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Assessment of chemotaxonomic relationship among five morphotypes of roselle (*Hibiscus sabdariffa* L.) using morphological and biochemical markers

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Abstract
Despite the reported intraspecific polymorphism among roselle plants, not much has been done to assess their intraspecific diversity in their morphological and biochemical markers. This study employed morphological and biochemical markers to assess the chemotaxonomic relationship among five roselle morphotypes growing in Wukari Local Government Area in Taraba State, Nigeria. The selected morphotypes were collected, identified, and evaluated in the field and laboratory using standard methods. Assessment of qualitative morphological traits revealed that the morphotypes were similar except for stem colour, petal colour, leaf lobe, stem surface, among others. Significant differences ($p < 0.05$) were observed for all quantitative traits studied with morphotype HS05 showing the highest mean performance for leaf area (214.52cm^2), plant height (246.63cm), number of nodes (295), number of branches (10.67). HS01 showed the least mean performance for leaf area (91.34cm^2), plant height (60.07cm), number of nodes (145.67), and number of branches (6.00) among others. Phytochemical analysis of calyxes revealed that all the morphotypes contained saponins and alkaloids while steroids were undetected. Analysis of numerical data from chromatographic profiles revealed that HS05 had the highest Paired Affinity value of 85.7% while the highest Group Affinity value of 313.4 was recorded for HS02. Pairwise, HS01 and HS02 as well as HS04 and HS05 clustered together. Results suggest that plant height and number of nodes are important descriptor traits and reveal HS01 and HS05 as the morphotypes with the best prospects for crop improvement. It also reveals HS03 as the morphotype with the most phytochemicals and highlights the potential of chromatography in the chemotaxonomy of intraspecies.

Keywords: roselle, hibiscus, chemotaxonomy, biochemical markers, TLC

Introduction
Roselle previously grown principally for their edible calyxes, leaves, fibres, and seeds, have gained much acceptance in sub-Saharan countries for their medicinal benefits. Research has revealed that the calyxes of roselle possess antispasmodic potential, lower blood pressure, balance cholesterol level in the body, and improve blood circulation (Raiz and Chopra 2018) [17]. Roselle have also been reported to be useful in reducing blood viscosity, stimulating peristaltic movement in the intestines as well as in improving the general health conditions of diabetics (Mardiah *et al.*, 2014) [13]. Furthermore, the exploration and collection of roselle from West Africa reveal that the region ranging from Northern Ghana to Mali through Burkina Faso is a centre of rich diversity of this species. In Northern Ghana, roselle have been characterised by their morphological traits which have been successfully correlated to genetic differences (Coffie 2016) [4]. However, extensive intraspecific variations reported in roselle have led to significant confusion regarding the identity of this plant giving rise to the need for taxa delineation. Although plants that share similar features in their facies are more likely to be closely related, distantly related species may also appear very much alike because of adaptation to similar environmental conditions during their evolution. In comparing variations both within and between species, taxonomists rely much on morphological, anatomical, and biochemical markers to discriminate between homologous and analogous structures. Morphological and biochemical markers are veritable tools with huge potentials in the chemotaxonomy of intra-species. These markers play vital roles in the identification of descriptor traits, selection of better performing taxa as well as the resolution of complexities associated with polymorphisms. Though morphological markers are known to be useful, they usually show limited application in the assessment of inter and intra-varietal polymorphism and hence, may not account for all the variations within and between species (Nadeem *et al.*, 2017) [14]. Characterizations of plants with biochemical markers are much more reliable in the identification of varieties, cultivars, and genotypes than morphological markers.

According to Mukesh *et al.* (2018) [11] biochemical data improve or even allow the elucidation of phylogeny and provide basic knowledge for understanding taxonomy, domestication, and evolution of plants. Despite the diversity among plants in the genus Hibiscus, different taxa are known to maintain their unique metabolic pathways which make them unique from members of other taxa (Kunaso *et al*., 2019) [9]. Study of plant metabolism among the roselle are useful in elucidating similarities as well as differences in the physiology of the plants while simultaneously highlighting biochemical uniqueness of each taxon, which may be of taxonomic significance. Chromatographic spot patterns are other excellent biochemical markers that find application in the evaluation of intraspecific variations as well as phylogenetic relationships among species and are reported to be important in plant taxonomy (Simon *et al*., 2018) [19].

