

Comparative Study on the Chemical Profile, Antioxidant Activity, and Enzyme Inhibition Capacity of Red and White *Hibiscus sabdariffa* Variety Calyces

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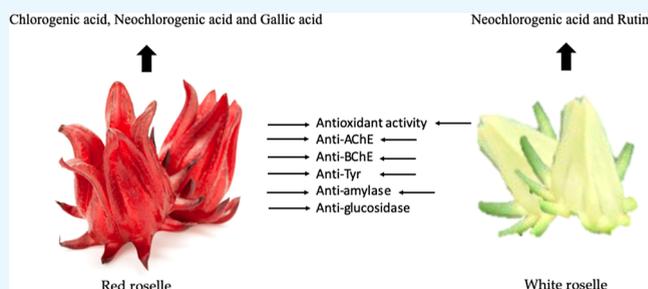
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ABSTRACT: *Hibiscus sabdariffa* L. (Family: Malvaceae) is believed to be domesticated by the people of western Sudan sometime before 4000 BC for their nutritional and medicinal properties. This study aimed to investigate the chemical profile, antioxidant activity, and enzyme inhibition property of extracts from red roselle (RR) and white roselle (WR) varieties grown in Sudan. Three aqueous extracts obtained by maceration, infusion, and decoction, in addition to the methanolic one, were prepared from the two roselle varieties. Results showed that the highest total phenolic and flavonoid contents of RR were obtained from the extracts prepared by infusion (28.40 mg GAE/g) and decoction (7.94 mg RE/g) respectively, while those from the WR were recorded from the methanolic extract (49.59 mg GAE/g and 5.81 mg RE/g respectively). Extracts of RR were mainly characterized by high accumulation of chlorogenic acid (6502.34–9634.96 mg kg⁻¹), neochlorogenic acid (937.57–8949.61 mg kg⁻¹), and gallic acid (190–4573.55 mg kg⁻¹). On the other hand, neochlorogenic acid (1777.05–6946.39 mg kg⁻¹) and rutin (439.29–2806.01 mg kg⁻¹) were the dominant compounds in WR. All extracts from RR had significant ($p < 0.05$) higher antioxidant activity than their respective WR except in their metal chelating power, where the methanolic extract of the latter showed the highest activity (3.87 mg EDTAE/g). RR extracts prepared by infusion recorded the highest antioxidant values (35.09, 52.17, 65.62, and 44.92 mg TE/g) in the DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), CUPRAC (cupric ion reducing antioxidant capacity), and FRAP (ferric reducing antioxidant power) assays, respectively. All aqueous extracts from the WR exerted significant ($p < 0.05$) acetylcholinesterase (AChE) inhibitory activity (3.42–4.77 mg GALAE/g; GALAE = galantamine equivalents), while only one extract, obtained by maceration, from RR exerted AChE inhibitory activity (4.79 mg GALAE/g). All extracts of the RR showed relatively higher BChE (butyrylcholinesterase) inhibitory activity (3.71–4.23 mg GALAE/g) than the WR ones. Methanolic extracts of the two roselle varieties displayed the highest Tyr (tyrosinase) inhibitory activity (RR = 48.25 mg KAE/g; WR = 42.71 mg KAE/g). The methanolic extract of RR exhibited the highest amylase (0.59 mmol ACAE/g) and glucosidase (1.46 mmol ACAE/g) inhibitory activity. Molecular docking analysis showed that delphinidin 3,5-diglucoside, rutin, isoquercitrin, hyperoside, and chlorogenic acid exerted the most promising enzyme inhibitory effect. In conclusion, these findings indicated that the chemical profiles and biological activity of roselle varied according to the variety, extraction solvent, and technique used. These two roselle varieties can serve as a valuable source for the development of multiple formulations in food, pharmaceutical, and cosmetic industries.



1. INTRODUCTION

Hibiscus sabdariffa L. (Family Malvaceae), commonly known as roselle and in Sudan as karkadeh, is widely distributed in tropical and subtropical regions and thought to be domesticated in western Sudan sometime before 4000 BC.^{1,2} The plant is considered an important economic crop in Sudan due to its many nutritional and pharmacological applications. It is also regarded as a famine food, and when drought is expected farmers prefer to cultivate roselle rather than cereals because of

its resistance to harsh environmental conditions.¹ Roselle is grown for its calyces which varied in color as red, dark red,

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green, or white. Red calyces have a high content of anthocyanins, whereas the white or green calyces have no or scarce amount of these flavonoids.^{3,4} In Sudan, there are red- and white-colored roselle types from which a cold or hot beverage is prepared.⁵ A comparative study on the red and white calyces from Sudan showed that the red and white calyces contained, respectively, 0.16 and 0.12% fat, 13.2 and 12% fiber, 7.88 and 7.35% protein, 57.16 and 61.55% carbohydrates, 11 and 15.5 mg/100 g of vitamin C, and 9 and 11 mg/100 g of titratable acidity.⁶ Calyces are combined with the fruits of *Adansonia digitata* and *Tamarindus indica* in traditional medicine to treat malaria.⁷ In addition, the calyx is used to treat hypertension, influenza, hemorrhoids, headache, fever, snakebite, scorpion sting, as a hypotensive and antispasmodic, and for uterine muscle relaxation.⁸

According to the review articles of Da-Costa-Rocha, Bonnlaender, Sievers, Pischel and Heinrich,⁹ and Riaz and Chopra,¹⁰ *H. sabdariffa* calyces are rich in phenols, mainly anthocyanins like delphinidin-3-glucoside, sambubioside, and cyanidin-3-sambubioside. Phenolic acids like neochlorogenic acid and chlorogenic acid, in addition to organic acids such as hydroxycitric acid, hibiscus acid, and hibiscus acid hydroxyethyl ester were also identified. Few phytochemical studies were conducted on the white calyx, and phytochemical screening showed that polyphenols, flavonoids, organic acids, sterols, and saponins were detected.¹¹ Pharmacologically, *H. sabdariffa* calyces have shown to possess antioxidant,¹² antiangiogenic,¹² antidiabetic,¹³ antihyperlipidemic,¹⁴ antiobesity,¹⁵ antihypertension,¹⁶ antidiuretic,¹⁷ and antibacterial¹⁸ activities among others.

The growing market of functional beverages and health promoting products based on plant extracts requires an in-depth characterization of their chemical constituents and biological properties. Biological activities of plants are linked to their phytochemical constituents,¹⁹ so it is essential to determine the appropriate extraction method and extractive solvents for a specific biological activity. Despite the facts that Sudan is considered as one of the principal producers of *H. sabdariffa* and it is frequently consumed as a hot or cold beverage and used in traditional medicine, there has been limited systematic study on the efficiency of different solvent extractions on bioactive molecules. Moreover, there are few studies on the chemical and biological activities of white roselle (WR) variety.^{4,11} In this context, the present study was designed to investigate the chemical profiles, antioxidant activity, and enzyme inhibition property of extracts from red roselle (RR) and WR varieties obtained through maceration, infusion, and decoction by using water and methanol as extraction solvents.

