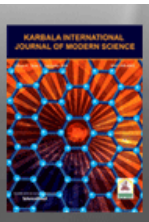
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Keywords

Hibiscus sabdariffa L.; infusion; maceration; maltodextrin; selectivity index

RESEARCH PAPER

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Keywords: *Hibiscus sabdariffa L.*, Infusion, Maceration, Maltodextrin, Selectivity index

1. Introduction

Roselle (*Hibiscus sabdariffa* L.) has long been consumed as a functional beverage ingredient and used as a natural food colorant in tropical regions. Its calyces are rich in secondary metabolites, particularly phenolic acids and flavonoids, as well as organic acids such as citric, malic, and tartaric acids. These constituents have been reported to possess strong antioxidant, antimicrobial, and cytotoxic activity [1–3]. Since these compounds differ in polarity and stability, the extraction method strongly influences the phytochemical profile and bioactivity of the resulting extract. Hot-water infusion is a simple, widely used method that reflects common consumer preparation practices. However, the elevated temperature involved may reduce the stability of sensitive pigments, especially

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anthocyanins [2], and may shift the extract composition toward highly polar non-pigment phenolics. On the other hand, ethanolic maceration improves the solubility and stability of anthocyanins under acidic conditions, typically resulting in higher anthocyanin content and antioxidant activity than the water extraction method [4–6]. Studies on various fruit matrices have shown that adding maltodextrin as a carrier to the extract, followed by drying to produce powder, can protect anthocyanins and phenolic compounds from degradation caused by oxygen, light, and high water activity. This method also improves powder quality, as indicated by high pigment retention and good functional stability, which are important for ingredient applications [2,7–9]. From a green extraction perspective, optimizing solvent selection and temperature is essential to obtain the desired chemical profile and target compounds while reducing energy consumption and solvent use [10,11].

Several studies have been carried out to measure and compare the phenolic content of roselle, but most have mainly focused on bulk total parameters, such as total phenolic content (TPC), total flavonoid content (TFC), and total anthocyanin content (TAC) [12]. Although some studies have applied more detailed separation and identification methods (e.g., HPLC, LC-HRMS), consistent profiling approaches across extraction methods and direct correlation of specific metabolites and chemical compounds with biological activities remain inconsistent across studies, making it difficult to consistently establish the contributions of key compounds to bioactivity [13]. Because biological activities (e.g., antioxidant, antibacterial, and cytotoxic effects) cannot always be predicted only from bulk total indicators, integrating chemical profiling with bioactivity assays is necessary to strengthen mechanistic interpretation and help identify metabolites contributing to the observed effects.

In this research, we compared the phytochemical profiles and bioactivities of roselle (*Hibiscus sabdariffa* L.) extracts obtained by three extraction methods: hot-water infusion, ethanolic maceration, and maltodextrin-assisted drying. The aim was to evaluate whether extraction-method-dependent selectivity influences the differences in bulk total (TPC, TFC, and TAC), chemical profile, and functional bioactivity, including antioxidant capacity (FRAP assay), antibacterial activity, and cytotoxicity (HT-29, 4T1, and T47D cancer cells; as well as Vero cells for the selectivity assay). In general, this study evaluated how extraction methods (low-cost hot water infusion, solvent-based maceration, maltodextrin-assisted powder formation) tune chemotypes and modulate the bioactivities of roselle extracts, providing fundamental insights into the optimized development of roselle-based beverages.

2. Materials and methods

2.1. Materials

Dried roselle calyces (*Hibiscus sabdariffa* L.) were sourced from Cae Tea Tisane (Malang, Indonesia) and authenticated by the Laboratory of Taxonomy, Structure, and Development of Plants, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia. They were stored in airtight amber containers at 20–25 °C and <60% relative humidity (RH), protected from light. Food-grade ethanol (96% v/v; Lab Alley, USA), maltodextrin (DE 15–20; Sigma–Aldrich, USA), citric acid (Merck, Germany), and Nutrient Agar (NA; Merck, Germany) were used as reagents and culture media.

HPLC-grade acetonitrile, formic acid, and ultrapure water (18.2 MΩ cm, Milli-Q system; Merck Millipore, Germany) were used for chromatographic and analytical procedures. The reagents for colorimetric assays included the Folin-Ciocalteu reagent, gallic acid, aluminum chloride ($AlCl_3$), sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), quercetin, 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and Trolox (all analytical grade; Sigma–Aldrich, USA).

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Vero, HT-29, 4T1, and T47D cell lines (ATCC) were cultured in DMEM (Sigma–Aldrich, USA), supplemented with 10% fetal bovine serum (FBS) (Sigma–Aldrich, USA) and 1% penicillin-streptomycin (Sigma–Aldrich, USA) at 37 °C in a humidified incubator with 5% CO_2. Light-sensitive reagents and extracts were handled under reduced-light conditions.

2.2. Preparation of roselle extracts

Roselle calyces (*Hibiscus sabdariffa* L.) were treated via three extraction techniques: hot-water infusion, ethanolic maceration, and maltodextrin-assisted powder formation. The resulting extracts were labeled EI, EM, and EP. Dried calyces were ground to a fine powder (≤60 mesh), and each extraction was carried out in separate triplicate batches (n = 3).

