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Phytochemicals of Gandarusa (*Justicia gendarussa*) and Its Preparations

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Abstract

Thirty-five metabolites of *Justicia gendarussa* (JG) leaves and its preparations were identified using LC-HR-MS/MS. Although alkaloids were detected in the leaves they were not identified in JG preparations using Smart Formula 3D software. This showed that an acidified extraction process used at the first stage of the purification procedure is able to remove the toxic alkaloids from the crude drug. The LC-MS/MS analyses showed that the main components of JG preparations were fatty acids and apigenin glycosides; it seemed that the fatty acids can be used for enhancing the dissolution of the polar glycosides. *T*-test calculation using Profile Analysis software showed that the acidified crude drugs, extract, granules of JG, and gendarussa capsules showed very similar LC-MS/MS profiles, which means that the biochemical components of JG are relatively stable during processing. Due to the lack of quality markers for these JG preparations, the application of metabolite profiling is recommended as the QC tool for commercial production by the pharmaceutical industry.

Keywords

alkaloids, apigenin glycosides, extract of gendarussa, gendarussa capsules, *Justicia gendarussa* , LC MS/MS profiling

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Justicia gendarussa Burm.f. (JG), family Acanthaceae, known as Gandarusa, is found in Indonesia and also in several other countries in Asia such as Sri Lanka, India, and Malaysia.[1] JG was used in Indian folk medicines for treating many diseases such as rheumatism, bronchitis, fever, eczema, and jaundice.[2] JG has been known and used as a traditional male antifertility drug in Papua, Indonesia.[3] In vitro and in vivo antifertility tests of *n*-butanol fractions of JG showed that the possible mechanism was through competitive and reversible inhibition of spermatozoa hyaluronidase.[4] The anti-fertility effects might be caused by the C-glycosyl flavone group with an apigenin base structure. Apigenin and its glycoside vitexin in JG can be used for their anti-inflammatory and antitumor activities.[5] Thus, JG herbal drug has the potential to be developed into a phyto-pharmaceutical product as a nonhormonal male contraceptive.[6-8] Capsules of JG leaf extracts have already been studied in clinical trials.[8]

Four new alkaloids {brazoides A, B, C (**3**), and D (**12**)} were isolated from leaves of JG and tested against three human cancer cell lines (glioblastoma, prostate, and colon), but unfortunately, none exhibited activity.[9] Also reported for JG leaves is 1,5-dideoxy-3-C-{[(5- hydroxy-2-{[(5-oxotetrahydro-2-furanyl) carbonyl] amino} benzyl) oxy] carbonyl} pentitol (**9**).[10,11] Justidrusamide A (**13**), B (**14**), C (**11**), D (**10**), E (**6**),[12,13] 6,8-di-C-α-L-arabinocylapigenin {gendarusin A (**16**)}, and 6-C-α-L-arabinocyl-8-C-β-arabinocylapigenin {gendarusin B (**17**)} were identified in ethanolic leaf extracts of JG originating from Indonesia,[6,13] and apigenin and its glycoside vitexin were isolated from leaves of JG from India.[2] An arylnaphthalene lignan, patentiflorin, was isolated from JG collected in Vietnam.[14] It seemed that the secondary metabolites of JG were biosynthesized in the leaves, then transported to the roots.[11]

It is well known that variability in the constituents of herbal medicines is influenced by various external factors. It has been reported that JG leaves grown in different places show different metabolite profiles using LC-MS/MS.[10]

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Metabolites **13**, **14**, and **16** occurred in different concentrations in batches of dried material obtained from different Indonesian regions.[13]

JG capsules contain ethanol extracts of the crude drug, and so contain numerous compounds (primary and secondary metabolites), but unfortunately the complete identification of the metabolites in the crude drug and its preparations has not yet been reported. The therapeutic and toxicological effects of herbal drugs depend on all chemical compounds in the preparations, and that is why it is important to identify all the metabolites of JG preparations, both qualitatively and quantitatively. This present work reports the qualitative identification, using an UHPLC-UHR-QTOF-MS, of all metabolites from each stage in the production process of JG capsules, that is, dried gandarusa leaves (DS), acidified dry leaves (A), ethanol extract (E), and granules (GR).

Base peak chromatograms (BPCs) of samples of DS, A, E, GR, and granules from Konimex capsules (GR K) are shown in Figure 1. The BPCs were evaluated from equivalent concentrations of all samples, based on either sample DS or A (see the Experimental section). Based on the visual examination of the BPCs and *t*-test results, DS showed a very different profile pattern of metabolites, while profiles of other samples (A, E, and GR) were similar (Table 1).

DS contained either alkaloids or other nitrogen containing compounds **3** to **15**. These compounds, mostly amino benzyl alkaloid derivatives, were not detected in other samples. Alkaloids **3**, **6**, and **9** to **14** have been previously reported in

