Identification Of Compounds Flavonoids Namnam Leaf Extract (Cynometra Cauliflora) As Inhibiting Α-Glucosidase

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Abstract. Plants namnam (Cynometra cauliflora) has the potential to be developed for a variety of bioactive ingredients in particular flavonoid compounds as inhibitors of α-glucosidase. These compounds can be extracted with various solvents and column chromatography, and can be also characterized by a variety of instruments. In this study, these compounds liquid-liquid extraction with n-butanol followed by column chromatography process. Meanwhile, characterization and separation of the most active fractions using TLC (Thin Layer Chromatography), FTIR (Fourier Transform Infrared) and LCMS (Liquid Chromatography Mass Spectrometry). Characterization of compounds with FTIR results show similarities with flavonoids for their -OH groups (u 3200-3450 cm-1), C = C aromatic (u 1500-1675 cm-1), -CH aliphatic (u 1350-1470 cm- 1), -CO (u 1080-1300 cm-1), and C = O (u 1690-1766 cm-1). While these results Characterization using LCMS showed flavonoid compounds such as xanthotoxin, Fraxetin, capensine, naringenin, malvidin, cyanidin, amorphigenin, nobiletin, isorhamnetin, epigallocatechin, gallate, apigenin and oenin. The compounds are alleged to have inhibitory activity against α-glucosidase among fraxetin, oenin, naringenin, malvidin, and cyanidin. Thus, the plant leaves Namnam has the potential to be developed as a natural therapy of diabetes mellitus through the mechanism of inhibition of α-glucosidase.

Keywords:α-glucosidase, antidiabetic, Cynometra cauliflora, FTIR, LCMS, Namnam

1. Introduction
Plants Namnam (Cynometra cauliflora) is a member of the nuts that are famili Fabaceae. This plant is widely available in south east asia and the peninsula of western and southern India. In Indonesia the plant is known in local languages as namu-namu (Manado and Maluku), namo - namo (Ternate), Namet (Halmahera), namute, lamute, klamute (Central Maluku), arepa (Bugis), puti anjeng (Makassar), Namnam (Sunda, Java, and Madura), Puci anggi (Bima) and kuwanjo (Bali). The plant is a fruit that is underutilized but has medical value as traditional medicine and is cultivated as an ornamental plant by the people in the countryside [1].
Results of previous studies show that the stem, leaves and, young leaves of plants grown Namnam containing bioactive components such as terpenoids, tannins, saponins, flavonoids and cardiac glycosides [2]. Sumarlin et al. [3] also showed that the methanol extract of the leaves of this plant contains flavonoids.

According to research Tadera et al. [4] in vitro, showed that the flavonoid compound is a compound antidiabetic agents through the mechanism of inhibition of α-glucosidase. Inhibition of carbohydrate hydrolysis to reduce postprandial hyperglycemia and slows the absorption of glucose [5] and reduced the induction of insulin [6]. That ability is caused by flavonoids what to have antihyperglycemic activity that affects the absorption of glucose (glucose uptake), glycogen synthesis and deposition of glycogen in different tissues as well as interact with the key enzyme in the glycolytic mice [7], [8].

That ability also is supported by Unnikrishnan et al. [9], which explained that flavonoids act as an antidiabetic agents. Ado et al. [10] have also reported that the extract of ethyl acetate (EtOAc) and n-butanol from Namnam leaf liquid-liquid partition had potential to inhibition activities to α-glucosidase.

Therefore it is necessary flavonoid compounds characterized extraction results, which assume have the ability bioactive especially as α-glucosidase inhibitors. This flavonoid compound characterization using FTIR (Fourier Transform Infrared) and LCMS (Liquid Chromatography Mass Spectrometry). The results will enrich the information about the plants’ NamNam (Cynometra cauliflora) potential as an antidiabetic agent through the inhibition of α-glucosidase.

2. Experimental Section
2.1. Materials
Leaves Namnam (Cynometra cauliflora) obtained from the Village Cintaratu, Pangandaran, West Java-Indonesia and identified in the Herbarium Bogoriense:Botany, Research Center for Biology LIPI, Bogor as the plant with the name Cynometra cauliflora L, ethyl acetate, chloroform, petroleum ether, acetone, quercetin standart, n - butanol, distilled water, dimethyl sulfoxide (DMSO), silica gel G60F254, powders KBr and Whatman filter No 1.All chemicals used pure analysis (p.a).

