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Research Article

The Use of LC/UV/MS for Qualitative and Quantitative Analysis of Phytochemicals in Roselle Hibiscus (*Hibiscus sabdariffa* L.) Leaves

Kit L Chin[1], Yadong Qi[1]*, Adolfo Pertuz[2], Julia Coppin[2], Qing-Li Wu[2] and James Simon[2]

[1]*Southern University Agricultural Research and Extension Center/College of Agriculture, Southern University Land Grant Campus, Baton Rouge, Louisiana, USA*

[2]*New Use Agriculture and Natural Plant Products Program, Department of Plant Biology, Rutgers University, New Brunswick, New Jersey, USA*

***Corresponding Author:** Yadong Qi, Southern University Agricultural Research and Extension Center/College of Agriculture, Southern University Land Grant Campus, Baton Rouge, Louisiana, USA.

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Abstract

A great deal of research focus has been placed on the calyx of roselle hibiscus due to its demonstrated medicinal properties by the phytochemicals present in them. Though roselle hibiscus leaves have been found to contain polyphenols and antioxidants, there is an increasing need to know the phytochemical profiles of antioxidants present in roselle hibiscus leaves. LC/UV/MS qualitative and quantitative analysis indicated that the amount of total polyphenolic contents and the individual phytochemical component of the leaves varied among five roselle accessions. Ten phytochemical compound were isolated and identified. This LC/UV/MS method was found to be a reliable analytical technique for determining the phytochemical structural components present in roselle hibiscus leaf extracts. It required a very small sample size and yet provided high resolution and sensitivity detection of phenolic acids and flavonoids along with rapid identification of individual phytochemical compound.

Keywords: Roselle; Hibiscus sabdariffa; Phytochemicals; LC/UV/MS; Qualitative and Quantitative Analysis

Abbreviations

LC/UV/MS: Liquid Chromatograph/Ultra Violet/Mass Spectrophotometry; 5-HMF: 5-(hydroxyethyl) Furfural; DAD: Diode Array Detector; ESI: Electrospray Ion Source; PDA: Photodiode Array

Introduction

More than 300 species of Hibiscus are found world-wide. Roselle (*Hibiscus sabdariffa* L.) is the only one species that has its calyce swollen and enclosed the fruits. It belongs to a Malvaceae family and has been used in traditional folk medicine for hypertension, inflammation and cancer prevention. This traditional folk has instigated a great deal of interest in the pigmented calyce of this plant. As a result of progressive research effort on its potential application as a natural product, roselle has been widely used and consumed because of its demonstrated medicinal properties which have been documented by many research articles. The extracts of the calyx were found to have effects on lowering blood pressure of mildly hypertensive patients [1-4], reducing serum cholesterol level in blood [5-8], exerting anti-inflammatory activity [9-11] and inhibiting cancer cell proliferation *in vitro* [5,8,12-14]. The leaves of this plant have been consumed widely by people in Africa as vegetable. Even though the leaf extracts have been found to possess many bioactivities such as anti-tumor [8,15], anti-parasitic [16], anti-oxidant [4,7], little pharmaceutical research has been carried out. After the harvest of the calyces, the leaves of this plant are mostly ignored or discarded in most countries. In recent years, our research team has discovered that the leaves of many accessions contain higher amount of total phenolic content and total antioxidant activities than the calyces. Such a discovery could make roselle hibiscus leaves as a new potential source of natural antioxidants for the food industry. There is need to conduct phytochemical profile evaluation of roselle accessions of different origin. In this paper, the LC/UV/MS method was developed to conduct simultaneous separation and determination of natural compounds present in the leaves of selected roselle hibiscus accession.