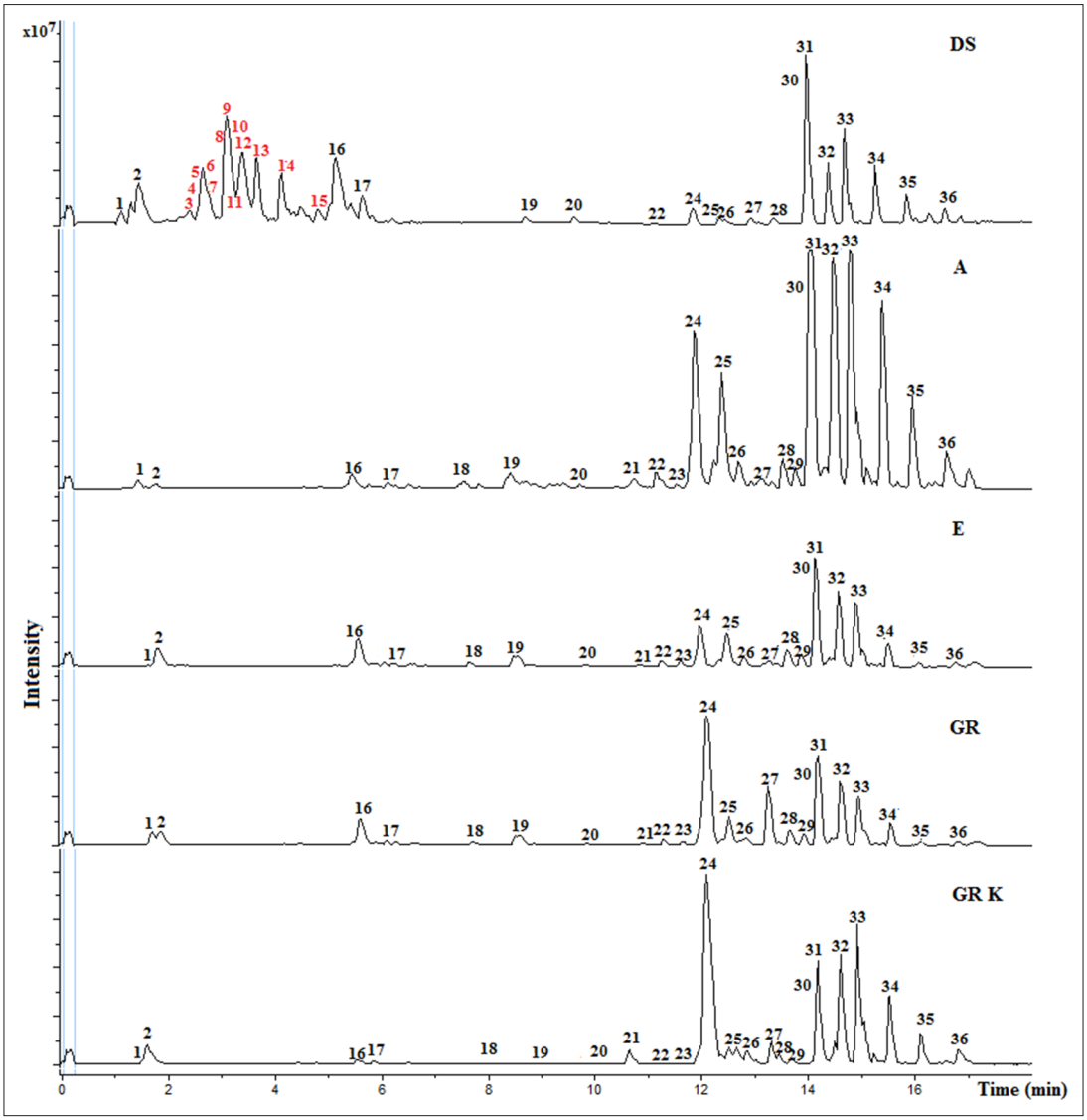

Figure 1. Base peak chromatograms of samples. Numbers (**1–36**) refer to metabolites as listed in Table 2. Red numbers represent alkaloids.

Table 1. . *P*-Value of Samples.

Sample	*P*-value (RT 1-10 min)	*P*-value (RT 1-20 min)
DS - A	0.81467	0.79201
A - E	0.98984	0.99502
A - GR	0.97957	0.98833
E - GR	0.99110	0.99673
GR - GR K	0.97029	0.97384

The results are based on *t*-test calculation by Profile Analysis software (Bruker Daltonik, Bremen, Germany).

JG leaves,[9–13,22] and metabolite **9** in the roots, stems, and leaves.[11] The molecular ions of **4**, **7**, and **8** were almost identical (< 5 ppm) to those of **6**, **10**, and **11**, respectively,[10,12,13] but their chemical structures were not identical (Table 2). Brazoides A and B[9] were not detected in this present study. This might be due to the differences in the ESI method and/or the different origin of the JG crude drug; brazoides A and B were previously detected using positive ESI mode.[9] Compound **5** was a carbamate pesticide, which is listed in the Shimadzu Pesticide MRM Library.[19] Thus the JG leaves had probably been contaminated with this pesticide, which was also found in JG fresh leaves from Gempol, but not in those from Surabaya.[23] It is known that the alkaloids in JG leaves can cause toxic effects,[4] and so it is necessary to process the leaves in order to remove the alkaloids. Compounds **3** to **15** could not be identified in A, E, GR, and GR K samples (by intensity of 10^7; Figure 1). However, when DS was diluted 10 times (DS1), all the alkaloids could still be detected using Smart Formula 3D (intensity 10^5) and their identity confirmed (Table 2), whereas in DS2 (DS1 was diluted 25 times), A, E, GR, and GR K, the alkaloids could not be detected (intensity 10^4).

The presence of alkaloids **13** and **14** in DS2, A, E, GR, and GR K could still be observed in the extract ion chromatograms (EIC), but with very low intensity (circa 0.2-0.5% to the intensity in DS). Other alkaloids showed identical results (data not shown). This showed that process II could be used effectively for the removal of most of the alkaloids from DS for producing A, E, GR, and GR K.

Apigenin glycosides **16** and **17**,[4,10,11,13] which are proposed active metabolites of JG leaves, were well detected in all samples. Metabolites **20** to **36** are dominated by fatty acids, which can enhance the solubility and dissolution rate of active polar compounds in herbal drugs[24] ; this might also be the case in JG preparations. Compound **34** has been reported previously in root cultures of JG.[11] Metabolite **26**, previously isolated from *Embelia ribes*, is a sesquiterpene benzoquinone (2,5-dihydroxy-3-tridecyl-1,4-benzoquinone).[20] Metabolite **28** is a hydroxylated fatty acid where the terminal (omega) carbon has been hydroxylated.[21]

Although metabolites **18**, **21**, **23**, and **29** were not detected in the DS using *Smart Formula 3D,* their EICs could still be obeserved at low intensity (10^4). Most of the observed metabolites (except **16** and **17**) showed relatively higher intensities in A, E, and GR compared with DS; this might be due to process II. It seemed that the water-soluble components of JG crude drugs were removed by process II, and so most of the other chemical components would be more concentrated in A. Unfortunately, **16** and **17** showed relatively lower intensities in A, which might be due to their water solubility. Process II should be further optimized for increasing their contents.