2.2. Equipment and instruments
The equipment used to do extraction and fractionation (isolation) are filter paper, Buchner funnel, Hirsch funnel, Erlenmeyer flask, pipette, spatula, measuring glass, vials, containers, separating funnel, and vacuum rotary evaporator, Fourier Transform Infrared/FTIR and Liquid Chromatography Mass Spectrometry/LCMS ESI and glass tools

2.3. Sample Preparation
Leaves Namnam (Cynometra cauliflora L.) washed with running water, sorted and then dried in direct sunlight for 30 hours until the moisture content 9-10%. Namnam leaf samples (Cynometra cauliflora), which had dried, the dried sorted and then crushed with a blender, to obtain a smooth powder. The small sample size (smooth powder) is expected to expand the material surface in direct contact with the solvent, so that the extraction process of bioactive components can run with the maximum [10].

Namnam leaf powder (Cynometra cauliflora) as much as 100 grams soaked in 500 mL of methanol p.a and do maceration for 24 hours. After 24 hours, the result of maceration is filtered with Whatman filter paper no.1, so that the filtrate obtained first. Then the residue leaves macerated Namnam back (remaceration) with methanol p.a were 250 mL for 9 hours, to obtain a second filtrate. Furthermore, the first filtrate and the second filtrate are mixed and concentrated using a vacuum rotary evaporator at a temperature of 45-50°C, so all that remains the leaf extract Namnam form viscous extract (crude extract). The results of this preparation process will continued for partition extraction.

2.4. Partition Extraction fot Crude Extract [10].
Leaves Namnam extracts (crude extract) through a fractionation process with a second liquid solvent (extraction method of liquid-liquid partition) using a separating funnel. After crude extracts macerated, they are fractionation with n-hexane, ethyl acetate, n-butanol, and water to form a 2-phase and
separated. That fractionation repeated until the upper phase is clear. Each phase obtained concentrated by rotary vacuum evaporator at a temperature of ± 70°C and then dried in an oven at a temperature more or less 50°C to obtain concentrated extract. The test results with a fraction of α-glucosidase inhibition highest in the later stages separated by Thin Layer Chromatography (TLC).

2.5. Columns Chromatography [11].
A total of 10 grams of silica gel 60 GF254, pore size 0.2-0.5 mm. That sample, then activated by heating in an oven for 2 hours at 110°C and then cooled in a desiccator. Column chromatography diameter of 2 cm and 30 cm high filled silica gel that activated to ¾ of the height in the column, then added eluent of TLC. Eluent used must be able to separate the sample well. Therefore, based on the best separation pattern on Thin Layer Chromatography. The procedure of column chromatography as follows:

a. Viscous samples or extracts as much as 1.5 grams dissolved in a solvent, and as much as 46 grams of silica gel was added and stirred until a thick extract becomes dry.
b. Dried silica gel was added to the column and compacted.
c. The Solvent added to the column and added dried extracts in that column.
d. Eluent acetone: petroleum ether = 70:30, 80:20, 90:10, 100: 0 added respectively.

A total of 500 mg of the extract is diluted with eluent and inserted into the column. Kran slightly opened, and eluent accommodated. Then carried out elution and eluates were collected in a sample bottle 2 mL per bottle, then each eluate was analyzed by TLC. The column stopped if the TLC plate already there is no staining. The results tested on plates Thin Layer Chromatography (TLC) will give the value of Rf (Retention factor) specific. The results Thin Layer Chromatography (TLC) that have similar Rf collected in the same fraction, namely fraction I (FI) and Fraction II (FII). Results maceration with methanol and fractionation some solvents such as n-hexane, ethyl acetate, and n-butanol, column chromatography (FI and FII) used for testing the activity of α-glucosidase inhibition.

2.6. Thin Layer Chromatography (TLC) [12].
Chromatography is performed to determine the profile TLC combined fractions obtained from the column. Eluent included approximately 10 mL into a vessel chromatography to high solvent reaches 0.5 cm to 1 cm, sealed and left until the system reaches equilibrium. Eluent used was ethyl acetate: chloroform = 8: 2. Spotted TLC plates separated by a distance of approximately 1.5 cm between the solutions of the substance examined. Then the plate is inserted in a vessel and sealed until the eluent propagate to a certain limit (approximately 0.2 cm below the top plate). After the eluent reached to limit, the plates dried by aerated. Spotting observed under UV 365 nm. Each result that showed the same value Rf (Retention factor) compacted then evaporated for further testing.

