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Phytoconstituents of *Zizyphus spina-christi* L. fruits and their antimicrobial activity

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Abstract

The crude protein of *Zizyphus spina-christi* seeds was found to be 15.9%. The percentage of essential amino acids was (12.8%) of total amino acids. Semi-essential was 17.1%, and non-essential represents 70% of the total mixture. Free sugars of the edible part of the fruit were isolated and identified by Paper Chromatography (PC) and High Performance Liquid Chromatograph (HPLC) and shown to be a mixture of fructose, xylose, glucose, and rhamnose. The yield of mucilage of the edible parts of the fruit of *Z. spina-christi* L. was 7.5%. PC and HPLC studies revealed that the hydrolysate of the mucilage of *Z. spina-christi* L. contains, mannose (93%), glucuronic acid (4.4%), rhamnose (1.24%) and galacturonic acid (0.37%). The lipid content of *Z. spina-christi* L. seeds was studied. Analysis of the fatty acids, by a Gas Liquid Chromatography (GLC) technique, revealed the presence of 13 fatty acids; linoleic acid represents 45% of the total mixture, followed by linolenic acid (20%). Cholesterol and β-sitosterol represent the major constituents of the unsaponifiable fraction (ca. 49%). This lipid fraction showed antimicrobial activity against G+ve *Bacillus subtilis*, and *Streptococcus pyogenes*, G−ve *Escherichia coli*. Fatty acid fraction showed high activity against *E. coli,* and *B. subtilis*.

Keywords: *Z. spina-christi*; L.; *Rhamnaceae*; Lipids; Mucilage; Antimicrobial activity

1. Introduction

The *zizyphus* species (*Rhamnaceae*) are commonly used in folklore medicine for the curing of various diseases (Bedevian, 1936; Kirtiker & Basu, 1984; Lindley, 1981; Quisumbing, 1978; The New Encyclopaedia Britannica, 1974), they are wide-spread in the Mediterranean region, Africa, Australia, and tropical America. *Z. spina-christi* L. is indigenous to Iraq, growing mainly in the Basra region (Duke, 1985). *Z. spina-christi* L. has been used in folk medicine as a demulcent, depurative, anodyne, emolient, stomachic, for toothaches, astringents, and as a mouth wash (Duke, 1985). The decoction of bark and fresh fruits is used to promote the healing of fresh wounds and also used as a body wash, while fruits are used for dysentery (Blatter, 1978; Irvine, 1961). The fruits are also used for bronchitis, coughs, and tuberculosis (Duke, 1985; Hutchens, 1973). The butanol extract of *Z. spina-christi* leaves and its main saponin glycoside (christinin-A) improved glucose utilization in diabetic rats (Glombitza, Mahran, Mirhom, Michel, & Motawi, 1994). *Z. spina-christi* was shown to contain betulic and ceanothic acid (Ikram & Tomillnson, 1976). Three cyclopeptide alkaloids (Shah, Ageel, Tariq, Mossa, & Al-yahya, 1986), as well as four saponin glycosides (Mahran, Glombitza, Mirhom, Hartmann, & Michel, 1994), and several flavonoids (Nawar, Ishak, Michel, & Buddrust, 1984) have been isolated from the leaves of *Z. spina christi* . Nothing has been reported about the protein, lipids and mucilage of *Z. spina-christi* seeds and fruits, therefore the present work is concerned with the study of the protein, lipids and their antimicrobial activity, free sugars and mucilage of *Z. spina-christi* fruits.

2. Materials and methods

2.1. Plant material

The fruits of *Z. spina-christi* were collected from the Orman garden, during December 1999 and kindly identified by Dr. M. El-Gebaly, Curator of the herbarium of National Research Centre. Seeds were manually isolated from the edible parts.

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2.2. Apparatus and techniques

A Pye Unicam gas chromatograph equipped with dual flame ionization detector (FID) and dual channel recorder was used for gas chromatographic analysis of fatty acid methyl esters and unsaponifiable matter.

High Performance Liquid Chromatograph (HPLC) was used for carbohydrate analysis .The liquid chromatograph consisted of an isocratic pump (model Lc.10As, Shimadzu, Japan), refractive index detector (RID-6A, Shimadzu, Japan) and arheodyne injector (Model 7161, Catati, California, USA) equipped with 20 μl injector loop (Model C-R7A, Shimadzu, Japan) and Phenomenex column USA, Kromasil 10 NH_2. (250×4.60i.d.) Ser.:12885. The sensitivity was set at 0.001 AUFS.

