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INTRODUCTION Melothria scabra Naudin belongs to the Cucurbitaceae family. It was originally from Mexico and Central America but has spread throughout Asia, including Indonesia. The plant also has some synonym names, which are M. donnell-smithii Cogn. Ex Donn.Sm., M. donnell-smithii var. hirtella Cogn., and M. donnell-smithii var. rotundifolia Cogn1. The fruit of M. scabra is slightly bittersweet and has a unique aroma. Morphologically, the fruit is oval with 3.5 in length and 2.5 cm in wide. It resembles a miniature version of watermelon and is locally known as "balongga" (Kolaka and Konawe) or "balongkha" (Buton).

The fruit is consumed directly or processed as pickles and vegetables by natives of Southeast Sulawesi; hence, it is often found in local markets2. In Sulawesi medicinal folklore, it is used to reduce blood pressure, while in Pakistan, it is applied as a mosquito repellant3. A previous study has reported the phytochemical screening and anti-diabetes activity of the ethanol extract of its leaves4. In the present study, we reported the phytochemical constituents of the ethanol extract of the fruits analyzed using LC-MS/MS spectrometry and evaluated the antioxidant activity and organic fractions using total phenolics, total flavonoids, and 2,2-diphenyl-1-picrylhydrazyl radical assays. The study of the fruits of M. scabra is reported for the first time in this paper. MATERIALS AND METHODS Materials Chemicals Methanol, ethyl acetate, and n-hexane were technical grades and distilled before being used.

Ethanol 96% was purchased locally. Folin-Ciocalteu reagent, aluminum chloride, gallic acid, quercetin, ascorbic acid, dimethyl sulfoxide (DMSO), and thin-layer chromatography (TLC) plate (silica gel 60 GF254, 0.25 mm) were purchased from Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from HiMedia (Mumbai, India). Plant material Fresh fruits of M. scabra (3.329 kg) were collected from Tanggetada District of Kolaka Regency, Southeast Sulawesi, Indonesia, in July 2021. The morphology of fruits, leaves, flowers, and seedlings of M. scabra was presented in Figure 1.

The fruit sample was identified, and its specimen was deposited at the Laboratorium Terpadu USN Kolaka with voucher number MS001. The collected fruits were washed with tap water and drained. Cleaned fruits were slidely cut and dried using the oven at 40°C for four days. Then, the dried samples were coarsely ground using a blender, weighted, and stored in an airtight plastic sealer until used. / Figure 1. Fruits (A), leaves (B), flower (C), and seedling (D) of M. scabra Methods Extraction and fractionation The coarsely powdered fruits of M. scabra (295 g) were macerated using ethanol 96% for 3 x 48 hours at room temperature.

The liquid extract was filtered using Whatman filter paper and concentrated using a

vacuum rotary evaporator, yielding a yellowish-brown crude ethanol extract. For fractionation using three different polarities of solvents (methanol, ethyl acetate, n-hexane), 10 g of crude ethanol extract was firstly reconstituted in methanol (250 mL) and successively partitioned with n-hexane and ethyl acetate each for 3 x 250 mL in a separating funnel. All solvents were then evaporated to yield organic fractions of methanol, ethyl acetate, and n-hexane. Both extract and organic fractions were stored in amber bottles and kept in the refrigerator at 4°C until used.

Phytochemical screening The phytochemicals contained in ethanol extract and organic fractions of M. scabra were screened according to our previous method5. The screening was carried out to detect the presence of alkaloids, tannins, flavonoids, terpenoids, steroids, and saponins. LC-MS/MS analysis The LC-MS/MS measurement of the ethanol extract of M. scabra was in collaboration with the Indonesian Institute of Sciences (LIPI). The method was adopted from our previous study with modifications6. The chemical constituents in the ethanol extract of M.

scabra were separated using a reverse-phase column (Waters HSS T3, 2.1 x 100 mm, 1.8 μm) and gradiently eluted with water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) at a flow rate of 0.3 mL/minute and column temperature of 40.0°C. The elution time was running for 16 minutes as follows: 95% A (0–1 minutes); 60% A (8 minutes); 100% B (11–13 minutes); and 5% B (16 minutes). The separated peaks were detected using a Xevo G2-XS QTof mass spectrometer. The mass experiments were carried out using a positive mode electrospray ionization (ESI) with acquisition parameters as follows: capillary voltage, 2 kV; cone voltage, 30 V; source temperature, 120°C; desolvation temperature, 500°C; cone gas flow, 50 L/h; desolvation gas flow, 1000 L/h. Experiments were carried out in two functions of MSE. Function one was carried out at a high CE ramp of 10 to 40 eV, cone voltage of 30 V, and collision energy of 6 eV.

