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PHYTOCHEMICAL STUDY FOR THE CONTENTS OF *HIBISCUS SABDARIFFA* L. SEEDS CULTIVATED IN EGYPT

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تم فى هذا البحث إجراء دراسة كيميائية على بذور نبات الكركدية من العائلة الخبازية بغرض التعرف على مكوناتها من البروتينات ، الأحماض الأمينية ، المعادن ، والدهون وأسفرت الدراسة عن:

نسبة البروتين (٣٣,٠٧٪) ووجود ستة عشر من الأحماض الأمينية بنسبة (٢١,٨٩٪) ووجد أرجينين ، ليسين ، فالين ، فينل ألانين بنسبة عالية (الأساسية) ، جليوتاميك ، أسبارتك ، جليسين ، سيرين ، ألانين (غير الأساسية).

كذلك وجد أن البذور غنية بعناصر الماغنسيوم (١,٣٠٪) ، البوتاسيوم (١,٠٢٪) ، الفوسفور (٠,٣٩٪) ، والكالسيوم (٠,٣٨٪).

وبدراسة مكونات الدهون للبذور أمكن التعرف على ألفا وبيتا أميرين ، بيتا سيتوستيرول وحمض الأوليانوليك وتسعة من الأحماض الدهنية لأستيرات الميثيل كانت أوليك (٣٨,٧٦٪) ، لين أوليك (٢٢,١٠٪) ، بالميتيك (١٤,٥٦٪) وأراشيدونك (٨,٦١٪) بنسبة عالية.

ومن خلاصة الأثير أمكن التعرف على مادة الجوسيبول وأمكن تقدير كميتها بواسطة كروماتوجرافيا السائل ذات الكفاءة العالية ووجدت (٠,٠٢٨٪).

A study for the protein, amino acids, minerals and lipids content for the seeds of Hibiscus sabdariffa L. was performed. Arginine, lysine, valine and phenylalanine were the major essential amino acids, besides glutamic, aspartic, glysine, serine and alanine. The seeds were found to be rich in magnesium (1.30%) and potassium contents (1.02%).. Gossypol (polyphenolic bisesquiterpene) was detected in the pet. ether and ether extracts of the seeds and estimated by HPLC ($2.8x10^{-3}$ g.% w/w).

INTRODUCTION

Hibscus sabdariffa L. (Malvaceae) is a herbaceous plant, widely cultivated in upper Egypt and tropical countries.[1,2] Its sepals are used in the form of decoction or infusion as a hypotensive drink and in various medicinal purposes.[3,4] The sepals and leaves of different species of Hibiscus were subjected to intensive studies for their contents from: flavonoids, anthocyandins, anthocyanins, mucilage and fatty acids.[5-16] The seeds are almost equivalent to dry sepals in weight, amounting for about 40% from the total weight of the fruits and about 200 kg per acre. To my knowledge, the seeds of *H. sabdariffa* L. have no use except in taking a part used in seedling and the rest is discard. Since this plant belongs to family Malvaceae which comprises: *Gossypium arboreum* L., *Gossypium herbaceum* L., *Gossypium barbadense* L. and *Gossypium hirsutum* L., seeds of which are used as a food for cattle. It is of interest to investigate the possibility of using the seeds of *H. sabdariffa* L. as source of cattle feeding too. The lipid content of the seeds of *Hibiscus sabdariffa* L. was studied previously and seven fatty acids were determined, besides α-amyrin and ß-sitosterol.[17]

The aim of the present work is to study the protein, amino acids, minerals, lipid and gossypol content of the seeds of *Hibiscus sabdariffa* L. cultivated in Assiut region.

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EXPERIMENTAL

Material

Samples of seeds of *H. sabdariffa* L. were collected in September 1996 from the plants cultivated in the Experimental Station of Medicinal Plants, Faculty of Pharmacy, Assiut University. The plant was previously authenticated by Prof. Dr. A.A. Fayed, Prof. of Plant Taxonomy, Dept. of Botany, Faculty of Science, Assiut University.

The mature seeds were separated and dried in hot air oven at 40°C, powdered and sieved. The powdered seeds was kept in a brown bottle and tightly firmed.

Apparatus and equipment

1- High performance amino acid analyzer (Beckman System 7300 and Data System 7000).
2- GLC (Shimadzu GC-14B 7343 Germany).
3- Reversed phase HPLC C-18, Knauer pump M-64, tunable UV detector (Knauer VO890), Schimadzu CR6A chromatopac data integrator.