2. MATERIALS AND METHODS

2.1. Plant Materials and Sample Preparation. In the month of September 2022, calyces of both RR and WR varieties were procured from a reputable local market situated in Omdurman, Sudan. To facilitate the subsequent analysis, a semipowdered form of each calyx type was meticulously obtained through a grinding process. The subsequent step in our methodology involved the extraction of bioactive compounds from these samples using both water and methanol (MeOH) as the extraction solvents. Precisely, an amount of 100 g from each sample was accurately weighed. To ensure an effective extraction process, 250 mL of the appropriate solvent was introduced to each sample, ensuring a comprehensive

coverage of the plant material. This meticulous preparation of samples and the extraction process formed the foundation of our subsequent analyses aimed at evaluating the chemical constituents and potential bioactivity of these roselle calyces.

2.2. Preparation of Extracts. The extraction process was carried out in strict adherence to the established protocols as outlined in the methodology originally described by Abubakar and Haque in their work, documented as ref 20.

2.2.1. Maceration Extraction. Prepared samples were soaked in distilled water or MeOH. Subsequently, these immersed samples were left to stand at room temperature for a duration of 1 h.

2.2.2. Infusion Extraction. This extraction was achieved by pouring boiling water over the samples and keeping them for 5 min.

2.2.3. Decoction Extraction. In the decoction extraction method employed, the prepared samples underwent a meticulously controlled process. First, they were subjected to a precise boiling procedure within a water bath, maintained for a duration of 5 min. Following this brief heating period, the samples were then allowed to naturally cool, thus ensuring the extraction of desirable compounds in a controlled and methodical manner. This extraction method was chosen for its effectiveness in yielding bioactive constituents while preserving the integrity of the extracted compounds.

Subsequently, all obtained extracts underwent a meticulous filtration process using Whatman no. 4 filter paper. For methanol (MeOH) extracts, a further concentration step was implemented. This was achieved by utilizing a rotary evaporator maintained at a constant temperature of 40 °C under reduced pressure. The purpose of this concentration step was to enhance the concentration of bioactive compounds within the MeOH extracts, facilitating more precise analyses. On the other hand, the aqueous extracts were subjected to a different preservation method, namely freeze-drying. This freeze-drying process enabled the removal of the moisture content from the extracts while retaining their beneficial components. Finally, after these meticulous extraction and concentration processes, the resulting extracts were accurately weighed and stored in a refrigerator. These storage conditions were chosen to maintain the stability and integrity of the extracts until they were used in subsequent analytical procedures.

2.3. Total Phenolic and Flavonoid Contents. The Folin–Ciocalteu and AlCl₃ assays, respectively, were utilized to determine the total phenolic and flavonoid contents, and the procedures are reported in our earlier paper.²¹

2.4. HPLC–ESI-MS/MS Analysis. HPLC–MS/MS analyses were conducted using an Agilent 1290 Infinity series instrument coupled with an Agilent Technology (Santa Clara, CA) triple quadrupole 6420 mass spectrometer equipped with an electrospray ionization (ESI) source and operated in both negative and positive ionization modes. All analytical details are given in our earlier paper.²²

2.5. Examination of Biological Potential. To assess the antioxidant potential of the extracts, a set of six complementary in vitro spectrophotometric tests were performed. Those included the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays, which examine the antioxidants' ability to neutralize free radicals. FRAP (ferric reducing antioxidant power) and CUPRAC (cupric ion reducing antioxidant capacity) assays, which evaluate the extract's reduction capabilities, as well as

metal chelating ability (MCA) and phosphomolybdenum (PBD) assays. Each of these assays, except for MCA, was evaluated by using the Trolox standard. The comparison for MCA was made in terms of equivalent EDTA per gram of extract. All used procedures are given in our previous work.²³ To assess the inhibitory effects of the tested extracts on various enzymes, we employed acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase (Tyr), amylase, and glucosidase. Details of the experimental procedures can be found in our prior publication.²³ We quantified AChE and BChE inhibition as milligrams of galantamine equivalents (GALAE) per gram of extract, Tyr inhibition as milligrams of Kojic acid equivalents (KAE) per gram of extract, and α -amylase and α -glucosidase inhibition as millimoles of acarbose equivalents (ACAE) per gram of extract. These measurements provide a standardized assessment of the inhibitory potential of extracts on these enzymes.

2.6. Molecular Modeling. AChE (PDB ID: 6O52),²⁴ α -amylase (PDB ID: 1B2Y),²⁵ and BChE (PDB ID: 6EQP)²⁶ and their prepared crystal structures were retrieved.²⁷ Human Tyr and α -glucosidase homology models constructed using the crystal structures of Tyr from *Priestia megaterium* (PDB ID: 6QXD)²⁸ and α -glucosidase from *Mus musculus* (PDB ID: 7KBJ),²⁹ respectively, were retrieved as well.³⁰ The ChEMBL database (<https://www.ebi.ac.uk/chembl/>) was used to download the 3D structures of all ligands whose geometry was optimized using UCSF Chimera.³¹ To generate docking input files, MGLTools 1.5.6 software was used. Within the program, a specific molecular processing step was executed, wherein all nonpolar hydrogen atoms were amalgamated, and Gasteiger charges were subsequently assigned to all atoms present. The Lamarckian genetic algorithm in AutoDock 4.2.6 (<https://autodock.scripps.edu>)³² was used to search for different ligand conformations in the active site of target enzymes, adopting a previously used protocol.³³ The interaction of selected compounds in RR and WR extracts with different target enzymes was analyzed and visualized using Biovia DS Visualizer, version 4.5 (BIOVIA, San Diego, CA, USA).

2.7. Statistical Analysis. Using XI Stat, statistical analysis was performed (version 16). All analyses were performed in triplicate ($n = 3$) and presented as means and standard deviations (mean std). The significance level for the one-way analysis of variance (ANOVA) and Tukey's post hoc test was set as $P < 0.05$ when comparing sample differences. To perform cluster analysis based on the chemical composition and biological abilities, SIMCA 14.0 was utilized.

3. RESULTS AND DISCUSSION

Aqueous and methanolic extracts of RR and WR were examined for their chemical content, antioxidant activity, and enzyme inhibition activity, and the extracts were coded as follows: RRM and WRM are extracts prepared by maceration, RRI and WRI by infusion, and RRD and WRD by decoction, while RRMe and WRMe represent the methanolic extracts.

