

Chemical Composition and Antimicrobial Activity of Sidr Honey

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A COMPARISON between two methods of extraction of Sidr honey was performed to elucidate the difference in chemical composition and antimicrobial activity. The results of the GC/MS studies of the n-hexane extracts by methods A (extraction without dilution) & B (extraction after dilution with water) for Sidr honey showed that method A contains all terpenoids and method B contains higher significant concentration of fatty acids. Some compounds were identified for the first time in Sidr honey. Piperitone, calamanene -1S-trans, 4-methoxy -butanoic acid, 2- pentanol -5- methoxy-2- methyl, Pyrido [3,2-d] pyrimidine - 4-ol, 2- ethyl- 5- tridecylpyrrolidine, quinoline -, 5,6,7,8-tetrahydro - 3-methyl and quinoline - 4,8-dimethyl. The antimicrobial activity varied according to the type of pathogen. However the method A showed the highest antibacterial activity, the method B had the highest antifungal activity.

Keywords : Sidr honey , Methods of extraction , GC-MS, Antimicrobial activity .

Unifloral honeys possess highly characteristic aromas indicating the presence of various volatile components some of which are probably derived from the sources of nectar; some will be dependant on the physiology of the bee and others arise during processing after harvest. Some work has been reported on the volatile constituents of various unifloral honeys. Volatile components of some unifloral Australian honeys as phenylacetaldehyde, benzyl alcohol and 2-phenylethanol were positively identified, linalool oxide and hexenyl butyrate only tentatively and some of the identified compounds were furan derivatives (Graddon *et al.*,1979). 2,6-dimethyl-3,7-octadien-2,6-diol and hotrienol were found in leatherwood honey (Rowland *et al.*, 1995) .

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Identification of different compounds using various methods have been reported. For example, dilution of honey with water (20g in 700ml water) prior to extraction with diethyl ether, where the dried extract was methylated prior to GC/MS analysis (Tan *et al.*, 1988). Directly extraction of honey with methylene chloride lead to the identification of some terpenoids by GC/MS analysis (Graddon *et al.*, 1979). Bicchi *et al.*, (1983), tried four methods for extraction and identified some terpenoids with GC/MS analysis. The discrepancy between the methods of extraction gave different identified compounds. Total extractable organics from honey have not been reported before. Thus the aim of the present work was to study the effect of method of extraction on chemical composition and antimicrobial activity of Sidr honey.

Material and methods

Honey sample

Sidr honey, unifloral honey produced from the nectar of *Ziziphus spina-christi*. It was kindly supplied from EL Yahia Apiry Farm, Kingdom of Saudi Arabia.

Extraction

Two equal weight samples of Sidr honey were extracted by two different methods in order to determine the suitable one. The first method of extraction (A) was performed according to (Graddon *et al.*, 1979), where 50 g honey were thoroughly mixed with 25 ml n-hexane and then decanted. This was repeated seven times with further 25 ml portions. Aliquots of solvent and the combined extract were concentrated by evaporating the solvent under vacuum at 25°C. The second method (B) was done according to (Tan *et al.*, 1988), where 50g honey were diluted with three volumes distilled water prior to n-hexane extraction. The extraction was repeated 7 times (25 ml. each).

Blank analysis (control) was performed by concentration of 100 ml of n-hexane under the same condition. The GC/MS analysis did not show any interfering components.

GC/MS analysis

A finnigan MAT SSQ 7000 mass spectrometer was coupled with a Varian 3400 gas chromatograph . DB-5 column , 30m x 0.32 mm (internal diameter). It was employed with helium as carrier gas and the temperature programmed from 40 to 260°C at 5°C / min (3-min initial hold , 10-min final hold) . The mass spectra were recorded in electron ionization (EI) mode at 70 eV, ion source temperature 150°C. The scan repetition rate was 0.5 s .

Identification of compounds

Peaks were identified by computer search of user generated reference libraries, incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity; mixed peaks were resolved by computer program aimed at resolving the mass spectral data of one compound from overlapping mass spectra of another.

