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# Screening for total antioxidant activity, flavonoids and phenolics variability in forty-five accessions of roselle (*Hibiscus sabdariffa* L.)

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**Build-up of free radicals in the human body can cause oxidative stress which may invariably lead to degenerative diseases and eventual death. Antioxidants such as polyphenols and flavonoids found in fruits and vegetables have been shown to protect the body against the damaging effects of these free radicals. Roselle contains high amounts of antioxidants and its leaves are often used for sauces or in herbal preparations to treat certain ailments. Several landraces of roselle occur in Ghana but have not been screened for antioxidant activity. Leaf samples of roselle acessions were harvested 60 days after sowing, freeze dried and analysed for total phenolic content (TPC), total flavonoid content (TFC) as well as total antioxidant activity (TAA) using UV-VIS Spectrophotometer (Shimadzu, 1201, Japan). No statistically significant differences were observed in TPC which ranged from 20 ± 0.6 µg/g (RNL) to 90 ± 0.6 µg/g (Sob-4). TFC and TAA in the leaf samples; however, showed statistically significant variation and ranged from 10.04 ± 0.31 µg/g (WH-S2V) to 49.79 ± 0.48 µg/g (Don-1) and 37.48% (Don-4) to 58.58% (WNL-HS), respectively. Higher leaf phenolic or flavonoid content did not necessarily translate into a higher antioxidant activity. This suggests that other forms of antioxidants other than phenolics or flavonoids might be responsible free radical scavenging activity in roselle leaves. Nevertheless, very high free radical percentage inhibition observed in the roselle leaves makes it an excellent material for mitigation against the adverse effect of free radicals. Nine promising accessions with high free radical scavenging activity have been identified for further improvement.**

**Key words:** *Hibisicus sabdariffa*, roselle, antioxidant activity, flavonoids, phenolic content.

## INTRODUCTION

Free radicals are unstable and highly reactive chemical species containing unpaired electrons in their atomic orbital (Lobo et al., 2010). They occur either naturally in the human body as products of normal biochemical processes or are derived from the effects of ionizing radiation. Their formation causes damaging activity in the

**Table 1.** Geographical distribution of the roselle accessions assembled for the study.

| Location | Landrace/Accession |
|---|---|
| Greater Accra | L-1, L-2, L-4A, L-4B, L-5B, L-5A, L-5C, L-7, S-1, Sob-1, Sob-2, Sob-3, Sob-4, Sob-5, Sob-6, Sob-7,Sob-8, Sob-9, Sob-10, RNL, RBL , WH-S2, WHN-S2BL, WNL-HLS, WNL-VSH2, WHN-HS, WH-S2VLPro, WNL-STT, WNL-H, WNL-VSHT, WNL-HS, WBL, WH-S2V |
| Central Region | WH-S2TP, WH-S2VL |
| Northern Region | Don-1, Don-2, Don-3, Don-4,N1-NL, N1-GP, N-3, N-4, N-5, S-2 |

body on important components such as carbohydrates, proteins, lipids and DNA. This damaging effect can cause oxidative stress which leads to degenerative diseases such as atherosclerosis, cancers and diabetes (Oboh and Rocha, 2006; Young and Woodside, 2001; Bagchi et al., 2000) which account for about 1.7 million (2.8%) deaths worldwide (WHO, 2016).

These diseases or death cases could be prevented by adequate intake of antioxidants which have been identified as compounds capable of inhibiting or delaying such stress processes. Antioxidants have been reported to provide body cells with significant protection against oxidative damage (Cid-Ortega and Guerrero-Beltrán, 2015; Carvajal-Zarrabal et al., 2012). Maintaining a body balance between them and pro-oxidative species such as peroxides, hydroxyl radicals and singlet oxygen is therefore, critical to ensuring good health status in humans. Important examples of antioxidants include polyphenols, lycopenes and flavonoids which can be obtained from fruits and vegetables (Karadag et al., 2009; Cai et al., 2004).