Authentic material

1- Amino acids were obtained from the central laboratory for Food and Feed, Agricultural Research Center, Giza.
2- Fatty acids were obtained from the Center of Applied Researches and Advanced Studies, Faculty of Pharmacy, Cairo University.
3- α & ß-amyrins, ß-sitosterol, and oleanolic acid were obtained from the Pharmacognosy Dept., Faculty of Pharmacy, Assiut University.
4- Gossypol (F.H. Smith) was obtained from the Pharmacognosy Dept., Faculty of Pharmacy, Cairo University.

Estimation of protein contents

Adopting the method of Bremner and Mulvaney (1982)[18] for determination the total nitrogen and total protein was estimated, (% of nitrogen x 6.25).

Determination of amino acids

Adopting the method of Winder and Eggum (1966),[19] amino acids were determined. A sample of 20-30 mg of the dried powdered seeds of *Hibiscus sabdariffa* L. seeds was weighed in a conical flask and 5 ml of performic acid was added. The flask was closed and placed in ice water bath for 16 h., sodium metabisulfate and 25 ml 6N HCl was added to the oxidized mixture.

The flask was placed in an oven at 110°C for 24 h. and the sample was evaporated to dryness in a rotary evaporator. A suitable volume of sodium citrate buffer (pH 2.20) was added to the dried film of hydrolysed sample. After dissolving all soluble materials, the sample is ready for analysis.

Column: Na/A/B/D 25 cm,
Sample volume: 50 μL

The amino acids were identified by comparing their retention times with those of authentic compounds and their quantities were determined according to measurements of peak area for each.[20] The results are listed in Table 1.

Determination of elements content

2 g. of the dried powdered seeds of *H. sabdariffa* L. was digested by nitric acid and perchloric acids according to Jakson method (1967).[21] Sodium and potassium were determined using flame photometer (corning 400). Calcium and magnesium were determined by the versene titration.[21] Phosphorous was determined colourimetrically by the chlorostannous reduction molybdophosphoric blue colour method in sulphuric acid system.[21] The results are listed in Table 2.

Preparation of the lipids

500 g. of the dried powdered seeds of *H. sabdariffa* L. was extracted with cyclohexane in a Soxhlet apparatus till exhaustion. The cyclohexane extract gave 100 g. (20%) of brownish yellow oily material.

Preparation of the saponifiable and unsaponifiable fractions

10 g. of the oil was subjected to

saponification adopting the standard procedure[22] to obtain the unsaponifiable matters (3.6 g.) and the fatty acid fraction (2.5 g.).

Table 1: Composition of the amino acid contents for the seeds of *Hibiscus sabdariffa* L.

Amino acid	% of amino acid per 100 g. of seeds
Aspartic	2.50
Threonine*	0.84
Serine	1.13
Glutamic	5.21
Glysine	1.18
Alanine	1.01
Cysteine	0.43
Valine*	1.05
Methionine*	0.54
Isoleucine*	0.81
Leucine*	1.58
Tyrosine	0.38
Phenylalanine*	1.03
Histidine	0.78
Lysine*	1.09
Arginine*	2.33
Total	21.89

*Essential amino acid

Table 2: Percentage of elements and phosphorus content calculated as g.% of dry weight material of *Hibiscus sabdariffa* L. seeds.

Element	Conc. in g.% of dry weight*
Sodium	0.12
Potassium	1.02
Calcium	0.38
Magnesium	1.30
Phosphourus	0.39

* Average of three determinations.

Investigation of the unsaponifiable fraction

The ether extract of the unsaponifiable matter was spotted on silica gel G plates alongside with authentic α & ß amyrins, ß-sitosterol and oleanolic acid. Two solvent system were used, benzene : ethyl acetate (4:1) and toluene : acetone (9:1). The spots were located by spraying with 10% H_2SO_4 in ethanol and heating for 10 min. at 110°C.

Investigation of the saponifiable matter

The obtained fatty acids were converted to their methyl esters[23] and analysed by GLC (Shimadzu 7343 GC-14 B) adopting the following conditions: Stationary phase SE-30 (1.5%) SW on chromosorb W.AW (60-80 mesh) DMCS, packed on coiled glass column 1 meter long, 0.2 mm inner diameter. The flow rates of: the carrier gas (N_2), H_2, and air were 40, 30 and 300 ml/min. respectively. The temperature of injection part was 280°C. The column temperature was programmed as follows: isothermal at 150°C for 30 minutes and from 150-240°C at a rate of 5°C/min. The injected volume was 2 μL.

Identification of the fatty acids was carried out by comparing the retention time of each methyl ester with those of authentic ones analyzed under the same conditions. The relative percentage of each fatty acid was determined by the use of electronic integrator and the results are listed in Table 3.