Antibacterial assay

Five bacterial strains were used: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*. The bacterial suspension was prepared and adjusted by comparison against 0.5 Mc-Farland turbidity standard (5×10^7 organisms / ml) tubes. It was further diluted to obtain a final concentration of 5×10^6 organisms / ml. *Staphylococcus aureus* was enriched on polymyxin agar (Finegold and Sweeny, 1961) as a selective media, while *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* were enriched on MacConkey broth. These organisms were subcultured on nutrient broth for further bacterial propagation (Cruickshank *et al.*, 1979). The broth was inoculated by the 0.20 ul/10 ml broth with either *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli* or *Pseudomonas aeruginosa*, then added 40 ul of 20 % honey or its extract. These tubes were incubated at 37°C for 24 hr. The growth of control bacterial strains as well as inhibition of the bacterial growth due to honey were measured, the optical density at 420 nm wave length by spectrophotometer. The mean values of inhibition were calculated from triple reading in each test. The minimal inhibitory concentration (MIC) of honey and its n-hexane extracts were determined by ten fold dilution method against bacterial strains in in-vitro (Hegazi, *et al.*, 1996a) . Data were analyzed statistically using student "T" test according to Snedecor (1961) .

Antifungal assay

The antifungal activity of tested crude Sidr honey and extraction with two methods was carried out against *Asprgillus ochraceus*, *Asprgillus niger*, *Asprgillus flavus*, *Asprgillus fumigatus*, *Mucor* and *Candida albicans* as described in British Pharmacopoeia. Sabouraud's glucose agar and broth inoculated by the spore suspension (0.20 ul/10 ml). Then added 40 ul of 20% crude Sidr honey, A or B extract. The tubes were incubated at 28°C for 48hr. The growth as well as inhibition were measured by Spectrophotometric assay as turbidity at 420 nm wave length .The mean value of inhibition were calculated from triple reading in each test. Data were analyzed statistically using student "T" test according to Snedecor (1961) .

Results

A comparison between two methods of extraction of Sidr honey was performed to elucidate the difference in chemical composition and antimicrobial activity. The results of the GC/MS studies of the n-hexane extracts by methods A (extraction without dilution) & B (extraction after dilution with water) for Sidr honey showed that the extracts are intricate mixtures containing various classes of compounds. The comparative studies between methods A&B revealed that method A contains all the terpenoids (monoterpenes and sesquiterpenes) and method B contains traces of few terpenoids as shown in Table 1 and Fig. 1,2. The major terpenoids were piperitone, citronellol, citronellyl formate, calamanene-1S-trans and eugenol acetate .

The GC/MS of the acidic compounds demonstrated that the majority of the fatty acids were present in the free form, with concentrations of 37.45 & 78.47 % in methods A & B respectively. Hexadecanoic, octadecanoic and oleic acids were significantly higher in method B (Table 1) .

There were some compounds identified for the first time in Sidr honey as follows: method A has a higher content of 4-methoxy-butanoic acid (5.01 %) than method B (1.20 %), 2-pentanol-5-methoxy-2-methyl (4.6%) in method A and (1.0%) in method B, pyrido[3,2-d] pyrimidine-4-ol, 2-ethyl-5-tridecylpyrrolidine, quinoline -5,6,7,8- tetrahydro-3- methyl and quinoline- 4,8-dimethyl in method A in concentration of 1.4, 0.15, 0.7and 0.12 while in method B 1.7, 0.12, 0.83 and 0.0 respectively.

The antibacterial and antifungal activities against Gram positive, Gram negative bacteria, moulds and yeast using two methods of extractions compared with Sidr honey itself were illustrated in Tables 2 & 3. The antibacterial activity varied according to the type of bacteria. The two methods of extraction revealed differences in the antibacterial activity against Gram positive and Gram negative bacteria. It was clear that method A showed the highest antibacterial activity. While method B showed the highest antifungal activity against *Mucor*, *Asprgillus flavus* and *Candida albicans*.The crud Sidr honey showed the highest antifungal activity against *Candida albicans*, *Asprgillus niger*, *Asprgillus ochraceus*, if compared with method A and B .