Materials and Methods

Chemicals and Reagents

Standard compounds including rutin hydrate, chlorogenic acid, kaempferol, formic acid, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), potassium persulfate were purchased from Sigma Aldrich of St. Louis, MO. Quercetin was purchased from ACROS of Geel, Belgium. Folin Ciocalteu's Phenol Reagent was purchased from MP Biomedicals of Solon, OH. HPLC grade solvents including water, methanol, acetonitrile and hydrochloric acid (HCl) were purchased from Fisher Scientific of Fair Lawn, NJ. Ethyl alcohol was purchased from Pharmco-AAPER of Brookfield, CT. Neochlorogenic acid, Cryptochlorogenic acid, and 5-HMF were not available to be used as standards at the time of analysis. Delphinidin sambubioside (dp-sam), Cyanidin sambubioside (Cy-sam) were obtained from Polyphenols Laboratories, Norway.

Plant samples

A total of five roselle hibiscus accessions were field trialed at the Horticulture Farm of Southern University. The seeds of these five accessions were germinated in the cell-pack tray in the greenhouse on April 1, 2010. One month after germination, seedlings were transplanted to the field with silty clay loam soil and the soil pH of 7.0. The between row spacing was 7 feet and the between plants spacing was 6 feet. Organic fertilizer 4-2-2 was applied at 4 kg/30m. A composite sample of both young and mature leaves within the top 30 cm shoot were collected from nine randomly selected plants and stored in a plastic bag in a cooler. Collected samples were then transported to the laboratory for oven=dry at 60^0C for 48 hours.

Sample preparation

Dried leaves were ground into powder using mini coffee grinder and stored in the ziploc bags. Approximately 100 mg grinded leaf powder was accurately weighed out and placed in a volumetric flask. The sample was extracted using 20 ml of 70% methanol/distilled water solution with 0.1% acetic acid under sonication for 10 minutes. Each sample was then conditioned at room temperature overnight [17,18]. The extract of each sample was then filtered through 0.45 um filter and used for qualification and quantification determination. Samples were injected into HPLC column immediately or stored in the freeze of -20^0C for further testing.

Methods

The qualitative identification was performed on Hewlett-Packard Agilent 1100 series HPLC-MS (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump system, a degasser, an autosampler, a DAD detector, an MSD trap with an electrospray ion source (ESI). The electro-spray ion mass spectrometer is under positive model or negative mode with scan range from m/z 100 to 800. The needle voltage is 3.5kV, nitrogen (99.999%) flow rate is 12 L/min and the capillary temperature is set as 350^0C. Nitrogen is set as nebulizer at 80 psi and helium as a collision gas. HP ChemStation software, Bruker Daltonics 4.2 and Data Analysis 4.2, were used in qualitative experiments. For quantitative analysis, the experiment was performed on Waters 2695 separation module equipped with a photodiode array detector (PDA). Solvents are degassed in a separate bath sonicator. For both analyses, the column used was Polaris TM Amide C18 column, 5 µm, 250 x 4.6 mm (Varian) at temperature of 25°C. The flow rate is 1.0 ml/min. The mobile phases consists of solvent A (formic acid/water (0.1%, v/v)), solvent B (formic acid/acetonitrile (0.1%, v/v)). The gradient liner gradient starts from 5 to 10% B in 0 to 10 minutes, from 10 to 34% B in 10 to 34 minutes, from 34 to 60% B in 34 to 35 minutes, and keeps at 60% B to 50 minutes. The flow rate is 1.0 ml/min. The spectral scan range of UV-DAD detector is 200 - 520 nm, and the detection wavelengths monitored at 280, 330, and 370 nm are used for the determination of phenolic acids and flavonoids, respectively.

Results and Discussion

The structure of the polyphenols was identified based on analysis of the MS and UV data. The content of individual compound was quantified using UV detection at 280 nm and 370 nm for phenolic acids and flavonoids, respectively. Figure 1 illustrated the representative HPLC/UV/MS chromatograms of Hibiscus leaf sample SHT-3 at 280 nm (A, for phenolic acids) and 370 nm (B, for flavonoids). Figure 2 shows the MS spectra of the major peaks of Chlorogenic acid and its isomers (A, B, C) Neodatelic acid (D) and Quercetin rutinoside (E).