In summary, 35 metabolites could be well identified in JG crude drugs and its preparations; chemical structures of some identified metabolites were presented in Figure 2 and metabolites **3**, **6**, **9** to **14**, **16**, **17**, and **34** have been identified previously in JG leaves.[9-13] This work showed that the phytochemicals of JG are mostly composed of alkaloids, apigenin glycosides, and fatty acids. Some previously identified metabolites, that is, apigenin, vitexin, and patentiflorin[2,5,22] were not detected in our JG leaves, which strengthens the claims that the origin of JG could affect its metabolite contents.[10,13] All 35 metabolites detected in DS were also identified in fresh leaves of JG,[23] and so it could be concluded that all 35 metabolites in FL were not degraded during the drying processes. As shown in Table 1, the chemical profiles of GR and GRK were almost identical to those of A and E, which meant that all metabolites were relatively stable during processes III to IV. Quality active marker(s) of JG have not yet been specified and so for QC purposes of JG preparations, a combination of chemical metabolite profiling and multivariate analysis (PCA, PLS-DA, SIMCA) must be applied. This can be used for ensuring the reproducibility, efficacy, safety, and the quality of JG herbal drugs.[25] This work is still in progress.

Experimental

Materials

JG fresh leaves were collected at Gempol-Surabaya (East Java) in July 2018. Samples were randomly collected from wild plants. Scientific identification was performed in the Department of Pharmacognosy and Phytochemistry, Airlangga University, Surabaya. A voucher sample (22/H3.1.5/DT/2018) of the leaves was deposited in the departement. Ammonium acetate (Sigma-Aldrich, St. Louis, Missouri, USA), methanol (Merck, Darmstadt, Germany), and pure water were of LCMS grade. Capsules of Gendarusa were provided by PT. Konimex, Solo, Indonesia. Methanol, ethanol, and formic acid {analytical reagent grade (Merck, Darmstadt, Germany)}, citric acid anhydrate (Weifang Ensign Industry, Weifang, Shandong, China), lactose monohydrate (Leprino Foods, Denver, USA), corn starch (Amylum

Table 2. Identified Metabolites.

Metabolites ; (retention time (min))	Measured *m/z* ; HRMS ions $[M-H]^-$ (*m/z* calc.)[a]	Detection in sample	Error (ppm) ions [a]	Probable elemental formulas [a]	Measured *M/z* HRMS fragment ions (*m/z* calc.)[a]	Error [mDa] ions [a]	Probable fragment formulas [a]	Metabolites	References
1 (1.37)	179.0559 (179.0561)	DS, A, E, GR, GR K	−1.2	$C_6H_{12}O_6$	163.0610 (163.0612) 161.0454 (161.0455) 149.0454 (149.0455) 89.0245 (89.0244) 59.0141 (59.0139)	−0.2 0.1 0.1 0.1 0.2	$[C_6H_{11}O_5]^-$ $[C_6H_9O_5]^-$ $[C_5H_9O_5]^-$ $[C_3H_5O_3]^-$ $[C_2H_3O_2]^-$	Glucose	15–18 [b,c,d]
