Phytochemical screening and chemical compound of green roselle (Hibiscus sabdariffa L.) and potential antibacterial activities

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Abstract. Herbal rosella plants (Hibiscus sabdariffa L.) are plants that have great potential in the pharmaceutical field. Indonesian Sweetener and Fiber Crops Research Institute (ISFCRI) has released four superior herbal rosella varieties, including Roselindo-1, Roselindo-2, Roselindo-3, and Roselindo-4. Of the four varieties, Roselindo 3 has different physical characteristics, namely green. The purpose of this study was to determine the chemical and phytochemical compounds contained in the green rosella extract. Green rosella extract was obtained using the Microwave-Assisted Extraction (MAE) method with ethyl acetate as a solvent. The results of phytochemical screening for green roselle extract contained flavonoids, tannins, and triterpenoids. Based on the results of Liquid Chromatograph Mass Spectrometry (LCMS) analysis, green roselle extract contains 323 compounds, including 5-hydroxymethyl-2-furaldehyde, 7-hydroxycoumarine, betaine, curcumin, nicotinamide, and jasmonic acid.

Keywords: green rosella, herbal extract, LC-MS, chemical compound

1. Introduction
Roselle (Hibiscus sabdariffa) is from the Malvaceae family, described as a bushy plant with a height of up to 2.5 m, characterised by smooth, reddish veins, cylindrical red stems, and long green leaves[1,2]. H. sabdariffa is a species of herbal roselle that has potential as a source of functional food, antioxidants, antibacterial, natural dyes, and uses in the health sector [3,4].

Indonesian Sweetener and Fiber Crops Research Institute (ISFCRI) in 2013 released four high-yielding varieties of herbal roselle, namely Roselindo 1 (Red Roselle), Roselindo 2 (Jamaica/Squid Purple Roselle), Roselindo 3 (Green Roselle), and Roselindo 4 (Ordinary Purple Roselle). Green roselle (Roselindo ) comes from Nigeria, morphological descriptions, have green fruit color, dark green epicalic color, dark brown seed color, 1000 seeds weight were 41.82 g(5). Hibiscus sabdariffa flower was used to make teas, jams, and juice. It has a sour flavour and contains high vitamin C, ranging from 188–2,033.52 mg/100 g dried petals [4,6].

Roselle has pharmacological activities including antioxidant[7], antibacterial[8,9], antifungal[10]. Many studies have been conducted on rosella extract, but most of them are on rosella plants with a red calyx color. There has not been much research done on the potential and content of chemical compounds in calyx green roselle. Therefore, the aim of this study was to determine the content of...
potential chemical compounds in green rosella (Roselindo 3) and to determine its potential utilization. The purpose of this study was to screen for phytochemical compounds in green rosella extract and determine the type of compound content by LC-MS/MS analysis.

2. Methodology
2.1. Time and place
This research was conducted in Plant Chemistry Laboratory and Phytopathology Laboratory, ISFCRI (Balittas) Malang, and the Central Laboratory for Biological Sciences (LSIH) Brawijaya University. The research was started in September 2019 – February 2020.

2.2. Material and methods
The materials used in the study included dry roselle powder obtained from ISFCRI (Balittas) Malang. All chemicals, including methanol, petroleum ether, ethyl acetate, n-butanol, diethyl ether, hydrochloric acid (HCl), sulfuric acid (H₂SO₄), ammonia, ethanol, chloroform, ferric chloride, were purchased from Merck (Darmstadt, Germany) in high-grade tissue roll, aluminum foil, plastic wrap, spirit, filter paper, disc paper (Blank disc Oxoid), Escherichia coli and Staphylococcus aureus bacteria.

2.3. Procedure
2.3.1. Plant material and preparation of sample. Plant material was collected from Karangploso Experimental Garden, ISFCRI at Malang district of East Java, Indonesia. Drycalyx of green roselle (Roselindo 3) was collected and ground to powder by using a grinder and shifted into 60 mesh.

2.3.2. Extraction of calyx green roselle (Hibiscus sabdarifa ,L). Dried powder of calyx green roselle samples (4 gram) was placed in a 350 ml Erlenmeyer flask, mix with 40 ml of ethyl acetate. The comparison between the samples (rosella petals powder) and the solvent was 1:10 (w/v). Then it was put into an extraction vessel and then irradiated using a Microwave-Assisted Extraction (MAE) at a temperature of 50°C for 5 minutes. After that, it was filtered using filter paper and concentrated using a 40°C rotary evaporator at a pressure of 60 rpm. An extract of green rosella was obtained.

2.3.3. Phytochemical screening of extract calyx green roselle (Hibiscus sabdarifa ,L). Phytochemical screening of green roselle extract was done qualitatively to detect the existence of alkaloids, saponins, steroids, flavonoids, tannins, and triterpenoids.