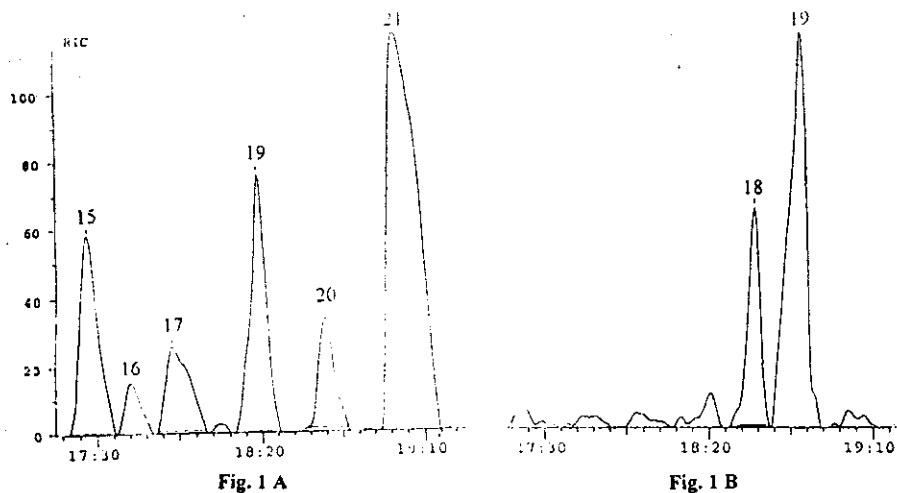


Fig .1. Reconstructed ion chromatogram of n-hexane extract of Sidr honey. The area shown (RT. 17: 30 -19 : 10) , contains citronellyl formate (15), geraniol (17), Bicyclo [2.2.1] heptane -7- methylene-2-(1- methylethyldiene) (20) and piperitone (21) in Fig. 1 A and nonanoic acid (br.) (18) and nonanoic acid (19) in Fig. 1 B; numbers in parentheses correspond to peaks.

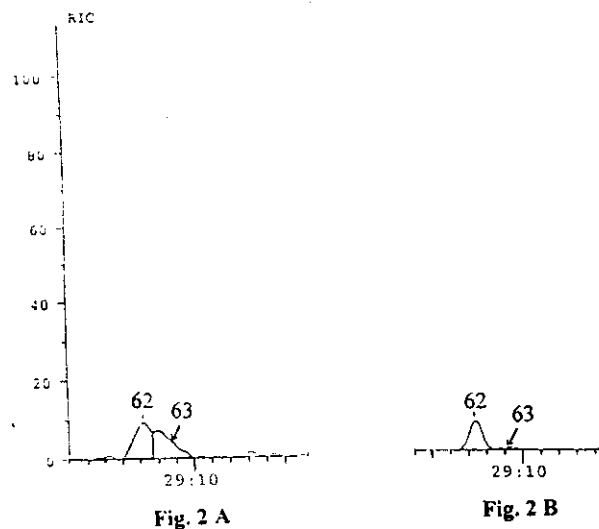


Fig .2. Reconstructed ion chromatogram of n-hexane extract of Sidr honey. The area shown (RT. 28:45 - 29 :15) , contains octadecene (62) and sesquiterpene alcohol (63) in Fig. 2 A and only octadecene (62) in Fig. 2 B; numbers in parentheses correspond to peaks.

TABLE 1. Compounds identified by GC/MS and analysis in Sidr honey .

Peak No	Compound	% TIC *	
		A	B
Fatty acids			
1	Butanoic acid-4-methoxy	5.01	1.20
5	Heptanoic acid	0.02	----
9	Octanoic acid	0.14	0.19
18	Nonanoic acid(br.)	0.17	-----
20	Nonanoic acid	-----	0.30
30	Decanoic acid	-----	0.29
48	Dodecanoic acid	0.27	1.04
64	Tetradecanoic acid(br.)	-----	0.03
65	Tetradecanoic acid(br.)	0.03	0.02
66	Tetradecanoic acid	1.10	3.00
70	Tetradecanoic acid-12methyl	0.16	0.30
72	Pentadecanoic acid	-----	0.07
77	Hexadecanoic acid(br.)	0.12	0.45
79	Hexadecanoic acid	12.40	29.35
87	Octadecanoic acid	12.40	20.20
88	Oleic acid	4.00	17.00
94	Eicosanoic acid	0.23	0.30
100	Docosanoic acid	0.72	0.11
107	Lignoceric acid	0.68	4.62
Fatty acid esters			
78	Ethyl palmitate	0.27	0.16
83	Linoleic acid methyl ester**	0.15	0.05
86	Ethyl oleate	0.60	1.15
110	Oleic acid octyl ester**	2.00	----
Oxygenated compounds			
3	2-Pentanol-5-methoxy-2-methyl**	4.60	1.00
41	Cyclooctane methanol- α - α -dimethyl	0.02	0.04
42	4-Methyl-2hydroxy cyclopent-2 en-1 one	0.04	0.03
67	Pentadecanol	0.07	0.04
91	Nonadecanol	0.08	----
105	Docosanol	0.01	0.12
2	Butane-1,3-dimethoxy**	0.03	0.04
56	Tridecanone-methyl**	0.05	0.02
Monoterpenes ,Sesquiterpenes			
7	trans- α - Dihydroterpineol	0.02	---
8	Isomenthone	0.03	---
10	Citronellol	0.36	---
11	Sabinene hydrate acetate -cis	0.02	0.05
13	4-Hepten-3one-5- ethyl-2-methyl	---	0.01
15	Citronellyl formate	0.14	---
17	Geraniol	0.08	---

TABLE 1 . Cont .