In Ghana, roselle (*Hibiscus sabdariffa* Linn.) which belongs to the family Malvaceae is one of the most important vegetables, especially popular among rural folks. Both leaves and calyces are prepared into sauces or herbal drinks which are believed to offer tremendous health benefits due to its phytochemical constituents, particularly antioxidants. Elsewhere, roselle extracts are, commonly used in traditional medicine because of their antihypertensive, hepatoprotective, cardio-protective and anti-cancerous properties (Odigie et al., 2003; Tseng et al., 1997).

In spite of its beneficial uses and potential as medicinal crop, not much research has been done on roselle in Ghana. Depending on genotype and environment, the phytochemical composition of roselle has been found to be quite variable (Burkill, 1997). However, no study has been conducted in Ghana to establish the variability among landraces. This study was, therefore, carried out to determine the phenolic content, total flavonoids and antioxidant activity in the leaves of 45 accessions of roselle in Ghana and to identify promising lines for use in future breeding programmes.

## MATERIALS AND METHODS

### Plant collection

Forty-five accessions of roselle were collected from three geographical regions (Table 1) of Ghana (Greater Accra, Central and Northern regions) between April and June 2013. These locations are areas where the crop is commonly grown and consumed as vegetable or processed into local beverage.

### Experimental site

The roselle accessions collected were cultivated at the Biotechnology and Nuclear Agriculture Research Institute (BNARI) which is located 76 m above sea level and situated on latitude 05° 40' N and longitude 0° 13' W in the coastal savannah agro-ecological zone.

### Cultivation

Seeds of each accession were sown in plastic containers (40 cm x 30 cm x 25cm) filled with a mixture of top soil and decomposed broiler manure in 6:1 ratio, respectively. Four seeds were sown per container and replicated 5 times in randomly complete block design (RCBD). Seedlings were thinned to 2, two weeks after emergence. Watering was done when necessary to prevent plants from wilting.

### Sample preparation

For each accession, fresh leaves were harvested from the mid-sections of 5 randomly selected plants at 60 days after emergence (DAE). The leaves were bulked and pulverized after freeze-drying. Pulverized samples were put into air-tight plastic bags and stored at 2°C until needed. For each sample, 0.05 g was weighed into a clean 15 mL Falcon tube and extracted serially with 5 mL of 60 % methanol and extraction was done four times, each time vortexing for 5 min. The supernatants were collected and stored for phytochemical analysis.

### Phenolic content determination

Polyphenolic contents were determined by a modified Folin-

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Ciocalteu method using gallic acid as standard (Singleton et al., 1999). A 50 µL portion of each of the extracted sample was mixed with 3 mL of distilled water ($dH_2O$) and 250 µL of a 1 in 10 diluted Folin-Ciocalteu Phenol Reagent. The mixtures were allowed to stand for 5 min, after which 750 µL of 20% $Na_2CO_3$ was added. This was thoroughly mixed and incubated for 30 min at room temperature (22 to 25°C) in the dark. Absorbance was measured at 760 nm using a UV-VIS Spectrophotometer (Shimadzu, 1201, Japan). A calibration curve was prepared using serial dilutions of 1 mg/mL gallic acid dissolved in water at the following concentrations: 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. A linear regression equation y = 1.1738x – 0.0041 with a regression coefficient of $R^2 = 0.9992$ was established for the curve. Total phenolic content (TPC) was determined and expressed as milligram gallic acid equivalent per gram of sample according to the formula:

$$\text{TPC (mg/g)} = [(\text{c} \times \text{v})/m]$$

Where, c = concentration of gallic acid (mg/mL); v = volume of sample extract (mL); m = load of sample extract (g).

### Flavonoid content determination

Total flavonoid content (TFC) was determined in the samples using the aluminium chloride colorimetric assay method (Zhishen et al., 1999). An aliquot of 500 µL sample extract was mixed with 1500 µL of 99.9% ethanol, 100 µL of 1.0 M potassium acetate, 100 µL of 10% aluminium chloride and 3000 µL of distilled water. After incubating the resulting mixtures for 30 min at room temperature, the corresponding spectrophotometric absorbance was measured at 415 nm. Standard calibration curve using quercetin standard solutions of 12.5, 25, 50, 75 and 100 µg/mL each time the samples were analysed, was constructed. For each standard, 500 µL was treated in the manner as indicated above for the samples. The linear regression equation y = 0.0055x +0.0026 with a regression coefficient, $R^2 = 0.99$, was derived for the calibration. Total flavonoid content (TFC), expressed as microgram of quercetin equivalent per gram of sample was determined from the expression:

$$\text{TFC (µg/g)} = [(c \times df \times v)/ w]$$

Where, c = concentration derived from standard curve (µg/mL); df = dilution factor; v = volume of stock solution (mL) and w = sample weight (g).