2.7. Analysis Using FTIR [13].
A total of 1 mg of extracts and fractions that have the greatest antidiabetic activity was mixed with 100 mg of KBr powder with a mortar agate or "vibrating ball mill" until homogeneous. After that the sample is placed on the FTIR spectrophotometer. Then recorded the spectrum at wave number 4000 - 450 cm\(^{-1}\) and analyzed further.

2.8. Spectroscopy Analysis Using LCMS
A total of 1 mg of extracts and fractions most active leaf Namnam (Cynometra cauliflora) test sample dissolved in methanol, and then injected as much as 20 mL into the LCMS system ESI (Electrospray Ionisation) models of positive ions, C18 column (RP18) superco column length 50 mm , internal diameter 2.1 mm column, particle size of 1.7 μm with a flow rate set 0.4 mL / min, the column temperature 40°C, 5μL injection with a span of 10 minutes. The measurement results presented in graphical form LC and MS.
3. Result And Discussions

3.1. Result of Thin Layer Chromatography (TLC).

Results of a preliminary analysis phytochemical test against leaf extract Namnam (*Cynometra cauliflora*) indicate that the extraction of maceration (methanol) and liquid-liquid (n-butanol) shows that it contains secondary metabolites include saponins, steroid or triterpenoid, phenolics, flavonoids, tannins, and quinones [3], [14]. At the same research has also found that the total phenolic and flavonoid methanol extract of leaves Namnam respectively 1.0096 ± 0.1170 mg GAE/g sample and 0.0962 ± 0.0049 mg QE / g sample (Sumarlin et al. 2015) and n-butanol extract of 52.47 ± 0.26 mg GAE/g sample and 4.1392 ± 0.3245 mg QE/g sample [14].

Results of thin-layer chromatography analysis of the butanol extract of leaves Namnam show eluent acetone: petroleum ether (7: 3 v/v) using UV365 rays obtained 3 spots at Rf respectively 0.825, 0.5 and 0.375 (*Figure 1a*). Each spot separated with both the looks of the value of Rf (Retention factor) which is a short distance. That showed the eluent acetone: petroleum ether (7: 3) can separate the compounds contained in extracts of butanol, which can be used as a mobile phase in the separation using column chromatography.

![Figure 1](image)

*Figure 1*. (a). TLC of Fraction n-butanol with Eluent Acetone : Petroleum Ether 7 : 3, (b). TLC from Column Chromatography Separation Results

The n-butanol extract was fractionated again using column chromatography. The mobile phase used was eluent provide optimal separation on TLC namely acetone: petroleum ether (7: 3 v/v). Meanwhile, the column chromatographic separation obtained 16 fraction A-P with eluent ethyl acetate: chloroform (8: 2 v/v) (*Figure 1b*). The results identified of these fractions show the similarity Rf fraction of A-J (0.7; 0.45; and 0.18) and fraction of K-P (0.65 and 0.375). Based on the similarities of the Rf, then that sample to merged into fractions I (A-J) and fraction II (K-P). Results of research Sumarlin et al. [14] also showed that the inhibition of α-glucosidase with value the highest according IC$_{50}$ is fraction I, IC$_{50}$ 54.30 ± 2.13 µg/ mL while the n-butanol extract IC$_{50}$ of 1.84 ± 0.88 µg/mL. This fraction is characterized by using FTIR and LCMS and compared with the fractionation pattern of n-butanol (Before column chromatography).

3.2. Results Identification of Functional Groups with FTIR spectrophotometer

FTIR spectroscopy has been widely used as one of the techniques used to analyze the components of bioactive products of natural materials [15]. The results of the characterization of functional groups by FTIR spectrophotometer showed the sample extract n-butanol and, comparison quercetin alike showed their functional groups O-H, C=C aromatic, C-O, C-OH cyclic, and = C-H (Table 1). The same thing when compared with Fraction I. However, in Fraction I did not show any C-OH cyclic. The assumption that because Fraction I is the separation of n-butanol extract so that the compound has a cyclic group C-OH separate and not detected at Fraction I (*Figure 2*).

