Optimization and Validation of RP-HPLC-UV/VIS Method for Determination Some Antioxidants in Dry Calyces of Iraqi Hibiscus Sabdraffia Linn

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Abstract:

A new (Reversed Phase- High Performance Liquid chromatography) RP-HPLC method with Ultraviolet-Visible spectrophotometry has been optimized and validated for the simultaneous extraction and determination of antioxidants present in Iraqi calyces of Hibiscus Sabdraffia Linn. The method is based on using ultrasonic bath for extracting antioxidants. Limit of detection in µg/ml of Vitamin C, Sabdaretine, Gossypetine, Hibiscetine, Anthocyanins, Dephinidin-3-glucoside were113.8294×10^{-6},123.0453×10^{-6},70.3681×10^{-6},59.6730×10^{-6},148.1710×10^{-6},and125.3481×10^{-6} respectively. The concentration of antioxidants found in dry spacemen of calyces of Iraqi Hibiscus Sabdraffia Linn. under study: Vitamin C, Sabdaretine, Gossypetine, Hibiscetine, Anthocyanins, and Dephinidin-3-glucoside are 258.3 µg/g, 225.51 µg/g, 154.975 µg/g, 111.407 µg/g, 439.442 µg/g, and 185.729 µg/g respectively.

Key words: antioxidants, Hibiscus Sabdraffia Linn.

Introduction:

Hibiscus Sabdraffia Linn. is a tropical plant and it belongs to malvacae family[1]. Calyces are important part of Hibiscus Sabdraffia Linn.it was found that when calyces is dried under sun light and air, contain organic acids (tartaric, citric, malic, and hibiscic) showing high stability under the mentioned drying process [2]. Many biological activity studies were conducted on H. sabdariffa and some of these compounds have been shown to have antibacterial, antifungal, antihypertensive, anti-inflammatory, antioxidant, antispasmodic, antitumor and hypoglycaemic [3] activities. Medical uses of Calyces are wide. Infusion of Calyces are regarded as diuretic, choleretic, febrifugal, hypotensive, decreasing the viscosity of blood and stimulating intestinal peristalsis [4], reduce hypertenstion [5]. Infusions of calyces are used as antiseptic, aphrodisiac, astringent, cholagogue, demulcent, diuretic, emollient, purgative, refreshment, sedative, stomachic, and tonic, traditionally [6]. Water extract of calyces is used for its astringent and digestive properties and also used for treating different type of cancer and reducing high cholestrol [7]. Calyx extract has also been used as an effective treatment for patients with kidney stones due to its uricosuric effect [8]. The red calyces contain antioxidants including flavonoids (gossypetine, hibiscetine, sabdaretine) [9], anthocyanins and vitamin C[10]. Calyx of Hibiscus Sabdariffa Linn. Contain also different anthocyanins as delphinindin-3-glucoside, delphinidin-3-xyloglucoside, delphinidin-3-sambubioside, cyaniding-3-xyloglucoside [11]. The antioxidants under study were considered as strong scavengers of free radicals which are unstable chemical species that react

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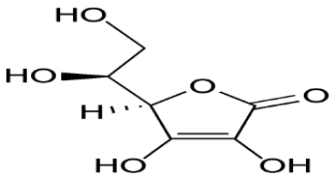
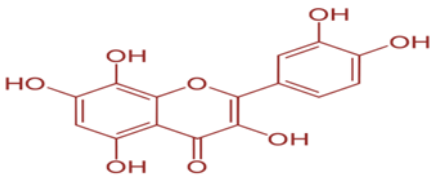
rapidly [12] with other chemical species in a biological system. Reactive species, such as superoxides ($O_2^{\cdot -}$ and $OOH^{\cdot}$), hydroxyl($OH^{\cdot}$), and peroxyl($ROO^{\cdot}$) radicals, can attack stable molecules in a healthy organism and produce illnesses[13]. Antioxidants can react with the radical, but rather than turning into another reactive molecule, these compounds are relatively stable in the presence of the radical electron. As a consequence, they scavenge the radical electrons, quench the chain reaction, and avoid further damage. The relative stability of antioxidants containing a radical electron is generally the result of the presence of conjugated bonds, so that the radical electron can be delocalized. As a consequence, aromatic compounds in general, and phenolic compounds in particular are very effective antioxidants [14]. Antioxidants provide protection against degenerative diseases. Such as cancer, hipercholestrol and coronary heart disease [15].