2.3.4. Test for alkaloids. Alkaloid compounds can be identified by adding Dragendorff, Mayer, and Wagner reagents to the raw material. The first stage in identifying alkaloids was putting 0.1 g sisal extract in a test tube, then add 2 ml of chloroform and 2 ml of ammonia, shake and add 2N HCl. Divide the solution into 3 test tubes. Next, in each test tube, add reagents in a row, namely Dragendorff, Mayer, and Wagner. Test results with Dragendorff reagents are positive containing alkaloids when red or orange deposits are formed. If it is identified using Mayer reagent, the positivity of containing an alkaloid can be seen when a yellowish-white precipitate is formed, and if using Wagner reagent, the presence of alkaloid can be seen when a brown precipitate is formed [11].

2.3.5. Test of saponins. Saponin compounds can be identified by weighing 0.5 grams of green roselle extract into a test tube. After that, add 2 ml of water until the extract part is submerged and shake it for 10 seconds. If, after shaking, the foam formed in a test tube and for 10 minutes the foam does not disappear, then it can be said that the extract contains saponin compounds [12].

2.3.6. Test of steroids. Green roselle extract (0.5 g) mixed with 10 ml chloroform (CHCl₃) boiled and filtered, and then added 1 ml of CH₃COOH and a few drops of sulfuric acid 37% (H₂SO₄) to the filtrate. A green ring indicates positive of steroid [13].
2.3.7. **Test of flavonoids**: Green roselle extract (0.1 g) was put into a test tube, and then added 0.5 mg of magnesium powder and three drops of concentrated HCl. A positive test for the presence of flavonoids is characterized by a change in the color of the solution to yellow, green, black, or orange [11].

2.3.8. **Test of triterpenoids**: Green roselle extract (1 g) was put in a test tube. Then, added 20 ml of ethanol, 2 ml of chloroform, and 3 ml of H$_2$SO$_4$. A positive test for triterpenoids was characterized by a change in the color of the solution to red [14].

2.3.9. **Test of tannins**: The steps taken to identify the tannin compound were to weigh as much as 0.5 grams of the extract and put it in a test tube. After that, 3 ml of warm water was added, and then 1-2 drops of 1% FeCl$_3$ were added. The extract or sample contains tannin compounds if a dark blue or green-black color is formed [12].

2.3.10. **Analysis of secondary metabolites by LC-MS/MS**. Samples of the green roselle extract were identified as secondary metabolites compound using Liquid Chromatography – High-Resolution Mass Spectrometry (LC-HRMS), and the molecular weight of the compounds present in the extract was processed using mz Cloud MS/MS Library software.

3. **Result and discussion**

3.1. **Phytochemical screening extract calyx green roselle** (*Hibiscus sabdarifa* L.)

Phytochemical screening is presented in Table 1. A phytochemical screening test is a qualitative test and is observed by visual or visual means so that it can determine whether secondary metabolites are present in the sample (positive) or not (negative). Some secondary metabolites such as flavonoids, triterpenoids, and tannins were presented in the extract.

| Phytochemical screening | Extract of Green Roselle |
|------------------------|--------------------------|
| Alkaloids              | -                        |
| Saponins               | -                        |
| Steroids               | -                        |
| Flavonoids             | +                        |
| Triterpenoids          | +                        |
| Tannins                | +                        |

Phytochemical screening of green roselle extract showed positive and negative results, where positive results were on flavonoid compounds, triterpenoids, and tannins. Tannins and their descendants are phenol constituents and have antioxidant activities [15]. The tannin test showed positive result if there is a change in the color of the sample to dark blue or blackish green [11]. Tannins are polymers of phenolic compounds that have an -OH group attached to an aromatic ring carbon [16]. The presence of tannin compounds in the sample has the potential as an antibacterial by precipitating proteins, inactivating bacterial cell adhesion, inactivating enzymes, and attacking cell wall polypeptides so that the formation of cell walls is not perfect [17].

Green roselle extract was also positive for flavonoids which were indicated by a change in the color of the sample to yellow, green, black, red, or orange[11]. Flavonoids are compounds that contain two aromatic rings and more than one hydroxyl group (-OH). The addition of concentrated HCl in the flavonoid test serves to hydrolyze flavonoids into their aglycones. Reduction with magnesium and concentrated HCl, the sample will change color[18]. Flavonoid compounds have antioxidants
activities [15]. Flavonoids have also exhibited a wide range of biological activities, such as antimicrobial, antioxidant, anti-inflammatory, analgesic, anti-allergic and cytostatic properties [19,20].