### Free radical scavenging assay

The determination of total antioxidant activity (TAA) was done by means of free radical scavenging assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Brand-Williams et al. (1995) with modification. Two hundred micro litres (200 µL) of extracts were each added to 3800 µL of 0.004% DPPH in methanol. After 60 min of incubation at room temperature in the dark, the absorbance was measured at 517 nm. The methanolic blank sample was used to set the spectrophotometer reading to zero. Absorbance readings were done in triplicates. The free radical scavenging activity, expressed as percent inhibition (I %) of the DPPH was computed using the equation:

$$I\ (\%) = [(A_0 – A_c) / A_0] \times 100$$

Where, $A_0$ = absorbance of the blank and $A_c$ = absorbance of the test sample (Brand-Williams et al., 1995).

ANOVA on TPC, TFC and TAA was performed using Statgraphics Centurion XVI, version 16.1.11. Duncan multiple range test, was used to separate means.

## RESULTS AND DISCUSSION

### Geographical distribution, phenolic and flavonoid contents of roselle leaves

Thirty-three accessions (73.3%) of roselle were collected from urban and peri-urban vegetable farms around the Greater Accra regional capital, Accra. Diversity in the accessions collected seemed to be greatest in this location probably because there is ready market for the fresh cut-foliage of roselle. As a result, farmers might have collected many accessions across the country and beyond for propagation. Only 12 accessions were collected from central and northern regions combined, where less diversity was observed.

Variations in phenolic and flavonoid contents of leaves of the 45 roselle (*H. sabdariffa* Linn) accessions are presented in Table 2. Total phenolic content (TPC) in the leaf samples ranged from 20 ± 0.6 (RNL) to 90 ± 0.6 µg/g (Sob-4). The highest and lowest contents were detected in accessions collected from the Greater Accra region. Accessions collected from the Central and Northern regions had TPC between 47 ± 0.6 (WH-S2VL) to 54 ± 0.3 (WH-S2TP) and 27 ± 0.4 (N1-NL) to 54 ± 3.4 (DON-1), respectively. Maximal TPC contents of samples from the Central and Northern regions were therefore 40% below the highest TPC obtained in the study. Statistical analysis, however, did not show significant differences ($p \geq 0.05$) among all the accessions in terms of TPC. Total phenolic contents observed in the current study were lower than values obtained in similar studies in ethanolic and aqueous extraction systems of roselle leaves in Brazil (Formagio et al., 2015) or elsewhere (Sirag et al., 2014; Al-Hashimi, 2012). The values are also lower than those obtained for moringa (Owusu-Ansah et al., 2011) and cassava leaves (Quartey et al., 2016) which are also used in preparation of herbal drinks and sauces for similar purposes as roselle leaves.

Total flavonoid content (TFC) of the roselle leaves varied significantly ($p \leq 0.05$) among the accessions. Don-1 recorded the highest value (49.79 ± 0.48 µg/g). The lowest TFC was observed in WH-S2V (10.04 ± 0.31 µg/g). These values are lower than those determined in Brazil by Formagio et al. (2015) in roselle leaves (140.29 ± 3.14 to 148 ± 2.42 mg/g) and calyces (95.85 ±1.85 to 104.52 ± 4.85 mg/g) possibly because the cultivation media were highly enriched with organic substrates or it is simply a factor of inherent genetic attribute of those varieties. Phenolic and flavonoid contents of plant materials have been shown to vary considerably depending on variety and environment (Yang et al., 2006; Burkill, 1997). Since the values reported in the current study are lower than those reported in other countries, it might be necessary to optimise agronomic and cultivation practices aside carrying out genetic improvement of the crop so as to obtain varieties with improved antioxidant potentials in Ghana.