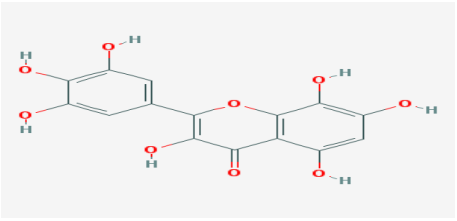
Reversed phase HPLC with UV/ Vis detector (RP-HPLC-UV/Vis) is an important analytical technique with strong chromospheres that absorb light in the wavelength region from 200 nm to 800 nm [16]. The objective of study is to simultaneous extraction, separation and determination of some antioxidants from calyces of Iraqi Hibiscus Sabdraffia Linn. by using modern technique of RP- HPLC- UV/ Visible detection technique. Antioxidants understudy are 1.Vitamin C, 2.Gossypetine, 3.Hibiscetine, 4.Delphinindin-3-glucoside, 5.Anthocyanins (as total), and 6.Sabdaretine (unknown structure) (see Figure 1)



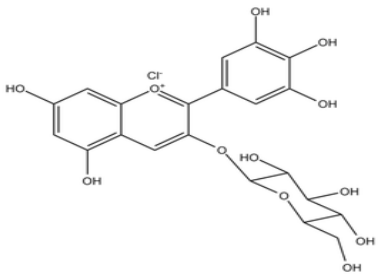
1. Vitamin C



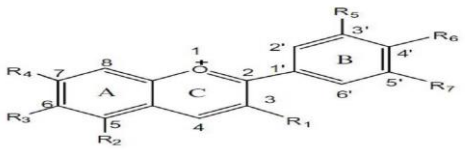
2. Gossypetine



3. Hibiscetine



4. Deiphinindin-3-glucoside



5. Anthocyanins

R1=O-sugare (glucose, arabinose, galactose).

R2, R4, R6= OH, R3, R5 and R7= H, OH, OCH_3

Fig.1: The Structure of Antioxidants in Calyces of Hibiscus Sabdraffia Linn.

Ijeoah et al. [17] evaluated total anthocyanin, total polyphenol, and ascorbic acid (vitamin C) contents in Hibiscus sabdraffia from Nigeria. Total anthocyanin contant was evaluated using the method of Fuleki and Francis

(1969). Anthocyanin content in this evaluation was 1.15 mg/g. total phenolic content was evaluated by using (Singleton et al, 1990) method. The total polyphenol content was 1.10 mg/g. the ascorbic acid contant of the sample was evaluated using AOAC (2000) method. The content of ascorbic acid was 16.7 mg/g.

Materials and Methods:

1. Hibiscus Sabdariffa Linn. sample.

Fresh Hibiscus Sabdariffa Linn. fruits were harvested from Baghdad, Iraq. After harvesting the fruits were washed with deionized water three times to clean them from dust, then seeds were removed to obtain fresh Hibiscus Sabdariffa Linn. calyces. Fresh Hibiscus Sabdariffa Linn. calyces were dried at a room temperature for 7 days. Dried Hibiscus Sabdariffa Linn. calyces were immediately packed in polyethylene jars. Dried Hibiscus Sabdariffa Linn. calyces also dried in oven at 35 C° for three hours until constant weight. Dryness Hibiscus Sabdariffa Linn. calyces were ground for 10 min using Agate mortar. The calyces powder were immediately packed in polyethylene jars and kept in refrigerator until used.

2. Chemicals.

All chemical reagents used for analysis antioxidants by RP- HPLC- UV/Vis were analytical Grad (99.99%) of BDH Company. The reagents include Acetonitrile, Deionize water, and Methanol. Standards of (1) Vitamin C (99.99%), (2) Gossypetin (99.9%), (3) Hibiscetin (99.9%), (4) Sabdaretine (99.9%), (5) Anthocyanins (99.8%), and (6) Delphinidin-3-glucoside (99.9%) were purchased from BDH Company.