Nevertheless, the existence of these groups showed a flavonoid compound. The data is consistent with the statement of Akbar [16], which concluded that the isolates from leaves Dandang gendis (*Clinacanthus nutans*) are a flavonoid compound shown by the presence of functional groups OH,
C=O, CO, C=C aromatic, and CH aliphatic recorded the FTIR spectrum. Even at a Fraction I showed weak absorption in wave number 1710.93 cm\(^{-1}\) indicate the presence of C = O group. According Sukadana [17] the carbonyl group is a common characteristic their flavonoid compound. According Easmin et al. [15] reported that functional groups such as -CHO, -COOH, -NO\(_2\), -NH and -OH induce inhibitory activity, especially inhibition of \(\alpha\)-glucosidase.

![Figure 2. Spectrum FTIR:Before Column Chromatography (extract n-butanol), After Column Chromatography (Fraction I)](image)

| Sample          | Numbers wave (cm\(^{-1}\)) | Reference (cm\(^{-1}\)) | Functional groups                              |
|-----------------|-----------------------------|--------------------------|-------------------------------------------------|
| Extract n-butanol | 3452,73                     | 3680-3650                | O-H Streching (bonded)                          |
|                 | 2925,17                     | 2850-2960                | CH\(_3\), \,-CH\(_2\)- Streching               |
|                 | 1717,68                     | 1690-1766                | C=O Streching                                   |
|                 | 1614,49; 1519,01            | 1500-1600                | C=C Streching aromatic                           |
|                 | 1447,64                     | 1350-1470                | C-H Bending aliphatics                          |
|                 | 1125,51                     | 1080-1300                | C-O Streching                                   |
|                 | 1006,89                     | 990-1060                 | C-OH Streching cyclic                           |
|                 | 677,04                      | 675-870                  | C=C Bending (aromatic substituted)              |
| Fraction I      | 3334,1                      | 3680-3650                | O-H Streching (Bondend)                         |
|                 | 2929,03                     | 2850-2960                | CH\(_3\), \,-CH\(_2\)- Streching               |
|                 | 1710,93                     | 1690-1760                | C=O Streching                                   |
|                 | 1628,95; 1517,08            | 1500-1675                | C=C Streching aromatic                           |
|                 | 1451,50; 1370,48            | 1350-1470                | C-H Bending aliphatics                          |
|                 | 1267,29; 1083,08            | 1080-1300                | C-O Streching                                   |
|                 | 818,82                      | 675-870                  | C=C Bending (aromatic substituted)              |
| Quercetin       | 3413,19                     | 3680-3650                | O-H Streching (bonded)                          |
|                 | 1666,57                     | 1647-1760                | C=O Streching                                   |
|                 | 1610,63; 1563,37; 1524,79   | 1500-1675                | C=C Streching aromatic                           |
|                 | 1265,36; 1203,63; 1170,84; 1133,23; 1095,61 | 1080-1300                | C-O Streching                                   |
|                 | 1014,60; 940,34             | 990-1060                 | C-OH Streching cyclic                           |
|                 | 869,93; 820,75; 726,33      | 675-870                  | C=C Bending (aromatic substituted)              |
According to the study, Jo S-H et al. [18] quercetin has the ability of α-glucosidase inhibitory activity. Research results also explain that quercetin had significant results in inhibits α-glucosidase compared to acarbose, which already used as antidiabetic drugs. Another reason the use of quercetin as a comparison, that is because the enzyme α-glucosidase used in the study came from Saccharomyces cerevisiae, so if you use acarbose as a comparison to be less sensitive to inhibit the activity of the enzyme α-glucosidase, this is because acarbose more active in inhibiting the enzyme α-glucosidase derived from mammals than α-glucosidase enzyme derived from bacteria and yeast [19]. Febriyanti [20] were able to compare the α-glucosidase activity of quercetin and acarbose with IC_{50} values in a row at 3.47 ppm and 129.75 ppm.