The identity of the peak was assigned by analyzing UV and MS data, and in comparison with the authenticated standards with the exception of Peak 1. Ten components (Table 1) were identified from peak 2 to peak 10 as Chlorogenic acid (C.A); Chlorogenic acid isomer 1 (C.A.1); Chlorogenic acid isomer 2 (C.A.2), Neodatteic acid (N.A.); Quercetin rutinoside (Q.R); Quercetin glucoside (Q.G); Kaempferol rutinoside (K.R), Kaempferol glucoside (K.G); Quercetin (Q); Kaempferol (K); delphinidin sambubioside (dp-sam), cyanidin sambubioside (Cy-sam). Figure 2 MS spectra of the major peaks of Chlorogenic acid and its isomers (A, B, C), Neodatelic acid (D) and Quercetin rutinoside (E).

Figure 1: Representative HPLC/UV/MS chromatograms of Hibiscus leaf sample SHT-3 at 280 nm(A, for phenolic acids) and 370 nm (B, for flavonoids). The peak identity was assigned using UV and MS data, and in comparison with the authenticated standards.

Figure 2: MS spectra of the major peaks of Chlorogenic acid and its isomers (A, B, C) Neodatelic acid (D) and Quercetin rutinoside (E).

Peak	[M+H]+/ [M-H]-(m/z)	MS fragment ion (m/z)	Identities
1	189		5-HMF**
2	353	191	Chlorogenic acid (C.A)
3	353	191	Chlorogenic acid (isomer 1) (C.A)
4	353	191	Chlorogenic acid (isomer 2) (Ch.A)
4a	335		Neodattelic acid (N.A)
5	611	465,303	Quercetin-rutinoside (Q.R)
6	465	303	Quercetin-glucoside (Q.G)
7	595	449,287	Kaempferol-rutinoside (k.R)
8	449	287	Kaempferol-glucoside (K.G)
9	303		Quercetin (Q)
10	287		Kaempferol (K)

Table 1: Peak assignments for the analysis of roselle hibiscus leaf sample, SHT-3.

** No authenticated standard was used in the analysis.

No authenticated standard was used for the compound assigned by peak 1 of figure 1. Its name is assumed to be 5-HMF (based on the research work by Zhen., *et al* [11]. Zhen., *et al.* [11] discovered the presence of 5-HMF in the roselle hibiscus leaves which might have been formed due to improper processing or storage of the leaf samples. It had been reported that the formation of 5-HMF was related to over drying, exposure to elevated temperature, pH value and storage conditions [19,20]. As per the findings of Zhen., *et al.* [11], Chlorogenic acid isomer 1 and chlorogenic acid isomer 2 could be referred as neochlorogenic acid and cryptochlorogenic acid, respectively. To confirm the identity of 5-HMF, chlorogenic acid isomer 1 and chlorogenic acid isomer 2, a further analysis with internal standards of 5-HMF, neochlorogenic acid and cryptochlorogenic acid will be needed. Table 2 and figure 4 show the variation of phytochemical components among five roselle accessions. Figure 3 illustrated the bar chart showing the total percentages of phytochemicals in the leaf sample of each accession.