Materials and Methods

Collection of plant materials and identification

The five selected morphotypes of *Hibiscus sabdariffa* used in this study were collected within Wukari Local Government Area in Southern part of Taraba State, Nigeria. HS01 and HS02 were collected from Ebenezer Primary School located at Latitude 7˚54′ 45″N and Longitude 9˚45′13″E. HS03, HS04 and HS05 were collected beside Redeemed Christian Church of God, Holy Parish situated at Latitude 7˚54′7″N and Longitude 9˚45′16″E all in the month of October 2018. The plants were identified and documented by a botanist in the Department of Biological Sciences, Federal University Wukari, Taraba State.

Study area

The study was carried out in Federal University Wukari, Taraba state which is located at a Latitude of 7˚50′37″N and Longitude 9˚46′30″E. Morphological traits was assessed in the field while the Thin Layer Chromatography (TLC) analysis were carried out in the Research Laboratory of the Department of Biological Sciences, Federal University Wukari, Taraba State all in the month of April 2019.

Examination of morphological traits

Field evaluation and data collection of the roselle morphotypes started at physiological maturity, 120 days after planting as recommended by Coffie (2016) [4]. The morphotypes were evaluated using 22 qualitative and 9 quantitative traits on three competitive plants. The qualitative traits assessed by visual inspection, included: stem colour, stem surface, branching pattern, growth habit as well as leaf characteristics such as shape, colour, blade, apex, arrangement, base, lobation, venation, margination, foliage cover, petiole surface and petiole pubescence. Floral characteristics such as petal colour, petal arrangement, sepal colour, stamen colour and flower shape were also evaluated. A meter rule graduated in centimetre was used to measure the quantitative vegetative traits such as leaf length, leaf width, height at first branch, plant height, length of internode, and length of petiole. Number of nodes per plant and number of branches were evaluated by count. The leaf area was calculated from the leaf length (L) using the formula recommended for roselle by Nnebue *et al*., (2015) [15] as:

$$\text{Leaf area} = 0.66 + 0.5968L^2$$

Methanolic extraction for phytochemical screening

The calyxes of each of the morphotypes were collected, washed with clean water, and sterilized with 70% ethanol before being air dried at room temperature until they became brittle. The dried samples were then pulverized aseptically with laboratory crucible to obtain a fine powder. The powder was stored at room temperature in labelled airtight containers and later extracted separately with absolute Methanol. For the extraction, 2 g of the pulverized samples each were extracted with 60 ml of absolute methanol by subjecting them to maceration at room temperature for 48 hours after which they were filtered with Whatman's filter paper. The extracts were then subjected to the various phytochemical tests using the methods adopted by Abu *et al.*, (2018) [1] with little modifications. The positive results were scored based on colour intensities.

Determination of Saponins (Froth Test)

To determine the presence of saponins, 2 mL distilled water was added to 3 mL of the extracts in each test tube and the solution was vigorously shaken before some drops of olive oil were added. The formation of stable foam was taken as an indication for the presence of saponins.

Detection of Alkaloids (Wagners Test)

A mL of each of the extracts was treated with 1 mL Wagner's reagent (Iodo-Potassium Iodide) in drop wise by the side of the test tube. The formation of a reddish-brown precipitate indicated the presence of alkaloids.

Detection of phytosterols (Salkowski's Test)

Two mL of each extracts were treated with 2 mL chloroform followed by few drops of Concentrated Sulphuric acid, shaken, and allowed to stand. The appearance of golden yellow colour indicated the presence of phytosterols.

Detection of phenols (Ferric Chloride Test)

Two mL of each of the extracts were treated with 3 to 4 drops of 10% ferric chloride solution. The formation of bluish black colour indicated the presence of phenols.

