.

Keywords: daphnane diterpenoids; LC-MS/MS; *Wikstroemia indica*; MS/MS fragmentation

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1. Introduction

Liquid chromatography coupled with high-resolution tandem mass spectrometry (LC-HR-MS/MS), usually equipped with an electrospray ionization source, has high adaptability across a broad spectrum of compounds, offering high mass accuracy and sensitivity. Moreover, it provides information-rich fragmentation through product ion spectra, thereby potentially revealing details about the molecular formula and structure of diverse secondary metabolites found in plants [1]. The conventional phytochemical research process often necessitates substantial amounts of accessible plant materials and time-consuming purification procedures, whereas applying LC-MS/MS analysis on crude plant extracts at the early stage of phytochemical investigations allows for the rapid identification of the compounds [2,3].

Daphnane diterpenoids, characterized by their *trans*-fused 5/7/6-tricyclic skeleton, have garnered attention for their diverse biological activities, including anticancer [4], anti-HIV [5], analgesic [6], anti-inflammatory [7], and neurotrophic activities [8,9]. These diterpenoids are predominantly found in plants of the Thymelaeaceae and Euphorbiaceae families, with the majority of them sourced from the Thymelaeaceae family [10]. Previous phytochemical investigations on plants of the Thymelaeaceae family have reported

the isolation of daphnane diterpenoids from 16 genera, such as *Daphne*, *Pimelea*, *Stellera*, and *Wikstroemia*. Among these, the *Wikstroemia* genus, comprising over 70 species, holds significant potential as a source of daphnane diterpenoids. Isolation of daphnane diterpenoids has hitherto been reported from a number of species, including *W. monticola* [11], *W. mekongenia* [12], *W. retusa* [13,14], *W. polyantha* [15], *W. chamaedaphne* [16–18], *W. chuii* [19], and *W. ligustrina* [20]. It is evident that the *Wikstroemia* genus remains relatively underexplored in the research into daphnane diterpenoids.

Wikstroemia indica (L.) C. A. Mey. is a semi-evergreen shrub mainly distributed in southeastern China, which has long been used as a traditional Chinese medicine for the treatment of bronchitis, hepatitis, and cancer [21]. Recent studies have revealed that the extract of this plant exhibited antiallergic [22], anti-inflammatory [23], and antineoplastic properties [24], therefore heightening interest in its pharmacological exploration. While previous phytochemical investigations of *W. indica* have yielded coumarins [25], flavonoids [26], lignans [27], and sesquiterpenoids [28], the presence of daphnane diterpenoids has yet to be documented.

During our ongoing research aimed at discovering biological diterpenoids from plants of the Thymelaeaceae family [5,20,29,30], this study comprehensively examined and identified daphnane diterpenoids in the stems of *W. indica* using LC-MS/MS analysis.

2. Results and Discussion

2.1. Detection of Daphnane Diterpenoids in W. indica by LC-MS/MS

Due to the limited availability of plant material, the presence of daphnane diterpenoids in *W. indica* was initially examined by LC-MS/MS analysis. The criteria for validating that the detected peaks represented daphnane diterpenoids were established based on a synthesis of our previous studies and literature review [20,31,32]. These criteria included: (1) In the mass spectra, protonated molecular ions ($[M + H]^+$) and/or ammonium adduct ions ($[M + NH_4]^+$) were observed in positive ion mode, while deprotonated molecular ions ($[M–H]^-$) and/or formate adduct ions ($[M + HCOO]^-$) were observed in negative ion mode. (2) In the product ion spectrum obtained from the protonated molecular ion as a precursor ion, a diagnostic ion at m/z 253 ($C_{17}H_{17}O_2$) or 269 ($C_{17}H_{17}O_3$) was observed in the positive ion mode [31]. (3) The characteristic C_{17} product ions derived from C_{20} skeletons with the neutral loss of $C_3H_4O_2$ were observed [32]. (4) When the ion peaks originated from a macrocyclic daphnane orthoester (MDO), the second and third criteria were not applicable. Instead, product ion peaks derived from continuous losses of H_2O and CO were observed at the mass range of m/z 250–350 and m/z 400–550, respectively [20].

To enhance the detecting sensitivity, a crude diterpenoid fraction was prepared from the 95% EtOH extract using a sequence of procedures, including EtOAc–H_2O partition and Diaion HP-20 column chromatography. Subsequent LC-MS/MS analysis of the crude diterpenoid fraction, guided by the aforementioned criteria, resulted in the detection of three major daphnane diterpenoid peaks (**15**, **20**, and **21**), strongly suggesting the occurrence of daphnane diterpenoids in *W. indica* stems. It was noteworthy that detecting daphnane diterpenoids can be challenging due to their chromatographic behavior, which was sometimes similar to common plant constituents, such as fatty acids, acylglycerols, and chlorophyll [33].

To further enhance the sensitivity of LC-MS/MS detection of daphnane diterpenoids, a portion of the crude diterpenoid fraction underwent additional fractionation through gradient HPLC. As a result, a total of 21 daphnane diterpenoid peaks (**1**–**21**) were detected from three out of twelve subfractions (Figure S1). Importantly, all these peaks were subsequently confirmed to be present in the crude diterpenoid fraction through extracted ion chromatogram (XIC) analysis (Figure 1, Table 1).