For hot-water infusion, 3 g of calyx powder was soaked in 1000 mL of distilled water at 70 °C for

5–10 min with gentle stirring, following a modified procedure described by Tenorio [14]. The slurry was separated by filtration using Whatman No. 1 paper, and the filtrate was cooled to 30 °C. Several aliquots were used immediately, while the rest were stored in airtight, light-protected containers.

Roselle calyx extraction was carried out by ethanolic maceration, following Kartini [15] with minor modifications. In brief, 100 g of roselle calyces were mixed with 1500 mL of 50% (v/v) ethanol, acidified with 2% citric acid. The mixture was homogenized using an overhead stirrer at 500 rpm for 60 min, then filtered and concentrated under reduced pressure at 50 °C with a rotary evaporator until a clear filtrate formed. This filtrate was further concentrated in a water bath to yield 30 mL of thick roselle extract.

To prepare the carrier-assisted powder, a portion of the concentrated EM extract was combined with maltodextrin at a 1:3 (w/w) ratio [15]. After thoroughly mixing, the mixture was ground with a mortar and pestle. Next, it was placed in a porcelain dish and dried in an oven at 60 °C for 24 h. After drying, the material was ground again and sifted through an 80-mesh sieve. Finally, the resulting powder was stored in amber vials at 4 °C.

For each extraction method, dry residue was determined by gravimetric analysis at 105 °C until a constant weight was obtained. Extract yield was calculated as a percentage and expressed as % (g extract/100 g dry roselle calyces). The mass of roselle calyces in each experiment was adjusted according to the objective of the extraction route. Extract yield was normalized to the dry weight of roselle calyces, and cell-based assays were conducted using equivalent extract concentrations. For further analysis, EI and EM were used as prepared, while EP was diluted to 10 mg mL^{-1} and vortexed for 1 min.

2.3. Determination of TPC

TPC was determined by the Folin-Ciocalteu method [16] and adapted to a 96-well microplate. Fresh gallic acid was prepared as a standard (2.5–30 μg mL^{-1}), and EI, EM, and EP were diluted with distilled water. In each well, 20 μL of standard/blank/sample was mixed with 100 μL of 10% FC reagent (v/v), incubated for 5 min, and then 80 μL of 7.5% (w/v) Na_2CO_3 was added.

After incubation (60 min, 22–25 °C, in the dark), the sample absorbance was measured at 765 nm (Spectrostar, BMG Labtech, Germany). The concentration was calculated from the gallic acid standard (quadruplicate; $R^2 \geq 0.98$), and the results were expressed as mg gallic acid equivalents (GAE) per gram of extract (dry basis) or mg GAE g^{-1}. Each sample (EI/EM/EP) was analyzed in triplicate ($n = 3$).

2.4. Determination of total flavonoid content (TFC)

TFC was measured using the $AlCl_3$ colorimetric assay [17–19], adapted to a 96-well microplate. Fresh quercetin was used as a standard (2.5–30 μg mL^{-1}), and EI, EM, and EP were diluted with distilled water. Reagents were added successively in each well up to a final volume of 200 μL: 20 μL of standard, blank, or sample was mixed with 60 μL of water, followed by 6 μL of 5% (w/v) $NaNO_2$ (incubated for 5 min), 6 μL of 10% (w/v) $AlCl_3$ (incubated for 6 min), and 108 μL of 1 M NaOH. The homogenized mixture was incubated for 5 min at 510 nm (Spectrostar, BMG Labtech, Germany). The concentration was determined from the quercetin standard (quadruplicate; $R^2 \geq 0.99$), and the results were reported in mg quercetin equivalents (QE) per g extract (dry basis) or mg QE g^{-1}. Analysis was repeated in triplicate for each sample (EI/EM/EP) ($n = 3$).

2.5. Determination of total anthocyanin content (TAC)

TAC analysis was performed using the pH- differential method adapted to a 96-well microplate [20]. Buffer solutions were prepared in advance: 0.025 M KCl (pH 1.0 ± 0.1) and 0.4 M sodium (or potassium) acetate (pH 4.5 ± 0.1), and EI, EM, and EP were diluted with distilled water. In a separate well, 200 μL of the sample was added to each buffer solution and incubated for 10–15 min (22–25 °C, in the dark). Absorbance was determined at 520 and 700 nm (Spectrostar, BMG Labtech, Germany). Monomer anthocyanins were calculated as cyanidin-3-glucoside (C3G) equivalents (MW = 449.2 g mol^{-1}, $\epsilon = 26{,}900$ L $mol^{-1}cm^{-1}$) and are expressed per gram extract (dry weight basis). Each sample (EI/EM/EP) was analyzed in triplicate ($n = 3$).

2.6. Liquid chromatography high-resolution mass spectrometry (LC-HRMS) analysis

Aliquots of each sample were diluted in a polar solvent (300 μL in 1.5 mL), vortexed (2000 rpm, 2 min), and then centrifuged (6000 rpm, 2 min). The filtrate was analyzed by LC using the Thermo Scientific Dionex Ultimate 3000 RSLCnano system equipped with an Accucore aQ column (50 × 2.1 mm, 2.6 μm). The mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in

acetonitrile. The flow was maintained at 0.1 mL min^{-1} with a gradient program over 30 min (5–90% B, then returning to the initial condition). The column temperature was maintained at 30 °C, and the injection volume was 5 μL [21].