3.1. Total Phenolic and Flavonoid Contents. The total phenolic content (TPC) in different extracts of RR was in the range of 24.32 and 28.40 mg GAE/g, with the highest content obtained from the RRI extract, while the TPC of WR was in the range of 7.43–49.59 mg GAE/g, with the highest amount recorded in the WRM extract (Table 1). The TPC in different extracts was observed in the following decreasing order: WRM > WRI > RRI > RRD > RRM > RRMe > WRMe > WRD. Although the TPCs in the four extracts of RR were slightly

Table 1. TPC and TFC of RR and WR Extracts^a

extract	TPC (mg GAE/g dc)	TFC (mg RE/g dc)
RRM	25.14 \pm 0.30 ^d	1.47 \pm 0.07 ^e
RRI	28.40 \pm 0.34 ^e	7.32 \pm 0.15 ^b
RRD	27.09 \pm 0.51 ^c	7.94 \pm 0.48 ^a
RRMe	24.32 \pm 1.04 ^d	3.54 \pm 0.09 ^d
WRM	49.59 \pm 0.75 ^a	1.32 \pm 0.04 ^e
WRI	42.66 \pm 0.64 ^b	nd
WRD	7.43 \pm 0.15 ^f	1.22 \pm 0.07 ^e
WRMe	15.73 \pm 0.52 ^c	5.81 \pm 0.08 ^c

^aRRM, red roselle extracted by maceration; RRI, red roselle extracted by infusion; RRD, red roselle extracted by decoction; RRMe, red roselle extracted by methanol; WRM, white roselle extracted by maceration; WRI, white roselle extracted by infusion; WRD, white roselle extracted by decoction; WRMe, white roselle extracted by methanol. Values are reported as mean \pm SD of three parallel measurements; dc: dry calyx; GAE: gallic acid equivalents; RE: rutin equivalents; nd: not detected; Different superscript letters in the same column indicate a significant difference ($p < 0.05$, Tukey's HSD multiple-range post hoc test).

varied, they were far lower than those of the WR obtained by maceration and infusion methods. In fact, the TPC in WRM and WRI extracts were 2.0- and 1.5-fold higher than those in their respective RR extracts. Furthermore, it was observed that high temperature (infusion and decoction) significantly increased ($p < 0.05$) the TPC of RR, supporting the finding of Duy and co-workers who reported that extraction temperatures exceeding 60 °C proved to be optimal for the extraction of TPC.³⁴ In contrast, this behavior was not observed in WR where maceration recovered the highest TPC.

The total flavonoid content (TFC) was in the range of 1.47 to 7.94 mg RE/g in the RR extracts and between 0 and 5.81 mg RE/g in the WR extracts, and the TFC was observed in the following decreasing order: RRD > RRI > WRMe > RRMe > RRM > WRM > WRD > WRI (Table 1). Comparing these results with previous studies showed some variations according to the studied organ and solvent extraction used.^{35–37} With the exception of the RRMe extract, all RR extracts contained a higher TFC than their respective extracts in the WR, with the RRD extract 6.5 times higher in TFC than that of the WRD one. Furthermore, although the infusion method also gave a high TFC in RR, it did not extract measurable flavonoids from the WR. In summary, it was observed that infusion and decoction by water recovered the highest TPC and TFC in RR. For WR, maceration and infusion extracted the highest TPC, while MeOH as a solvent extracted the highest TFC. This variation in TPC and TFC could be due to variable types of phenolics and flavonoids in both roselle varieties. In fact, many studies have substantial evidence, highlighting the intricate nature of the phenolic content within roselle. These investigations have revealed that several critical factors exert a profound influence on the phenolic composition of this botanical specimen. Among the pivotal determinants are the specific varieties or cultivar types of roselle utilized, the prevailing environmental conditions during cultivation, the methodologies employed for harvesting and postharvest handling, and, significantly, the extraction techniques employed.^{18,38}

3.2. Chemical Profile. From the 38 standard compounds, the highest number of compounds in RR extracts were identified in RRM (25), followed by RRD (23), RRI (22), and RRMe (15) (Table 2). The number of identified compounds

Table 2. Chemical Profile of RR and WR Extracts^a

no.	compounds	concentration (mg kg ⁻¹ dry calyx)							
		RRM	RRI	RRD	RRMe	WRM	WRI	WRD	WRMe
1	gallic acid	2317.96	1719.57	190	4573.55	37.98	39.50	95.80	125.74
2	neochlorogenic acid	8949.61	937.57	8676.59	6793.08	1777.05	2673.98	6489.45	6946.39
3	delphinidin-3-galactoside	1113.05	1522.65	1152.12	n.d.	n.d.	n.d.	n.d.	n.d.
4	(+)-catechin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	procyanidin B2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	914.55
6	chlorogenic acid	9219.52	9634.96	8719.24	6502.34	215.60	282.46	986.01	870.63
7	<i>p</i> -hydroxybenzoic acid	134.90	119.15	102.10	264.62	n.d.	n.d.	n.d.	n.d.
8	(-)-epicatechin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	cyandin-3-glucoside	757.06	1079.60	1005.69	n.d.	n.d.	n.d.	n.d.	n.d.
10	petunidin-3-glucoside	4.57	6.80	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	3-hydroxybenzoic acid	236.66	180.64	167.32	n.d.	n.d.	n.d.	n.d.	n.d.
12	caffeic acid	1109.25	899.81	820.38	1220.57	156.57	277.48	982.60	941.12
13	vanillic acid	102.62	n.d.	n.d.	218.08	n.d.	n.d.	n.d.	n.d.
14	resveratrol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	pelargonidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	pelargonidin-3-rutinoside	10.69	16.64	13.23	n.d.	n.d.	n.d.	n.d.	n.d.
17	malvidin-3-galactoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	syringic acid	414.54	459.51	453.19	816.44	181.98	107.50	323.20	510.10
19	procyanidin A2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	131.27
20	<i>p</i> -coumaric acid	108.52	109.51	127.58	188.50	14.54	19.06	51.62	81.29
21	ferulic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22	3,5-dicaffeoylquinic acid	9.45	n.d.	10.39	n.d.	n.d.	n.d.	n.d.	10.56
23	rutin	684.22	758.45	649.18	575.69	1765.17	439.29	1309.09	2806.01
24	hyperoside	147.38	172.89	133.08	604.55	958.79	285.56	326.84	1933.27
25	isoquercitrin	667.62	716.03	647.28	411.45	765.23	155.34	580.42	1165.66
26	delphinidin-3,5-diglucoside	596.80	625.71	558.49	353.32	621.85	134.29	521.94	961.58
27	phloridzin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
28	quercitrin	6.10	n.d.	5.02	n.d.	n.d.	n.d.	n.d.	n.d.
29	myricetin	502.06	670.50	387.78	n.d.	n.d.	n.d.	n.d.	n.d.
30	naringin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
31	kaempferol-3-glucoside	101.37	123.48	117.29	86.58	37.09	7.32	n.d.	69.70
32	hesperidin	86.65	95.29	78.26	n.d.	n.d.	n.d.	n.d.	n.d.
33	ellagic acid	1281.31	1383.25	1045.73	298.66	136.78	n.d.	n.d.	597.72
34	<i>trans</i> -cinnamic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
35	quercetin	1250.51	1416.77	994.48	310.53	84.15	29.55	116.18	623.64
36	phloretin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
37	kaempferol	65.28	69.59	49.94	n.d.	60.03	n.d.	72.28	n.d.
38	isorhamnetin	n.d.	n.d.	n.d.	n.d.	1.50	n.d.	n.d.	n.d.
	total content	29877.71	22718.38	27814.36	23217.96	6814.32	4451.31	11855.45	18689.23