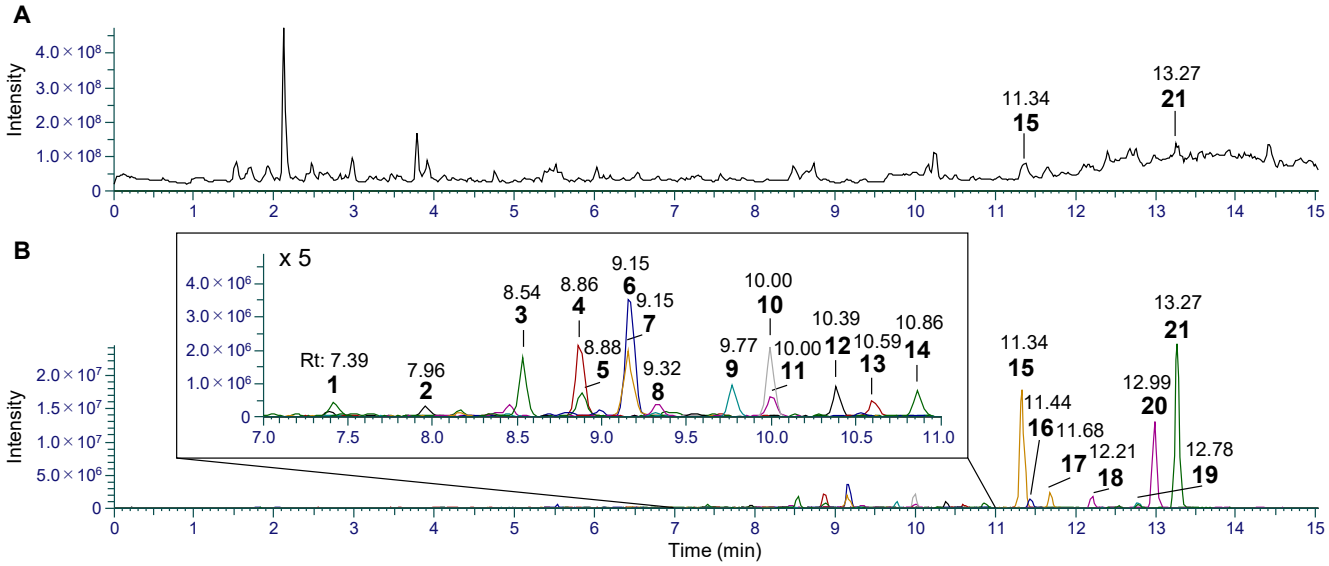

Figure 1. (**A**) Total ion chromatogram in the positive ion mode and (**B**) extracted ion chromatogram from the crude diterpenoid fraction of the stems of *W. indica*.

Table 1. Daphnane diterpenoids **1**–**21** identified from the stems of *W. indica*.

No.	Rt (min)	Molecular Formula	$[M + H]^+$ (*m*/*z*)		ESI-MS/MS (*m*/*z*) (%) [c]	Identification
			Detected Mass (*m*/*z*)	Error (ppm)		
1 [a]	7.39	$C_{55}H_{62}O_{18}$	1011.3984	−2.97	793 (36), 775 (22), 765 (19), 747 (20), 731 (34), 703 (23), 689 (16), 671 (47), 659 (12), 653 (17), 643 (17), 625 (24), 609 (18), 567 (24), 549 (100), 531 (15), 521 (39), 507 (36), 503 (37), 493 (10), 489 (66), 479 (18), 477 (18), 471 (37), 461 (68), 459 (23), 443 (80), 433 (24), 431 (25), 425 (23), 415 (31), 403 (27), 397 (19), 375 (22), 363 (18), 339 (23), 325 (16), 307 (37), 295 (22), 291 (21), 279 (37), 263 (22), 221 (21), 183 (18), 181 (47), 153 (16), 141 (25), 105 (65)	daphneodorin C [29]
2 [a]	7.96	$C_{55}H_{62}O_{18}$	1011.3984	−2.43	793 (52), 775 (30), 765 (19), 747 (19), 731 (22), 707 (16), 689 (19), 671 (57), 653 (23), 643 (20), 629 (21), 625 (23), 611 (28), 593 (18), 583 (17), 549 (72), 531 (17), 521 (22), 507 (29), 503 (20), 489 (73), 479 (22), 471 (54), 461 (68), 453 (22), 443 (56), 425 (18), 415 (19), 375 (15), 363 (29), 307 (37), 291 (26), 279 (16), 221 (25), 181 (47), 163 (41), 141 (22), 105 (100)	daphneodorin B [29]
3 [a]	8.54	$C_{34}H_{50}O_9$	603.3527	0.36	585 (29), 361 (10), 343 (23), 325 (20), 315 (6), 313 (5), 307 (10), 297 (16), 295 (5), 279 (11), 271 (7), 269 (6), 267 (8), 253 (15), 207 (100), 203 (5), 107 (8), 95 (8), 81 (6)	wikstroelide M [14]

Table 1. *Cont.*

No.	Rt (min)	Molecular Formula	$[M + H]^+$ (*m*/*z*)		ESI-MS/MS (*m*/*z*) (%) [c]	Identification
			Detected Mass (*m*/*z*)	Error (ppm)		
4 [a]	8.86	$C_{30}H_{42}O_9$	547.2888	−2.45	529 (35), 511 (30), 501 (32), 493 (27), 483 (59), 467 (29), 465 (64), 449 (21), 447 (29), 439 (25), 437 (40), 423 (23), 421 (25), 419 (29), 405 (28), 395 (20), 341 (27), 323 (24), 295 (26), 283 (29), 255 (29), 239 (21), 236 (23), 235 (100), 233 (47), 227 (22), 215 (36), 203 (28), 199 (21), 193 (24), 187 (27), 161 (29), 135 (36), 133 (33)	pimelotide C [34]
5	8.88	$C_{37}H_{50}O_{10}$	655.3469	−1.24	619 (10), 515 (28), 497 (79), 479 (92), 469 (38), 467 (14), 461 (21), 451 (100), 443 (16), 439 (11), 433 (71), 423 (24), 421 (12), 415 (20), 405 (27), 367 (12), 311 (19), 293 (45), 275 (16), 265 (31), 263 (10), 251 (14), 247 (10), 225 (10), 211 (16), 133 (18), 123 (11), 105 (77)	kraussianin [35]
6 [a]	9.15	$C_{40}H_{46}O_{12}$	719.3062	−1.33	507 (3), 489 (4), 359 (4), 341 (12), 323 (18), 311 (3), 305 (4), 295 (12), 277 (4), 269 (8), 177 (75), 149 (95), 121 (11), 107 (100), 81(3)	acutilobin C [36]
7 [a]	9.15	$C_{30}H_{44}O_8$	533.3104	−1.00	515 (36), 497 (66), 479 (100), 469 (28), 467 (16), 461 (66), 451 (53), 449 (25), 443 (20), 433 (59),425 (24), 423 (13), 421 (16), 415(21), 407 (14), 405 (21), 403 (16), 309 (14), 291 (12), 281 (13), 263 (12), 211 (13), 187 (19), 185 (16), 159 (13), 135 (13), 133 (27)	pimelea factor S_6 [37]
8 [a]	9.32	$C_{44}H_{54}O_{12}$	775.3690	0.20	635 (15), 617 (21), 599 (22), 545 (63), 527 (33), 495 (47), 477 (85), 459 (100), 449 (21), 447 (28), 441 (37), 431 (50), 429 (20), 423 (90), 419 (18), 413 (29), 405 (24), 401 (23), 319 (18), 309 (24), 291 (24), 281 (18), 279 (23), 263 (19), 251 (18), 151 (27), 105 (88)	stelleralide H [38]
9 [a]	9.77	$C_{39}H_{46}O_{11}$	691.3133	−2.63	509 (4), 505 (2), 491 (5), 359 (7), 341 (20), 323 (21), 313 (4), 311 (3), 305 (3), 297 (2), 295 (13), 277 (4), 269 (9), 267 (3), 261 (3), 151 (100), 147 (75), 133 (3)	daphneodorin D [29]
10 [a]	10.00	$C_{40}H_{48}O_{12}$	721.3203	−1.60	509 (4), 491 (7), 359 (6), 341 (22), 323 (23), 313 (3), 311 (3), 305 (5), 295 (16), 277 (4), 269 (11), 267 (4), 261 (3), 177 (95), 151 (100), 95 (6), 81 (6)	acutilobin D [36]
11 [a]	10.00	$C_{44}H_{54}O_{12}$	775.3687	−0.09	563 (28), 545 (100), 513 (35), 495 (60), 477 (41), 467 (45), 465 (24), 449 (78), 441 (16), 437 (35), 431 (34), 425 (20), 423 (20), 421 (24), 419 (19), 391 (18), 309 (17), 291 (19), 263 (19), 105 (40)	gnidimacrin [39]
12 [b]	10.39	$C_{51}H_{58}O_{14}$	912.4147	−1.98	773 (48), 651 (47), 633 (58), 615 (34), 543 (100), 511 (57), 493 (82), 481 (20), 475 (58), 465 (54), 463 (36), 447 (90), 435 (43), 429 (38), 421 (47), 419 (21), 417 (22), 327 (23), 309 (40), 291 (26), 279 (22), 105 (55)	stelleralide G [38]