Mass spectrometry (MS) was performed on a Thermo Scientific Q Exactive spectrometer in the positive ion mode using a Full-Scan dd/MS workflow. MS1 and MS2 data were recorded in 70,000 FWHM (*m/z* 50–750) and 17,500 FWHM, respectively, with nitrogen as the collision gas. The data were processed using Compound Discoverer 3.3. The metabolite assignments reported in this study were considered tentative and were based on LC-HRMS accurate-mass matching and database-assisted annotation. Definitive identification would require further confirmation using authentic reference standards.

2.7. Antioxidant capacity: ferric reducing antioxidant power (FRAP) assay

Antioxidant activity was analyzed following the reported method by Benzie and Strain (1996) [22] with some modifications (adopted in a 96-well microplate). EI, EM, and EP were diluted with distilled water in advance. A total of 20 μL of each extract was mixed with 180 μL of FRAP working solution, formulated by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 (v/v/v) ratio, then pre-heated to 37 °C. After 10 min in the dark (37 °C), the absorbance at 593 nm was measured using a microplate reader (Spectrostar, BMG Labtech, Germany). FRAP was determined from Trolox standards (100–1000 μM; quadruplicate; $R^2 \geq 0.995$). Results are expressed as μmol Trolox equivalents (TE) per g extract (dry weight basis) or μmol TE g^{-1}. Analysis was carried out in triplicate for each extract in three independent batches (n = 3) [23–26].

2.8. Antibacterial activity

Antibacterial activity was screened using an agar well diffusion method adapted from Refs. [27,28]. The assay was carried out on Mueller-Hinton agar plates (MHA; pH 7.2–7.4, temperature 25 °C, depth ± 4 mm). Inocula of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were adjusted to 0.5 McFarland in 0.85–0.90% NaCl, and plates were swabbed uniformly within 15 min. Each 90-mm plate contained five 6-mm wells (≥24 mm center-to-center well distance; ≥15 mm from the edge) of three extracts (EI, EM, and EP), distilled water (negative control), and 50 μL of amoxicillin (positive control) at designated concentrations. The assay plates were permitted to pre-diffuse for 30 min, followed by inverted incubation at 35 ± 2 °C for 18–24 h. The inhibition zone diameter was measured as the total diameter, encompassing the 6-mm wells. All tests were performed in triplicate across three independent runs (n = 3 biological replicates).

2.9. Cell viability assay (MTT)

MTT assay was used to evaluate the cytotoxicity of the extracts (EM, EI, and EP) against 4T1 and T47D breast cancer cell lines, as well as the HT-29 colorectal cancer cell line, using Vero cells as a selectivity control. Cells were cultured in RPMI-1640 medium enriched with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin, then incubated at 37 °C and 5% carbon dioxide. Cells were seeded at a density of (6–10) × 103 cells per well with an incubation time of 24 h. Twelve wells per plate were allocated as controls (medium-only, cells-only, solvent-only).

The extracts (EI, EM, and EP) were dissolved in DMSO and diluted with culture medium, resulting in a final DMSO concentration of ≤0.5% (v/v) per well. Treatments were applied in a twofold dilution series at seven concentration points (1000–15.625 μg mL^{-1}) in triplicate, and cells were exposed to the samples. In parallel with the MTT assay, cell morphology was observed after 24 h of treatment using an inverted microscope and documented as representative micrographs at × 40 magnification. Morphological observations were performed to identify signs of cytotoxicity, including cell rounding, shrinkage, membrane blebbing, loss of adhesion, and cell detachment, relative to the untreated control group. After 24 h of exposure, wells were rinsed with 100 μL of PBS, and 100 μL of MTT solution (0.5 mg mL^{-1} in medium) was added to each well. The plates were incubated for 3–4 h, protected from light. Formazan crystals were dissolved in 100 μL of 10% w/v SDS solution prepared with 0.01 M HCl, and the mixture was incubated at room temperature overnight in the dark. Absorbance was read at 595 nm (test wavelength; 630 nm as the reference wavelength) using a microplate reader (Spectrostar, BMG Labtech, Germany).

The blank sample consisted of MTT-treated culture medium, but without cells. A blank absorbance value was subtracted from all sample readings as a correction. Cell viability was calculated as the percentage of vehicle-treated cells, after correction for blanks. IC_{50} values were determined by fitting

viability percentage data against log10 concentration using least-square linear regression (Microsoft Excel). Selectivity index (SI) values were calculated as the ratio of IC_{50} in Vero cells to IC50 in cancer cells. Each concentration was carried out in triplicate, and results were summarized from three independent runs (n = 3 biological replicates), expressed as means ± SDs [29].

2.10. Statistical analysis

Each measurement was performed at least three times, and the results are reported as mean ± standard deviation. Differences between groups were assessed using one-way ANOVA with Tukey's HSD post hoc test ($p < 0.05$), while the relationship between TPC, TFC, TAC, and FRAP was calculated using Pearson's correlation coefficient. All analyses were conducted with IBM SPSS v.29.

3. Results and discussion

3.1. Phytochemical composition

All data were reported per gram of extract on a dry weight basis: TPC (mg GAE g^{-1}), TFC (mg QE g^{-1}), and TAC (mg C3G g^{-1}). One-way ANOVA revealed no significant differences in TPC and TFC among the extraction methods ($p > 0.05$), while TAC showed significant variation ($p < 0.05$). Tukey's test indicated that TAC values in EM and EP were higher than those in EI (Fig. 1).