A Lc 3000 Amino acid Analyzer, Shimadzu, Japan, was used.

2.3. Preparation of the protein

The crude protein was isolated according to the method described by Ibrahim and El-Eraqy (1996). Twenty grams of the defatted powdered seeds were stirred in 10% sodium chloride solution for 1 h, then filtered. An equal volume of 10% trichloroacetic acid (TCA) solution was added to the filtrate. A white precipitate was formed, collected by centrifugation, washed with 5% TCA solution, then washed with ether and absolute ethanol, then dried in a vacuum desiccator. The crude dried protein was dialyzed by parchment membrane. The non-dialyzable fraction was collected and dried.

2.4. Amino acid analysis

The protein (about 40 mg) was hydrolyzed with 6N HCl at 105 °C for 24 h in a sealed tube under N_2, adopting the method of (Bailey, 1967). After cooling and filtering, the residue left after filtration was washed with distilled water and the combined filtrates were made up to 50 ml in a volumetric flask. A portion of the filtrate (10 ml) was evaporated to dryness at room temperature in a desiccator under vacuum. The residue was dissolved in a 5 ml buffer (0.2 N sodium citrate, pH 2.2) and the solution was filtered through 0.22-μm membrane. Twenty microlitres of the final filtrate were injected into the instrument for quantitative determination of the amino acid.

2.5. Isolation of lipid fraction

About 100 g of dried powdered seeds of *Z. spina-christi* were exhaustively extracted with petroleum ether (40–60 °C) in a Soxhlet extractor. The extract was filtered and the filtrate was evaporated under vacuum at 40 °C. A residue of 2.1 g was left, which was saponified in the usual way (N/2) methanolic KOH (Nazif, 1994). The unsaponifiable fraction of the total lipids about 0.9 g was subjected to GLC analysis. Pye Unicam gas chromatograph was used under the following conditions: column: OV 17 Methyl phenyl silicone (1.5 m×4 mm). The column oven temperature was programmed at 10 °C/min, from 70 to 270 °C; detector temperature, 300 °C, injector temperature 250 °C, nitrogen flow rate, 30 ml/min, hydrogen flow rate 33 ml/min. Air flow rate was 330 ml/min, chart speed 0.5 cm/min FID. Results are illustrated in Table 1.

The residual aqueous solution was acidified with HCl and the liberated fatty acids were extracted with ether and subjected to methylation using dry HCl (4.5%) and MeOH (Nazif, 1994). They were analyzed on GLC, using a Pye Unicam PU 4550 equipped with a column, PEGA 10% (1.5 m×4 mm). The column oven temperature was programmed at 8 °C/min from 70 to 190 °C, detector temperature, 210 °C, injector temperature, 210 °C nitrogen flow rate, 30 ml/min, hydrogen flow rate, 33 ml/min air flow rate, 330 ml/min; chart speed, 0.5 cm/min. Results are shown in Table 2.

2.6. Isolation of free sugars

Fifty grams of fruits (deprived of seeds) of *Z. spina-christi* L. were exhaustively extracted by maceration in 1 l of 95% boiling ethanol; after complete exhaustion, the ethanolic extract was concentrated under vacuum at 40 °C to release ethanol; then the concentrated aqueous extract was applied to PC, using n-butanol:benzene:-pyridine:water (5:1:3:3) v/v as an eluting solvent; the chromatogram was visualized by spraying with aniline oxalate reagent and heating to 105 °C for 5 min. Ali-

Table 1
GLC analysis of unsaponifiable fraction of *Zizyphus spina-christi*. L. seeds

Peak no.	RRT[a]	Relative (%)	Component
1	0.19	0.148	Dodecane
2	0.22	4.13	Tridecane
3	0.26	2.43	Tetradecane
4	0.28	1.07	Hexadecane
5	0.33	4.26	Octadecane
6	0.36	4.47	–
7	0.38	3.97	Eicosane
8	0.41	1.60	–
9	0.43	2.02	Docasane
10	0.47	1.55	Tetracosane
11	0.53	12.9	Hexacosane
12	0.57	5.49	Octacosane
13	0.69	0.466	Tricontane
14	0.74	0.304	Squalene
15	0.81	21.7	Cholesterol
16	0.85	0.447	Stigmasterol
17	1	27.1	β-sitosterol

[a] Relative to β-sitosterol.

quots of 10 μl were injected into the HPLC column. The results are shown in Table 3.