Table 1. *Cont.*

No.	Rt (min)	Molecular Formula	$[M + H]^+$ (*m*/*z*)		ESI-MS/MS (*m*/*z*) (%) [c]	Identification
			Detected Mass (*m*/*z*)	Error (ppm)		
13 [a]	10.59	$C_{37}H_{46}O_{11}$	667.3107	−0.93	545 (100), 527 (66), 509 (59), 499 (24), 491 (37), 483 (26), 481 (52), 465 (55), 463 (37), 453 (23), 447 (28), 445 (31), 435 (35), 419 (21), 417 (21), 357 (23), 321 (27), 295 (22), 235 (96), 231 (61), 203 (54), 185 (27), 173 (20), 153 (21), 105 (45)	stelleralide C [5]
14 [a]	10.86	$C_{39}H_{44}O_{10}$	673.2999	−1.27	359 (1), 341 (3), 323 (4), 295 (3), 277 (1), 269 (2), 149 (100), 131 (6), 107 (21)	12-*O*-(*E*)-cinnamoyl-9,13,14-ortho-(2*E*,4*E*,6*E*)-decatrienylidyne-5*β*,12*β*-dihydroxyresiniferonol-6*α*,7*α*-oxide [40]
15 [b]	11.34	$C_{30}H_{44}O_8$	533.3107	−0.23	361 (9), 343 (24), 325 (49), 307 (35), 297 (28), 279 (30), 267 (58), 253 (100), 203 (16), 155 (6)	simplexin [41]
16	11.44	$C_{32}H_{44}O_8$	557.3109	−0.08	361 (8), 343 (23), 325 (33), 313 (7), 307 (30), 297 (19), 295 (11), 285 (6), 279 (20), 277 (6), 267 (55), 261 (6), 253 (77), 251 (7), 249 (11), 225 (6), 179 (100)	wikstrotoxin B [11]
17 [b]	11.68	$C_{39}H_{46}O_{10}$	675.3151	−1.85	675 (9), 509 (2), 507 (2), 491 (2), 359 (5), 341 (15), 323 (21), 313 (4), 311 (3), 305 (4), 295 (13), 277 (5), 269 (11), 267 (3), 265 (2), 261 (2), 249 (2), 241 (2), 237 (2), 209 (2), 151 (100), 133 (4)	12-*O*-(*E*)-cinnamoyl-9,13,14-ortho-(2*E*,4*E*)-decadienylidyne-5*β*,12*β*-dihydroxyresiniferonol-6*α*,7*α*-oxide [40]
18 [a]	12.21	$C_{37}H_{50}O_9$	639.3527	−0.10	621 (12), 499 (30), 481 (93), 463 (100), 453 (32), 445 (35), 436 (23), 435 (57), 417 (34), 407 (15), 311 (5), 293 (16), 265 (23), 105 (67)	wikstromacrin [20]
19 [a]	12.78	$C_{36}H_{50}O_{10}$	643.3458	−2.97	365 (2), 359 (4), 341 (11), 323 (11), 313 (3), 305 (2), 295 (10), 277 (4), 269 (6), 267 (3), 207 (100), 189 (3), 107 (3)	wikstroelide A [13]
20 [b]	12.99	$C_{37}H_{50}O_9$	639.3525	−0.28	621 (16), 499 (28), 481 (73), 463 (100), 453 (31), 451 (12), 445 (55), 435 (55), 433 (14), 423 (12), 417 (34), 409 (12), 407 (10), 405 (16), 311 (13), 293 (24), 275 (12), 265 (24), 251 (12), 133 (12), 105 (99)	pimelea factor P_2 [41]
21 [b]	13.27	$C_{34}H_{48}O_8$	585.3409	−2.14	361 (14), 343 (20), 325 (41), 313 (10), 307 (25), 297 (23), 295 (13), 279 (25), 267 (59), 253 (99), 249 (16), 207 (100)	huratoxin [42]

[a] Identifications were confirmed with the daphnane diterpenoids isolated in our previous studies. [b] Isolated daphnane diterpenoids in this study. [c] ESI-MS/MS of $[M + H]^+$ ion for peaks **1**–**11** and **13**–**21** and ESI-MS/MS of $[M + NH_4]^+$ ion for peak **12**.