^aRRM, red roselle extracted by maceration; RRI, red roselle extracted by infusion; RRD red roselle extracted by decoction; RRMe red roselle extracted by methanol; WRM, white roselle extracted by maceration; WRI, white roselle extracted by infusion; WRD, white roselle extracted by decoction; WRMe, white roselle extracted by methanol; n.d.; not detected.

in WR extracts was less than those in RR extracts, with the highest number obtained for WRMe (16), followed by WRM (15), WRI, and WRD (12). The total content of the identified compounds was in the following descending order: RRM (29877.71 mg kg⁻¹) > RRD (27814.36 mg kg⁻¹) > RRMe (23217.96 mg kg⁻¹) > RRI (22718.38 mg kg⁻¹) > WRMe (18689.23 mg kg⁻¹) > WRD (11855.45 mg kg⁻¹) > WRM (6814.32 mg kg⁻¹) > WRI (4451.31 mg kg⁻¹). Thus, it is clear that RR extracts had a higher content of compounds than the extracts of WR. Furthermore, aqueous extracts prepared by maceration and decoction were the best methods to recover the identified compounds from RR, while methanol as the solvent as well as decoction by water were the best techniques for the WR. For both roselle varieties, infusion was the method that recovered the least total content. This variation indicated that the best method for recovering phenolic compounds

depends on the nature and structure of the target components.³⁹ Generally, extracts of RR were characterized by the high accumulation of chlorogenic acid (≤ 9634.96 mg kg⁻¹), neochlorogenic acid (≤ 8949.61 mg kg⁻¹), gallic acid (≤ 4573.55 mg kg⁻¹), quercetin (≤ 1416.77 mg kg⁻¹), delphinidin-3-galactoside (≤ 1522.65 mg kg⁻¹), ellagic acid (≤ 1383.25 mg kg⁻¹), and caffeic acid (≤ 1220.57 mg kg⁻¹). On the other hand, neochlorogenic acid (≤ 6946.39 mg kg⁻¹), rutin (≤ 2806.01 mg kg⁻¹), hyperoside (≤ 1933.27 mg kg⁻¹), and isoquercitrin (≤ 1165.66 mg kg⁻¹) were the dominant compounds in WR. In addition, procyanidin B2 (914.55 mg kg⁻¹), procyanidin A2 (131.27 mg kg⁻¹), were present in relatively considerable concentration in the WRMe extract and absent in all other extracts from the two roselle varieties. Delphinidin-3-galactoside (≤ 1522.65 mg kg⁻¹), *p*-hydroxybenzoic acid (≤ 236.66 mg kg⁻¹), cyanidin-3-glucoside (≤ 1079.60

Table 3. Antioxidant Activity of RR and WR Extracts^a

samples	DPPH (mg TE/g dc)	ABTS (mg TE/g dc)	CUPRAC (mg TE/g dc)	FRAP (mg TE/g dc)	MCA (mg EDTAE/g dc)	PBD (mmol TE/g dc)
RRM	33.53 ± 3.77 ^a	46.89 ± 0.18 ^c	56.75 ± 0.37 ^b	40.76 ± 2.75 ^a	na	0.65 ± 0.02 ^b
RRI	35.09 ± 1.09 ^a	52.17 ± 0.61 ^a	65.62 ± 2.78 ^a	44.92 ± 0.94 ^a	na	0.72 ± 0.02 ^{ab}
RRD	33.69 ± 0.34 ^a	50.10 ± 0.29 ^{ab}	64.15 ± 0.27 ^a	42.57 ± 1.54 ^a	na	0.70 ± 0.02 ^b
RRMe	28.30 ± 0.35 ^b	48.22 ± 1.53 ^{bc}	60.38 ± 2.09 ^b	34.81 ± 3.14 ^b	0.78 ± 0.16 ^b	0.79 ± 0.06 ^a
WRM	6.44 ± 0.20 ^d	7.13 ± 0.26 ^{ef}	17.25 ± 0.43 ^{de}	11.97 ± 0.18 ^d	na	0.28 ± 0.01 ^e
WRI	7.08 ± 0.47 ^d	6.03 ± 0.67 ^f	15.36 ± 0.56 ^e	11.87 ± 0.11 ^d	na	0.26 ± 0.01 ^e
WRD	10.11 ± 0.44 ^{cd}	9.32 ± 0.16 ^e	19.35 ± 0.46 ^d	15.86 ± 0.29 ^d	na	0.37 ± 0.01 ^d
WRMe	12.95 ± 0.79 ^c	21.52 ± 0.27 ^d	35.97 ± 0.49 ^c	22.03 ± 0.66 ^c	3.87 ± 0.39 ^a	0.55 ± 0.03 ^c

^aRRM, red roselle extracted by maceration; RRI, red roselle extracted by infusion; RRD red roselle extracted by decoction; RRMe red roselle extracted by methanol; WRM, white roselle extracted by maceration; WRI, white roselle extracted by infusion; WRD, white roselle extracted by decoction; WRMe, white roselle extracted by methanol. Values are reported as mean ± SD. DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), CUPRAC: cupric ion reducing antioxidant capacity, FRAP: ferric reducing antioxidant power, MCA: metal chelating activity, PBD: phosphomolybdenum. TEs, Trolox equivalents; EDTAEs, disodium edetate equivalents; dc: dry calyx; na: not active. Different superscript letters in the same column indicate a significant difference ($p < 0.05$, Tukey's HSD multiple range post hoc test).

Table 4. Enzyme Inhibitory Activity of RR and WR Extracts^a

samples	AChE (mg GALAE/g dc)	BChE (mg GALAE/g dc)	Tyr (mg KAE/g dc)	amylase (mmol ACAE/g dc)	glucosidase (mmol ACAE/g dc)
RRM	4.79 ± 0.00 ^a	3.77 ± 0.38 ^{ab}	18.49 ± 1.20 ^c	0.30 ± 0.05 ^d	0.36 ± 0.02 ^c
RRI	na	4.23 ± 0.43 ^a	15.95 ± 0.55 ^{cd}	0.42 ± 0.08 ^{bc}	0.44 ± 0.01 ^c
RRD	na	3.71 ± 0.45 ^{ab}	18.44 ± 1.17 ^c	0.46 ± 0.03 ^b	0.77 ± 0.02 ^b
RRMe	na	4.17 ± 0.23 ^a	48.25 ± 1.05 ^a	0.59 ± 0.03 ^a	1.46 ± 0.02 ^a
WRM	3.42 ± 0.12 ^b	1.81 ± 0.07 ^c	13.98 ± 1.19 ^d	0.02 ± 0.00 ^e	na
WRI	4.76 ± 0.01 ^a	2.11 ± 0.02 ^c	13.36 ± 0.44 ^d	0.04 ± 0.01 ^e	na
WRD	4.77 ± 0.01 ^a	3.02 ± 0.09 ^b	15.01 ± 0.33 ^d	0.27 ± 0.04 ^d	na
WRMe	na	3.63 ± 0.19 ^{ab}	42.71 ± 1.37 ^b	0.33 ± 0.03 ^{cd}	na

^aRRM, red roselle extracted by maceration; RRI, red roselle extracted by infusion; RRD red roselle extracted by decoction; RRMe red roselle extracted by methanol; WRM, white roselle extracted by maceration; WRI, white roselle extracted by infusion; WRD, white roselle extracted by decoction; WRMe, white roselle extracted by methanol. Values are reported as mean ± SD; GALAEs, galantamine equivalents; KAEs, kojic acid equivalents; ACEs, acarbose equivalents; dc: dry calyx; na, not active. Different superscript letters in the same column indicate significant difference ($p < 0.05$, Tukey's HSD multiple-range post hoc test).