2 (1.46)	415.1097 (415.1093)	DS, A, E, GR, GR K	0.9	$C_{14}H_{24}O_{14}$	177.0403 (177.0405) 163.0614 (163.0612) 161.0452 (161.0455) 159.0303 (159.0299) 119.0346 (119.0350) 101.0246 (101.0244) 89.0246 (89.0244) 59.0141 (59.0139) 44.9984 (44.9982)	−0.2 −0.2 0.4 −0.4 −0.4 0.2 −0.2 0.2 0.2	$[C_6H_9O_6]^-$ $[C_6H_{11}O_5]^-$ $[C_6H_9O_5]^-$ $[C_6H_7O_5]^-$ $[C_4H_7O_4]-$ $[C_4H_5O_3]-$ $[C_3H_5O_3]-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	2-[[3,4-Dihydroxy-5-(hydroxymethyl)-2-[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxyoxolan-2-yl] methylperoxy] acetic acid	15 [c],16
3 (2.62)	413.1567 (413.1566)	DS	−0.3	$C_{18}H_{26}N_2O_9$	369.1287 (369.1303) 163.0611 (163.0612) 147.0662 (147.0663)	−1.7 −0.1 0.1	$[C_{16}H_{21}N_2O_8]^-$ $[C_6H_{11}O_5]^-$ $[C_6H_{11}O_4]^-$	1,5-Dideoxy-3-C-({[2-(γ-glutamylamino)-5-hydroxybenzyl]oxy}carbonyl) pentitol or Brazoide C	9,15 [d],16
4 (3.01)	368.1350 (368.1351)	DS	−0.2	$C_{17}H_{24}NO_8$	147.0449 (147.0452) 59.0140 (59.0139)	−0.3 −0.2	$[C_9H_7O_2]^-$ $[C_2H_3O_2]^-$	2-Amino-3-[4-[2-[(2*S*,3*R*,4*R*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]acetyl]phenyl] propanoic acid	15 [c],16
5 (3.02)	222.0772 (222.0772)	DS	0.0	$C_{11}H_{13}NO_4$	147.0449 (147.0452)	−0.3	$[C_9H_7O_2]^-$	Bendiocarb	15,19 [b,c,d],16
6 (3.03)	368.1350 (368.1351)	DS	0.4	$C_{17}H_{24}NO_8$	222.0770 (222/0772) 175.0610 (175.0612) 164.0705 (164.0717) 163.0611 (163.0612) 101.0242 (101.0244) 59.0141 (59.0139) 44.9984 (44.9982)	−0.0 −0.0 −1.0 −0.0 −0.10.4 0.3	$[C_{11}H_{12}NO_4]^-$ $[C_7H_{11}O_5]^-$ $[C_9H_{10}NO_2]^-$ $[C_6H_{11}O_5]^-$ $[C_4H_5O_3]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	(3*R*)−5-Hydroxy-2-(2-hydroxy-5-oxopyrrolidin-1-yl)benzyl 2,3-dihydroxy-2-((R)−1-hydroxyethyl)butanoate or Justidrusamide E	13,16
7 (3.18)	384.1297 (384.1300)	DS	−0.9	$C_{17}H_{23}NO_9$	370.1138 (370.1144) 326.12 38 (326.1245) 222.0762 (222.0772) 206.0821 (206.0823) 101.0245 (101.0244) 59.0140 (59.0139) 44.9983 (44.9982)	−0.5 −0.7 −0.9 0.2 0.1 −0.2 0.1	$[C_{16}H_{20}NO_9]^-$ $[C_{15}H_{20}NO_7]^-$ $[C_{11}H_{12}NO_4]^-$ $[C_{11}H_{12}NO_3]^-$ $[C_4H_5O_3]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	2-*O*-(2-{[4-(Carboxymethyl)benzyl] amino}−2-oxoethyl)-α-D-glucopyranose	15 [c,d],16
8 (3.21)	384.1301 (384.1300)	DS	−0.2	$C_{17}H_{23}NO_9$	370.1154 (370.1144) 238.0723 (238.0721) 222.0770 (222.0772) 163.0610 (163.0612) 59.0140 (59.0139) 44.9983 (44.9982)	−1.0 −0.2 −0.2 0.2 −0.2 0.1	$[C_{16}H_{20}NO_9]^-$ $[C_{11}H_{14}NO_5]^-$ $[C_{11}H_{12}NO_4]^-$ $[C_6H_{11}O_5]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	(1*R*)−1,5-Anhydro-1-{3-[(4-carboxybenzoyl) (hydroxy)amino] propyl}-D-mannitol	15 [d],16
9 (3.28)	396.1299 (396.1300)	DS	0.4	$C_{18}H_{23}NO_9$	368.1352 (368.1351) 250.0724 (250.0721) 163.0610 (163.0612) 129.0550 (129.0557)	0.1 0.4 0.2 −0.7	$[C_{17}H_{22}NO_8]^-$ $[C_{12}H_{12}NO_5]^-$ $[C_6H_{11}O_5]^-$ $[C_6H_9O_3]^-$	1,5-Dideoxy-3-C- {[(5-hydroxy-2-{[(5-oxotetrahydro-2-furanyl) carbonyl] amino}benzyl) oxy]carbonyl}pentitol	10,11,15 [d],16