Meanwhile, function two was carried out at a high CE ramp of 10 to 40 eV, cone voltage of 30 V, and collision energy of 10 to 40 eV. For both functions, the acquisition time ran for 16 minutes with detection of mass-to-charge ratio (m/z) starting from m/z 50 to m/z 1200. The obtained m/z values of separated peaks were identified using the UNIFI software and analyzed using online mass databases, including the MetFrag, METLIN, PubChem, mzCloud dan ChemSpider.

Total phenolics assay According to our previous study with modifications5,7, total phenolics in the ethanol extract and organic fractions of M. scabra fruits were evaluated spectrophotometrically using the Folin-Ciocalteu (FC) method. A linear gallic acid standard curve was first established within a concentration range of 6.25 to 100 μg/mL

versus absorbance of molybdenum-tungsten blue complex at 635 nm. Equation 1 obtained as follows: ??????= 0.0511×[???????????? ???????? ???? ???? ????] +0.1036 ?? 2 =0.990 [1] Stock solutions of samples (10 mg/mL) were prepared in DMSO, and the final concentration in the reaction mixture was 1 mg/mL. A volume of 0.2

mL of sample solution was triplicately transferred into vials and incubated with 0.2 mL of FC reagent for 5 minutes at room temperature. The mixture was then added with 0.8 mL NaHCO3 solution (7.5%, w/v) and re-incubated for 30 minutes in the dark at room temperature. The absorbance was read against a sample blank containing sample solution and water. The total phenolics in the sample were obtained by the interpolation of absorbance in the equation and expressed as mg of gallic acid equivalent for each gram of sample (mg GAE/g).

Total flavonoids assay Total flavonoids content in the ethanol extract and organic fractions of M. scabra fruits was evaluated spectrophotometrically using the aluminum chloride method as previously reported with modifications5,7. A linear quercetin standard curve was first established within a concentration range of 1.56 to 100 µg/mL versus the absorbance of the yellow complex at 425 nm. Equation 2 obtained as follows: ??????= 0.0799×[?????????????????? ???? ???? ????] -0.0688 ?? 2 =0.999 [2] Stock solutions of samples (10 mg/mL) were prepared in DMSO, and the final concentration in the reaction mixture was 1 mg/mL.

A volume of 1 mL of sample solution was triplicately transferred into vials and incubated with 1 mL of AlCl3 solution (2%, w/v) for 15 minutes at room temperature. The absorbance was read against a sample blank containing sample solution and water. The total flavonoids in the sample were obtained by the interpolation of absorbance in the equation and expressed as mg of quercetin equivalent for each gram of sample (mg QE/g). DPPH assay DPPH assay was carried out to determine the radical scavenging activity of ethanol extract and organic fractions of M. scabra fruits towards free radicals5,7.

The assay was performed qualitatively using dot-blot staining on TLC plate and quantitatively using the spectrophotometric method. For the qualitative assay, samples solutions (2 mg/mL) were prepared in their soluble solvents and spotted (20 µL) on a TLC plate with a final concentrations range of 6.2 to 100 µg/spot. After drying the solvents, the TLC plate was dipped into DPPH methanolic solution (0.5 mM) for 10 seconds, shortly dried using an air-dryer, and incubated for 30 minutes in the dark at room temperature.

The radical scavenging activity was analyzed by observing the clear zone around the

spot under daylight against the purple background of DPPH. For quantitative analysis, samples stock solutions (10 mg/mL) were prepared in DMSO and serially diluted into eight concentrations ranging from 100 to 0.8 µg/mL. Triplicate volumes (1 mL) of diluted solutions were transferred into vials and incubated, each with 1 mL of DPPH solution (0.25 mM) for 15 minutes in the dark at room temperature.

The absorbance was recorded at 515 nm against samples and blank containing DMSO. Sample absorbance (Abssample) was obtained after blank sample correction. The 50% radical scavenging concentration (SC50) values of ethanol extract, organic fractions, and positive controls (ascorbic acid, gallic acid, quercetin) were calculated using GraphPad Prism 5 software (GraphPad Inc., California, US). The radical scavenging activity (%RSA) was calculated using Equation 3 as follows: %??????= ?????? ???????? ?????????????? - ?????? ???????????? ?????? ???????? ?????????????? ×100 [3] Statistical analysis Data for all assays were presented as mean ± standard deviation (SD), which was obtained from three replications. Data were statistically analyzed using the GraphPad Prism 5 Software. Differences among groups were evaluated using Analysis of Variance (ANOVA) with Tukey's Test (p <0.05).