2.7. Isolation of mucilage

Twenty grams of fresh fruit (deprived of seeds) of *Z. spina-christi* L. were macerated in 1 l of distilled water slightly acidified to pH 4 with diluted HCl, stirred for 3 h at 25 °C, and left overnight. The solution was filtered and the process repeated twice again; the combined filtrates were concentrated under vacuum to about 200 ml, then mucilage was precipitated from the aqueous extract by adding slowly, while stirring, 4 volumes of absolute ethanol. The precipitate was separated by centrifugation, then washed several times with absolute ethanol, followed by acetone with stirring and filtered, and kept in a vacuum desiccator over anhydrous calcium chloride. The isolated mucilage was an odourless substance with mucilaginous taste, swelling in water; it gives a colloidal viscous, non-adhesive solution, insoluble in ethanol, ether and chloroform.

Table 2
GLC analysis of fatty acid methyl esters of *Zizyphus spina-christi* L. seeds

Peak no.	RRT[a]	Relative (%)	Constituents
1	0.286	0.106	Caprylic ($C_{10:0}$)
2	0.420	0.129	Lauric ($C_{12:0}$)
3	0.494	0.689	Myristic ($C_{14:0}$)
4	0.528	0.322	–
5	0.568	0.223	–
6	0.642	0.359	–
7	0.675	10.0	Palmitic ($C_{16:0}$)
8	1	45.4	Linoleic ($C_{18:2}$)
9	1.099	20.0	Linolenic ($C_{18:3}$)
10	1.339	4.59	Arachidic ($C_{20:0}$)
11	1.469	4.26	($C_{20:0}$)
12	1.591	12.5	($C_{20:2}$)
13	1.739	1.13	Arachidonic($C_{20:4}$)

[a] Relative to linolenic acid.

Table 3
HPLC analysis of free sugars and the mucilage hydrolysate of *Zizyphus spina-christi* L. fruits

No. of spots	RT[a] (min)	Authentic sugars	Percentage	
			Free sugars	Mucilage hydrolysates
1	1.59	Unidentified	–	0.80
2	1.79	Galacturonic acid	–	0.36
3	2.5	Glucuronic acid	–	4.40
4	2.9	Rhamnose	2.6	1.24
5	3.5	Xylose	5.7	–
6	3.9	Mannose	–	93
7	4.2	Glucose	6.2	–
8	4.5	Galactose	–	–
9	6.3	fructose	78	–

[a] Retention time in minutes

2.8. Hydrolysis of mucilage

Part of the obtained mucilage (100 mg) was heated in 2 ml of 0.5 M sulphuric acid in a sealed tube for 20 h in a boiling water bath. At the end of hydrolysis, a brown flocculent precipitate was noticed, which was filtered off and the filtrate was freed (SO_4^{-2}) by precipitation with barium carbonate. The filtrate was evaporated under vacuum and the residue dissolved in 10% isopropyl alcohol and chromatographed on PC. Also, the residue dissolved in bidistilled water and acetonitrile (1:3), and an aliquot of 20 μl was injected into an HPLC column using the following conditions:

Column:Kromasil 10 NH_2, (250×4.6 mm i.d.); Mobile phase was acetonitrile:water (75:25) v/v.

Flow rate was 1 ml/min; retention times and areas were determined using refractive index detector and c-R7A model injector. All assays were performed at ambient conditions.

2.9. Test for antimicrobial activity

The lipid content of seeds of *Z. spina-christi* L., its saponifiable fraction and its unsaponifiable fraction were tested for their antimicrobial activity against certain bacteria (G+ve and G−ve), yeast and fungi by using diffusion assay method (Hammoud & Lambert, 1978). The micro-organisms were obtained from the centre of culture at the National Research Centre. The

Table 4
Antimicrobial activity of lipid content of *Z. spina-christi* L. seeds

Micro-organisms	Inhibition zone (cm)				
	Total pet. ether extract	Total fatty acid fraction	Unsaponifiable fraction	Ampicillin	Canesten
1-*Bacillus subtilis*	0.9±0.1	1.3±0.15	–	3.9±0.1	–
2-*Streptococcus pyogenes*	2.1±0.15	2.6±0.1	–	4.2±0.15	–
3-*E. coli*	1.9±0.1	2.5±0.1	0.7±0.15	3.5±0.1	–
4-*Sacchromyces cereviciae*	–	–	–	–	2.5±0.15
5-*Aspergillus niger*	–	–	–	–	3.5±0.14
6-*Aspergillus flavus*	–	–	–	–	3.1±0.15

Results are mean of two replicates: 1 g% ampicillin (Wyeth); 1 g% canesten (Bayer), 0.1 g/ml of both fatty acids mixture and unsaponifiable matter; 0.15 g/ml of total pet. ether extract.