2.2. LC-MS Guided Isolation and Structural Determination of Major Daphnane Diterpenoids

An LC-MS guided isolation was carried out to obtain daphnane diterpenoids. The crude diterpenoid fraction was subjected to ODS column chromatography and eluted with a stepwise gradient of MeOH–H_2O. The fractions, in which daphnane diterpenoids were detected by LC-MS/MS analysis, were subjected to silica gel column chromatography and

eluted with a gradient of *n*-hexane–EtOAc–MeOH–HCOOH. Those fractions containing daphnane diterpenoids were further purified by preparative HPLC and resulted in the isolation of six major daphnane diterpenoids (**12**, **15**, **17**, **18**, **20**, and **21**) (Figure 2).

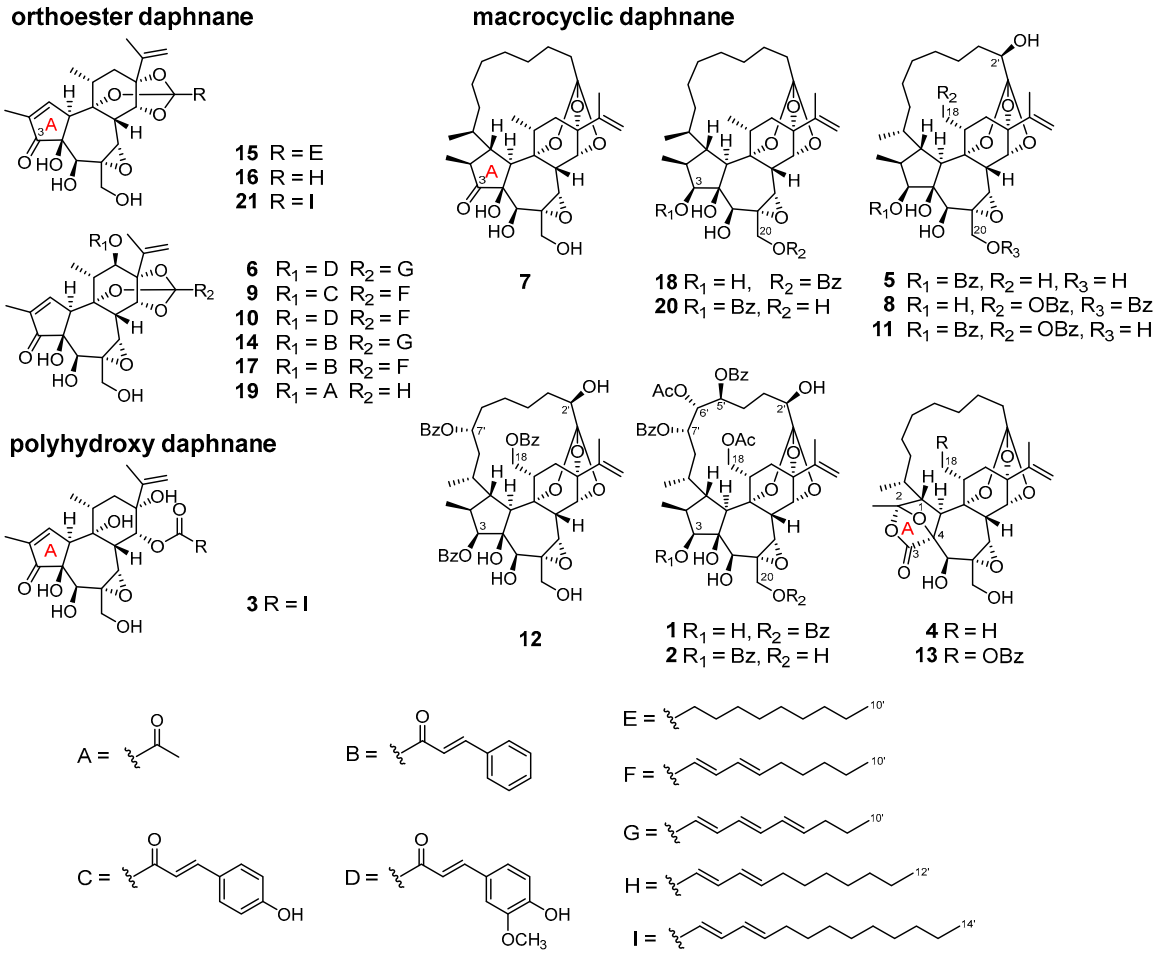

Figure 2. Structures of daphnane diterpenoids **1**–**21**.

The isolated compounds were identified by detailed NMR and MS spectroscopic analyses. In the ^{1}H- and ^{13}C-NMR spectra, the characteristic resonances for an isopropenyl moiety at δ_H 4.83–4.99 (H_a-16), 4.94–5.17 (H_b-16), and 1.72–1.84 (H_3-17), an epoxy group at δ_H 3.32–3.57 (H-7), δ_C 60.0–61.0 (C-6), and 63.5–64.4 (C-7), and an orthoester group at 116.5–119.8 (C-1′), indicated that they were daphnane diterpenoids (Tables S1 and S2) [10].

For compounds **15**, **17**, and **21**, the characteristic resonances of *α*,*β*-unsaturated carbonyl moiety at δ_H 7.56–7.61 (H-1), δ_C 160.5–161.4 (C-1), 136.6–136.9 (C-2), and 209.5–209.9 (C-3) indicated they belong to orthoester daphnane diterpenoids (Table S1). The presence of a decanoate moiety at C-1′ in **15** was deduced from the proton resonance for the aliphatic methylene multiplets at δ_H 1.23–1.93 and a terminal methyl triplet at δ_H 0.86 (H_3-10′). The 2*E*,4*E*-tetradecadienylidyne moiety of **21** was defined from the proton resonances for a conjugated diene at δ_H 5.89 (d, *J* = 15.5 Hz, H-2′), 6.68 (dd, *J* = 15.5, 10.6 Hz, H-3′), 6.04 (dd, *J* = 15.2, 10.6 Hz, H-4′), and 5.83 (dt, *J* = 15.2, 7.2 Hz, H-5′), and a *n*-nonyl moiety including eight methylenes at δ_H 1.24–2.07 and a terminal methyl group at δ_H 0.86 (t, *J* = 6.9 Hz, H_3-14′). The presence of cinnamoyloxy moiety of **17** was deduced from a *trans*-olefinic moiety at δ_H 6.35 (d, *J* = 15.9 Hz, H-2″) and 7.62 (d, *J* = 15.9 Hz, H-3″), and a phenyl moiety at δ_H 7.37–7.51 (each multiplet, H-5″ to H-9″), as well as the carbon resonance for an ester carbonyl at δ_C 165.8 (C-1″), and a 2*E*,4*E*-decadienylidyne moiety was confirmed by the proton resonances at δ_H 5.65 (d, *J* = 15.4 Hz, H-2′), 6.66 (dd, *J* = 15.4, 10.6 Hz, H-3′), 6.04 (dd, *J* = 15.1, 10.6 Hz, H-4′), and 5.85 (dt, *J* = 15.1, 7.0 Hz, H-5′), and a