Isolation and determination of gossypol

Adopting the method of Marcelle *et al.* (1985),[24] gossypol was isolated from the ether extract of the defatted powdered seeds of *H. sabdariffa* L. It was crystallized from pet. ether giving yellow crystals (yield 8 mgs from 500 g. of the seeds), m.p. 180°-182°, $[\alpha]_D^{25}$ +392° (c: 0.058, $CHCl_3$). TLC was performed on silica gel G (254 nm) film using solvent system $CHCl_3$-EtOAc-HCOOH (18:2:1) using standard (±) gossypol. The spots were visualized with acidified phloroglucinol in EtOH (29 g. phloroglucinol + 95 ml alc. + 5 ml conc. HCl) giving red spots after heating at 105°C. TLC: 1) $CHCl_3$: HOAc (9:1) R_F: 0.62, 2) EtOAc : Pet.ether (3:1) R_F: 0.70.

Table 3: GLC analysis of fatty acid methyl esters derivatives of *Hibiscus sabdariffa* L. seeds.

No.	Fatty acid methyl ester	R_t	Relative %
1	Myristic acid	11.1	0.34
2	Plamitic acid	14.1	14.56
3	Palmitolenic acid	14.6	0.47
4	Oleic acid	18.0	38.76
5	Linoleic acid	18.6	22.10
6	α-Linolenic acid	19.2	5.04
7	Unknown	21.0	3.30
8	Unknown	22.4	2.25
9	Unknown	23.8	0.81
10	Arachidic acid	25.1	3.46
11	Arachidonic acid	28.9	8.61
12	Behenic acid	32.8	0.15

R_t: Retention time in minutes

Quantitative estimation of gossypol in the ether extract of the seeds of *H. sabdariffa* L. was performed by means of reversed phase HPLC. The HPLC system consisted of a Knauer HPLC delivery pump 64, a Knauer variable wavelength spectrophotometric detector monitored at 254 nm, C-R6A chromatopac recording integrator, a stainless steel (25x0.5 cm i.d.) C-18 Eurosepher 80 (5 μm particles) column, attached to a cartridge guard column. The mobile phase was CH_3CN-H_2O-HOAc (75:24:1), flow rate 1.5 ml/min. and UV detection at wavelength 254 nm. Injected volume of the sample was 20 μL.

RESULTS AND DISCUSSION

In this study the nitrogen content of the seeds of *H. sabdariffa* L. could be estimated (5.29%) and the protein reached to (33.07%). The high % of protein is of great importance if it compared to that found in Soyabean (30-35%).

From Table 1, the amino acids totally amounting to (21.89%). The major essential were: Arginine (2.33%), leucine (1.58%), lysine (1.09%), valine (1.05%) and phenylalanine (1.03%), in addition to threonine (0.84%), isoleucine (0.81%), and methionine (0.54%).

The non essential amino acids were: glutamic (5.21%), aspartic (2.50%), glysine (1.18%), serine (1.13%), alanine (1.01%), besides histidine (0.78%), cysteine (0.43%) and tyrosine (0.38%). Since the essential amino acids are needed for the synthesis of body proteins at all times, arginine (2.33%) and histidine (0.78%) should be required during the periods of rapid tissue growth characteristic of childhood or recovery from illness.[25]

From Table 2 magnesium represents the major element in the seeds (1.30%), followed by potassium (1.02%), calcium (0.38%), phosphorous (0.39%) and sodium (0.12%). The highest % of potassium and the lowest one of sodium may suggest the use of seeds as hypotensive agent as well as a nutritive supplement for minerals.

TLC analysis of the unsaponifiable matter revealed the presence of six sharply separated spots, four of them were identified as: α & ß amyrin, ß-sitosterol and oleanolic acid.

GLC analysis of the fatty acid methyl esters of *H. sabdariffa* L. seeds is given in Table 3. It revealed the presence of 12 fatty acids, 9 of them could be identified. The majors were: oleic (38.76%), linoleic (22.10%), palmitic (14.56%), arachidonic (8.61%), α-linolenic (5.04%),

arachidic (3.46%), besides palmitolenic (0.47%), myristic (0.34%), and behenic (0.15%).

Study of the ether extract of the defatted seeds revealed the presence of gossypol (polyphenolic bisesquiterpene), which could be identified by means of its melting point, mixed melting point, elemental analysis, PMR, MS and comparison of UV and IR spectra with authentic sample spectra reported for gossypol.[26,27]

Quantitative estimation of gossypol by HPLC in *H. sabdariffa* L. seeds was found: 2.8×10^{-3} g.% w/w.

From the previous results one can conclude that, the seeds of *H. sabdariffa* L. can be used as a source for protein and minerals for nutritive purposes of economical value. Additionally, the seeds can be used as a source for gossypol which initiates its use as a male fertility regulating agent.[28-30]

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