Determination of Flavanoids

1ml of 10% ammonia and 1 ml of concentrated sulphuric acid was added to 3 ml of each extract. Appearance of yellow colour indicated the presence of flavonoids.

Detection of proteins and amino acids (Xanthoproteic Test)

Two millilitres (2 ml) of each extract were treated with few drops of concentrated Nitric acid. Formation of yellow colour indicates the presence of proteins.

Coumarin Glycosides (Ferric chloride test)

To 3 ml of each of the concentrated methanolic extract, few drops of 10% Ferric chloride solution were added to each. Formation of a deep green colour, which turned yellow on addition of concentrated Nitric acid, indicated the presence of coumarin glycosides.

Determination of Steroid (Liebermann Burchard Reaction)

Two millilitres of each extract were added with 1 ml of chloroform and a few drops of concentrated Sulphuric acid along the sides of the test tubes. The formation of a reddish-brown precipitate at the bottom of the test tubes indicated the presence of steroids.

Determination of Anthraquinones (Borntrager's Test)

To 3 ml of each extract, dilute sulphuric acid was added,

boiled, and filtered. To the cold filtrate equal volume of chloroform was added. The organic layer was separated, and ammonia was added. The presence of anthraquinones was observed with the appearance of pink or red colour.

Determination of Phlobatannins

Two millilitres of each extract were boiled with 1% aqueous hydrochloric acid; the formation of red precipitate indicates the presence of phlobatannins.

Thin Layer Chromatography

Chromatographic plate measuring 6 cm by 10 cm and coated with silica gel 60 F_{254} on an aluminium sheet of 1 mm thick was used for this study. The plate was activated in an oven at 110^{0}C for 30 minutes as recommended by the plate manufacturer. Fresh leaves from the apical part of the plants were crushed in laboratory crucibles in Methanol and the extracts were applied at the starting point 1 cm above the edge of the plate using capillary tubes and then air dried. The plate was developed in the solvent system of ethyl acetate, acetic acid and water (volume per volume) in the ratio 6:2:3. The plate was placed in a glass chamber containing the solvent and allowed to develop chromatograms. The chromatograms were observed under visible light and obvious spots were traced, scored and recorded. The spots and retention factors were used as basis for comparison of various phenolic compounds observed. On the basis of colour and position, spots assumed to be identical in two or more morphotypes were assigned the same score.

Data Analysis

Analysis of Morphological and phytochemical data

The qualitative and phytochemical data were scored into binary and used to draw a dendrogram with Euclidean distance using Past3 statistical software version 4.0.1 (Hammer *et al*., 2020) [5]. While the quantitative morphological data were subjected to One-way Analysis of Variance (ANOVA) to compare means with significant differences ($P < 0.05$) probability level and the SNK's Post-Hoc test was employed to separate significant means using the Statistical Package for Social Science (SPSS) software version 23.0.0 (1BM, 2015).

Analysis of biochemical data

The method described by Madani (2015) [12] was adopted to make suitable comparisons in the form of qualitative relationships. Selected morphotypes were compared with regard to their biochemical affinities. Values of Paired Affinity (PA), Group Affinity (GA) and Isolation Value (IV) were calculated using the following formulas:

$$PA = \frac{\text{Spots common to var A and B}}{\text{total spots in A and B}} \times 100$$

$$GA = \text{Total PA} + 100$$

$$IV = \frac{\text{Number of unique spots in a varity}}{\text{total number of spots in species}} \times 100$$

Results

Table 1 shows result for qualitative morphological traits for the five morphotypes used in this study. All the morphotypes were similar in 13 out of the 22 traits assessed including growth habit, petiole surface, pubescence, flower shape and petals arrangement. They were also similar in their leaf characteristics such as shape, surface, arrangement, colour, apex, base, blade as well as venation. Variations were observed in the branching pattern, stem surface, foliage cover, leaf lobation and margination as well as in the colour of stem, petals, sepal, and stamen. Dark red colour and glabrous surface of stem, pinkish petal, dark red sepal and low foliage cover were unique to HS01 while the pentalobed leaf margin was unique to HS05. Based on the qualitative morphological traits, the morphotypes divided into two major groups with linkage between HS01 and HS02 while HS03, HS04 and HS05 also clustered together (Figure 1).