n-pentyl moiety including four methylenes at δ_H 2.08 (H-6′), 1.37 (H-7′), and 1.27 (H-8′, 9′), and a terminal methyl group at δ_H 0.87 (t, J = 6.8 Hz, H_3-10′). The location of the cinnamoyl moiety at C-12 was confirmed by the HMBC correlation from H-12 to C-1″. Thus, **15**, **17**, and **21** were determined as simplexin (**15**) [41], 12-*O*-(*E*)-cinnamoyl-9,13,14-ortho-(2*E*,4*E*)-decadienylidyne-5*β*,12*β*-dihydroxyresiniferonol-6*α*,7*α*-oxide (**17**) [40], and huratoxin (**21**) [42].

On the other hand, compounds **12**, **18**, and **20** belong to MDOs since the resonances of H-1 were observed as the methine proton at δ_H 2.11–2.97 and the methyl proton resonances at δ_H 0.79–1.26 of H_3-10′ were observed as doublet (Table S2) [10]. The presence of benzoyl moieties of **12**, **18**, and **20** were determined by the aromatic proton resonances at δ_H 8.02–8.16 (H-2′,6′), 7.35–7.47 (H-3′,5′), and 7.53–7.59 (H-4′). The locations of benzoyl moieties at C-3, C-18, and C-7′ of **12**, C-20 of **18**, and C-3 of **20** were confirmed by the HMBC experiment. Thus, **12**, **18**, and **20** were determined as stelleralide G (**12**) [38], wikstromacrin (**18**) [20], and pimelea factor P_2 (**20**) [41].

2.3. Identification of Minor Daphnane Diterpenoids by MS/MS Fragmentation Elucidation

To identify the minor daphnane diterpenoids (**1**–**11**, **13**, **14**, **16**, and **19**), which could not be isolated, MS/MS fragmentation elucidation was performed. These daphnane diterpenoids exhibited abundant ions in the product ion spectra derived from the protonated molecular ion as a precursor ion. Consequently, a detailed interpretation of the MS/MS fragmentation pathways in positive mode for these peaks was conducted (Figures 3 and 4). The identification of those peaks was confirmed by the LC-MS data which were in full accordance with the corresponding compounds isolated in our previous studies (Table 1) [5,29,30].

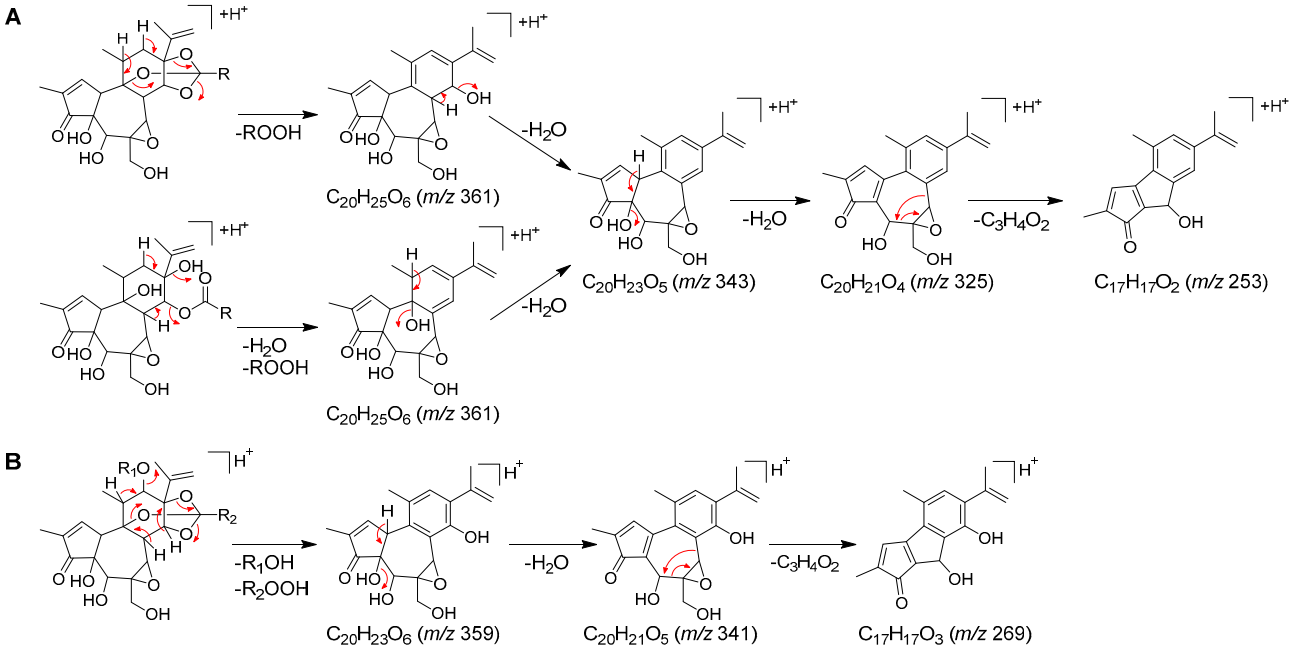

Figure 3. Proposed ESI-MS/MS fragmentation pathways for minor daphnane diterpenoids in positive mode. (**A**) peaks **3** and **16**, and (**B**) peaks **6**, **9**, **10**, **14**, and **19**.