These results indicate that phenolic compounds remained stable across extraction methods, whereas anthocyanins were more sensitive to solvent type and extraction process. This observation aligns with the known phytochemical characteristics of roselle.

Meanwhile, pigmented fractions, such as delphinidin, cyanidin, and sambubiosides, are more sensitive than non-pigmented fractions, notably caffeoylquinic acids (CQA) and rutin [30]. This is confirmed by the LC-HRMS profile interpretation and data in Table 1. The LC-HRMS results demonstrated that EI was dominated by non-pigmented phenolics (higher CQA signal; rutin detected), whereas EM and EP, as hydroalcoholic extracts, had higher signal intensity for small organic acids and matrix-derived components, such as citric acid, 2-methylcitric acid, galacturonic acid derivatives, and sugar-degradation products like furans. This implies that hydroalcoholic extraction improves matrix permeability and compound solubility, resulting in extracts rich in organic acids [1].

The intense evaporation process used in maltodextrin-assisted drying (EP) and the heating process in all applied extraction methods may have led to sugar/anthocyanin degradation (gallic acid, methyl gallate, 5-HMF, 5-hydroxy-2-furoic acid, and 2,5-furandicarboxylic acid; Table 1), which may explain the low TAC results.

Additionally, EI was more selective toward polar non-pigmented phenolics, while EM (using an acidified hydroalcoholic solvent) promoted pigment solubility and stabilized the flavylium cation [14,31].

3.2. Antioxidant capacity

The FRAP results indicated that antioxidant capacity was relatively comparable across the extraction methods. Although FRAP means varied, following the order EP ≥ EI ≥ EM, the one-way ANOVA results showed no significant differences ($p > 0.05$; Fig. 2), indicating a small range on the dry weight basis (1.38 ± 0.15–1.78 ± 0.17 μmol TE g^{-1}). The FRAP results were relatively similar across all extraction methods, as was the case with TPC and TFC, although TAC showed a significant difference.

This finding indicates that anthocyanins are less stable during extraction, suggesting a small

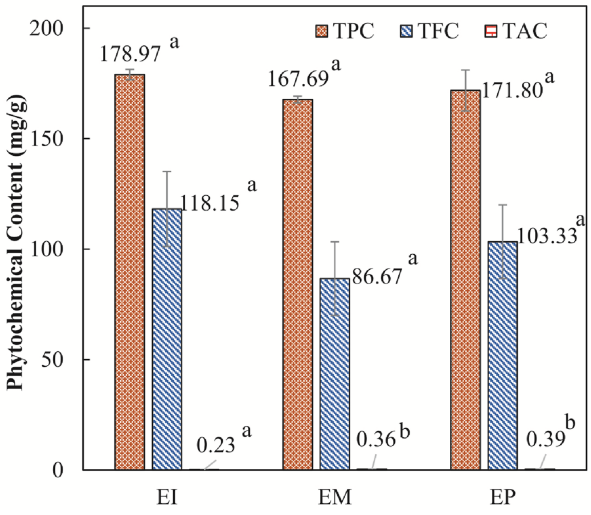

Fig. 1. Phytochemical contents of roselle extracts (EI, EM, and EP). Bars show mean ± SD (n = 3, dry basis). One-way ANOVA followed by Tukey's HSD (α = 0.05): total phenolic content (TPC) and total flavonoids (TFC) were not significantly different across routes ($p > 0.05$), whereas total anthocyanin content (TAC) differed ($p < 0.05$) with EI < EM = EP. Different superscript letters within each parameter indicate significant differences; identical letters indicate non-significant differences.

Table 1. Differences in metabolite compounds of roselle extracts prepared from hot-water infusion (EI), ethanolic maceration (EM), and maltodextrin-assisted powdering (EP) using LC-HRMS Analysis.