3. Preparation of Standard Solution.

Preparation of standard stock and working solution are carried out by the following method. To prepare 1000 ppm stock standard of any antioxidants, 1 gram was transferred into a volumetric flask of 1000 ml and diluted to mark by using deionized water. A quantity of 10 ml is pipetted from the above stock solution and transferred into a volumetric flask of 100 ml and diluted with deionized water to the mark in order to obtain 100 ppm. A working standard solution is prepared by transferring of 25 ml of diluted standard solution in a volumetric flask of 100 ml and diluted to the mark with deionized water to obtain a working standard solution of 25 ppm. These working standard solutions were kept refrigerated in ambered containers.

4. Extraction Procedure.

Calyces liquid extracts were carried by using deionized water: methanol solutions in different ratio, table (1), were used for the extraction. Calyces powder (1 g) was placed in glass beakers, and 10 ml of extracting agent was added. The beakers were placed in the Ultra sonic bath for 15 min, the mixtures were allowed to stand for 5 min. The supernatant was filtered through Whatman paper No.2 and also filtered through micro filter (0.45 μm). The filtrate was kept in polyethylene tubes and preserved in fridge.

Table (1): Different Deionized Water- Methanol Ratio Used in Extraction.

Samples	Deionized water- methanol ratio	
	Deionized water%	Methanol%
Sample 1	100	0
Sample 2	80	20
Sample 3	60	40
Sample 4	50	50
Sample 5	40	60
Sample 6	20	80
Sample 7	0	100

5. HPLC Analysis.

The quantitative and qualitative analysis of antioxidants was performed

on Shimadzu HPLC system model LC-6A equipped with two pumps as solvent delivery model LC-10A from Shimadzu Corporation and A Shimdzu SPD-6A ultraviolet –visible variable wave length (190-800 nm) detector. 20 µl samples was injected, and the chromatographic separation was performed on a RP-C_{18} Phenomenex (3 µm) column, 50mm × 4.6 mm. After optimizing the instrumental and sample parameter, the HPLC analysis condition for the sample were Deionized water acidified with acetic acid(0.1)% and Acetonitrile (20:80)% as mobile phase, 1.0 ml/min as flow rate, and using 254 nm as maximum wavelength.

Results and Discussion :

1. Optimization of Organic Modifiers of Mobile Phase in Reversed Phase HPLC.

This study was done by using deionized water acidified with acetic acid (0.1%) and acetonitrile in different ratio, this stage was done by system of HPLC. So, in order to study the effect of acetonitrile as an organic modifier on the separation efficiency, several experiments were carried out through changing the quantities of acidified deionized water and acetonitrile as following (Table 2) : (100%:0%, 95%: 5%, 90%: 10%, 80%: 20%, 75%: 25%, 70%: 30%, and 65%: 35% as acidified deionized water: acetonitrile). During the optimization process, it was found that on using 0%, 5% and 10% ACN, the obtained base line is very noisy, wide peak shape and tailed except Sabdaretine and Dephinidin-3-glucoside showed a symmetrical peak especially in 0% ACN. In 25%, 30% and 35% ACN, showed a good base line, but the peaks of anthocyanins and Dephinidin-3-glucoside showed a symmetrical peaks at 30% ACN. At 35% ACN Vitamin C and Gossypetine showed also a symmetrical peak. Finally it was found that on using 20% ACN and 0.1% acetic acid provide a good resolution as shown in Figure (2).

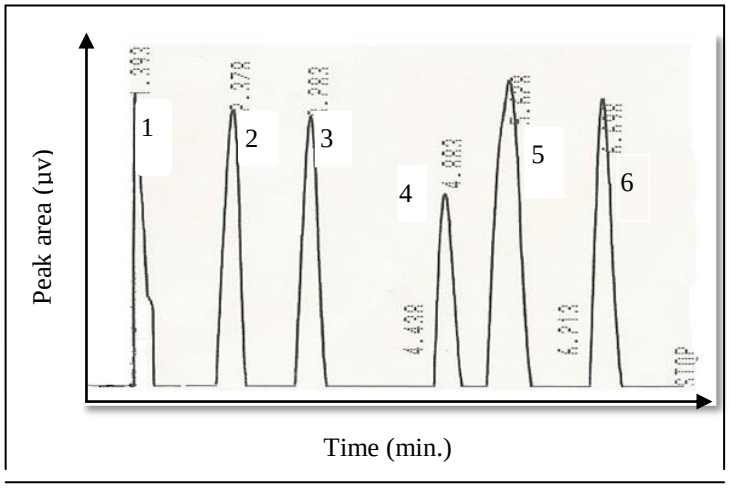


Fig. 2: As a mobile phase chromatogram Separation of Antioxidants using 20%ACN, the sequence of the peaks is as follow: 1.Vitamin C, 2. Sabdaretine, 3. Gossypetine, 4.Hibiscetine, 5.Anthocyanins, 6.Dephinidin-3-glucoside.