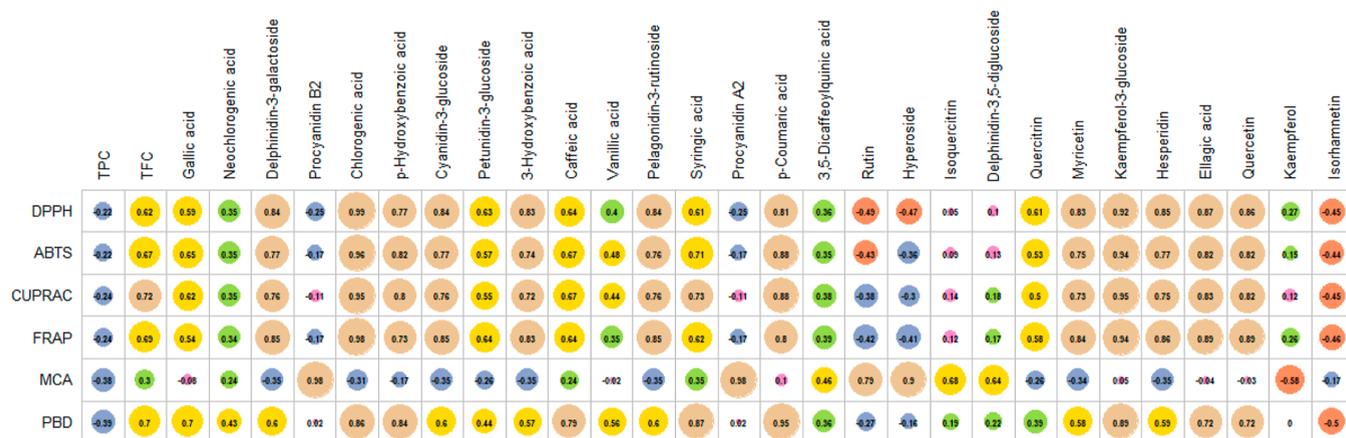


Figure 1. Pearson correlations between total and individual bioactive compounds and antioxidant properties.

mg kg⁻¹), 3-hydroxybenzoic acid (≤ 236.66 mg kg⁻¹), myricetin (≤ 670.50 mg kg⁻¹), vanillic acid (≤ 218.08 mg kg⁻¹), and hesperidin (≤ 95.29 mg kg⁻¹) were only identified in RR extracts. Comparing these results with previous studies on roselle samples collected from different regions in the world, it was noted that most of these compounds were also previously identified but with varied concentrations and mainly in RR.^{9,10,40} On the other hand, some compounds, such as ferulic acid, (+)-catechin, (-)-epicatechin, pelargonidin-3-glucoside, and malvidin-3-galactoside, were commonly identified in RR but were not detected in the present study.

Variation on the level of individual phenolics in different roselle varieties could be attributed to many factors like genetic factors, environmental conditions, postharvest processing, and methods of extraction.^{18,38}

On the other hand, few studies reported the chemical profile of WR. For example, El-Naeem, Abdalla, Ahmed, and Alhassan¹¹ identified cyanidin-3-glucoside, pelargonidin-3-glucoside, delphinidin-3-sambubioside, cyanidin-3-rhamnoside, and delphinidin-3-rhamnoside in both RR and WR samples collected from Sudan. However, the first two compounds were used as the standard in the present study and were identified in

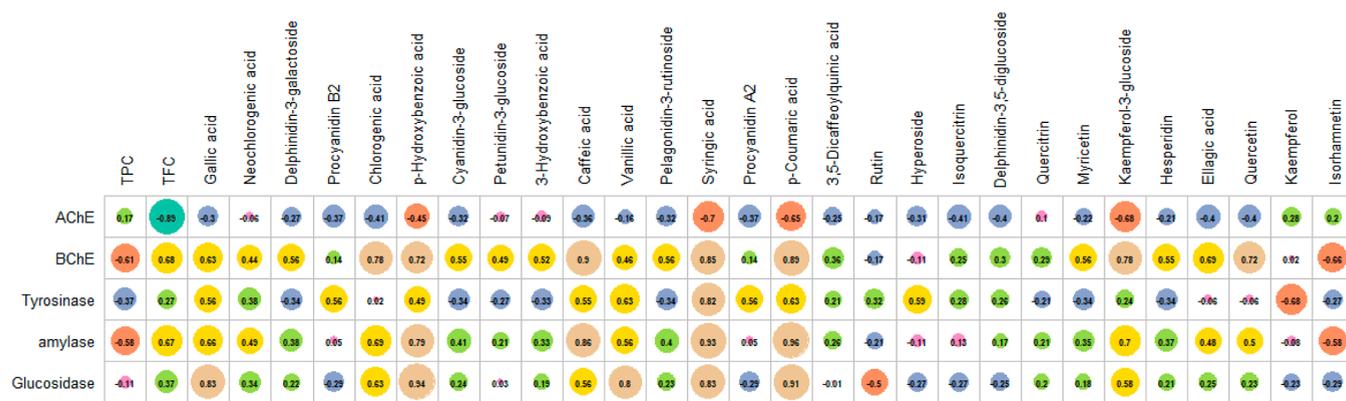


Figure 2. Pearson correlations between total and individual bioactive compounds and enzyme inhibitory effects.