No	Compound	Formula	Molecular Weight (MW) (g/mol)	RT (min)	Area (%)			Compound Class
					EI	EM	EP	
1	Betaine	$C_5H_{11}NO_2$	117.15	1.31	28.01	–	19.21	Alkaloid
2	Choline	$C_5H_{13}NO$	103.10	0.96	–	–	0.94	Quaternary amine
3	L-Proline	$C_5H_9NO_2$	115.13	0.96	4.97	–	0.93	Amino acid
4	Adenine	$C_5H_5N_5$	135.05	1.06	–	0.15	0.23	Purin
5	L-aspartic acid	$C_4H_7NO_4$	133.03	0.95	–	0.42	–	Amino acid
6	2-Acetamido-2-deoxy-L-galacturonic acid	$C_8H_{13}NO_7$	235.07	1.01	–	–	0.04	Pectin derivative
7	Dihydroxyfumaric acid	$C_4H_4O_6$	148.00	1.04	–	0.02	–	Organic acid
8	L-Valine	$C_5H_{11}NO_2$	117.15	1.26	0.32	3.64	0.23	Amino acid
9	Succinic acid	$C_4H_6O_4$	118.09	1.26	0.02	–	0.01	Organic acid
10	5-Hydroxymethylfurfural (5-HMF)	$C_6H_6O_3$	126.11	1.27	0.72	–	–	Sugar degradation
11	5-Hydroxymethyl-2-furaldehyde	$C_6H_6O_3$	126.11	1.27	2.96	–	2.07	Sugar degradation
12	Glutaric acid	$C_5H_8O_4$	132.12	1.15	0.16	0.72	0.79	Decarboxylic acid
13	Trigonelline	$C_7H_7NO_2$	137.14	1.38	2.46	0.34	1.10	Alkaloid
14	L-Phenylalanine	$C_9H_{11}NO_2$	165.07	1.46	0.24	–	0.11	Amino acid
15	7-Hydroxycoumarin (Umbelliferone)	$C_9H_6O_3$	162.14	2.06	0.94	–	–	Phenolic
16	trans-Aconitic acid	$C_6H_6O_6$	174.01	1.19	–	1.07	1.22	Organic acid
17	(Dehydro)ascorbic/Erythorbic acid	$C_6H_8O_6$	176.12	1.31	0.03	0.06	0.09	Ascorbic acid
18	4-O-Acetyl-D-galacturonic acid	$C_8H_{12}O_8$	236.05	1.20	–	2.97	–	Pectin derivative
19	DL-Tyrosine	$C_9H_{11}NO_3$	181.07	1.20	–	–	0.10	Amino acid
20	N-Acetyl-L-glutamic acid	$C_7H_{11}NO_5$	189.06	1.20	–	–	0.01	Amino acid
21	Oxalosuccinic acid	$C_6H_6O_7$	190.01	1.22	1.24	–	0.10	Organic acid
22	α-ketoadipic acid	$C_6H_8O_5$	160.03	1.26	–	0.19	–	Organic acid
23	2-Methylcitric acid	$C_7H_{10}O_7$	206.04	1.27	–	4.66	–	Organic acid
24	Methyl gallate	$C_8H_8O_5$	184.03	2.12	–	0.37	–	Phenolic
25	Gallic acid	$C_7H_6O_5$	170.12	1.30	0.29	0.19	–	Phenolic
26	Dehydroquinic acid	$C_7H_{10}O_6$	172.03	1.38	–	0.34	–	Quinic acid derivative
27	5-Hydroxy-2-furoic acid	$C_5H_4O_4$		2.10	–	–	1.58	Sugar degradation
28	(E)-Ferulic acid	$C_{10}H_{10}O_4$	194.19	2.06	0.01	–	0.01	Phenolic
29	Caffeoylquinic acid	$C_{16}H_{18}O_9$	354.09	2.09	0.86	–	0.46	Phenolic acid
30	2,5-Furandicarboxylic acid	$C_6H_4O_5$	156.00	2.12	–	0.50	0.65	Dicarboxylic acid
31	5-(Methoxymethyl)-2-furoic acid	$C_7H_8O_4$	156.04	2.12	–	0.76	–	Furan derivative
32	Shikimic acid	$C_7H_{10}O_5$	174.05	2.12	–	0.41	–	Organic acid
33	Hydroxycitric acid	$C_6H_8O_8$	208.12	0.95	4.24	1.01	0.41	Organic acid
34	Citric acid	$C_6H_8O_7$	192.12	1.27	–	32.50	18.68	Organic acid
35	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	464.09	10.60	–	–	0.02	Flavonoid glycoside
36	Rutin	$C_{27}H_{30}O_{16}$	610.52	2.00	0.02	–	–	Flavonoid glycoside
37	Delphinidin 3,3′,5-tri-O-β-D-glucopyranoside	$C_{33}H_{40}O_{22}$	789.67	1.97	–	–	0.01	Anthocyanin

Note: All compounds listed in Table 1 were tentatively annotated based on LC-HRMS accurate-mass matching and database-assisted analysis, and were not confirmed using authentic standards.

contribution to the FRAP. On the other hand, the phenolic compounds, which act as primary electron donors, appeared relatively stable across all extraction methods [32–35]. This is further supported by the FRAP results for EM and EP, which showed no significant increase.

This is consistent with the LC-HRMS profile (Table 1). EP and EI were enriched in caffeoylquinic acids and flavonol glycosides, such as rutin, which are effective electron donors at acidic pH and elicit a strong response with Fe(III)-TPTZ [36–39]. These compounds may contribute to the relatively higher FRAP responses observed in EI and EP compared with EM. Caffeoylquinic acids have been reported as the key driver of ferric reduction capacity in natural plant-based beverages, where FRAP closely correlates with total chlorogenic acids, and phenolic acids account for most of the response [40]. In EP, caffeoylquinic acids were detected together with flavonol glycosides (e.g. quercetin-3-glucoside/isoquercitrin) and minor phenolic acids, all embedded in a protective carrier matrix [41,42]. High content of caffeoylquinic acids has also been reported in roselle decoctions [14]. On the other hand, EM consisted of a lot of small organic compounds (e.g., citric and 2-methylcitric acids, aconitic and hydroxycitric acids), matrix-derived galacturonic species, and sugar-degradation products, which contributed less to the FRAP response than phenolic compounds acting as strong reducing