Table 2: The Effect of Organic Modifier on RT and Peak Area of Antioxidant.

Antioxidant	*Acetonitrile %*	*Retention time (min.)*	*Peak area (µv)*
Vitamin C	0	1.883	30764
	5	1.617	25751
	10	1.638	28406
	20	1.393	19793
	25	1.242	26358
	30	1.142	33081
	35	1.022	21831
Sabdaretine	0	2.618	25947
	5	2.462	9427
	10	2.328	24418
	20	2.378	19932
	25	2.155	14266
	30	1.922	30414
	35	1.81	35571
Gossypetine	0	3.772	37753
	5	4.14	25014
	10	3.35	35716
	20	4.883	13164
	25	3.082	14516
	30	2.758	30382
	35	2.642	18506
Hibiscetine	0	6.748	43167
	5	7.115	33648
	10	4.56	3695
	20	4.883	13164
	25	4.9	24541
	30	3.413	29166
	35	3.29	25757
Anthocyanins	0	8.388	55675
	5	8.1	2446
	10	5.442	43105
	20	5.628	21424
	25	5.82	14135
	30	3.928	44685
	35	3.8	27887
Dephinidin-3-glucoside	0	10.37	15759
	5	8.573	41957
	10	7.467	32284
	20	6.698	14992
	25	6.07	5615
	30	4.39	29380
	35	4.283	27574

2. The Effect of Extraction Mixture on Extraction of Antioxidants.

Seven kinds of solvent extracts from calyces of H. Sabdraffia Linn. were used to examine the effects of extraction solvent mixtures on antioxidant concentration. All calyx extracts are rich source in antioxidants but the best extraction occurs in 100% deionized water: 0% methanol as extraction agent. Figure 3 shows the best separation.

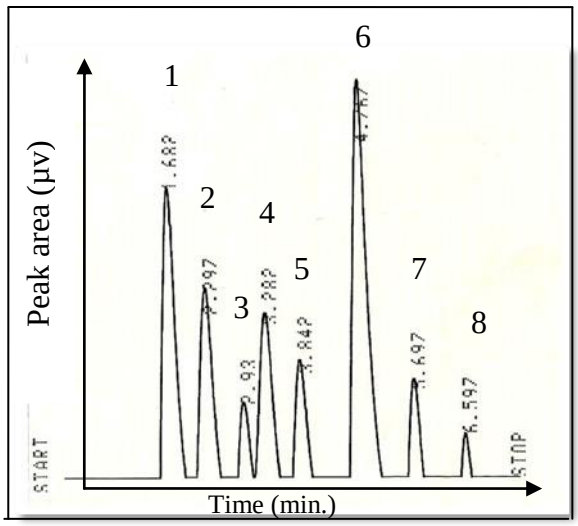


Fig. 3: The Best Separation of Antioxidant in Calyces Sample.

According to above figure and retention time, the sequence of the peaks is as follow: 1.Vitamin C, 2. Sabdaretine, 3.Unknown peak, 4. Gossypetine, 5.Hibiscetine, 6.Anthocyanins, 7.Dephinidin-3-glucoside, 8. Unknown peak.

Validation Method

The validation study for Vitamin C, Sabdaretine, Gossypetine, Hibiscetine, Anthocyanins, and Dephinidin-3-glucoside using RP-HPLC-UV/Vis was performed under the optimized conditions at 254nm as maximum wavelength, 1.0 ml/min as flow rate of mobile phase, and mixture phase A (acidified deionized water) with phase B (acetonitrile) as a mobile phase with elution ratio (80A: 20 B; v/v) during analysis time (7 minutes).

1. Linearity and Limit of Detection (LOD).

Six standards solutions of antioxidants were prepared in the following concentration: 25, 12.5, 6.25, 3.25, 1.62 and, 0.78 in µg/ml. The calibration curve obtained by plotting the peak area of chromatograms for antioxidants against the concentration, with three replicates (n = 3). Table 3 shows the validation of analytical method obtained from the calibration curves of antioxidants analysed on RP-HPLC-UV/Vis.