**Table 2.** Antioxidant activity, total phenolics and flavonoids in the leaves of 45 roselle accessions.

| Accession number | Accession | Phenolics | | Flavonoids | | Antioxidant activity | |
|---|---|---|---|---|---|---|---|
| | | Total content, TPC (µg /g) | Ranking | Total content, TFC (µg/g) | Ranking | Inhibition (%), TAA | Ranking |
| 1 | RNL | 90±0.6$^{a}$ | 1 | 49.27±3.10$^{y}$ | 2 | 45.17±0.00$^{s}$ | 3 |
| 2 | S-1 | 88±0.6$^{a}$ | 2 | 44.00±0.00$^{x}$ | 3 | 41.17±1.17$^{opqr}$ | 7 |
| 3 | L-4A | 74±1.9$^{a}$ | 3 | 30.01±1.35$^{tu}$ | 8 | 27.20±0.71$^{fg}$ | 31 |
| 4 | RBL | 68±1.3$^{a}$ | 4 | 31.89±0.54$^{v}$ | 5 | 20.52±0.14$^{ab}$ | 43 |
| 5 | SOB-1 | 62±2.4$^{a}$ | 5 | 28.06±0.38$^{rs}$ | 11 | 20.73±0.21$^{ab}$ | 42 |
| 6 | SOB-3 | 58±0.3$^{a}$ | 6 | 29.89±0.74$^{tu}$ | 9 | 37.31±2.12$^{klmn}$ | 15 |
| 7 | DON-1 | 54±3.4$^{a}$ | 7 | 49.79±0.48$^{y}$ | 1 | 37.18±0.18$^{klmn}$ | 16 |
| 8 | WH-S2TP | 54±0.3$^{a}$ | 8 | 19.11±0.15$^{mn}$ | 19 | 38.14±4.20$^{klmnop}$ | 11 |
| 9 | N1-GP | 50±0.4$^{a}$ | 9 | 30.89±0.35$^{uv}$ | 6 | 32.90±0.35$^{hij}$ | 24 |
| 10 | WH-S2VL | 47±0.6$^{a}$ | 10 | 22.38±0.64$^{q}$ | 13 | 37.74±0.74$^{klmnop}$ | 13 |
| 11 | N-3 | 46±1.7$^{a}$ | 11 | 26.28±0.48$^{r}$ | 12 | 33.07±0.17$^{hij}$ | 23 |
| 12 | L-2 | 46±0.3$^{a}$ | 12 | 17.50±0.24$^{klm}$ | 23 | 39.20±2.75$^{nopqr}$ | 8 |
| 13 | WBL | 45±1.4$^{a}$ | 13 | 22.10±0.37$^{pq}$ | 14 | 27.38±1.83$^{fg}$ | 30 |
| 14 | SOB -8 | 44±0.9$^{a}$ | 14 | 20.99±0.25$^{opq}$ | 15 | 27.60±1.12$^{fg}$ | 29 |
| 15 | DON-2 | 43±3.5$^{a}$ | 15 | 35.60±0.55$^{w}$ | 4 | 36.11±3.89$^{ijklmn}$ | 19 |
| 16 | L-5B | 42±0.0$^{a}$ | 16 | 19.21±0.45$^{mno}$ | 18 | 22.41±1.47$^{abcd}$ | 37 |
| 17 | DON-4 | 39±2.3$^{a}$ | 17 | 30.53±0.67$^{uv}$ | 7 | 37.48±1.76$^{klmno}$ | 14 |
| 18 | DON-3 | 39±0.9$^{a}$ | 18 | 28.55±0.20$^{st}$ | 10 | 23.07±1.26$^{bcde}$ | 35 |
| 19 | WHN-S2BL | 38±1.1$^{a}$ | 19 | 16.05±0.23$^{jk}$ | 27 | 21.16±0.36$^{ab}$ | 41 |
| 20 | L-7 | 38±0.8$^{a}$ | 20 | 17.75±0.18$^{klm}$ | 22 | 38.87±0.94$^{mnopq}$ | 9 |
| 21 | N-4 | 38±0.0$^{a}$ | 21 | 17.24±0.53$^{kl}$ | 24 | 21.46±0.94$^{abc}$ | 40 |
| 22 | S-2 | 37±1.6$^{a}$ | 22 | 17.90±0.07$^{lm}$ | 20 | 35.28±0.81$^{ijklm}$ | 20 |