With the minute quantity of leaf samples used in this LC/UV/MS analysis, the instrument was not only able to quantify the total polyphenol content of each roselle hibiscus accession (Figure 3), but could also separate and identify individual phytochemical

Accession	5-HMF	C.A	C.A (Isomer)	C.A (Isomer 2)	N.A	Q.R	Q.G	K.R	K.G	Q.U	K	Dp-sam	Cy-sam	Total
Senegal	0.07	0.92	0.12	0.15	0.08	2.32	0.21	0.13	0.37	0.14	0.01	0.04	0.04	4.60
Malaysia	0.06	0.71	0.12	0.14	0.07	0.85	0.48	0.30	0.16	0.00	0.00	0.03	0.04	2.93
Jamaica	0.13	0.98	0.14	0.20	0.09	2.96	0.28	0.17	0.41	0.20	0.02	0.04	0.04	5.64
Nigeria	0.09	1.03	0.13	0.17	0.09	2.82	0.29	0.18	0.50	0.11	0.01	0.04	0.05	5.49
Liberia	0.03	0.88	0.12	0.16	0.09	1.88	0.22	0.13	0.26	0.00	0.00	0.03	0.05	3.84

Table 2: Table showing the total percentage of phytochemical components found in leaf sample of each accession.

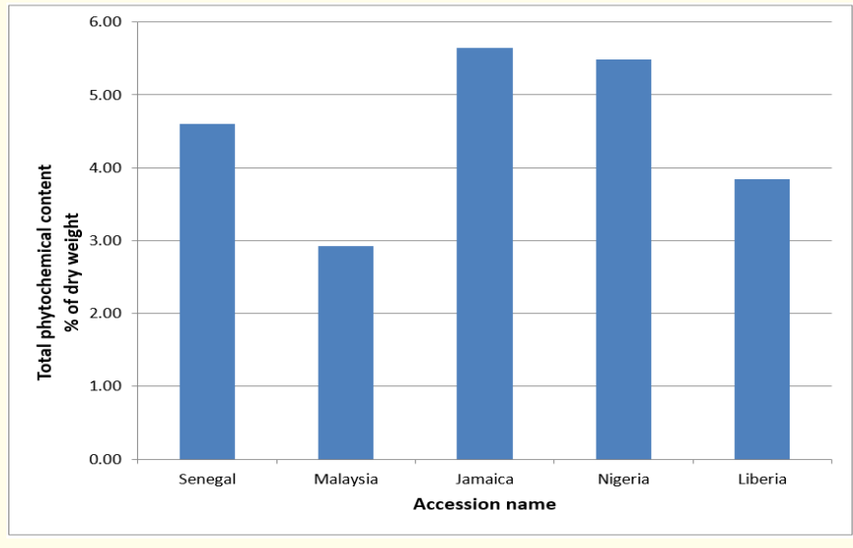
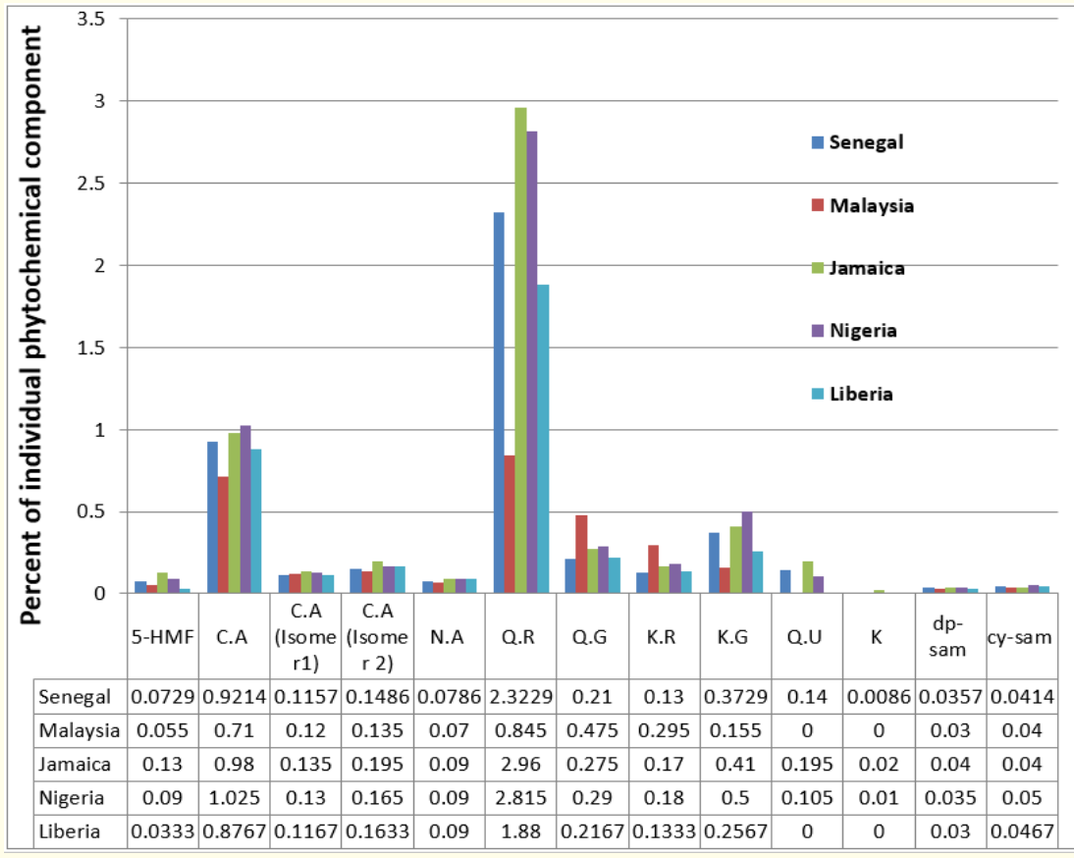