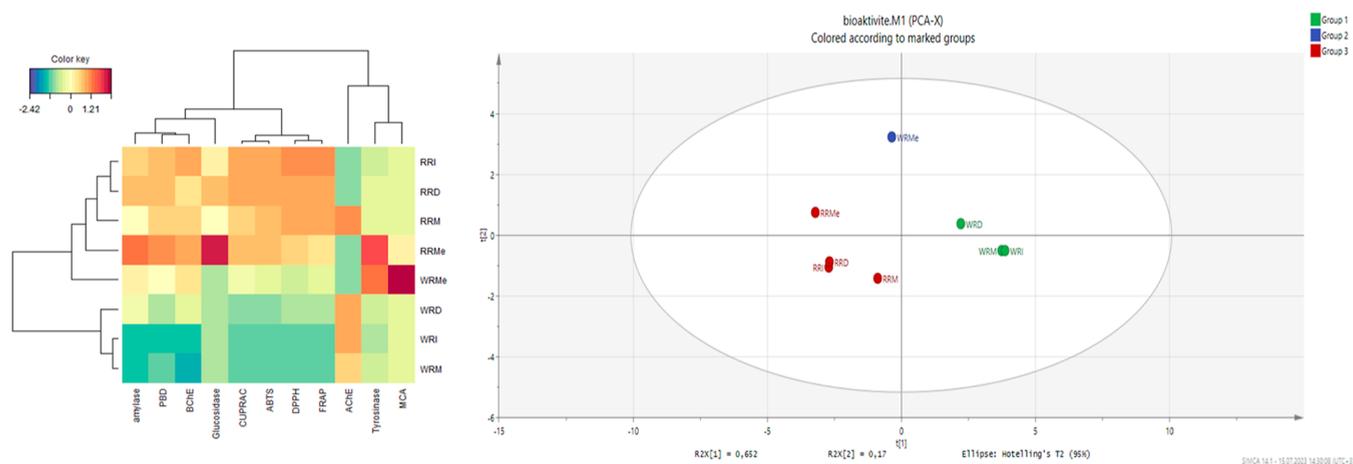


Figure 3. Cluster analysis of the tested extracts based on biological activities.

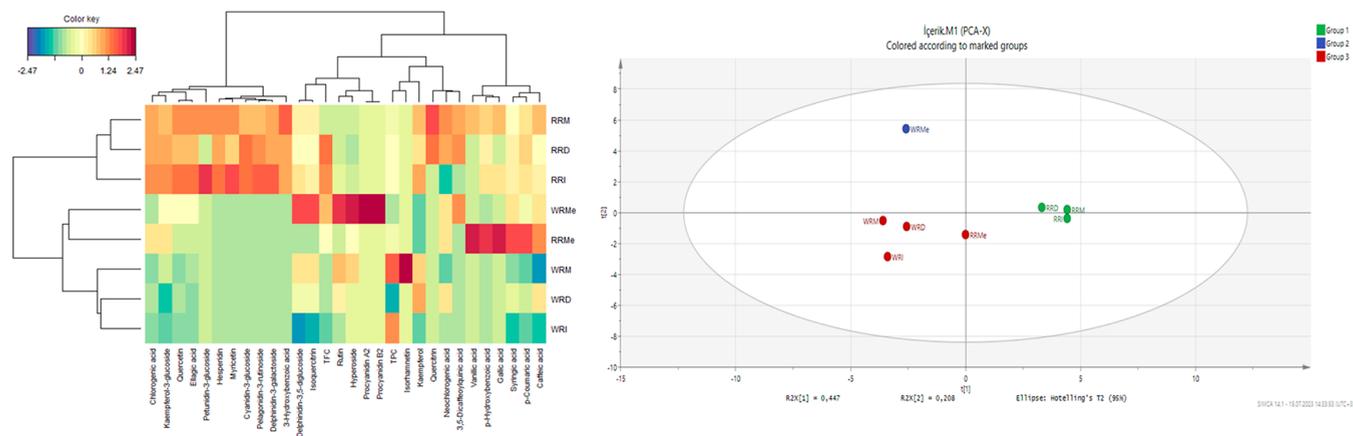


Figure 4. Cluster analysis of the tested extracts based on chemical composition.

RR extracts but not in WR ones. This difference could be in part due to extraction method performed; El-Naeem, Abdalla, Ahmed and Alhassan¹¹ prepared roselle extracts from the Soxhlet apparatus using acidified methanol. Reyes-Luengas, Salinas-Moreno, Ovando-Cruz, Arteaga-Garibay, and Martinez-Peña⁴ found that the caffeic acid content was relatively highly accumulated in WR than in the RR. This was not the case in the present study except for WRD which recovered slightly higher quantity of caffeic acid ($982.60 \text{ mg kg}^{-1}$) than that in RRD ($820.38 \text{ mg kg}^{-1}$). Thus, it was clear that both roselle varieties have their characteristic chemical profile in

terms of type and amount of individual phytoconstituents and accordingly might influence their biological properties.

3.3. Antioxidant Activity. Antioxidants counteract the oxidative damage of cell structures and molecules by reactive oxygen species and ultimately contribute to the prevention from a wide range of diseases.⁴¹ In the present study, 6 complementary assays were performed to evaluate the antioxidant activity of roselle extracts, and results are presented in Table 3. All RR extracts exhibited higher antioxidant activity than their respective WR ones with values significantly ($p < 0.05$) far higher in all assays except in their metal chelating

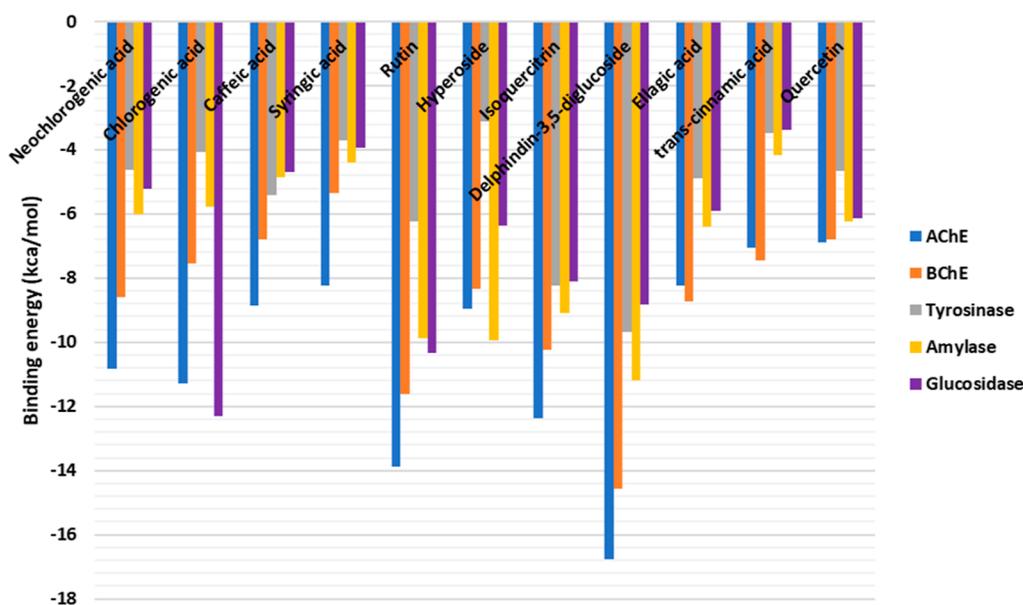


Figure 5. Docking score of the compounds present in large amounts in RR and WR extracts.