(Continued)

Table 2. Continued

Metabolites ; (retention time (min))	Measured *m/z* ; HRMS ions [M-H]$^-$ (*m/z* calc.)[a]	Detection in sample	Error (ppm) ions [a]	Probable elemental formulas [a]	Measured *M/z* HRMS fragment ions (*m/z* calc.)[a]	Error [mDa] ions [a]	Probable fragment formulas [a]	Metabolites	References
10 (3.51)	384.1302 (384.1300)	DS	−0.5	$C_{17}H_{23}NO_9$	222.0770 (222.0772)	−0.2	$[C_{11}H_{12}NO_4]^-$	4-((2-((((R)-2,3-dihydroxy-2-((R)−1-hydroxyethyl)butanoyl)oxy)methyl)−4-hydroxyphenyl)amino)-4-oxobutanoic acid or Justidrusamide D	10,12,13,16
					163.0609(163.0612)	0.3	$[C_6H_{11}O_5]^-$		
					101.0244 (101.0244)	0.1	$[C_4H_5O_3]^-$		
					59.0141 (59.0139)	−0.2	$[C_2H_3O_2]^-$		
					44.9984 (44.9982)	0.2	$[CHO_2]^-$		
11 (3.54)	384.1304 (384.1300)	DS	1.1	$C_{17}H_{23}NO_9$	338.1239 (338.1245)	−0.6	$[C_{16}H_{20}NO_7]^-$	4-((2-((((2 s,3S)−2,3-dihydroxy-2-((R)-1-hydroxyethyl)butanoyl) oxy)methyl)-4-hydroxyphenyl) amino)-4-oxobutanoic acid or Justidrusamide C	10,12,13,16
					253.1079 (253.1081)	−0.2	$[C_{13}H_{17}O_5]^-$		
					238.0715 (238.0721)	−0.6	$[C_{11}H_{12}NO_5]^-$		
					222.0774 (222.0772)	0.2	$[C_{11}H_{12}NO_4]^-$		
					163.0610 (163.0612)	0.2	$[C_6H_{11}O_5]^-$		
					157.0502 (157.0506)	0.4	$[C_7H_9O_4]^-$		
					149.0453 (149.0455)	0.3	$[C_5H_9O_5]^-$		
					101.0244 (101.0244)	0.1	$[C_4H_5O_3]^-$		
					59.0140 (59.0139)	0.2	$[C_2H_3O_2]^-$		
12 (3.66)	397.1617 (397.1616)	DS	0.1	$C_{18}H_{26}N_2O_8$	163.0609 (163.0612)	0.3	$[C_6H_{11}O_5]^-$	1,5-Dideoxy-3-C-({{[2-(γ-glutamylamino) benzyl]oxy}carbonyl)-L-arabinitol or Brazoide D	9,15 [d],16
					44.9984 (44.9982)	−0.3	$[CHO_2]^-$		
13 (4.00)	368.1353 (368.1351)	DS	−0.6	$C_{17}H_{23}NO_8$	354.1198 (354.1194)	−0.4	$[C_{16}H_{20}NO_8]^-$	4-((2-((((2 s,3S)−2,3-dihydroxy-2-((R)-1-hydroxyethyl)butanoyl) oxy)methyl) phenyl)amino)-4-oxobutanoic acid or Justridusamide A	10-13,15 [d],16
					352.1408 (352.1402)	0.6	$[C_{17}H_{22}NO_7]^-$		
					222.0773 (222.0772)	0.1	$[C_{11}H_{12}NO_4]^-$		
					206.0819 (206.0823)	0.4	$[C_{11}H_{12}NO_3]^-$		
					163.0612 (163.0612)	0.0	$[C_6H_{11}O_5]^-$		
					101.0242 (101.0244)	−0.2	$[C_4H_5O_3]^-$		
					59.0140 (59.0139)	0.2	$[C_2H_3O_2]^-$		
					44.9982 (44.9982)	0.0	$[CHO_2]^-$		
14 (4.34)	368.1354 (368.1351)	DS	−0.9	$C_{17}H_{23}NO_8$	222.0771 (222.0772)	0.1	$[C_{11}H_{12}NO_4]^-$	4-((2-((((R)−2,3-dihydroxy-2-((R)1-hydroxyethyl)butanoyl) oxy)methyl) phenyl)amino)-4-oxobutanoic acid or Justridusamide B	10-13,15 [c],16
					163.0611 (163.0612)	0.1	$[C_6H_{11}O_5]^-$		
					101.0245 (101.0244)	0.1	$[C_4H_5O_3]^-$		
					59.0142 (59.0139)	0.3	$[C_2H_3O_2]^-$		
					44.9985 (44.9982)	−0.3	$[CHO_2]^-$		
15 (4.94)	352.1402 (352.1402)	DS	0.0	$C_{17}H_{23}NO_7$	236.0923 (236.0928)	−0.5	$[C_{12}H_{14}NO_4]^-$	6-{[(Benzyloxy) carbonyl]amino} -6-deoxy-1,2-O-isopropylidene-α-D-glucofuranose	15 [d],16
					222.0766 (222.0772)	0.6	$[C_{11}H_{12}NO_4]^-$		
					206.0808 (206.0823)	1.5	$[C_{11}H_{12}NO_3]^-$		
					174.0554 (174.0561)	−0.7	$[C_{10}H_8NO_2]^-$		
					135.0445 (135.0452)	−0.6	$[C_8H_7O_2]^-$		
16 (5.00)	533.1297 (533.1301)	DS, A, E, GR, GR K	0.6	$C_{25}H_{26}O_{13}$	161.0242 (161.0244)	−0.2	$[C_9H_5O_3]^-$	6,8-Di-C-alpha-L-arabinopyranosylapigenin or Gendarusin A	10,11,13,15 [c],16
					117.0348 (117.0346)	0.2	$[C_8H_5O]^-$		
					89.0245 (89.0244)	−0.1	$[C_3H_5O_3]^-$		
					59.0142 (59.0139)	−0.3	$[C_2H_3O_2]^-$		
17 (5.65)	533.1300 (533.1301)	DS, A, E, GR, GR K	−0.2	$C_{25}H_{26}O_{13}$	145.0298 (145.0295)	−0.3	$[C_9H_5O_2]^-$	6,8-Di-C-β-D-arabinopyranosylapigenin or Gendarusin B	10,11,13,15 [d],16
					89.0242 (89.0244)	−0.2	$[C_3H_5O_3]^-$		
					59.0141 (59.0139)	0.2	$[C_2H_3O_2]^-$		
18 (7.29)	273.1713 (273.1707)	A, E, GR, GR K	2.2	$C_{14}H_{26}O_5$	255.1599 (255.1602)	−0.3	$[C_{14}H_{23}O_4]^-$	6-(2-Ethyl-5-hydroxy-hexoxy)-6-oxo-hexanoic acid	15 [c],16
					213.1141 (213.1132)	−0.8	$[C_{10}H_{17}O_4]^-$		
					201.1132 (201.1134)	−0.2	$[C_{18}H_{17}O_4]^-$		
					125.0965 (125.0972)	0.6	$[C_8H_{15}O_1]^-$		
					59.0143 (59.0139)	0.4	$[C_2H_3O_2]^-$		

(Continued)