| 23 | L-4B | 37±0.3$^{a}$ | 23 | 14.99±0.28$^{ij}$ | 28 | 21.81±0.15$^{abcd}$ | 39 |
| 24 | N-5 | 35±0.0$^{a}$ | 24 | 16.88±0.06$^{kl}$ | 25 | 22.77±0.66$^{abcde}$ | 36 |
| 25 | L-5C | 34±0.9$^{a}$ | 25 | 19.81±0.12$^{no}$ | 17 | 49.75±0.77$^{t}$ | 2 |
| 26 | L-1 | 33±1.0$^{a}$ | 26 | 13.64±0.27$^{efghi}$ | 33 | 20.24±0.28$^{ab}$ | 44 |
| 27 | WH-S2 | 33±0.9$^{a}$ | 27 | 16.21±0.05$^{jkl}$ | 26 | 41.28±1.86$^{pqr}$ | 6 |
| 28 | WNL-HS | 32±1.3$^{a}$ | 28 | 20.31±0.91$^{nop}$ | 16 | 58.58±1.11$^{u}$ | 1 |
| 29 | SOB-9 | 32±0.5$^{a}$ | 29 | 14.64±0.48$^{hij}$ | 29 | 34.84±1.07$^{ijj}$ | 22 |
| 30 | WNL-HLS | 31±0.4$^{a}$ | 30 | 13.86±0.27$^{fghi}$ | 32 | 35.02±1.07$^{ijkl}$ | 21 |
| 31 | SOB-7 | 31±0.0$^{a}$ | 31 | 11.95±0.00$^{bcde}$ | 40 | 36.47±1.64$^{jklmn}$ | 18 |
| 32 | SOB-6 | 30±1.5$^{a}$ | 32 | 12.76±0.04$^{cdefg}$ | 36 | 38.01±0.09$^{klmnop}$ | 12 |
| 33 | WNL-H | 30±0.0$^{a}$ | 33 | 12.30±0.70$^{cdef}$ | 39 | 25.41±1.08$^{def}$ | 33 |
| 34 | WNL-STT | 28±0.2$^{a}$ | 34 | 14.18±0.24$^{ghi}$ | 31 | 29.63±1.40$^{gh}$ | 26 |
| 35 | WNL-VSH2 | 27±0.5$^{a}$ | 35 | 12.84±0.15$^{defgh}$ | 35 | 22.10±0.15$^{abcd}$ | 38 |
| 36 | N1-NL | 27±0.4$^{a}$ | 36 | 17.81±0.65$^{klm}$ | 21 | 28.55±0.32$^{fg}$ | 27 |
| 37 | L-5A | 26±0.8$^{a}$ | 37 | 11.48±0.40$^{abcd}$ | 41 | 32.56±1.03$^{hi}$ | 25 |
| 38 | WNL-VSHT | 25±1.1$^{a}$ | 38 | 11.02±0.44$^{abc}$ | 42 | 25.17±0.08$^{cdef}$ | 34 |
| 39 | WH-S2VLPro | 25±0.2$^{a}$ | 39 | 14.58±0.46$^{hij}$ | 30 | 28.39±0.32$^{fg}$ | 28 |
| 40 | WH-S2V | 23±0.5$^{a}$ | 40 | 10.04±0.31$^{a}$ | 45 | 26.41±0.70$^{efg}$ | 32 |
| 41 | SOB-5 | 22±1.6$^{a}$ | 41 | 10.49±0.15$^{ab}$ | 44 | 38.77±0.47$^{lmnopq}$ | 10 |
| 42 | WHN-HS | 22±1.1$^{a}$ | 42 | 10.98±0.12$^{abc}$ | 43 | 42.94±0.00$^{rs}$ | 4 |
| 43 | SOB-2 | 22±0.2$^{a}$ | 43 | 13.31±0.18$^{efghi}$ | 34 | 42.24±0.30$^{qrs}$ | 5 |
| 44 | SOB-10 | 22±0.1$^{a}$ | 44 | 12.45±0.16$^{cdefg}$ | 37 | 37.09±0.46$^{klmn}$ | 17 |
| 45 | SOB-4 | 20±0.6$^{a}$ | 45 | 12.45±0.00$^{cdefg}$ | 38 | 19.12±0.97$^{a}$ | 45 |

Values within a column followed by the same superscript are not significantly ($p \leq 0.05$) different according to Duncan multiple range test.