Figure 3: Bar chart showing the total percentages of phytochemicals in the leaf sample each accession



	5-HMF	C.A	C.A (Isomer1)	C.A (Isomer 2)	N.A	Q.R	Q.G	K.R	K.G	Q.U	K	dp-sam	cy-sam
Senegal	0.0729	0.9214	0.1157	0.1486	0.0786	2.3229	0.21	0.13	0.3729	0.14	0.0086	0.0357	0.0414
Malaysia	0.055	0.71	0.12	0.135	0.07	0.845	0.475	0.295	0.155	0	0	0.03	0.04
Jamaica	0.13	0.98	0.135	0.195	0.09	2.96	0.275	0.17	0.41	0.195	0.02	0.04	0.04
Nigeria	0.09	1.025	0.13	0.165	0.09	2.815	0.29	0.18	0.5	0.105	0.01	0.035	0.05
Liberia	0.0333	0.8767	0.1167	0.1633	0.09	1.88	0.2167	0.1333	0.2567	0	0	0.03	0.0467

Figure 4: Comparative percentage of each phytochemical component among five roselle hibiscus accessions. (5-HMF: 5-(Hydroxyethyl) Furfural; C.A: Chlorogenic Acid; N.A: Neodattelic Acid; Q.R: Quercetin Rutinoside; Q.G: Quercetin Glucoside; K.R: Kaempferol Rutinoside; K.G: Kaempferol Glucoside; Q: Quercetin; K: Kaempferol; dp-sam: Delphinidin Sambubioside, Cy-sam: Cyanidin Sambubioside).

Conclusion

LC/UV/MS method is a reliable analytical technique for the analysis of phytochemical structural components present in roselle hibiscus leaf extracts. It requires a very small sample size and provides a high sensitivity detection of non-anti-oxidants such as 5-hydroxymethyl) furfural, phenolic acids and flavonoids. The method also provides high resolution and rapid identification of individual phytochemical compound without the need for isolating individual compound individually. The method has aided in obtaining desired data for the profiling of phytochemical components of five roselle hibiscus accession leaves. With this method, a total of 13 distinct compounds were identified belonging to the anthocyanidin, phenolic acid, and flavonoid family. The high amount of antioxidants of these identified compounds has rendered the leaves of roselle hibiscus for potential uses in many therapeutic applications. The identified compounds of the roselle hibiscus leaves can serve as an supplementary sources of natural product antioxidants in the functional food sector.

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