Phytochemical screening for triterpenoids is characterized by a color change to red, brown, or violet [11]. The color change is due to the oxidation of steroid or triterpenoid compounds through the formation of conjugated double bonds. In the triterpenoid compound test, there was the release of H₂O and the incorporation of carbocations which caused electrophilic addition, followed by the release of hydrogen, where hydrogen and its electrons were released so that they experienced conjugation extension [21].

### 3.2. Analysis of secondary metabolites by LC-MS/MS

Analysis using LC-MS/MS aims to determine secondary metabolites contained in green rosella extract. This analysis will produce two data, namely LC (Liquid Chromatography) and MS (Mass Spectrometry) data. LC (Liquid Chromatography) displays chromatogram data (Figure 1) which contains peaks indicating the compounds contained in the extract. MS (Mass Spectrometry) data shows the molecular weight and molecular structure of each peak that appears. LC/MS-MS is an analytical technique that separates the components of the sample, and then charged ions are detected by the mass spectrometer. LC-MS data was presented by chromatogram and contained information about molecular structure, molecular weight, and quantity of sample components.

![Chromatogram LC-MS/MS extract calyx green roselle.](image)

**Figure 1.** Chromatogram LC-MS/MS extract calyx green roselle.

**Table 2.** Result of LC-MS/MS Analysis of Extract Calyx Green Roselle (*Hibiscus sabdarifa*L.).

| Chemical Compound                  | Chemical Formula | Molecular Weight | RT [min] | Area (Max.) | Mz     |
|-----------------------------------|------------------|------------------|----------|-------------|--------|
| 5-Hydroxymethyl-2-furaldehyde     | C₆H₅O₃           | 126,0311         | 2.073    | 999,029,558,74 | 95,1   |
| 7-Hydroxycoumarine                | C₆H₅O₃           | 162,0307         | 3.066    | 113,631,138,65 | 71,1   |
| Betaine                           | C₂H₁₀NO₂         | 117,0785         | 0.958    | 29,137,035,61 | 93,6   |
| Curcumin                          | C₂₁H₂₂O₆         | 368,1204         | 6.510    | 12,077,328,67 | 74,3   |
| Nicotinamide,                     | C₆H₈N₂O         | 122,0475         | 1.382    | 7,572,839,28  | 82,7   |
| Jasmonic acid                     | C₁₇H₁₉O₅         | 210,1251         | 8.668    | 2,701,051,87  | 81,1   |
In this study, the results of extract analysis using LC-MS/MS showed that green rosella extract contained 323 compounds (Figure 1) which were a mixture of various types of chemical compounds. From the number of compounds identified, six compounds with the largest peak area were selected, namely 5-Hydroxymethyl-2-furaldehyde, 7-Hydroxycoumarine, Betaine, Curcumin, Nicotinamide, Jasmonic acid (Table 2).

Table 2 showed that the six compounds selected from the LC-MS/MS analysis had different molecular weights or mass, identified time (Retention Time), max area, and similarity index (mz cloud). These compounds are bioactive compounds that have antibacterial abilities. Single chromatogram data of each of these compounds can be seen in the figure below.

**Figure 2.** Single chromatogram 5-Hydroxymethyl-2-furaldehyde compound.

5-Hydroxymethyl-2-furaldehyde (C$_6$H$_6$O$_3$) is an organic compound formed by the dehydration of reducing sugars. The molecule contains both aldehyde and alcohol functional groups with a furan ring. Figure 2 shows that the 5-Hydroxymethyl-2-furaldehyde compound analyzed using LC-MS/MS was identified at a retention time (RT) of 2.073 minutes, area (max) 999,029,558.74 with a similarity index (mzCloud) of 95.1%.

**Figure 3.** Single chromatogram 7-Hydroxycoumarine compound.

7-Hydroxycoumarine (C$_9$H$_6$O$_3$) were coumarin derivatives that are substituted by a hydroxy group at position 7. Coumarin is a secondary metabolite compound of the phenolic group that is commonly found in plants [22]. Figure 3 shows that the 7-Hydroxycoumarine compound analyzed using LC-MS/MS was identified at a retention time (RT) of 3.066 minutes, area (max) 113,631,138.65 with a similarity index (mzCloud) of 71.1%. Compounds belonging to coumarin derivatives have
antibacterial abilities, which were first recognized in 1945 by [23]) who conducted research on dicoumarol compounds and have the ability to inhibit the growth of several bacterial strains. Research by [24] also explained that 42 coumarin-derived compounds, including 7-Hydroxycoumarine, have an antibacterial activity that can inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis*, and *Salmonella Typhimurium*. Therefore, the results of the research on antibacterial activity test with the formation of a clear zone, one of which is due to the role of the 7-Hydroxycoumarine compound, which has an antibacterial ability. According to [25], the mechanism of inhibition of coumarin compounds in inhibiting bacteria is by breaking the peptidoglycan bond in the bacterial cell wall and damaging the hydrophobic bonds of the cell membrane, which causes changes in cell wall permeability so that bacterial cell growth is inhibited or the cells die.