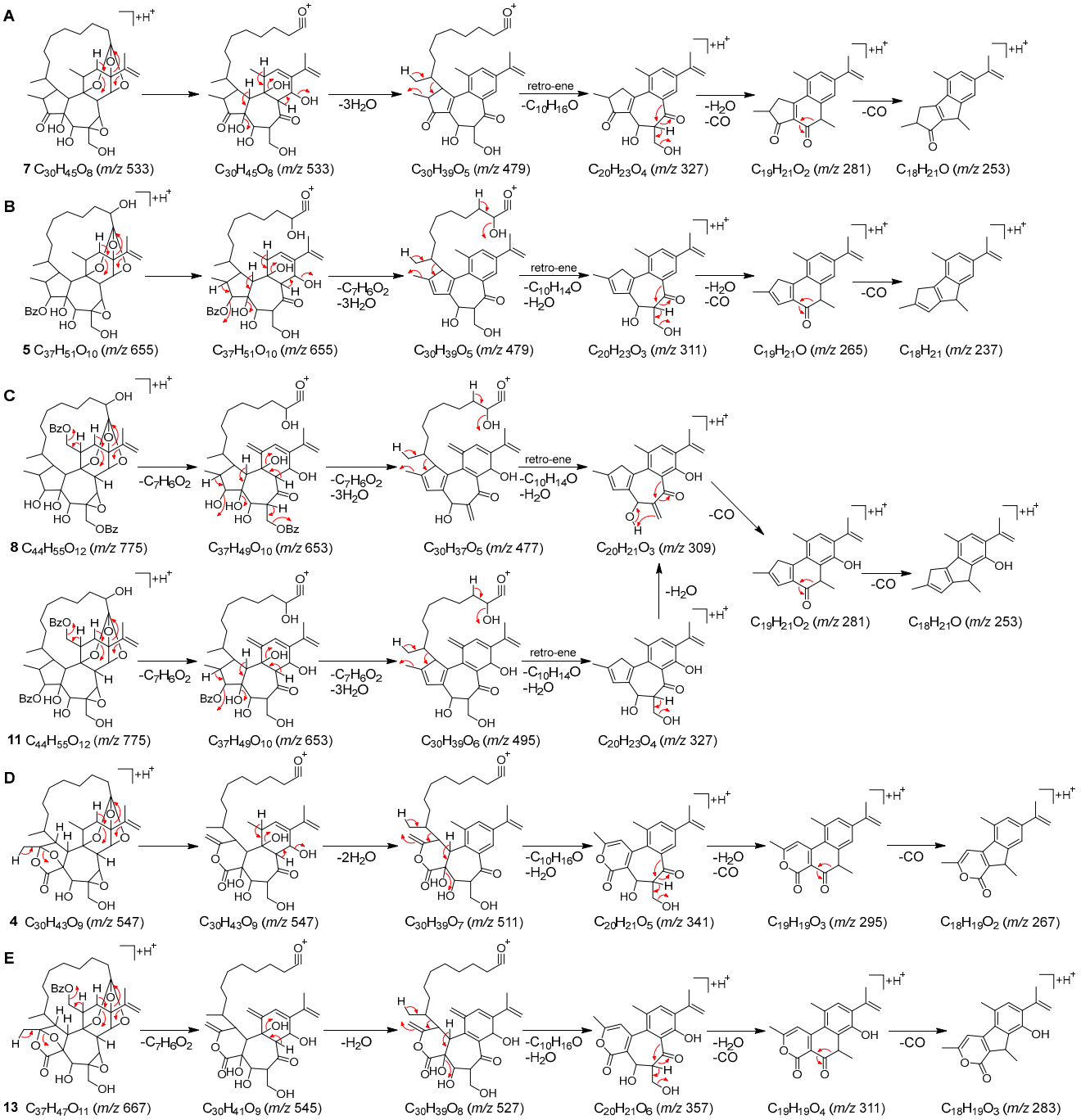

Figure 4. Proposed ESI-MS/MS fragmentation pathways for minor daphnane diterpenoids in positive mode. (**A**) peak **7**, (**B**) peak **5**, (**C**) peaks **8**, **11**, (**D**) peak **4**, and (**E**) peak **13**.

In the product ion spectra of peaks **3** and **16**, the characteristic product ion was observed at *m*/*z* 253 ($C_{17}H_{17}O_2$), which was produced by the loss of the 6,7-epoxy moiety, along with the oxymethylene at C-20, as a $C_3H_4O_2$ unit due to cleavage occurring at the B-ring. This observation suggested that both **3** and **16** were daphnane diterpenoids lacking a substituent at C-12 (Figures 3A and S22). Furthermore, the product ions at *m*/*z* 207 ($C_{14}H_{23}O$), 95 (C_6H_7O), and 81 (C_5H_5O) for peak **3**, and at *m*/*z* 179 ($C_{12}H_{19}O$), 95 (C_6H_7O), and 81 (C_5H_5O) for peak **16** indicated that a 2*E*,4*E*-tetradecadienoyl moiety was ester-linked to the C-ring in peak **3** and a 2*E*,4*E*-dodecadienoyl moiety was ester-linked in peak **16**. However, the molecular formula of peak **3** was 18 Da (H_2O) larger than that

of the orthoester daphnane, huratoxin (**21**) [42] and the fragment ion of $[M + H–H_2O]^+$ appeared with greater intensity in the mass spectrum of peak **3** (Figure S23). Based on these observations, it was concluded that peak **3** represented a polyhydroxy daphnane type compound, which lacks the orthoester moiety at the C-ring. Thus, peaks **3** and **16** were identified as wikstroelide M (**3**) [14] and wikstrotoxin B (**16**) [11], respectively.