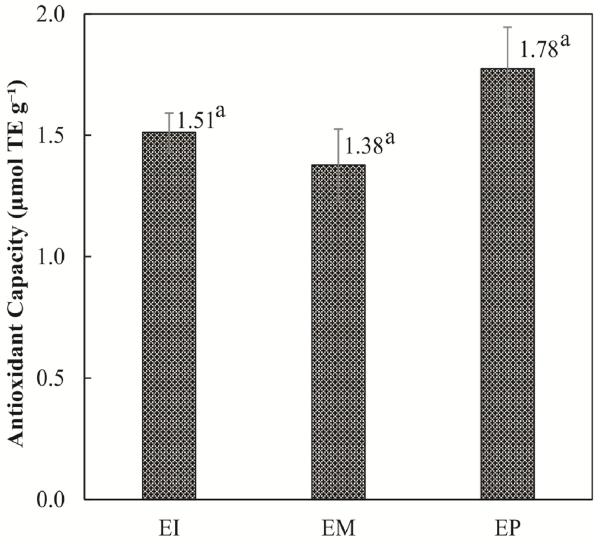

Fig. 2. Ferric reducing antioxidant power (FRAP) of roselle extracts prepared by hot-water infusion (EI), ethanolic maceration (EM), and maltodextrin-assisted powder (EP). Bars show mean ± SD (n = 3), expressed as Trolox equivalents per gram extract (dry basis). Bars sharing the same letter are not significantly different at p < 0.05. Since no significant differences were observed among the extracts, all bars are labeled with the same superscript.

agents. Despite their role in sensory evaluation and biological activity, organic acids are weaker reducing agents, as shown by FRAP assays [43,44]. It is also important to note that betaine, detected prominently in EI and EP, may contribute to antioxidant-related effects in vivo through indirect cellular protective mechanisms, even though its direct contribution to the FRAP response is likely limited. This interpretation is consistent with reports showing that betaine has little direct free-radical-scavenging ability but can still exert antioxidant effects through cellular defense mechanisms [45,46]. Therefore, the FRAP assay should be interpreted primarily as an index of direct ferric-reducing capacity rather than as an overall measure of the extracts' biological antioxidant potential.

3.3. Antibacterial activity

Under the current agar well diffusion conditions (50 μL/well of 4 mg mL-1; ≈200 μg/well), no zones of inhibition were observed for *Escherichia coli* or *Staphylococcus aureus* with any of the extracts (EI, EM, and EP), with 0.0 ± 0.0 mm beyond the well edge. In contrast to all the extracts, amoxicillin, as a positive control (100 ppm), generated a clear zone (5.04 ± 1.27 mm for *S. aureus*; 3.10 ± 0.25 mm for *E. coli*), validating the applied procedure. Agar well diffusion is not sensitive to high-molecular-weight or highly polar glycosylated phenolics (e.g., CQA isomers, rutin, and isoquercitrin), whereas broth microdilution is more reliable for detecting the growth inhibition of these compounds [47].

Furthermore, the barriers of the gram-negative outer membrane reduce the apparent activity and often require permeabilizers (e.g., EDTA) to disclose latent effects in *E. coli* [48,49]. Weak organic acids are highly pH-dependent, and their antimicrobial activity typically decreases under near-neutral conditions because a smaller fraction remains in the undissociated form [50,51]. In our assay, no inhibition zone was observed, despite LC-HRMS evidence of citric acid and related organic acids. Overall, the limitations of the agar well diffusion method, the outer membrane resistance, and pH effects were considered in interpreting the 0-mm findings. Moreover, the positive control validated the antibacterial assay.

3.4. Cytotoxicity of roselle extracts on 4T1 and T47D (breast) and HT-29 (colorectal) cancer cells

Cell viability assays evaluated the ability of each extract to reduce cell viability in cancer and Vero cells. In T47D cells, EP demonstrated the greatest decline in cell viability compared to EI and EM. This contrasted with statistical test results showing a significant difference between EP and EM ($p < 0.05$). In HT-29 cells, EI showed the most potent cytotoxicity, followed by EM and EP, respectively. All pairs of extract means (EI-EM, EI-EP, EM-EP) indicated statistically significant differences ($p < 0.05$).

In 4T1 cells, the extract cytotoxicity followed the order EM > EP > EI, with all differences statistically significant ($p < 0.05$) (Fig. 3). Although the cell viability data revealed a dose-dependent response, IC_{50} values were used as a standard measure to compare potency across all extracts and cell lines and to assess the selectivity of cancer cells against normal cells. The dose–response curves for HT-29, T47D, and 4T1 are displayed in Table 2. In general, cell viability declined significantly in a concentration-dependent manner across all extracts, as indicated by IC_{50} values.

The selectivity index ($SI = IC_{50}(Vero)/IC_{50}(cancer)$) results demonstrated a certain response of each particular cell line to the extracts. The SI values obtained exceeded two ($SI > 2$) for EM and EI against HT-29 cells, for all extracts against 4T1 cells, and for EI and EP against T47D cells, indicating strong selectivity toward cancer cells (Table 3). Additionally, the SI values of EP against HT-29 cells and EM against T47D cells were above 1 ($SI > 1$), implying