Significant differences ($P < 0.05$) were observed among all morphotypes for quantitative vegetative traits assessed as shown in Table 2. For leaf length and leaf area, HS05 recorded the highest mean value of 18.73 cm and 214.53 cm^2 while HS01 has the least mean value for leaf length and leaf area (12.20 cm and 91.34 cm^2), respectively. HS05 also recorded the highest mean value for leaf width (20.94 cm), number of branch (10.67) and number of nodes (295.33). However, HS04 had the least mean value for leaf width (10.62) while the least mean value for number of branches (6.00) as well as number of nodes were recorded for HS01. Although HS05 had the highest mean plant height (246.63 cm), HS01 recorded the longest mean petiole (15.22 cm). HS01 and HS03 recorded the least mean value (60 cm) and (7.19 cm) for plant height and length of petiole, respectively. For height at first branch, the highest mean (17.33 cm) was recorded for HS05 while HS04 recorded the least mean height at first branch (4.57 cm).

Table 3 presents data on the phytochemicals and their relative abundance in all the morphotypes under study. The result indicates that all the morphotypes contained alkaloids and saponins while steroids were not observed in their calyxes. Other phytochemicals such as phenols, phytosterols, Proteins and flavonoids were present in HS03, HS04 and HS05 but undetected in HS01 and HS02 while phlobatanins was present in HS01, HS02 and HS03 but undetected in HS04 and HS05. Coumarin glycosides were undetected in HS01 but present in all other morphotypes while anthraquinones was present in HS01 and HS02 but undetected in the other morphotypes. The morphotypes HS03 contained the highest number of phytochemical classes (7) while HS01 and HS02 contained the fewest (5) classes of phytochemicals. Based on the phytochemical data, the morphotypes were grouped into two major branches with HS01 and HS02 clustering in the first branch while HS03, HS04 and HS05 clustered together in the second branch figure 2. The chromatographic spots, colour, and Retention Factor for all the morphotypes are shown in Tables 4. HS05 had the highest number of spots (4) while HS01 had the least (1). However, no visible spot was observed in HS03. Pairwise, HS01 and HS02 as well as HS04 and HS05 clustered together as shown in Figures 3. Analysis of numerical data from chromatographic spots shown in Table 5 revealed the Paired Affinity value for all the morphotypes. HS04 and HS05 had the highest Paired Affinity value of 85.7% while HS01 and HS05 had the least Paired Affinity of 40.0%. Table 6 presents the Group Affinity values, unique spots as well as isolation values. The highest Group Affinity values of 313.4 was recorded for HS02 with a unique spot in HS05 having Isolation Value 25%.

Table 1: Qualitative morphological traits for vegetative structures among the five morphotypes