In the product ion spectra of peaks **6**, **9**, **10**, **14**, and **19**, the product ion generated by the loss of the $C_3H_4O_2$ unit was consistently observed at m/z 269 ($C_{17}H_{17}O_3$), indicating that these peaks corresponded to orthoester daphnanes with a substituent attached to C-12 (Figures 3B and S24). The product ions corresponding to substituents observed in these peaks were assignable as follows: a cinnamoyl moiety at m/z 131 (C_9H_7O), a coumaroyl moiety at m/z 147 ($C_9H_7O_2$), a feruloyl moiety at m/z 177 ($C_{10}H_9O_3$), a 2*E*,4*E*-decadienoyl moiety at m/z 151 ($C_{10}H_{15}O$), 95 (C_6H_7O), and 81 (C_5H_5O), a 2*E*,4*E*,6*E*-decatrienoyl moiety at m/z 149 ($C_{10}H_{13}O$) and 107 (C_9H_9O), and a 2*E*,4*E*-dodecadienoyl moiety at m/z 179 ($C_{12}H_{19}O$), 95 (C_6H_7O), and 81 (C_5H_5O). Namely, the feruloyl and 2*E*,4*E*,6*E*-decatrienoyl moieties were present in peak **6**, the coumaroyl and 2*E*,4*E*-decadienoyl moieties in peak **9**, the feruloyl and 2*E*,4*E*-decadienoyl moieties in peak **10**, and the cinnamoyl and 2*E*,4*E*,6*E*-decatrienoyl moieties in peak **14**. In peak **19**, only the product ions due to the 2*E*,4*E*-dodecadienoyl moiety were observed, but the molecular formula and the observation of product ions derived from the neutral loss of $C_2H_4O_2$ suggested the presence of the acetyl moiety. Thus, peaks **6**, **9**, **10**, **14**, and **19** were identified as acutilobin C (**6**) [36], daphneodorin D (**9**) [29], acutilobin D (**10**) [36], 12-*O*-(*E*)-cinnamoyl-9,13,14-ortho- (2*E*,4*E*,6*E*)-decatrienylidyne-5β,12β-dihydroxyresiniferonol-6α,7α-oxide (**14**) [40], and wikstroelide A (**19**) [13].

Peaks **1**, **2**, **4**, **5**, **7**, **8**, **11**, and **13** were identified as MDOs by their characteristic MS/MS fragmentation patterns. Although the number of oxygen functional group varied among these compounds, they were all characterized by the abundance of C_{30} to C_{28} product ions observed in the range of m/z 400–550. The molecular formula of peak **7** indicated the absence of acyl groups. In the product ion spectrum, the neutral loss associated with the macrocyclic ring was assigned to be $C_{10}H_{16}O$ as in compounds **18** and **20**. In addition, a series of C_{20} to C_{18} product ions were observed with successive losses of H_2O and CO from m/z 327 ($C_{20}H_{23}O_4$) (Figures 4A and S25). Peak **7** was suggested to possess the cyclopentanone A-ring structure based on the degree of unsaturation and was identified as pimelea factor S_6 (**7**) [37]. Peak **5** had a molecular formula that was 16 Da (OH) larger than peaks **18** and **20**. The product ion spectrum of peak **5** exhibited a series of C_{20} to C_{18} product ions below m/z 350, as observed in **18** and **20** (Figures 4B and S25). However, the neutral loss associated with the macrocyclic ring differed from **18** and **20**, where it was $C_{10}H_{16}O$ rather than $C_{10}H_{14}O$ in **5**. These observations indicated that **5** possesses an additional hydroxyl group at C-2′ of the macrocyclic ring compared to **18** and **20**, and was further identified as kraussianin (**5**) [35].

The pair of peaks **1** and **2**, as well as the pair of peaks **8** and **11**, had the same molecular formula and exhibited similar product ion spectra, indicating that each pair, like compounds **18** and **20**, was in a regioisomeric relationship. The product ion spectra of peaks **1** and **2** revealed three molecules of $C_7H_6O_2$ neutral loss originating from benzoyl acids and two molecules of $C_2H_4O_2$ neutral loss originating from acetic acids, as well as the neutral loss of $C_{10}H_{10}O$ associated with the macrocyclic ring. In addition, a series of C_{30} to C_{28} product ions were observed with successive losses of H_2O and CO from m/z 507 ($C_{30}H_{35}O_7$) (Figures 4C and S26). These observations suggested that peaks **1** and **2** correspond to daphneodorin B (**1**) or daphneodorin C (**2**) with the same molecular formula and combinations of acyl groups. By comparison of retention times, peaks **1** and **2** were identified as daphneodorin C (**1**) and daphneodorin B (**2**), respectively [29]. The product ion spectra of peaks **8** and **11** revealed the elimination of two molecules of $C_7H_6O_2$, indicating the presence of two benzoyloxy moieties (Figures 4C and S26). Additionally, the neutral loss associated with the macrocyclic ring was assigned to be $C_{10}H_{14}O$ as in **5** and

the product ion pattern below m/z 350 was the same as **12**, which were identified as the regioisomer, stelleralide H (**8**) [38] and gnidimacrin (**11**) [39], respectively.

Peak **4** had a molecular weight 14 Da greater than peak **7** but the neutral loss associated with the macrocyclic ring was assigned to be $C_{10}H_{16}O$ as in peak **7**, suggesting that the daphnane skeleton was different from peak **7** (Figures 4D and S27). Based on the molecular formula and degree of unsaturation, peak **4** was identified as pimelotide C [34], which has the bicyclo[2.2.1]heptane A-ring structure. In the product ion spectrum of peak **13**, the observation of a loss of $C_7H_6O_2$ suggested the presence of a benzoyloxy moiety (Figures 4E and S27). Furthermore, the product ions observed within the range of m/z 400–550 were 2 Da smaller than those of peak **4**. Thus, peak **13** was identified as stelleralide C [5], which shared the bicyclo[2.2.1]heptane A-ring structure of peak **4** and a benzoyloxy moiety attached to C-18.

3. Materials and Methods

3.1. General Experimental Procedures

The NMR spectra were collected on a JEOL ECA-500 spectrometer (JEOL Ltd., Tokyo, Japan) with the deuterated solvent used as the internal reference. The ^{1}H-NMR spectra were performed at 500 MHz, and the ^{13}C-NMR spectra were generated at 125 MHz. HRESIMS was conducted using a Q-Exactive Hybrid Quadrupole Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA). The following columns were utilized for column chromatography: Diaion HP-20 (Mitsubishi Chemical Corporation, Tokyo, Japan), ODS (Chromatorex DM1020T, Fuji Silysia Chemical Ltd., Aichi, Japan) and silica gel (Chromatorex PEI MB 100-40/75, Fuji Silysia Chemical Ltd., Aichi, Japan) columns. For gradient HPLC, two JASCO/PU-2080 Plus Intelligent HPLC pumps (JASCO Corporation, Tokyo, Japan), equipped with an MX-2080-32 dynamic mixer (JASCO Corporation, Tokyo, Japan), a JASCO UV-970 Intelligent UV/vis detector, and an SSC-6800 fraction collector (JASCO Corporation, Tokyo, Japan), were utilized. For preparative HPLC, a Waters 515 HPLC pump (Waters Corporation, Massachusetts, USA), equipped with an ERC RefractoMax520 differential refractometer detector (Thermo Scientific, Waltham, MA, USA) and a Shimadzu SPD-10A UV-vis detector (Shimadzu, Kyoto, Japan), was utilized. For normal-phase HPLC separations, a silica gel column (YMC-Pack SIL, 5 μm, 250 × 20 mm) was utilized with a flow rate of 5.0 mL/min. For reversed-phase HPLC separations, an RP-C_{18} silica gel column (YMC-Actus Triart C_{18}, 5 μm, 150 × 20 mm) was utilized, with a flow rate of 8.0 mL/min.