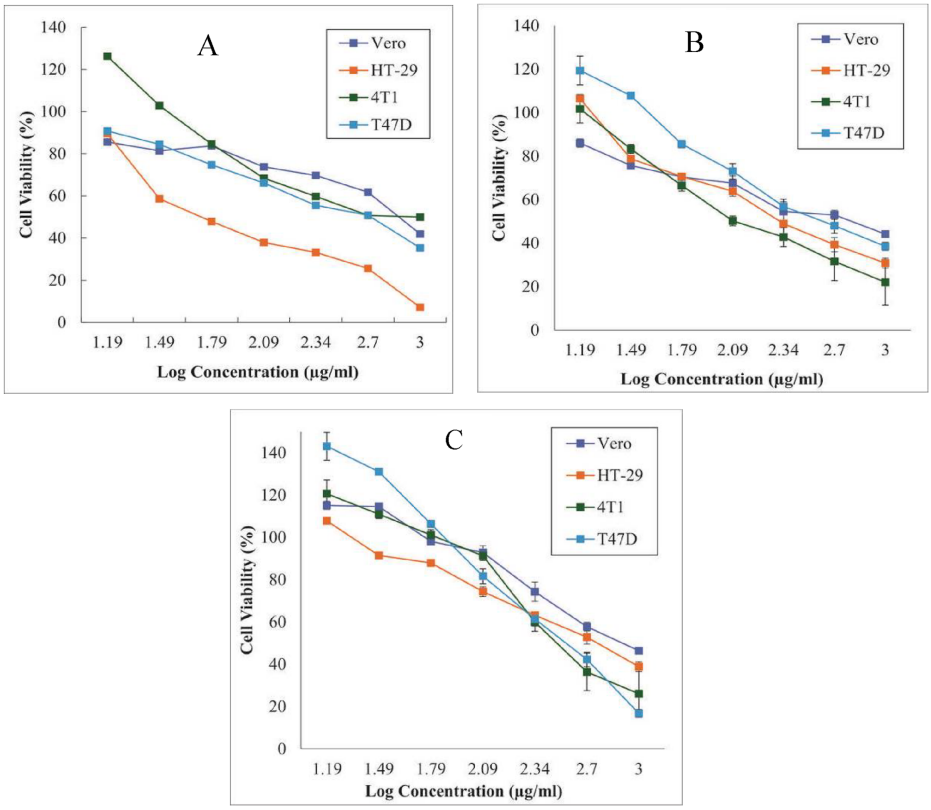

Fig. 3. Cell viability of (a) hot-water infusion (EI); (b) ethanolic maceration (EM); and (c) maltodextrin-assisted powder (EP) for the four cell lines Vero, HT29, 4T1, and T47D.

greater toxicity against cancer cells than normal cells, yet it was considered relatively low.

As with the IC_{50}/SI data, Fig. 4 reveals apoptosis-like morphological changes, including cell rounding and shrinkage, membrane blebbing, loss of adherence, and detachment. This was recognized in EI-treated HT-29 cells, EM-treated 4T1 cells, and EP-treated T47D cells. These morphological changes supported the high SI results, affirming the extracts' preferential cytotoxicity toward cancer cells. The alignment among IC_{50}, SI, and morphological changes supports the explanation that the extracts' chemotypes selectively modulate the cancer cell viability response within the achieved dose range [52].

This is further supported by the distinct chemotypes of each extract (EI, EM, and EP) and their redox capacities, as identified by LC-HRMS and FRAP analyses, respectively. The stronger response

Table 3. Selectivity index (SI=IC_{50}(Vero)/IC_{50}(cancer)) of roselle extracts prepared by hot-water infusion (EI), ethanolic maceration (EM), and maltodextrin-assisted powder (EP). Values > 2 indicate selective cytotoxicity toward cancer cells.

Tested Samples	Cell lines		
	HT-29	4T1	T47D
EI	14.49	2.12	2.91
EM	2.13	3.00	1.26
EP	1.65	2.35	2.54

Note: Bold indicates SI > 2.

Table 2. IC50 Values of hot-water infusion (EI), ethanolic maceration (EM), and maltodextrin-assisted powder (EP) on one normal cell line (Vero) and three human cancer cell lines (HT-29, 4T1, T47D).

Tested Samples	µg/mL-1µg/mL-1)50 IC			
	Vero	HT-29	4T1	T47D
EI	1157.3 ± 327.8^{b}	79.9 ± 1.38^{a}	546.0 ± 42.1^{c}	397.6 ± 17.6^{ab}
EM	552.1 ± 48.7^{a}	258.8 ± 15.5^{b}	183.9 ± 50.7^{a}	436.6 ± 39.6^{b}
EP	906.8 ± 61.0^{ab}	549.4 ± 90.6^{c}	385.9 ± 47.5^{b}	356.9 ± 32.5^{a}

Different superscript letters within each cancer-cell column indicate Tukey HSD groups at $p < 0.05$.