**Figure 4** Single chromatogram *Betaine* compound.

*Betaine* (C₅H₁₁NO₂) is an alkaloid in small amounts that can be found in plants. Alkaloids are secondary metabolites that contain one or more carbon, hydrogen, nitrogen atoms and generally contain oxygen[18]. Figure 4 shows that the betaine compound analyzed using LC-MS/MS was identified at a retention time (RT) of 0.958 minutes, area (max) 29,137,035.61 with a similarity index (mzCloud) of 93.6%. *Evolvulus alsinoides* L. plant contains a bioactive alkaloid compound in the form of betaine which has antibacterial activity that can inhibit the growth of *Staphylococcus aureus*, *Bacillus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, and *Salmonella typhii* bacteria [26]. Therefore, the results of the research on antibacterial activity test with the formation of a clear zone, one of which is due to the role of the alkaloid compound in the form of betaine which has an antibacterial ability. The mechanism of inhibition of alkaloid compounds is by interfering with the constituent components of peptidoglycan in bacterial cells, which causes the cell wall layer to not form completely so that bacterial cells die[18].

**Figure 5** Single chromatogram *Curcumin* compound.
Curcumin (C_{21}H_{20}O_{6}) is a small molecular weight polyphenolic compound and lipophillic in nature, hence insoluble in water and also in ether but soluble in ethanol, dimethylsulfoxide, and other organic solvents. Figure 5 shows that the curcumin compound analyzed using LC-MS/MS was identified at a retention time (RT) of 6.51 minutes, area (max) of 12,077,328.67 with a similarity index (mzCloud) of 74.3%. Based on the structural analysis of curcumin, it is known that its pharmacological activity is related to its functional groups such as double - bonds in the middle chain, diketone groups, and phenolic hydroxy groups. The active functional group has a role in causing its pharmacological activity. Some of the biological activities of curcumin include anti-inflammatory, antioxidant, anticancer, antifungal, antibacterial, antimutagenic, antiviral/anti-HIV, antiparasitic, anticoagulant, antidiabetic, anticholesterol, antiproliferative, anti-infective, and antifertility[27]. Curcumin can exist in the form of two tautomeric forms, keto, and enol. The enol form is more energetically stable in the solid phase and in solution. Curcumin has anti-inflammatory, antioxidant, antiviral, and antifungal actions. Curcumin is not toxic to humans. Curcumin exerts anti-inflammatory activity by inhibiting of a number of different molecules that play an important role in inflammation [28].

Nicotinamide (C_{6}H_{6}N_{2}O) is the water-soluble amide isotype of vitamin B3[29]. Figure 6 shows that the nicotinamide compound analyzed using LC-MS/MS was identified at retention time (RT) 1.382 minutes, area (max) 7.572.839.28 with similarity index (mzCloud) 82.7%. Nicotinamide is mainly involved in DNA repair, cellular energy metabolism, and in the regulation of the transcription process. Nicotinamide has been shown to be beneficial in treating hyperpigmentation and melasma and in abrogating features of aging, with trials reporting a reduction in objective indices, which included wrinkles, improvement in elasticity, and lentigines following topical nicotinamide application [30].

Jasmonic Acid (C_{12}H_{20}O_{3}) is derivative of fatty acids. The chemical structure of jasmonic acid was 3-oxo-2-20 -cis-pentenyl-cyclopentane-1-acetic acid, an endogenous signaling molecule involved in
diverse developmental processes that were originally considered stress-related hormone in higher plants [31]. Figure 7 shows that the jasmonic acid compound analyzed using LC-MS/MS was identified at a retention time (RT) of 8.668 minutes, area (max) 2.701.051.87 with a similarity index (mzCloud) of 81.1%. Jasmonic acid is a plant-signaling molecule closely associated with plant resistance to abiotic stress. In abiotic stress, JA is usually involved in physiological and molecular responses. Physiological responses often include activation of the antioxidant system (superoxide anion radical, NADPH-oxidase, peroxidase), accumulation of soluble sugars and amino acids (isoleucine and methionine), and regulation of stomatal opening and closing. Jasmonic acid also has antimicrobial and antioxidant activities [32].

4. Conclusion
The green rosella calyx extract contains chemical compounds, including flavonoids, triterpenoids, and tannins. The results of the LC-MS/MS analysis of the green rosella extract contain 323 compounds, and among them are 5-hydroxymethyl-2-furaldehyde, 7-hydroxycoumarine, betaine, curcumin, nicotinamide and jasmonic acid. Based on the results of previous studies, these compounds have pharmacological activities, including antibacterial, antifungal, and antioxidant. Further research needs to be done, namely testing the pharmacological activity of green calyx rosella extract.

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