20	Bicyclo[2.2.1]heptane-7-methylene-2-(1-methylethylidene)	0.07	---
21	Piperitone	0.48	---
31	1-Oxaspiro[4.5]deca-3,6 diene,6,10,10,trimethyl	0.09	0.07
32	Sesquiterpene hydrocarbon	0.03	---
33	Bicyclo[3.1.1]hept-2-ene-3-propanoic acid, β ,6,6 trimethyl	---	0.21
34	Eugenol acetate	0.12	0.03
35	Calamanene-1S- trans	0.14	---
63	Sesquiterpene alcohol	0.03	---
98	Azuleno[6.5-b] furan-2,6-(3H,4H) dione,3a,7,7a,8,9,9a- hexahydro	0.05	---
<i>Nitrogen Compounds:</i>			
51	Quinoline-4,8-dimethyl	0.12	---
69	Diphenylamine	0.05	0.04
74	Pyrido[3,2-d]pyrimidine-4-ol **	1.40	1.70
75	Quinoline,5,6,7,8-tetrahydro-3- methyl	0.70	0.83
81	Benzene,1-(1,1-dimethyl ethyl) 3,5- Dimethyl-2,4,6-trinitro	0.17	---
92	2-Ethyl-5-tridecylpyrrolidine **	0.15	0.12
<i>Aromatic Compound</i>			
80	bis(2-Methylethyl) phthalate	0.31	---
84	1,2-Benzene dicarboxylicacid butyl cyclohexyl ester	0.07	0.05
106	bis-(2-Ethylhexyl)phthalate	0.80	0.20

* The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation .

** Identified for the first time in honey . (br.) = branched .

TABLE 2. Antibacterial activity of Sidr honey and its different extracts (optical density measurements at 420nm) .

Treatment	<i>Staphylococcus aureus</i>		<i>Streptococcus pyogenes</i>		<i>Klebsiella Pneumoniae</i>		<i>Pseudomonas aeruginosa</i>		<i>Escherichia coli</i>	
	Growth inhibition	MIC (ug/ml)	Growth inhibition	MIC (ug/ml)	Growth inhibition	MIC (ug/ml)	Growth inhibition	MIC (ug/ml)	Growth inhibition	MIC (ug/ml)
Bacterial Normal growth	1.55± 0.05	----	1.40 ± 0.001	----	0.9 ± 0.15	----	1.70± 0.15	----	1.55 ± 0.05	----
Sidr honey	0.0104± 0.0005	1600	0.0500± 0.008	1600	0.078± 0.003	1200	0.097± 0.0025	2100	0.173± 0.004	2800
Method A	0.155 ± 0.0005	2800	0.0257± 0.0002	2400	0.06 ± 0.001	1000	0.067± 0.0010	1600	0.014 ± 0.0009	2100
Method B	0.0552± 0.0002	3600	0.3117± 0.0002	3600	0.201± 0.001	2400	0.201± 0.0009	1800	0.510 ± 0.012	3800
Tetracycline (50ug)	0.095 ±0.0001	1000	0.0057± 0.0002	2100	0.349 ± 0.0001	1800	0.969 ± 0.0004	3200	1.449 ± 0.0003	4400

* Growth Inhibition = Inhibition of the growth measured by optic density at 420 nm analyzed by spectrophotometer.

** MIC: Minimal inhibition concentration)

TABLE 3. Antifungal activity of Sidr honey and its different extracts (optical density measurements at 420nm) .