The significances among parameters (total phenolics, total flavonoids, DPPH) were measured using the Pearson correlation8. RESULTS AND DISCUSSION Extraction yield and phytochemical screening The extraction of fruits of M. scabra was done by using ethanol 96% using the maceration technique. Aqueous ethanol was considered a less toxic solvent for further in vitro and in vivo studies based on cells and animal models9. The extract was obtained as a yellowish-brown gum with a percentage yield of 16.8%, showing a high number of polar compounds. This amount was observed in the yield of methanol fraction with a percentage yield of 67.9% compared to the ethyl acetate (27.0%) and hexane (5.0%) fractions, as presented in Table I. Fruits of M. scabra also showed the presence of alkaloids, tannins, flavonoids, terpenoids, and saponins. Meanwhile, steroids were not detected, probably due to their low amounts.

Distributions of all present groups of compounds varied among organic fractions, but alkaloids, terpenoids, and saponins seem dominants. Flavonoids represented as antioxidants10 were detected in polar and semipolar solvents extraction, including ethanol extract, methanol, and ethyl acetate fractions. The occurrence of alkaloids and flavonoids has been reported from the leaves of this plant, along with glycosides, carbohydrates, and proteins4. Table I. Phytochemical groups detected in the ethanol extracts and organic fractions of M.

scabra fruits Samples _Yield (g) _Phytochemical groups _ _Ethanol extract _49.53 _Alkaloids, tannins, flavonoids, terpenoids, saponins _ _Metanol fraction _6.79 _Alkaloids,

tannins, flavonoids, terpenoids, saponins _ _Ethyl acetate fraction _2.70 _Alkaloids, flavonoids, terpenoids, saponins _ _n-hexane fraction _0.50 _Alkaloids, terpenoids, saponins _ _ Identification of chemical constituents The chromatographic separation of chemical constituents in the ethanol extract of M. scabra fruits was analyzed using the ultra-performance liquid chromatography (UPLC) method, which separated various peaks starting from retention time (Rt) of 1.5 to 12.5 minutes. Some major peaks were observed at the initial retention time from 1.5

to 2.5 minutes, indicating the composition of polar compounds. The UPLC chromatogram of these peaks and other minors is displayed in Figure 2. Further detection using a mass detector yielded the separated peaks' mass-to-charge ratio (m/z). As many as six compounds (1-6) were successfully identified from the ethanol extract of M. scabra fruit, and most were polar compounds (1-5). The LC-MS/MS identification of these compounds was displayed in Table II, and their two-dimensional structures were illustrated in Figure 3. / Figure 2. UPLC chromatogram of separated peaks of compounds in the ethanol extract of M.

scabra fruits Two major peaks were observed at the retention time of 1.7 and 1.80 minutes, having protonated pseudo-molecular ion of m/z 266 and m/z 1.80. The fragmentation of these ions resulted in the identification of compounds 1 and 2, classified as Heyns compounds containing reducing sugar (fructose) and amino acid11. As far as our concern, both compounds have yet previously reported from plants of the Cucurbitaceae family but found as the main components in the jujube (Rhamnaceae) and mulberry (Moraceae) fruits analyzed using a high-sensitive HPLC-ESI-Q-ToF-MS/MS12.

Compound 1 was also found in the cured tobacco leaves (Solanaceae) as well as stored apricots and peaches fruits (Rosaceae)13. Meanwhile, compound 2 was predicted as a product of the Maillard reaction of fructose with primary or secondary amines, in which its exact structure needs further identification12. Table II. Chemical constituents of the ethanol extract of M. scabra fruits identified using LC-MS/MS Rt (min) _(+)-ESI _Identified constituents _MS/MS fragmentation (m/z) _ _1.73 _266.12362 [M+H]+ _D-1-[(3-Carboxypropyl) amino]-1-deoxyfructose (1) C10H19NO7 MW 265.2604 _248/206/104 _ _1.80 _234.09724 [M+H]+ _Fructose-C3H5NO (2) C9H15NO6 MW 233.2185 _216/159 _ _1.85 _118.08588 [M+H]+ _Valine (3) C5H11NO2 MW 117.14634 _102/100/86/84 _ _1.93 _305.13435 [M+Na]+ _1ß,3a,9ß-Trihydroxyeudesma-5,11(13)-dien-12-oic acid (4) C15H22O5 MW 282.3322 _305/227/191 _ _7.85 _865.42170 [M]+ _Cucurbitacin B-2-O-a-L-rhamnopyranosyl-ß-D-glucopyranoside (5) C44H66O17 MW 866.9846 _663/501/483/465/ 441/336/177 _ _10.27 _260.16444 [M+H]+