![Figure 3. Spectrum FTIR :Quercetin Standart](image)

3.3. Results Analysis Using LCMS
The results of LCMS analysis of n-butanol extract showed the separation of the three peaks (Figure 4) and, a compound suspected in this fraction also dominated by flavonoid compounds (Table 2). Analysis of the n-butanol extract can be presumed that the compound has inhibitory activity against α-glucosidase is fraxetin and oenin. Shen et al. [21] suggested that fraxetin derivative (7,8-dihydroxy-6-metoxicumarin) be developed as an inhibitor of α-glucosidase. Murali et al. [22] also suggested that fraxetin can be used to treat diabetes and potentially as antihyperglycemic.

![Figure 4. Chromatogram LCMS Extract n-butanol](image)

Similarly, derivatives oenin (malvidin-3-glucosidase) have demonstrated their potential as antidiabetes because it is able to inhibit α-amylase (IC_{50} = 1.91 (1.89 to 1.94) mg/mL) [23]. Additionally, Everette et al. [24] showed that the derivative is oenin (malvidin) can inhibit α-glucosidase 33.54%.
Table 2. Results of Analysis of Active Compounds LCMS n - butanol extract

| Retention Time | Molecular Mass [M+H]^+ | Reference (m/z) | Compounds | Formula Molecule |
|----------------|------------------------|-----------------|-----------|-----------------|
| 0.31           | 217,0641               | [25]            | Xanthotoxin | C_{12}H_{16}O_{5} |
| 0.98           | 209,1487               | [25]            | Fraxetin   | C_{10}H_{16}O_{6} |
| 9.83           | 494,5849               | [26]            | Oenin      | C_{23}H_{24}O_{12} |

The results of LCMS analysis of Fraction I showed several peaks (Figure 5), this means the first faction is generated by column chromatography not pure. Based on data from LCMS, chromatogram obtained several compounds that can identify. The identification based on mass spectra recorded on specific retention time (Figure 5).

The results of LCMS analysis of the faction I also dominated by the compound of the flavonoid (Table 3). Research Tadera et al. [6] report that the group anthocyanin flavonoids, flavonols, flavones, isoflavones, and flavanones were able to inhibit the activity of the enzyme α-glucosidase and α-amylase.

Table 3. Results of Analysis of Active Compounds LCMS Fraction I

| Retention Time | Molecular Mass [M+H]^+ | Reference (m/z) | Compounds          | Formula Molecule |
|----------------|------------------------|-----------------|--------------------|-----------------|
| 0.31           | 277,0899               | [25]            | Capensine          | C_{4}H_{16}O_{6} |
| 3.52           | 273,1343               | [25]            | Naringenin         | C_{13}H_{12}O_{5} |
| 6.30           | 332,2078               | [26]            | Malvidin           | C_{12}H_{16}O_{7} |
| 7.39           | 288,2544               | [26]            | Cyanidin           | C_{15}H_{18}O_{6} |
| 7.82           | 411,3470               | [25]            | Amorphigenin       | C_{23}H_{22}O_{7} |
| 8.00           | 403,2335               | [27]            | Nobiletin          | C_{12}H_{22}O_{8} |
| 8.24           | 317,2879               | [26]            | Isonhamnetin       | C_{16}H_{12}O_{7} |
| 8.31           | 459,3504               | [26]            | Epigallocatechin Gallate | C_{22}H_{16}O_{11} |
| 8.42           | 271,1538               | [26]            | Apigenin           | C_{15}H_{10}O_{5} |
| 9.41           | 494,5566               | [26]            | Oenin              | C_{23}H_{24}O_{12} |

The results of the identification of compounds with a spectrophotometer LCMS Fraction I discovered several compounds putatively inhibit α-glucosidase namely malvidin, IC_{50} 0.36 mg / mL [28], naringenin, IC_{50} 6.52 mg / mL [29], and cyanidin, IC_{50} of 0.04 mg / mL [30]. Akkarachiyasit et al. [31] states that cyanidin-3-rutinoside can slow the absorption of carbohydrates through the inhibition of pancreatic α-amylase so that the potential for the prevention and treatment of diabetes mellitus.
Figure 6. Structure of Compound Malvidin (18), Cyanidin (19), Naringenin (20)

Figure 6 shows that all of the carbon atoms form a ring A, B, and C, undergo sp² hybridization forms that allow these atoms to form a large conjugated system. Thus suspected that the large conjugated system framework (large-conjugated system) is required to inhibit α-glucosidase [32].