	TRAIT	HS-01	HS-02	HS-03	HS-04	HS-05
1	Stem colour	Dark red	Light red	Reddish green	Green	Green
2	Branching pattern	Dense	Diffuse	Dense	Diffuse	Dense
3	Growth habit	Shrub	Shrub	Shrub	Shrub	Shrub
4	Stem surface	Glabrous	Scurfy	Scurfy	Scurfy	Scurfy
5	Leaf blade	Lobed	Lobed	Lobed	Lobed	Lobed
6	Leaf colour	Green	Green	Green	Green	Green
7	Leaf shape	Palmate	Palmate	Palmate	Palmate	Palmate
8	Leaf surface	Glabrous	Glabrous	Glabrous	Glabrous	Glabrous
9	Leaf margin	Dentate	Dentate	Dentate	Serrate	Serrate
10	Leaf apex	Acute	Acute	Acute	Acute	Acute
11	Leaf lobe	Trilobed	Tetralobed	Tetralobed	Trilobed	Pentalobed
12	Leaf arrangement	Alternate	Alternate	Alternate	Alternate	Alternate
13	Leaf base	Acute	Acute	Acute	Acute	Acute
14	Leaf venation	Palmate	Palmate	Palmate	Palmate	Palmate
15	Petal colour	Pink	Pale yellow	Creamy	Creamy	Creamy
16	Petal arrangement	Touching	Touching	Touching	Touching	Touching
17	Stamen colour	Red	Red	Yellow	Yellow	Yellow
18	Flower shape	Trumpet	Trumpet	Trumpet	Trumpet	Trumpet
19	Sepal colour	Dark red	Light red	Green	Green	Green
20	Foliage cover	Low	Very high	Very high	Very high	Very high
21	Petiole surface	Pubescent	Pubescent	Pubescent	Pubescent	Pubescent
22	Petiole pubescence	Puberlulent	Puberlulent	Puberlulent	Puberlulent	Puberlulent

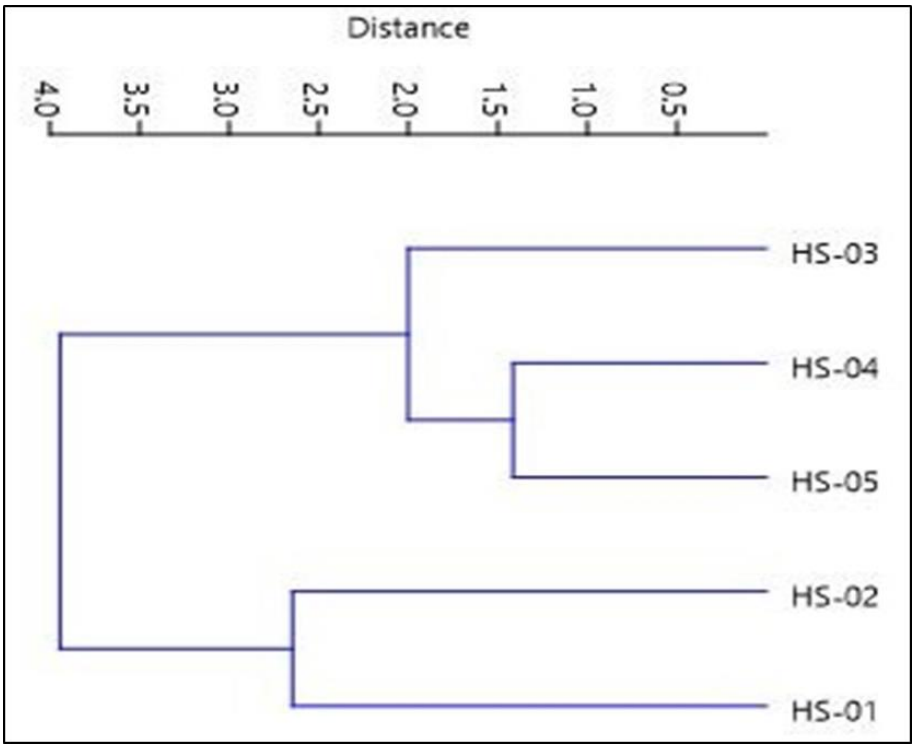

Fig 1: Dendrogram explaining linkages among morphotypes based on morphological variations using Euclidean distances.

Table 2: Quantitative vegetative traits (mean ±SE)