power where the WRMe extract showed higher activity. The RR extracts exerted higher ABTS radical scavenging activity than DPPH ones. The highest significant ($p < 0.05$) values in the ABTS assay were recorded from the RRI (52.17 mg TE/g) and RRD (50.10 mg TE/g) extracts, while the 3 aqueous extracts (33.53–35.09 mg TE/g) showed the best DPPH scavenging activity. Furthermore, all RR extracts showed higher capacity to reduce the Cu^{2+} ions than the Fe^{3+} ones with RRI (65.62 and 44.92 mg TE/g, respectively) and RRD (64.15 and 42.57 mg TE/g, respectively) extracts, revealing the utmost values. All extracts either showed no or weak metal chelating capacity. The RRMe (0.79 mmol TE/g) and RRI (0.72 mmol TE/g) extracts exhibited the best total antioxidant activity from the phosphomolybdenum assay. Highest significant ($p < 0.05$) antioxidant activity of WR extracts was mainly recorded in the WRMe extract in all assays. These results supported the study of Tahir and co-workers who reported that red varieties of roselle exerted higher ferric reducing antioxidant power and radical scavenging activity compared to the white variety.⁵ Furthermore, it was reported that roselles with a dark-red calyx color had specific anthocyanin levels and higher antioxidant activity.^{5,15,42} In the present study, anthocyanins like delphinidin-3-galactoside and cyanidin-3-glucoside were not detected in the WR extracts but were present in considerable amount in the RR extracts and eventually might contribute to the observed higher antioxidant activity of the latter. Additionally, other phenolics known for their antioxidant property like phenolic acids (chlorogenic acid, neochlorogenic acid, caffeic acid, and ellagic acid) as well as flavonoids (quercetin, rutin and hesperidin) were also identified in the present study.^{43,44} However, WR extracts showing lower antioxidant capacity than RR ones despite the presence of many of these antioxidant compounds in considerable content suggested the presence of antagonistic phytoconstituents as well.⁴⁵

3.4. Enzyme Inhibition Activity. Enzymes are readily susceptible to inhibition by small metabolites and thus represent a potential therapeutic alternative for some human diseases like Alzheimer's disease, diabetes, and some skin disorders.⁴⁶ In the present study, extracts of the two roselle

varieties were also evaluated for their capacity to inhibit the AChE, BChE, Tyr, α -amylase, and α -glucosidase enzymes. Results are presented in Table 4. The aqueous extracts from WR showed significant ($p < 0.05$) AChE inhibitory activity in the range of 3.42–4.77 mg GALAE/g with the highest almost equal inhibitory potential recorded from WRD (4.77 mg GALAE/g) and WRI (4.76 mg GALAE/g) extracts. For RR extracts, only one extract, namely RRM (4.79 mg GALAE/g), showed high AChE inhibitory activity with a value comparable to those exerted by the WRD and WRI extracts. The methanolic extracts from both roselle varieties did not exert AChE inhibition activity. On the other hand, all extracts of the two roselle varieties showed considerable BChE inhibitory activity in the following decreasing order: RRI > RRMe > RRM > RRD > WRMe > WRD > WRI > WRM. All four RR extracts revealed almost the same ($p > 0.05$) BChE inhibitory activity and higher values (3.71–4.23 mg GALAE/g) than those displayed by the WR extracts (1.81–3.63 mg GALAE/g). These results supported the study of Oboh, Adewuni, Ademiluyi, Olasehinde, and Ademosun,⁴⁷ demonstrating the in vitro effect of *H. sabdariffa* calyx on the AChE and BChE in rat brain. Furthermore, the phenolic acids, *p*-coumaric, *p*-OH-benzoic, ferulic, gallic and ellagic acids, which were identified in the present study, were proven to act as efficient cholinesterase inhibitors.^{48,49} Both RR and WR methanolic extracts displayed significantly higher ($p < 0.05$) Tyr inhibitory activity (48.25 and 42.71 mg KAE/g, respectively) than their different aqueous extracts. Previous study on the methanolic extract of the leaves revealed weak Tyr inhibitory activity (5%).³⁵ Most extracts from both roselle varieties revealed moderate α -amylase inhibition activity in the range of 0.30–0.59 and 0.02–0.33 mmol ACAE/g in the RR and WR, respectively. Considering the capacity of roselle extracts to inhibit the α -glucosidase enzyme, it was observed that only the RR extracts displayed α -glucosidase inhibitory activity with highest significant ($p < 0.05$) value recorded from RRMe (1.46 mmol ACAE/g). The antidiabetic activity of roselle extracts was demonstrated previously, and many phenolics including caffeic acid⁵⁰ and gallic acid⁵¹ were proven to be responsible for this activity.