_2-Heptyl-3-hydroxy-4(H)-quinolone (6) $C_{16}H_{21}NO_2$ MW 259.3434 _173/146 _ _ The fruits of M. scabra also produced a significant count of an essential amino acid valine (3), observed as one of the major peaks at a retention time of 1.85 minutes and had an m/z 118 [M+H]+. Hence, the fruits of M.

scabra could be developed as a source of nutritive essential amino acids. Another major peak was observed at a retention time of 1.93 minutes, with an adducted ion m/z 305 [M+Na]+. The compound was identified as trihydroxyeudesma-5,11(13)-dien-12-oic acid (4) and classified as a sesquiterpenoid. The presence of this compound might be attributed to a sweet and unique aroma of the fruits of M. scabra. _ Figure 3. Chemical compounds identified in the ethanol extract of M. scabra fruits The occurrence of a saponin (5) was detected at a retention time of 7.85 minutes and had an m/z 865 [M]+.

The fragmentation of this compound yielded fragment ions at m/z 663, 603, and 501 (Figure 4), which correspond to the successive eliminations of rhamnosyl and acetyl groups and fission of a glucose ring, following its loss (Figure 5). The loss of the acetyl group (-C_2H_3O) suggested the main skeleton of this compound as cucurbitacin B glycoside14,15, which is further confirmed by fragment ions at m/z 483 and 465 due to the loss of two hydroxyl groups. The cucurbitacins (curcubitane-type) are highly oxygenated tetracyclic triterpenoids with a wide range of promising biological activities, including cytotoxicity, hepatoprotective, antidiabetic effects, cardiovascular protection, antioxidant, and anti-inflammatory15.

This group of compounds is considered as chemical markers for the Cucurbitaceae family16. / Figure 4. LC-MS/MS spectrum of compound 5 _ Figure 5. The plausible MS2 fragmentation of compound 5 Compound 5 was further identified as cucurbitacin B-2-O-a-L-rhamnopyranosyl-ß-D-glucopyranoside. The occurrence of cucurbitacin B-2-O-ß-D-glucopyranoside has been reported from the leaves of Citrullus colocynthis and showed the inhibitory effect of the growth of human breast cancer cells17. However, the occurrence of compound 5 as cucurbitacin B diglycoside has not been previously reported. Nonetheless, its exact structure needs further studies by isolation and identification using more spectroscopic measurements.

One non-polar peak at a retention time of 10.27 minutes had an m/z value of 260 [M+H]+ as the base peak. The peak showed fragment ions at m/z 173 and 146, corresponding to the loss of heptyl and hydroxyl side groups from the quinolone skeleton. This peak was identified as 2-heptyl-3-hydroxy-4(H)-quinolone (6) and belonged to the quinolone alkaloid group. The occurrence of compound 6 in the fruits of M. scabra is considered unique since the compound has been acknowledged as a quorum sensing (QS) produced by plant growth-promoting bacteria such as

Pseudomonas and Paenibacillus species.

We suggested compound 6 as a diffusible signal in communication between M. scabra and soil bacteria, resulting in the protection of the fruits from other pathogenic bacteria and fungi since they are growing on the soil's surface18. In addition, as a QS, quinolones were also produced by plants of the Rutaceae family which exhibited remarkable antimicrobial activity19. Hence, further studies on the antimicrobial activity of M. scabra fruits could be developed. Total phenolics and total flavonoids The fruits of M. scabra contained a high amount of phenolics, especially in the methanol and ethyl acetate fractions (Table III).