Trait/Taxa	HS01	HS02	HS03	HS04	HS05
LL(cm)	12.20±0.33^{a}	16.16±0.54^{b}	13.48±0.54^{a}	13.53±0.32^{a}	18.73±0.28^{c}
LW(cm)	14.35±0.38^{b}	12.22±0.49^{a}	11.48±0.57^{a}	10.62±0.44^{a}	20.94±0.53^{c}
HFB(cm)	6.57±0.32^{a}	11.43±0.69^{b}	11.87±0.86^{b}	4.57±0.73^{a}	17.33±0.45^{c}
PH(cm)	60.07±1.75^{a}	92.63±1.22^{b}	76.53±1.93^{ab}	149.33±11.15^{c}	246.63±2.14^{d}
LI(cm)	2.73±0.16^{a}	4.70±0.30^{b}	3.62±0.26^{a}	5.13±0.65^{b}	5.34±0.35^{b}
LP(cm)	15.22±0.42^{d}	8.07±0.47^{ab}	7.19±0.32^{a}	8.97±0.33^{bc}	9.44±0.36^{c}
LA(cm^{2})	91.34±4.57^{a}	160.05±9.56^{b}	114.27±9.13^{a}	111.86±4.95^{a}	214.52±11.89^{c}
NB	6.00±0.57^{a}	7.67±1.45^{ab}	8.00±0.57^{ab}	9.67±0.88^{ab}	10.67±0.88^{b}
NN	145.67±27.82^{a}	236.33±26.12^{ab}	207.67±28.90^{ab}	252±13.32^{ab}	295.33±26.27^{b}

LL: Leaf Length, LW: Leaf Width, LA: Leaf Area, NB: Number of Branch, HFB: Height at First Branch, PH: Plant Height, LI: Length of Internode, NN: Number of Nodes, LP: Length of Petiole. a,b,c: means along the same row having different superscripts differ significantly (P<0.05).

Table 3: Phytochemical screening of calyx methanolic extracts of roselle calyxes in Wukari, Taraba State.

SN	Phytochemical	HS01	HS02	HS03	HS04	HS05
1	Alkaloids	++	+	+	+	+
2	Saponins	+	+	+	+	+
3	Phytosterols	-	-	++	+	+
4	Phenols	+	-	-	-	-
5	Flavonoids	-	-	++	+	+
6	Proteins	-	-	+++	+	++
7	Coumarin glycoside	-	+++	++	+	+
8	Steroids	-	-	-	-	-
9	Anthraquinones	+	+	-	-	-
10	Phlobatanins	+++	++	+	-	-

+ Present; ++ moderately present; +++ highly present; - undetected.

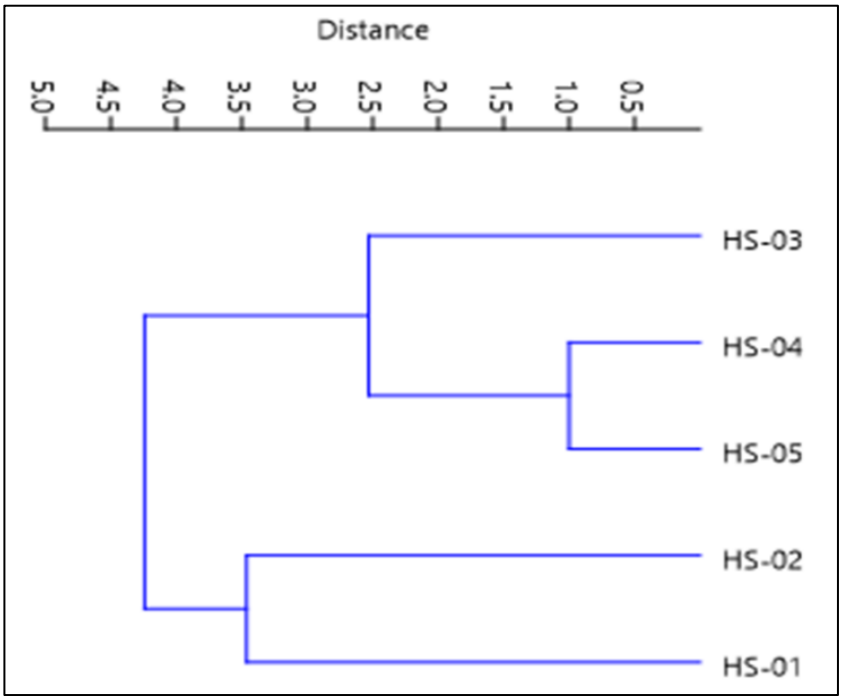

Fig 2: Dendrogram explaining linkages of morphotypes based on phytochemicals in calyxes