Zhang et al. [33] through his research interest Callistephus chinensis discovered several compounds that have the ability as α-glucosidase inhibitors such as those found in this study include apigenin (IC₅₀ 25.47 μg/mL), apigenin-7-O-β-D-glucoside (29.73 μg/mL), and naringenin (16.97 μg/mL). Cazarolli et al (2009a; 2009b) have also found that apigenin derivatives such as apigenin-6-C-(2"-O-α-L-ramnopiranosil)-β-L-fucopyranoside and apigenin-6-C-β-L-fucopyranoside can act as insulin secretagogues or agents insulin mimetic. Tambunan et al. [34] also state that the rod Brotowali (Tinospora crispa Miers) contains flavonoids such as apigenin O-glycoside so as antidiabetic potential through α-glucosidase inhibitory activity.

Figure 7. Structure of Compound Capensine (8), Naringenin (9), Malvidin (10), Cyanidin (11), Amorphigenin (12), Nobiletin (13), Isorhametin (14), Epigallocatechin Gallate (15), Apigenin (16), Oenin (17).
The relationship structure of flavonoids to the inhibitory activity of α-glucosidase can be summed up into three things, among others, (1) a hydroxyl at position 3 of the flavonoids play an important role in inhibiting the activity of α-glucosidase, this explained on the compound naringenin does not have the 3-OH show the weaker inhibition with IC$_{50}$ value of 6.51 mg/mL compared malvidin compound (IC$_{50}$ = 0.36 mg/mL) and cyanidin (IC$_{50}$ = 0.04 mg/mL). (2) inhibition of α-glucosidase activity increases based on the number of hydroxyls contained in ring C, 4-hydroxylated (malvidin and naringenin) have IC$_{50}$ values were higher than 4'-5'-dihydroxylatic (cyanidin). (3) A group of sugar substituted at position C-3 flavonoid does not give effect to the inhibition of α-glucosidase [32].

![Figure 8. Scheme Assumption Mechanism Active Substance with α-glucosidase](image)

Damazio et al. [35] suggest that flavonoids can inhibit the activity of α-glucosidase, unsaturated ring C, 3-OH, 4-CO, linked to the B ring at position 3, and the substitution hydroxyl of ring B. The results Easmin et al. [15] concluded that polyphenols, flavonoids, carbohydrates, sterols, terpenes amines and allegedly contributed to the inhibition of α-glucosidase extract of P. macrocarpa. Furthermore, Li et al. [36] that the C-3'-OH of the B ring flavones, which cause increase in α-glucosidase inhibitory activity, whereas the glycosylated C on C-6 or C-8 of the A ring weaken inhibitory activity.

According to Kim et al.[37], most of the α-glucosidase inhibitors work by mimicking the position of the transition unit piranosidik of natural glucosidase substrate, so that the mechanism of inhibition is thought to be a competitive inhibition. In this case the α-glucosidase inhibitor bound to the enzyme through the formation of covalent bonds. The scheme modeling mechanism between compounds with α-glucosidase inhibitor can looked in Figure 8.

α-Glucosidase inhibitor compounds such as flavonoids work inhibiting the hydrolysis reaction disaccharide into glucose. In the early stages of a carboxylic acid residue, alkaline will form hydrogen bonds with the hydroxyl group at C ring flavonoids and experienced stabilization through hydrophobic interactions between rings C and Leu313, so that the formation reaction of glucose and other monosaccharides can be slow [38]. Thus flavonoids will work to depend on amount, the type and position of components glycosyl residue of natural materials [39].

4. Conclusion

Based on the results of the characterization of the leaf extract Namnam (Cynometra cauliflora) with FTIR (Fourier Transform Infrared) and LCMS (Liquid Chromatography Mass Spectrometry), it can be concluded that Leaves Namnam (Cynometra cauliflora) extract in liquid-liquid extraction with n-butanol or column chromatography process is dominated by the flavonoid compound. The flavonoid compounds that assumption have α-glucosidase inhibitory activity is fraxetin and oenin (n-butanol extract) and naringenin, malvidin, cyanidin and apigenin (Extracted Fraction I of column chromatography). And the plant leaves Namnam (Cynometra cauliflora) has a wide variety of flavonoids that potential to be developed as a natural therapy of diabetes mellitus through the mechanism of inhibition of α-glucosidase.
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