3.2. Plant Material

The stems of *W. indica* were collected at Guangxi Province, People's Republic of China in February 2018 and identified by Dong Liang (Kunming Plant Classification and Biotechnology Co., Ltd., Kunming, China). A voucher specimen (accession number: 20201021) had been deposited in the herbarium of Shenyang Pharmaceutical University.

3.3. Extraction and Isolation

The air-dried whole plants of *W. indica* (1000 g) were cut into small pieces and extracted with 95% EtOH at room temperature to give an EtOH extract and a residue. The EtOH extract was concentrated (63.0 g), suspended in H_2O, and then partitioned with EtOAc. The EtOAc fraction (30.0 g) was subjected to Diaion HP-20 column chromatography, eluted with a stepwise gradient of MeOH/H_2O (from 5:5 to 10:0, v/v) to afford three fractions (E1 to E3). The E3 fraction (10.8 g) was subjected to ODS column chromatography, eluted with a stepwise gradient of MeOH–H_2O (from 7:3 to 10:0, v/v) to afford four subfractions (E3-1 to E3-4). Subfraction E3-2 (1840.3 mg) was subjected to silica gel column chromatography, eluted with a gradient of *n*-hexane–EtOAc–MeOH–HCOOH, to afford four subfractions (E3-2-1 to E3-2-5). Subfraction E3-2-3 (193.4 mg) was purified by RP-HPLC (70% CH_3CN, 80% CH_3CN) to give **12** (0.6 mg). Subfraction E3-2-2 (73.0 mg) was purified by RP-HPLC (85% CH_3CN) to give six subfractions (E3-2-2-1 to E3-2-2-6). Subfraction E3-2-2-3 (16.6 mg)

was purified by RP-HPLC (80% CH_3CN), followed by NP-HPLC (*n*-hexane/AcOEt, 3:7) to give **15** (7.6 mg), **17** (1.6 mg), and **18** (1.3 mg). Subfraction E3-2-2-4 (4.4 mg) was purified by RP-HPLC (85% CH_3CN) to give **20** (1.9 mg) and **21** (1.0 mg).

3.4. LC-MS/MS Conditions

The LC-MS/MS analysis was performed using the same instruments and column as in previous experiments [20]. For LC conditions, the mobile phase comprised eluent A (distilled water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), programmed as follows: 0–15 min, a linear gradient from 50% to 100% B, 15–18 min, 100% B, followed by column re-equilibration at 50% B for 10 min before the subsequent injection. For MS conditions, the in-source CID was set at 0 eV, and the resolution was 70,000 for full MS and 35,000 for full MS/data dependent (dd)-MS/MS modes. The AGC was established at 1E6 for full MS and 1E5 for dd-MS/MS. Data-dependent scanning was performed using HCD with the normalized collision energy at 15 eV. The extracted ion spectra were generated by extracting the following base peaks of each compounds within ± 5 ppm mass tolerance: m/z 1028.4283 $[M + NH_4]^+$ (**1**), m/z 1028.4264 $[M + NH_4]^+$ (**2**), m/z 585 $[M + H–H_2O]^+$ (**3**), m/z 547.2888 $[M + H]^+$ (**4**), m/z 655.3469 $[M + H]^+$ (**5**), m/z 719.3062 $[M + H]^+$ (**6**), m/z 533.3104 $[M + H]^+$ (**7**), m/z 775.3690 $[M + H]^+$ (**8**), m/z 691.3133 $[M + H]^+$ (**9**), m/z 721.3203 $[M + H]^+$ (**10**), m/z 775.3687 $[M + H]^+$ (**11**), m/z 912.4147 $[M + NH_4]^+$ (**12**), m/z 667.3107 $[M + H]^+$ (**13**), m/z 673.2999 $[M + H]^+$ (**14**), m/z 533.3107 $[M + H]^+$ (**15**), m/z 557.3109 $[M + H]^+$ (**16**), m/z 675.3151 $[M + H]^+$ (**17**), m/z 639.3527 $[M + H]^+$ (**18**), m/z 643.3458 $[M + H]^+$ (**19**), m/z 639.3525 $[M + H]^+$ (**20**), and m/z 585.3409 $[M + H]^+$ (**21**). All data collected in the profile mode were acquired and processed using Thermo Xcalibur 4.1 software.

4. Conclusions

This study represents the first comprehensive identification of 21 daphnane diterpenoids from the stems of *W. indica* through a combination of LC-MS guided isolation and MS/MS fragmentation elucidation. The investigation revealed that *W. indica* contained structurally diverse daphnane diterpenoids, including orthoester daphnane type, polyhydroxy daphnane type, and macrocyclic daphnane orthoester type compounds. The application of MS/MS fragmentation elucidation for structural analysis enabled the rapid and precise identification of these diterpenoids within crude plant extracts. This methodology holds great promise for future research endeavors aimed at discovering bioactive diterpenoids from plants of the Thymelaeaceae family.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants12203620/s1, Figure S1: Total ion chromatograms and extracted ion chromatograms in the positive ion mode from the (A) E3-2 fraction, (B) E3-3 fraction, and (C) E3-4 fraction; Tables S1 and S2: ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR data of compounds **12**, **15**, **17**, **18**, **20**, and **21** ($CDCl_3$); Figures S2–S21: NMR spectra and HRESIMS data of compounds **12**, **15**, **17**, **18**, **20**, and **21**; Figures S22–S27: Product ion spectra of peaks **1**–**11** and **13**–**21** obtained from the protonated molecular ion peak in positive ion mode, and **12** obtained from the ammonium adduct ion peak positive ion mode.

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