Table 4: Colour, RF and Abundance of Spots from Thin Layer Chromatography

SPOT	COLOUR	RF	HS01	HS02	HS03	HS04	HS05
1	Yellow	0.14	-	-	-	-	+
2	Green	0.24	-	+	-	+	+
3	Yellow	0.28	-	-	-	+	+
4	Green	0.33	+	+	-	+	+

Solvents: Ethyl acetate, acetic acid and water (6:2:3 v/v)

Table 5: Values Paired affinity

Taxa	HS01	HS02	HS03	HS04	HS05
HS01	-	-	-	-	-
HS02	66.70%	-	-	-	-
HS03	0%	0%	-	-	-
HS04	50%	80%	0%	-	-
HS05	40.00%	66.70%	0%	85.70%	-

Solvents: Ethyl acetate, acetic acid and water (6:2:3 v/v)

Table 6: Group Affinity, Isolation Value and Unique Spots

Taxa	Group Affinity	unique Spots	Isolation Value (%)
HS01	256.7	0	0
HS02	313.4	0	0
HS03	166.7	0	0
HS04	252.4	0	0
HS05	292.4	1	25

Solvents: Ethyl acetate, acetic acid and water (6:2:3 v/v)

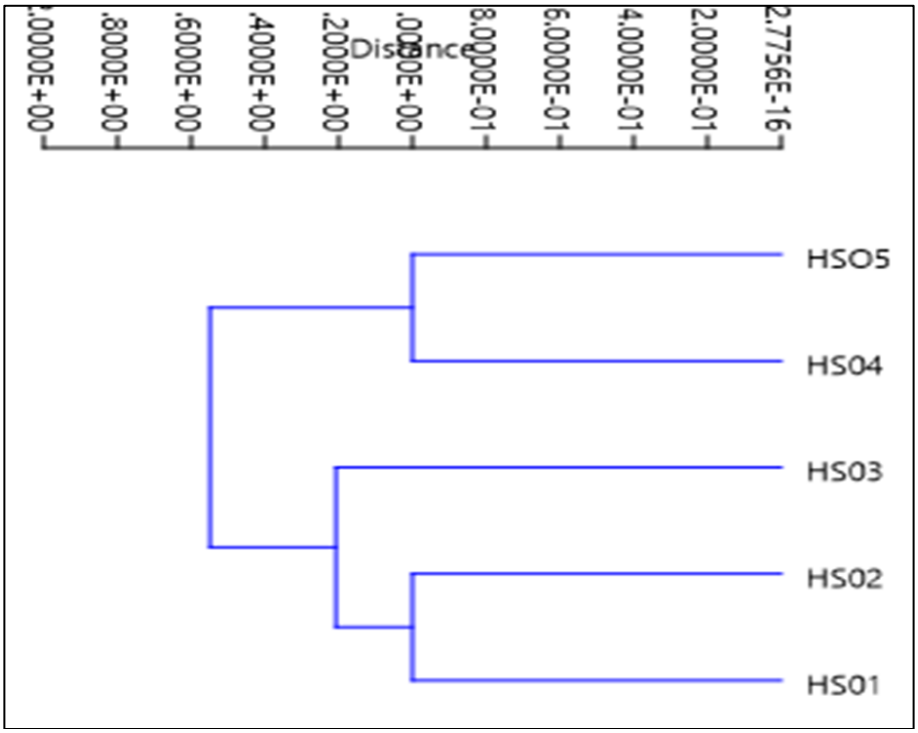

Fig 3: Dendrogram explaining chromatographic variations from first solvent system.