Table 3: Validation of analytical method for antioxidants by RP-HPLC-UV/Vis.

Antioxidants	Calibration equation	R^2	RSD%	LOD(µg/ml)
Vitamin C	Y=2530.1x+148.5	0.9980	0.013334	113.8294×10^{-6}
Sabdaretine	Y=2340.6x+5281	0.9914	0.008592	123.0453×10^{-6}
Gossypetine	Y=3240.1x+917.27	0.9987	0.010393	70.3681×10^{-6}
Hibiscetine	Y=4826.3x-1048.5	0.9975	0.01242	59.6730×10^{-6}
Anthocyanins	Y=1943.7x+2908.5	0.9974	0.011975	148.1710×10^{-6}
Dephinidin-3-glucoside	Y=2297.6x+1910.8	0.9943	0.014	125.3481×10^{-6}

2. Mean concentration of antioxidants in Calyces of Iraqi Hibiscus Sabdraffia Linn.

Table 4 shows the mean concentration of antioxidants for best separation from the calyces extract in ratio (100% deionized water: 0% methanol). Triplicate measurement was done on the best extract sample.

Table 4: Mean Concentration of Antioxidants.

Antioxidants	Mean concentration ± SD (µg/ml)
Vitamin C	25.830± 1.9
Sabdaretine	22.5510± 2.0
Gossypetine	15.4975± 1.8
Hibiscetine	11.1407± 2.1
Anthocyanins	43.9442± 1.7
Dephinidin-3-glucoside	18.5729± 2.1

3. Concentration of Antioxidants in Dry Spacemen of Calyces of Hibiscus Sabdraffia Linn.

The following table 5 shows the concentrations of the substances which was studies by this research in the dry spacemen of calyces.

Table 5: Concentration of Antioxidants

Antioxidants	Concentration (µg/g)
Vitamin C	258.3
Sabdaretine	225.51
Gossypetine	154.975
Hibiscetine	111.407
Anthocyanins	439.442
Dephinidin-3-glucoside	185.729

Conclusions:

This proposed analytical method by RP- HPLC- Uv/ Vis for simultaneous separation and determination of six antioxidants naturally present in Hibiscus sabdraffia calyces was highly convenient for evaluation the level concentration of the compounds under study. The obtained results show also, that the Iraqi Hibiscus Sabdraffia calyces are rich in these compounds and can be used usefully for human consumption.

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أمثلة طريقة كرموتوغرافيا السائل عاليه الاداء- طور معاكس مع مطيافية الأشعة فوق البنفسجية-المرئية والتحقق من صحتها لتعيين بعض مضادات الأكسدة في زهرة نبات الكجرات العراقي

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الخلاصه.

طريقة كرموتوغرافيا السائل عالية الأداء- طور معاكس الجديدة (RP-HPLC) مع مطيافية الأشعة فوق البنفسجية- المرئية (Ultraviolet- Visible spectrophotometry) تم أمثلتها وأثبتت لأستخلاص والتعيين في وقت واحد لمضادات الأكسدة الموجودة في زهرة نبات الكجرات العراقية.

الطريقة أسست على أستخدام حمام مائي فوق صوتي لأستخلاص المركبات العضوية. عوامل النموذج والجهاز تم أمثلتها بطريقة الأمثلة التقليدية (عامل واحد كل مرة). حدود التحسس لمضادات الأكسدة وهي بوحدة µg/ml: Vitamin C, Sabdaretine, Gossypetine, Hibiscetine, Anthocyanins, Dephinidin-3-glucoside were 113.8294×10^{-6}, 123.0453×10^{-6}, 59.6730×10^{-6}, 70.3681×10^{-6}, and 125.3481×148.1710 10^{-6} respectively.

تركيز مضادات الأكسدة التي تم دراستها لنموذج زهرة الكجرات الجافة هي كالاتي:

Vitamin C, Sabdaretine, Gossypetine, Hibiscetine, Anthocyanins, and Dephinidin-3-glucoside are 258.3µg/g, 225.51µg/g, 154.975µg/g, 111.407µg/g, 439.442µg/g, and 185.729µg/g respectively.