Table 2. Continued

Metabolites ; (retention time (min))	Measured *m/z* ; HRMS ions [M-H]$^-$ (*m/z* calc.)[a]	Detection in sample	Error (ppm) ions [a]	Probable elemental formulas [a]	Measured *M/z* HRMS fragment ions (*m/z* calc.)[a]	Error [mDa] ions [a]	Probable fragment formulas [a]	Metabolites	References
19 (8.08)	299.0565 (299.0561)	DS, A, E, GR, GR K	1.3	$C_{16}H_{12}O_6$	269.0461 (269.0455)	0.6	$[C_{15}H_9O_5]^-$	3′-*O*-Methylluteolin or Chrysoeriol	15 [b,c,d],16
20 (9.45)	267.1966 (267.1966)	DS, A, E, GR, GR K	0.0	$C_{16}H_{28}O_3$	221.1552 (221.1547) 143.1079 (143.1078) 59.0140 (59.0139) 44.9984 (44.9982)	0.5 0.2 0.2 −0.2	$[C_{14}H_{21}O_2]^-$ $[C_8H_{15}O_2]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	11-(2-Oxocyclopentyl) undecanoic acid	15 [d],16
21 (10.46)	325.2030 (325.2020)	A, E, GR, GR K	2.9	$C_{18}H_{30}O_5$	307.1923 (307.1915) 291.1965 (291.1966) 291.1608 (291.1602) 265.1815 (265.1809) 251.1662 (251.1653) 211.1345 (211.1340) 197.1182 (197.1183) 171.1027 (171.1027) 59.0141 (59.0139) 44.9983 (44.9982)	0.8 0.1 0.7 0.6 −0.9 −0.5 −0.1 −0.1 0.3 −0.1	$[C_{18}H_{27}O_4]^-$ $[C_{18}H_{27}O_3]^-$ $[C_{17}H_{23}O_4]^-$ $[C_{16}H_{25}O_3]^-$ $[C_{15}H_{23}O_3]^-$ $[C_{12}H_{19}O_3]^-$ $[C_{11}H_{17}O_3]^-$ $[C_9H_{15}O_3]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	9-(3-Heptanoyl-2-oxyranyl)-9-oxononanoic acid	15 [d],16
22 (10.84)	291.1971 (291.1966)	DS, A, E, GR, GR K	1.8	$C_{18}H_{28}O_3$	59.0141 (59.0139) 44.9984 (44.9982)	−0.2 −0.2	$[C_2H_3O_2]^-$ $[CHO_2]^-$	12-Oxo-phytodienoic acid	15 [b,c] ,16
23 (11.22)	485.2552 (485.2545)	A, E, GR, GR K	−1.5	$C_{28}H_{38}O_7$	441.2656 (441.2646) 289.1804 (289.1809) 59.0142 (59.0139) 44.9986 (44.9982)	1.0 0.6 0.3 0.4	$[C_{27}H_{37}O_5]^-$ $[C_{18}H_{25}O_3]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	20-(Carboxymethyl)-6-methoxy-2,5,17-trimethyl-2,4,8,10,14,18,20-docosaheptaenedioic acid	15 [b,c],16
24 (11.56)	293.2128 (293.2122)	DS, A, E, GR, GR K	−1.9	$C_{18}H_{30}O_3$	275.2021 (275.2017) 171.1028 (171.1027) 59.0143 (59.0139)	0,5 −0.1 0.4	$[C_{18}H_{27}O_2]^-$ $[C_9H_{15}O_3]^-$ $[C_2H_3O_2]^-$	(10E,12Z)-9-Oxooctadeca-10,12-dienoic acid or 9-OxoODE	15 [b,c,d],16
25 (12.05)	295.2287 (295.2279)	DS, A, E, GR, GR K	2.9	$C_{18}H_{32}O_3$	277.2180 (277.2173) 59.0143 (59.0139) 44.9986 (44.9982)	0.7 −0.5 −0.4	$[C_{17}H_{29}O_2]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	(9Z,11E)-(13S)-13-Hydroxyoctadeca-9,11-dienoic acid or 13(S)-HODE	15 [b,c,d],16
26 (12.40)	321.2078 (321.2071)	DS, A, E, GR, GR K	2.2	$C_{19}H_{30}O_4$	293.2129 (293.2122) 275.2024 (275.2017)	−0.7 −0.7	$[C_{18}H_{29}O_3]^-$ $[C_{16}H_{27}O_2]^-$	Rapanone	15,20 [b,c],16
27 (12.89)	323.2232 (323.2228)	DS, A, E, GR, GR K	−1.3	$C_{19}H_{32}O_4$	307.2282 (307.2279) 277.2180 (277.2173) 89.0250 (89.0244) 59.0141 (59.0139) 44.9983 (44.9982)	0.3 0.7 0.6 −0.3 −0.1	$[C_{19}H_{31}O_3]^-$ $[C_{18}H_{29}O_2]^-$ $[C_3H_5O_3]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	Dihydromonacolin L acid	15 [b],16
28 (13.16)	271.2286 (271.2279)	DS, A, E, GR, GR K	−2.7	$C_{16}H_{32}O_3$	225.2233 (225.2224) 59.0142 (59.0139) 44.9985 (44.9982)	−0.9 −0.4 0.3	$[C_{15}H_{29}O]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	16-Hydroxyhexadecanoic or Juniperic acid	15,21 [b,c],16
29 (13.48)	297.2439 (297.2435)	A, E, GR, GR K	−1.3	$C_{18}H_{34}O_3$	59.0143 (59.0139) 44.9985 (44.9982)	−0.5 −0.3	$[C_2H_3O_2]^-$ $[CHO_2]^-$	Ricinoleic acid or 12-Hydroxy-9-octadecenoic acid	15,21 [b,c] ,16
30 (13.68)	277.2183 (277.2173)	DS, A, E, GR, GR K	−3.5	$C_{18}H_{30}O_2$	59.0144 (59.0139) 44.9986 (44.9982)	−0.6 −0.4	$[C_2H_3O_2]^-$ $[CHO_2]^-$	gamma-Linolenic acid	15[b,c,d],17
31 (13.69)	227.2023 (227.2017)	DS, A, E, GR, GR K	−2.9	$C_{14}H_{28}O_2$	59.0144 (59.0139) 44.9986 (44.9982)	−0.6 −0.4	$[C_2H_3O_2]^-$ $[CHO_2]^-$	Myristic acid	15 [b,c,d],17
32 (14.11)	241.2173 (241.2173)	DS, A, E, GR, GR K	0.0	$C_{15}H_{30}O_2$	59.0143 (59.0139) 44.9985 (44.9982)	−0.4 −0.3	$[C_2H_3O_2]^-$ $[CHO_2]^-$	Pentadecylic acid or Pentadecanoic acid	15 [b,c,d],17

(Continued)