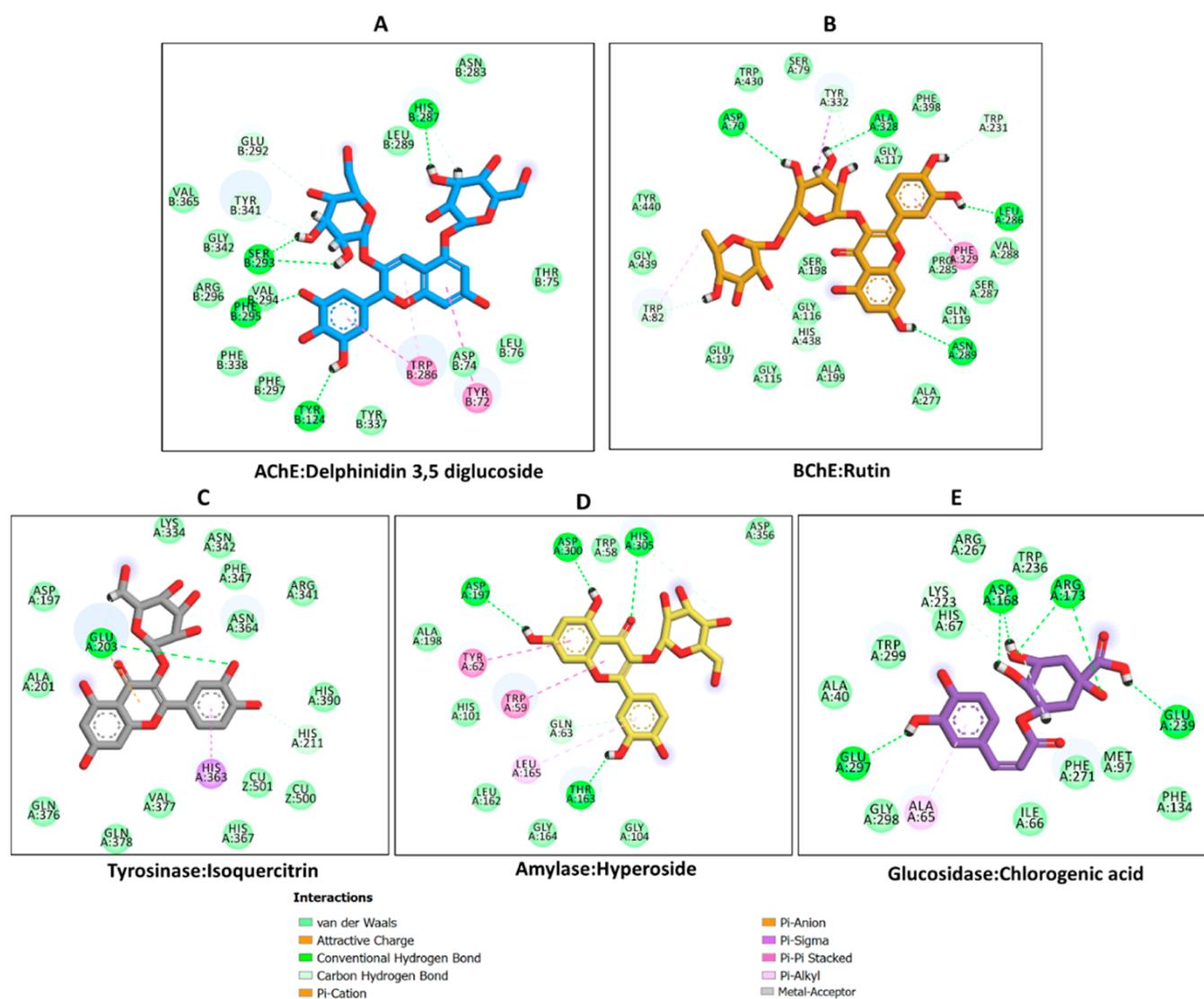


Figure 6. Interaction between target enzymes and compounds in large amount in RR and WR extracts: (A) AChE and delphinidin 3,5-diglucoside, (B) BChE and rutin, (C) Tyr and isoquercitrin, (D) amylase and hyperoside, and (E) glucosidase and chlorogenic acid.

3.5. Multivariate Analysis. To understand the relationship between the structure and activity, we performed Pearson's correlation analysis. First, we detected a possible correlation between total/individual bioactive compounds and antioxidant properties (Figure 1). Interestingly, no positive correlation was observed between the total bioactive compounds and the antioxidant property in our study. This fact can be explained by the limitations of spectrophotometric assays. However, some compounds were strongly correlated with the antioxidant property. In particular, chlorogenic acid, *p*-coumaric acid, and kaempferol-3-glucoside can be regarded as the main contributors to the radical scavenging activity and reducing ability. Regarding the metal chelating ability, the ability can be attributed to the presence of procyanidin (A2-B2) and hyperoside ($R > 0.9$). Our findings were also confirmed by some researchers who reported the antioxidant property of these compounds in *in vitro* and *in vivo* systems.^{52–56} Taken together, the obtained results can be useful for the development of functional foods with increased antioxidant capacity.

Figure 2 shows the correlation values between the total and individual bioactive compounds and the enzyme inhibitory

effects. No correlation was observed between the total bioactive compounds and enzyme inhibitory potentials in our study. Furthermore, no correlation was reported for AChE inhibition, and the observed ability can be explained by the presence of nonphenolic inhibitors such as terpenes. However, some compounds were linked to BChE inhibitory effects. For example, caffeic, syringic, and *p*-coumaric acids showed high correlation values for the BChE effect. Syringic acid strongly correlated with the Tyr inhibitory effect. In addition to syringic acid, some compounds (vanillic acid, hyperoside, *p*-coumaric acid, etc.) moderately correlated with Tyr inhibition. For the antidiabetic enzymes, syringic and *p*-coumaric acids exhibited a linear correlation between these enzyme inhibitions. In general, *p*-coumaric and syringic acids were main contributors to the tested enzyme inhibition and have reported as potent inhibitors in previous studies.^{57–61}

In addition to Pearson's correlation, we performed hierarchical cluster analysis for biological activities and chemical profiles. The results are shown in Figures 3 and 4. Based on the chemical composition and biological activity results, the tested samples were divided into three groups. First, the samples were separated based on the colors of calyx

(white or red). Maceration, decoction, and infusion were distributed in the same plots. But the methanolic extracts of white and red calyces were very different from others. Based on the chemical profiles, the macerated, infused, and decocted samples were very tightly distributed in both calyces. From this point, we concluded that the choice of solvent is a crucial step in preparing functional applications from *H. sabdariffa*.

3.6. Molecular Docking. This study analyzed the binding mode and interaction of selected compounds in RR and WR extracts against different potential target enzymes. The predicted binding propensity in terms of the calculated binding energy for each ligand against different proteins is given in Figure 5. The resulting protein–ligand interaction was visualized for some selected docking complexes (Figure 6). Delphinidin 3,5-diglucoside exhibited strong binding to the AChE active site mainly via H-bonds and π – π stacked interactions and supporting van der Waals interactions (Figure 6A). Similar patterns of interaction were observed between rutin and BChE (Figure 6B). This is consistent with the findings of a previous study on this compound.⁶² Isoquercitrin appeared to bind to the active site of Tyr via H-bonding, π -sigma, and multiple van der Waals interactions all over the channel (Figure 6C). The key interactions between hyperoside and amylase appeared to be H-bonds and π – π -stacked interactions with amino acid residues located deep in the active site of the enzyme (Figure 6D). Chlorogenic acid was buried in the catalytic site of glucosidase via mainly multiple H-bonds and van der Waals interactions (Figure 6E). Together, these may be binding mechanisms by which the selected compounds in RR and WR extracts inhibit the biological activity of the target enzymes.

4. CONCLUSIONS

The present study provides better insight into the influence of the type of solvent extraction and technique on the chemical profile in terms of type and amount of individual phytoconstituents in red and white *H. sabdariffa* calyces and their impact on their antioxidant and enzyme inhibition properties. It was observed that the chemical profiles and biological activities varied according to the variety, extraction solvent, and technique used. Extracts of WR obtained by maceration and infusion recorded the highest content in TPC, while those from RR obtained by infusion and decoction had the highest content of TFC. Both varieties varied in their chemical profiles and accordingly their antioxidant and enzyme inhibition capacities. Maceration and decoction were the best methods to extract the standard compounds from RR, while methanol as the solvent as well as decoction by water were the best techniques for the WR. Generally, RR extracts contained higher concentrations of chlorogenic acid, neochlorogenic acid, and gallic acid, while WR accumulated higher contents of neochlorogenic acid and rutin. Extracts of RR had higher antioxidant activity (5/6 assays) than their respective WR ones. Methanolic extracts displayed better enzyme inhibition activity against BChE, Tyr, and α -amylase enzymes than different aqueous extracts with extracts obtained from the RR, showing significantly highest inhibition activity. The three aqueous extracts of WR exerted significantly high AChE activity in contrast to only one extract from RR obtained by maceration (RRM). Only the RR extracts inhibited the α -glucosidase enzyme. Therefore, RR and WR can serve as potential sources for varied pharmaceutical and nutraceutical applications. The extraction solvent methods used in this study

could be adopted for producing phenolic-rich roselle extracts with interesting biological activity. Further in vivo and clinical studies are highly needed to examine the potential of these two roselle varieties.

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Notes

The authors declare no competing financial interest.

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