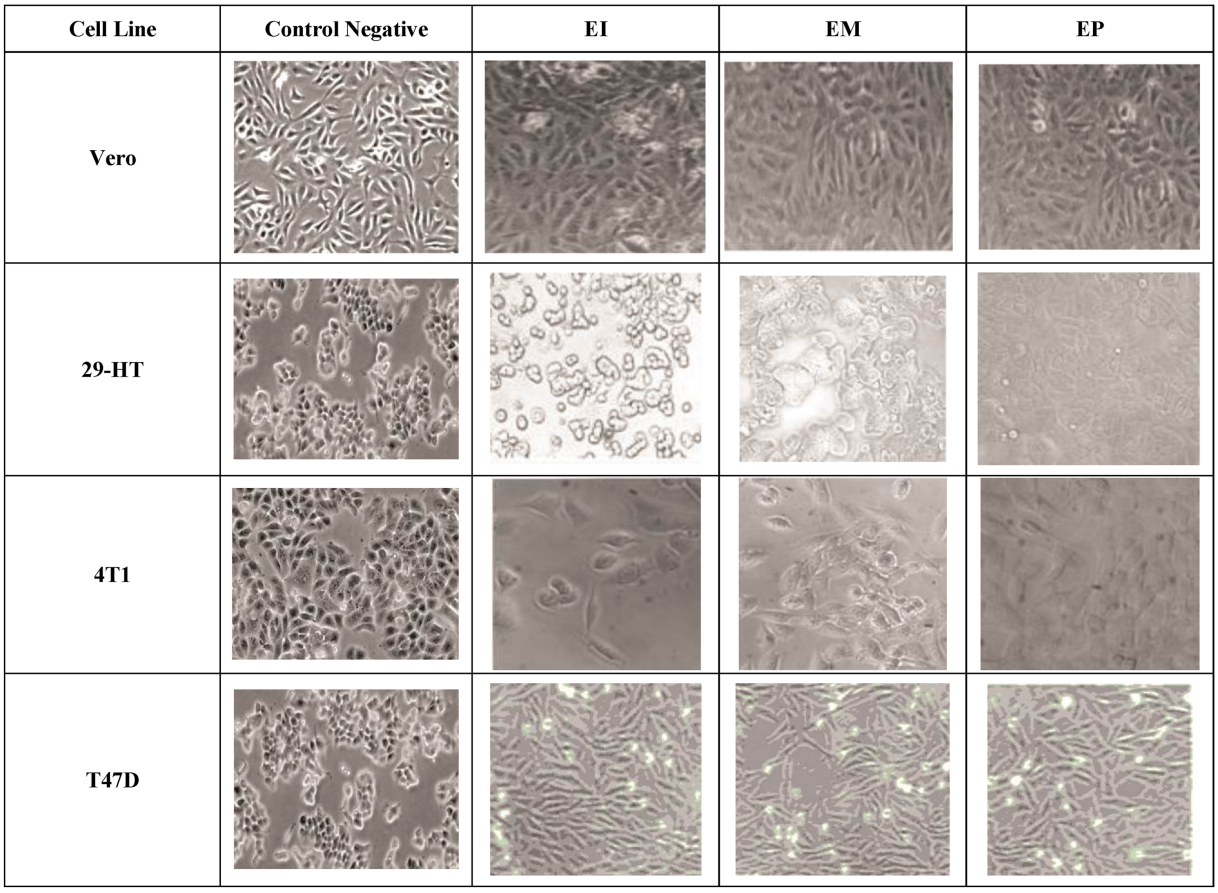

Fig. 4. Representative morphology (magnification x40) of normal cell line (Vero), colon cancer cell line (HT-29), and breast cancer cell line (4T1 and T47D) after 24 h treatment with hot-water infusion (EI), ethanolic maceration (EM), and maltodextrin-assisted powdering (EP) at selected concentrations.

of EI in HT-29 may be associated with the presence of caffeoylquinic acid-related features (ΣCQA) observed in the extract. These compounds have been reported to be associated with redox-mediated mitochondrial stress, cell cycle arrest, and apoptosis in colon cancer models [53–55].

On the other hand, EP revealed the least potency against HT-29 cells, despite the presence of ΣCQA, which may reflect matrix effects during powder formation, where carrier-based spray-dried systems can influence the apparent bioactive contribution measured in assays [41,42]. Although betaine was detected prominently in EI and EP, its specific role in the cytotoxic response cannot be concluded from the present data. Previous studies suggest that betaine may influence cancer cell proliferation, apoptosis, and oxidative status in a context-dependent manner [56]. However, the cytotoxicity observed here is more likely associated with the combined action of multiple metabolites in each extract. Therefore, further mechanistic studies, including ROS measurement, apoptosis assays, and cell-cycle analysis, are required to clarify the contribution of betaine.

In 4T1 cells (murine triple-negative breast cancer), EM exhibited the greatest potency. This finding is consistent with reports that polyphenol-rich fractions can exhibit antitumor and anti-metastatic activities in murine breast cancer models [57–59].

4. Conclusion

The evaluation of three distinct extraction methods revealed that (i) TPC and TFC exhibited no significant differences among the extraction methods used, (ii) TAC was sensitive to the extraction method applied (EI < EM ≈ EP), and (iii) FRAP showed no significant difference despite a modest trend (EP ≥ EI ≥ EM). In agar well diffusion, polar roselle extracts showed no zone of inhibition against *E. coli* and *S. aureus* due to the method's limitation with high-polarity phenolics.

Based on the IC_{50} results, the cytotoxic effects were selective and cell-line-dependent: EI was

effective against HT-29, EM against 4T1, and EP against T47D. High SI values were obtained in EM and EI against HT-29 cells, all extracts against 4T1 cells, and EI and EP against T47D cells (SI > 2).

These results were further supported by the LC-HRMS profiles of the extracts (e.g., ΣCQA/rutin in EI; organic acid/matrix enrichment in EM/EP), suggesting that differences in metabolite composition may provide a more informative basis for interpreting bioactivity variation than bulk total compounds (TPC/TFC/TAC) alone. Future studies are required to validate these findings by incorporating non-linear dose–response modeling and mechanistic biomarkers (apoptosis/ROS). In summary, this study demonstrates that the applied extraction method can be tailored to selectively obtain target biological activities and chemotypes.

Ethics information

All plant materials used in this study were identified and authenticated by the Laboratory of Taxonomy, Structure and Development of Plants, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya. The identification reference numbers are as follows: *Hibiscus sabdariffa* L. (No. 01305/UN10.F0916/B/TA.00.02.3/2025).

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Conflicts of interest

The authors declare that they have no competing interests.

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