Moreover, the ethyl acetate fraction contained more flavonoids than other fractions, indicating the accumulation of semi-polar flavonoids in the ethanol extract of M. scabra fruits20. High contents of phenolics and flavonoids might be contributed to the antioxidant activity10. Table III. Total phenolics and total flavonoids in the ethanol extract and organic fractions of M. scabra fruits Samples _Total phenolics (mg GAE/g, mean ± SD, n =3) _Total flavonoids (mg GAE/g, mean ± SD, n =3) _ _Ethanol extract _86.3 ± 3.4 _15.5 ± 0.3 _ _Metanol fraction _259.1 ± 8.4 _17.2 ± 0.4 _ _Ethyl acetate fraction _242.9 ± 3.3 _22.4 ± 0.2

_ _n-hexane fraction _54.2 ± 2.4 _1.6 ± 0.2 _ _ DPPH radical scavenging activity The ability of M. scabra fruits to scavenge DPPH radicals was evaluated using qualitative and quantitative methods. Qualitatively, potent scavenging activity against DPPH radical was shown by the ethanol extract, methanol, and ethyl acetate fractions compared to ascorbic acid as the positive control (Figure 6). Meanwhile, the hexane fraction showed weak antioxidant activity. Their activities were concentration-dependent at a concentration range of 100 to 6.2 µg/spot. / Figure 6. DPPH dot-blot staining of ethanol extract and organic fractions of M.

scabra fruit In line with the qualitative assay, the ethanol extract and organic fractions (methanol and ethyl acetate) of M. scabra fruits quantitatively exhibited potent antioxidant activity compared to the hexane fraction (Table IV). The ethyl acetate fraction was considered the most potent radical scavenger with an SC50 value of 20.7 µg/mL, followed by the methanol fraction (SC50 28.6 µg/mL) and the ethanol extract (SC50 37.5 µg/mL). As the positive controls, their activities were comparable to ascorbic acid, gallic acid, and quercetin. Table IV. DPPH radical scavenging activity of the ethanol extract and organic fractions of M.

scabra fruits Samples _%RSA (at 100 µg/mL, mean ± SD, n =3) _SC50 (µg/mL) _ _Ethanol extract _62.7 ± 0.6* _37.5 _ _Metanol fraction _67.6 ± 0.9* _28.6 _ _Ethyl acetate fraction

_80.5 ± 2.0* _20.7 _ _n-hexane fraction _49.1 ± 0.2* _476.3* _ _Ascorbic acid _98.8 ± 0.6 _2.8 _ _Gallic acid _97.2 ± 0.3 _3.9 _ _Quercetin _98.1 ± 0.6 _9.4 _ _*Values are significantly different compared to positive controls (p <0.05) based on Tukey's test. The correlations of phenolics and flavonoids in the fruits of M. scabra at high concentration (100 µg/mL) were considered moderate analyzed using the Pearson correlation with r values of 0.8274 (p = 0.1726) and 0.8226 (p = 0.1774), respectively (Table V). However, these contents found not to correlate with the SC50 values (r = -0.6920, p = 0.3080 for phenolics and r = -0.3695, p = 0.6305 for flavonoids).

Results indicated that phenolics and flavonoids did not solely determine the radical scavenging activity of the ethanol extract and its organic fraction. Other compounds may also contribute to the activity, either scavenging the radical or assisting the radical activity21. Hence, further compounds isolation and structure elucidation as well as assessments of antioxidant activity using other assays, especially in vitro cell-based assay and in vivo assay using animal models, need to be carried out. Table V.

Pearson correlation (r) and linear regression curve (r2) between total phenolics and total flavonoids and DPPH (p<0.05, two-tailed) Content _%RSA (100 µg/mL) _SC50 _ _ _Pearson (r) _Linear curve (r2) _Pearson (r) _Linear curve (r2) _ _Total phenolics _0.8274 _0.6847 _-0.6920 _0.4789 _ _Total flavonoids _0.8226 _0.6766 _-0.3695 _0.1365 _ _CONCLUSION This study concludes that the fruits of M. scabra contained alkaloids, tannins, flavonoids, terpenoids, and saponins.

Among these, six compounds were identified as D-1-[(3-carboxypropyl)amino]-1-deoxyfructose (1), fructose-C3H5NO (2), valine (3), 1ß, 3a, 9ß-trihydroxyeudesma-5,11(13)-dien-12-oic acid (4), cucurbitacin B-2-O-a-L-rhamnopyranosyl-ß-D-glucopyranoside (5), and 2-heptyl-3-hydroxy-4(H)-quinolone (6). These compounds in M. scabra are reported for the first time in this study. The fruits also contained a high amount of phenolics and flavonoids, which moderately correlated to their radical scavenging activity. The ethanol extract, methanol, and ethyl acetate fractions showed potent antioxidant activity, which could be developed as a source of natural antioxidant agents.

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