Table 5
Amino acid content of *Zizyphus spina-christi* L. seeds

Name of amino acid	R.T. (min)	% Of each amino acid w.r.t. total
Aspartic acid	11	0.122
Serine	15.9	10.8
Proline	20.53	11.3
Glycine	25.12	17.1
Alanine	26.2	7.45
Valine	30.80	4.42
Methionine	32.75	0.747
Isoleucine	334.7	1.15
Leucine	35.47	4.75
Tyrosine	38.52	1.15
Phenylalanine	40.53	1.75
Histidine	48.43	12.3
Lysine	51.22	1.92
NH_4^+	54.65	20.0
Arginine	59.02	4.85

diameter of the cups made was 0.8 cm and concentrations used were 1 g% ampicillin (Wyeth) and 1 g% canesten (Bayer); 0.1 g/ml of both fatty acids mixture and unsaponifiable matter and 0.15 g/ml of total petroleum ether extract, were suspended in chloroform as diluent vehicle. Results are shown in Table 4 (see below).

3. Results and discussion

The total protein content of *Z. spina-christi* seeds was found to be 15.9%, determined by using the macro-Kjeldal method (AOAC, 1984). The essential amino acids were: valine (4.42%), methionine (0.747%), isoleucine (1.15%), leucine (4.75%), phenylalanine (1.75%). Semi-essential amino acids were: histidine (12.3%) and argenine (4.85%). Non-essential amino acids: were aspartic acid (0.122%), serine (10.8%), proline (11.3%), glycine (17.1%), alanine (7.45%), tyrosine (1.15%) and the main amino compound was $NH4^+$ (20.0%). The analysis of amino acids (Table 5) revealed the presence of 15 amino acids. The percentage of essential amino acids was (12.8%) of total amino acids. Semi-essential were 17.1% and non-essential were 70% of the total mixture.

The lipid content of *Z. spina-christi* L. seeds amounted to 2.3% of dry weight. GLC of unsaponifiable fraction Table 1 revealed the presence of a mixture of hydrocarbons ranging from n-C_{12} to n-C_{30}, representing 50.7% of total unsaponifiable matter. Hexacosane (n-C_{26}) represents 12.9% of total unsaponifiable matter. Cholesterol and β-sitosterol represent (21.7%) and (27.1%), respectively.

Analysis of the fatty acid methyl esters, by GLC, revealed the presence of 13 fatty acids. Unsaturated fatty acids represent the major components (83.5%; Table 2).These unsaturated fatty acids may be responsible for the broad spectrum antimicrobial activity of the plant (Hashem & Saleh, 1999), while the saturated fatty acids represent 16.5%. Linoleic acid $C_{18:2}$ amounted to 45% of the total fatty acids, followed by linolenic acid, $C_{18:3}$, (20.01%); palmitic acid represents 10% of total fatty acids mixture.

The mucilage of *Z. spina-christi* L. fruits deprived of the seeds was studied. The mucilage content amounted to 7.5%. This high content of mucilage makes it promising as a demulcent and emolient in folk medicine (Duke, 1985) . PC and HPLC studies revealed that the hydrolysate of the mucilage contains mannose (93%) as a major component, also glucouronic acid (4.4%), rhamnose (1.24%), and galacturonic acid (0.37%) were found in the HPLC of the mucilage hydrolysate (Table 3).

Free sugars of the fruits of *Z. spina-christi* L. (deprived of seeds) were found to be a mixture of glucose (6.2%), rhamnose (2.6%), xylose (5.7%) and the main sugar was found to be fructose (78% of the total mixture of free sugars). This relatively high content of fructose makes this fruit useful for diabetic persons.

The antimicrobial activity of different extracts of fruits and seeds of *Z. spina-christi* L. revealed the highest activity of fatty acid fraction of lipids of seeds of *Z. spina-christi* L. against *Bacillus subtilis*, *E. coli* and *Streptococcus pyogenes,* as illustrated in Table 4. This result is supported by Hashem and Saleh (1999).

Also a moderate activity against the same microorganisms was noticed in the total petroleum ether fraction. Slight activity against *E. coli* was noticed in the unsaponifiable fraction.

This result was found to be in accordance with the use of a decoction of fresh fruits to promote the healing of fresh wounds and use as a body wash in folk medicine (Blatter, 1978; Irvine, 1961).

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