Table 2. Continued

Metabolites ; (retention time (min))	Measured *m/z* ; HRMS ions $[M-H]^-$ (*m/z* calc.)[a]	Detection in sample	Error (ppm) ions [a]	Probable elemental formulas [a]	Measured *M/z* HRMS fragment ions (*m/z* calc.)[a]	Error [mDa] ions [a]	Probable fragment formulas [a]	Metabolites	References
33 (14.47)	255.2337 (255.2330)	DS, A, E, GR, GR K	2.9	$C_{16}H_{32}O_2$	59.0143 (59.0139) 44.9985 (44.9982)	−0.4 −0.3	$[C_2H_3O_2]^-$ $[CHO_2]^-$	Palmitic acid	15 [b,c,d],17
34 (15.10)	283.2633 (283.2643)	DS, A, E, GR, GR K	−3.5	$C_{18}H_{36}O_2$	59.0139 (59.0139) 44.9981 (44.9982)	0.0 -0.1	$[C_2H_3O_2]^-$ $[CHO_2]^-$	Stearic acid	15 [b,c,d],17
35 (15.62)	311.2966 (311.2956)	DS, A, E, GR, GR K	3.5	$C_{20}H_{40}O_2$	283.2645 (283.2643) 59.0141 (59.0139) 44.9986 (44.9982)	-0.2 0.3 0.4	$[C_{18}H_{35}O_2]^-$ $[C_2H_3O_2]^-$ $[CHO_2]^-$	Arachidic acid	15 [b,c,d],17
36 (16.30)	339.3276 (339.3269)	DS, A, E, GR, GR K	−2.3	$C_{22}H_{44}O_2$	59.0143 (59.0139) 44.9984 (44.9982)	0.5 0.2	$[C_2H_3O_2]^-$ $[CHO_2]^-$	Docosanoic acid or Behenic acid	15 [b,c,d],17

[a]Smart Formula 3D (elemental formulas were confirmed from their isotope patterns).
[b] MetFrag (KEGG database).
[c] MetFrag (Pubchem database).
[d] Metfrag (Chemspider database).

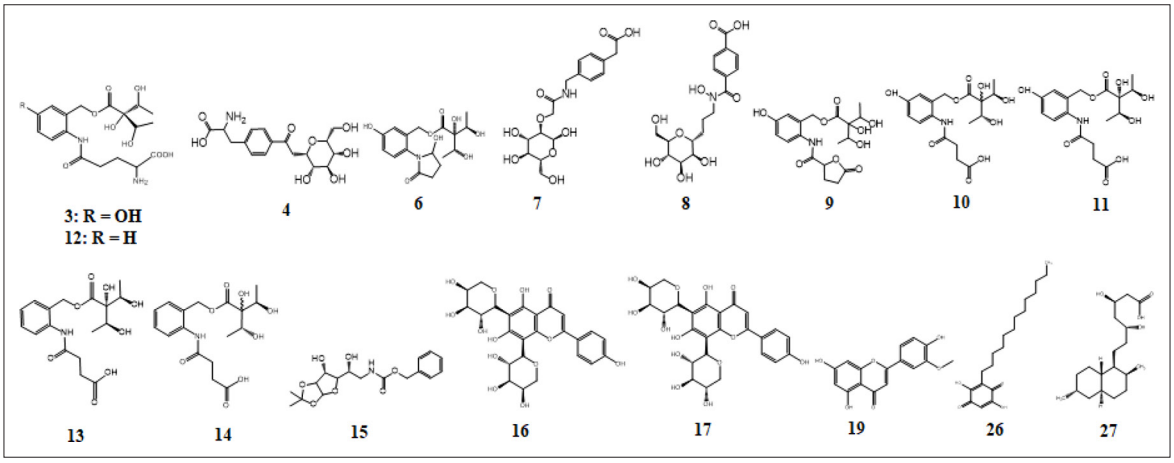

Figure 2. Chemical structures of some identified compounds. Numbers refer to metabolites as listed in Table 2.

Maydis, Cargill Bio-Chemical China), Cab-O-Sil® (Pluronic F-68, Sigma Life Science, St. Louis, Missouri, USA), and sodium lauryl sulfate (PT Hawwari Trading Apriansyah, Bogor, Indonesia) were of pharmaceutical grade.

Moisture Content Determination

Moisture content (MC) of each sample was measured using a Moisture Analyzer HB43-S (Mettler Toledo, Columbus, OH, USA). The MC values listed are the average value (n = 3).

Preparation of Granules

(I) Five kg fresh JG leaves (MC 68.2%) were sorted, washed, air-dried (28°C ± 3°C), and powdered (DS, 1.052 kg, MC 8.36%). **(II)** DS was acidified with anhydrous citric acid to pH ±3 for 3 × 24 hours, then filtered. The residue was mixed with distilled water, pH ±7, then filtered and the filtrate dried (A, 652.0 g, MC 11.5%). **(III)** A was macerated with 70% EtOH for 3 × 24 hours. The extract was collected, concentrated using a rotary evaporator, and dried in an oven (E, 10.53 g, MC 4.68%).

(IV) E (6750.3 mg) was added to 3008.1 mg lactose, 3009.8 mg corn starch, 451.5 mg Cab-O-Sil®, and ca. 133.5 mg sodium lauryl sulfate and mixed until homogeneous. The granule mass was sieved through a mesh no. 10 and dried in an oven at 50°C for ±6 hours. The dry granules were sieved through a mesh no. 20. GR (13.33 g) was obtained with MC 2.55%. GR was physically tested for granule quality according to the Indonesian Pharmacopeia V.[26] Twenty capsules from PT. Konimex with an average weight of 0.4392 g were taken and mixed homogeneously (GR K, MC 2.23%).

Sample Preparation for UHPLC-UHR-QTOF-MS Analysis

Two mL MeOH containing 0.1% formic acid was added to each sample (circa 250.0 mg for DS and A; circa 100.0 mg for E; circa 200.0 mg for GR and GR K, respectively; accurately weighed). The samples were vortexed for 15 seconds, sonicated for 10 minutes, and then centrifuged at 4000 rpm for 10 minutes. The extraction process was repeated 3 times. Supernatants were collected and dried using N_2. The residue (extract) was dissolved in a calculated equivalent of MeOH (for DS and A 200 µL), vortexed for 30 seconds, and ultrasonicated for 1 minute until dissolved completely, filtered and 1 µL injected into the UHPLC-UHR-QTOF-MS. Each sample was replicated at least 3 times.