**Table 3.** Correlation coefficients and p-values among phyto-chemicals and antioxidant activity in roselle leaf samples of 45 accessions of roselle.

| Antioxidant activity | Total phenolic content | Total flavonoids content | Total antioxidant activity |
|---|---|---|---|
| Total phenolic content | - | | |
| Total flavonoid content | 0.4495 (0.00) | - | |
| Total antioxidant activity | 0.0681 (0.5235) | 0.1856 (0.0798) | - |

Figures in parenthesis are p-values.

## Free radical scavenging activity in the roselle leaf landraces

The total antioxidant activity (TAA) determined in the roselle accessions are presented in Table 2. The highest TAA detected in the roselle leaf samples was 58.58 ± 1.11% (WNL-HS) which differs significantly ($p \leq 0.05$) from the least value of 19.12 ± 0.97% recorded for Sob-4. Roselle leaves exhibit a considerably high degree of free radical scavenging activity. Of the accessions studied, 15.56% showed free radical scavenging capacities above the highest value detected in 14 accessions of moringa (39.48%). This indicates that roselle leaves could constitute an excellent recipe for making herbal drinks aimed at combating the adverse effects of free radicals in the body via its antioxidant activity.

Indeed, ethanolic and aqueous extracts of roselle leaves showed a reduced lipid peroxidation in rats which confirmed their antioxidant properties (Ochani and D'Mello, 2009). Extracts evaluated for *in vitro* anti-cancerous activity against several cultured human cancer lines also demonstrated significant selective activity against leukemia lines (Formagio et al., 2015). Flavonoids and polyphenols are powerful free radical scavengers which regulate the activities of most enzyme systems as they interact with other biomolecules. Dietary inclusion of antioxidants can therefore prevent oxidative damage to biological molecules which can cause harmful diseases (Essa and Shubramanian, 2006; Rice-Evans et al., 1996). The results of the current study therefore, suggest that roselle leaves as vegetables or herbal drink may be consumed to scavenge free radicals and boost the body's immune system.

## Relationship among phenolics, flavonoids and total antioxidant activity in roselle leaves

Correlations among TPC, TFC and TAA are shown in Table 3. A positive correlation (0.4495) was found between TPC and TFC. Positive correlations also existed between TFC and TAA as well as TPC and TAA. However, though not statistically significant, the relationship between TFC and TAA (0.1856) was relatively stronger than what was observed between TPC and TAA (0.0681). Flavonoids are the principal phytochemicals found in roselle (Tsai et al., 2002). This implies that higher proportion of the free radical scavenging activity in the leaf samples could be attributable to flavonoid rather than phenolic antioxidant activity as also suggested by Formagio et al. (2015).

## Conclusions

Leave samples of the 45 roselle accessions tested exhibited wide variations in TPC, TFC and TAA. Though TPC and TFC observed were below ranges determined in roselle elsewhere, the roselle leaves showed very high free radical scavenging activity, suggesting that they may be excellent ingredients in anti-aging and therapeutic herbal preparations to combat various forms of cancers and heart related diseases. Total flavonoid content correlated more strongly with TAA (0.1856) than with phenolics (0.0681). Much of the free radical scavenging activity in the leaves could be mainly attributed to its flavonoid content.

A twenty percent selection intensity imposed on the 45 roselle accessions yielded 9 promising lines with high total antioxidant activity ranging from 38.87 ± 0.94 to 58.58 ± 1.11% identified for future genetic improvement. These include two accessions which rank very high for both TPC and TFC (RNL and S-1).

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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