Discussion

Assessment of intra-specific variations is an effective approach for identifying descriptor traits and high performing taxa in plants. Results from the assessment of qualitative morphological variations among the morphotypes revealed differences in the traits assessed. This result agreed with those reported by Coffie (2016) [4] for leaf margin, foliage cover and stem colour. However, the divergence in petal and sepal colours between the present study and that of Coffie (2016) [4] may have been due to the differences in the morphotypes assessed in the two studies. The clustering of HS01 with HS02 as well as HS04 with HS05, as observed in the dendrogram for qualitative data, suggest that the paired morphotypes might hybridise for heterotic effect. The significant differences ($P < 0.05$) recorded for quantitative morphometric traits confirm the presence of much intra-specific variations among the morphotypes studied. Although the result of the quantitative morphological traits for plant height, numbers of nodes and leaf length varied from those reported by Coffie (2016) [4], they agreed with those reported by Thirupathi *et al*. (2015) [20]. This variation in plant height, and leaf length could be attributed to differences in environmental factors like soil fertility, moisture, planting season and climate between locations. This position is also supported by Mansoor *et al*. (2017) [10] who stated that some morphological traits of roselle such as plant height, leaf size among others vary from region to region and among species due to environmental factors and hybridization. It is also probable that the differences might have arisen through mutation which is the ultimate source of all genetic variability in a population.

The results of phytochemical screening of the calyxes revealed that they are abundant in saponins, alkaloids, phenol, anthraquinones, flavonoids, phytosterols, coumarin glycosides and phlobatanins but scarce in phenols and steroids. The presence of phenols, flavonoids and alkaloids agrees with the findings of Okereke *et al.* (2015) [16] and Abba *et al.* (2015) [2]. Howeverr, the presence of alkaloids, anthraquinones, flavonoid and saponins contradict the findings of Ajoku *et al.* (2015) [3]. The presence of these phytochemicals is indicative that the calyxes of roselle can play vital roles in nutraceutical and pharmaceutical industries. This corroborates the findings of Okereke *et al.* (2015) [16] and Islam (2019) [7] who reported the calyxes of roselle as good sources of food ingredient and as medicine in treating cancers, diabetes, and cardiovascular disorders. Dendrogram representing the linkages of the morphotypes based on the phytochemicals in the calyxes shows isolation of HS03 which indicates that it might possesses molecules that are unique to it.

The TLC analysis revealed differences in the number of spots among the morphotypes based on the solvent system employed in this study. This result varied from the findings by Jin-wang *et al.* (2015) [8] and Shilpi and Madhavi (2016) who independently reported five and two spots for roselle plants. The differences in the number and pattern of spots could stem from the fact that Jin-wang *et al.* (2015) [8] used different solvent systems while Shilpi and Madhavi (2016) used *Hibiscus rosa-sinensis* in their analyses. In this study, the unique spot revealed for HS05 appeared to be the characteristic for the roselle morphotypes. The PA of 85.7% between HS04 and HS05 indicates close affinity hence relationship between these two morphotypes and suggest them as suitable candidate in breeding for calyx traits. This agreed with the report of Coffie (2016) [4] who reported these two as morphotypes of the same species. The clustering pattern in the dendrograms representing data from the TLC revealed the pairing of HS01 with HS02 as well as HS04 with HS05 which might be an indication of close relationship in the chemotaxonomy of these intra-species.

Conclusion

Roselle is one of the indigenous vegetables with high potential for food and medicine widely consumed in Sub-Sahara Africa. Extensive intra-specific polymorphism associated with roselle plant makes the identification of descriptor traits and best performing morphotypes difficult. The findings from this study revealed HS05 to be the best performing morphotype for vegetative traits such as plant

height, number of nodes and branches, as well as leaf area with HS01 Showing least performance for the same traits. The study suggests HS01 and HS05 to have the best prospects for crop improvement and breeding purposes in roselle. Findings from the phytochemical screening in the calyxes showed that the calyxes of HS03 are rich reservoir of phytochemicals which may be essential for drug discovery. Results from the Thin Layer Chromatography revealed the difference between HS01 and HS05 as well as from other morphotypes which further supported the uniqueness of the two morphotypes and highlight the potential of chromatography in the chemotaxonomy of intraspecies.

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