Example: Calculation of the amount of MeOH for dissolving A and E that have equivalent concentrations.

For 250.0 mg DS (MC 8.36%), dry weight DS = 229.0 mg, extract DS dissolved in 200 µL MeOH (using Socorex micropipette, Ecublens, Switzerland).

Equivalent volume of MeOH for dissolving A (weight = 258.9 mg, MC 11.55%):

$$\frac{88.45}{100} \times 258.9\text{mg; Volume MeOH} = \frac{228.9971}{229.0} \times 200\mu\text{L} = 200\mu\text{L}$$

100.8 mg E (MC 4.68%), total weight E = 10.5307 g; total weight A = 652.0 g.

Equivalent weight of E to A:

$$\frac{0.1008\text{g}}{10.5307\text{ g}} \times 652.0\text{ g} = 6.228\text{ g}$$

Equivalent volume of MeOH for dissolving E:

$$\frac{95.32}{100} \times 6.228\text{ g; Volume MeOH} = \frac{5.937}{0.229} \times 200\mu\text{L} = 5.185\text{mL}$$

Table 3. .The Mobile Phase Program and Flow.

Time (min)	Flow (mL/min)	%A	%B
0.0	0.200	99.0	1.0
0.1	0.200	99.0	1.0
1.0	0.200	99.0	1.0
3.0	0.200	61.0	39.0
14.0	0.400	0.1	99.9
16.0	0.480	0.1	99.9
16.1	0.480	99.0	1.0
19.0	0.480	99.0	1.0
20.0	0.200	99.0	1.0

Liquid Chromatography-Mass Spectrometry

A Dionex Ultimate 3000 RSLC UHPLC (Dionex, Thermo Scientific, Garmening, Germany) was used, coupled with a QTOF Bruker Maxis Impact HD (Bruker Daltonik, Bremen, Germany), equipped with electrospray ionization operating in negative ion mode. The capillary voltage was 2500 V, dry N_2 gas flow of 8.0 L/min (200 C), nebulizer pressure 2.0 bars, end plate offset 500 V. The MS/MS analysis was performed by auto fragmentation (auto MS/MS), where the 3 most intensive peaks were fragmented. Mass Range *m/z* 50-1000; Quadropole ion energy was 5 EV and collision energy 10 EV (80-120%); Spectra rate: 2 Hz (MS), 2 Hz (MS/MS low), 8 Hz (MS/MS, high) total time cycle 0.9-2 s; Mass calibration was performed using 1 mM sodium formate/acetate in 50% isopropanol with 0.2% formic acid, HCOO (NaCOOH)1 (*m/z* 112.9856), Ac(NaAc)1 (*m/z* 141.0169), and Ac(NaF)1 (*m/z* 127.0013). Chromatographic separation was carried out using an Acclaim RSLC 120 C18 column (2.2 µm 120 Å 2.1 × 100 mm) (Dionex, Thermo Fischer Scientific, Sunnyvale, CA, USA). The mobile phase consisted of (A) 5 mM ammonium acetate in methanol (10:90 v/v), and (B) 5 mM ammonium acetate in methanol under a gradient program and flow (Table 3).

Data Analysis, Processing, and Identification of Metabolites

Data analysis was performed using the following software: Data Analysis 4.1 (Smart Formula, Smart Formula 3D, Isotope Pattern, and Fragmentation Explorer), Profile Analysis 2.1 (*t*-test), Metabolite Detect 2.0 (Bruker Daltonik, Bremen, Germany), and Chemdraw Ultra 12.0.2.1047 (CambridgeSoft, Perkin Elmer Inc, Akron, OH, USA); online MS databases: MetFrag (version 2010),[15] METLIN,[17] MassBank of North America (MoNA),[18] CFM-ID.[16] The 3 most intensive molecular ions were automatically selected by auto MS/MS from each BPC peak. Only molecular ions that could be observed and detected by Smart Formula 3D were further analyzed. The proposed molecular formula was predicted using Smart Formula based on the exact mass (<5 ppm measured to calculated) and was confirmed using isotopic pattern; the fragmentation of the compound was generated using Smart Formula 3D. Verification of the MS/MS ion fragments (daughter ions) were based on their EIC. The fragmentation patterns of the compounds were evaluated by using MetFrag,[15] METLIN,[17] and MoNA.[18] All compounds (except **6**, **10**, **11**) predicted by Metfrag were based on the highest score and the most explained peaks (fragments); Metfrag was set for biological compounds only. Metabolites which were predicted by databases[15,17,18] were confirmed by using CFM-ID[16] ; the SMILE format (calculated by Chemdraw) of the predicted compounds was inserted into CFM-ID for generating the MS/MS pattern; the patterns of the MS/MS fragmentations of CMF-ID (CID 10 EV) were then compared with the measured data. MS/MS of metabolites **6**, **10**, and **11**, which showed no results by using the databases,[15,17,18] could be well predicted using CFM-ID. Inserting MS/MS of other metabolites into CFM-ID yielded identical predicted compounds with databases.[15,17,18] Predicted fragmentation from all databases was further evaluated and confirmed by Fragmentation Explorer. Confirmations of the identity of the predicted compounds were performed using the identification point (IP) system according to EC/657/2002; all compounds showed IP > 4.5.[27] Ratio of the intensity of the molecular ion to the intensity of the most prominent fragment for compounds **1** to **36** was less than ±30% (measured data to CFM-ID; data not shown).[28] By these data the identity of compounds **1** to **36** that are listed in Table 2 could be well confirmed.

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Declaration of Conflicting Interests

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