Treat ment	<i>Asprgillus ochraceus</i>		<i>Asprgillus niger</i>		<i>Asprgillus flavus</i>		<i>Asprgillus fumigatus</i>		<i>Mucor</i>		Candida albicans	
	Growth inhibition	MIC (ug/ml)	Growth inhibition	MIC (ug/ml)	Growth inhibition	MIC (ug/ml)	Growth inhibition	MIC (ug/ml)	Growth inhibition	MIC (ug/ml)	Growth inhibition	MIC (ug/ml)
Normal growth of fungi	0.855± 0.003	---	0.736± 0.006	---	0.808± 0.046	---	0.743± 0.0013	---	0.852± 0.019	---	0.771± 0.074	---
Sidr honey	0.190± 0.001	2400	0.163± 0.008	2400	0.231± 0.008	1600	0.172± 0.011	2800	0.425± 0.008	3200	0.155± 0.023	1600
Method A	0.195± 0.006	1800	0.217± 0.005	2100	0.151± 0.091	1200	0.91± 0.003	5600	0.154± 0.027	2800	0.323± 0.068	2100
Method B	0.238± 0.001	2800	0.222± 0.001	2100	0.94± 0.008	4200	0.258± 0.001	3600	0.92± 0.003	4800	0.251± 0.009	1800
Ketoco nazole (50 ug)	0.095 ± 0.001	3200	1.233 ± 0.004	8400	0.469 ± 0.0003	3400	1.700 ± 0.002	6400	1.270 ± 0.0011	5600	0.638 ± 0.003	2400

Discussion

The results of identification of monoterpenes and sesquiterpenes extracted by either method A or B elucidated that method A contains all the terpenoids while method B contains traces. It could be concluded that the dilution of honey with water prior to solvent extraction makes it impossible to detect monoterpenes and sesquiterpenes. This is similar to Graddon's method which succeeded to identify terpenoids (Graddon *et al.*, 1979), while Tan's method failed to identify any (Tan *et al.*, 1988). All the fatty acids present in Sidr honey observed in different types of unifloral honeys as clover, Manuka and Kanuka (Tan *et al.*, 1988), heather honey (Tan *et al.*, 1989) and thyme & willow honeys (Tan *et al.*, 1990). The nitrogen compounds present in Sidr honey were reported for the first time in this investigation. Some authors identified other quinoline derivatives, pyridine, cinnamaldehyde and cinnamyl alcohol in coriander, lime and sunflower honeys (Artem'ev and Chepurnoi, 1984), while N-beta-phenylethyl formamide, N-beta-phenylethyl-2-formyl pyrrole, N-beta-phenylethyl acetamide, indol, methyl anthranilate and methyl-N-acetyl anthranilate were identified in some Japanese and Chinese honeys (Tsuneya and Shiga, 1988). The study of antibacterial activity revealed that method A showed the highest antibacterial activity in comparison with method B and the crude honey. The variation in the antibacterial activity is attributed to the chemical composition of different methods of extraction. Similar results were also obtained from different types of honey by other authors (Molan, 1992; Hegazi *et al.*, 1996 b and Hegazi, 1998). The antifungal activity of Method A or B was varied according to the type of pathogen. It was found that terpene hydrocarbons and sesquiterpenes showed antimicrobial activity against *Aspergillus niger*, *Aspergillus ochraceus*, *Penicillium Rubrum* and *P. spinulosum* (Kalodera *et al.*, 1994). Garg and Muller (1993) reported that the antifungal activity decreased with increasing carbon chain length in the even-numbered carbon chain series, while odd-numbered carbon fatty acids showed irregularities. Undecanoic acid (C11:0) was the most toxic in the C7:0 -C18:0 series. Polyunsaturated fatty acids were more toxic than their corresponding saturated acids. It is clear from the results that the difference in antifungal activity of method A or B are reflected to the participation of different groups of compounds in Sidr honey as well as the differential concentration of these groups in method A and B, which showed irregularities in the antifungal

activity against different fungi. Such findings was observed (Toth, 1986) where there is a connection between aromatic content of honey and beneficial effects of honey consumption in some cases of diseases of upper respiratory tract caused by *Candida albicans*, *Klebsiella pneumoniae* and *E. coli*.

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التركيب الكيميائي والكفاءة المضادة للميكروبات لعسل السدر

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لقد أجريت دراسة لعمل مقارنة بين طريقتين من طرق الاستخلاص لإستبيان التركيب الكيميائى لعسل السدر وأثرها على الكفاءة المضادة للميكروبات حيث أسفرت نتائج دراسة التركيب الكيميائى لعسل السدر المستخلص ن - هكسان بالطريقة أ (دون تخفيف) وبالطريقة ب (المستخلصة بعد تخفيف العسل بالماء) بواسطة كروماتوجرافيا الغاز وطيف الكتلة أن الطريقة أ تظهر كل التربينات بينما الطريقة ب تحتوى على أحماض دهنية بنسبة جوهريه عالية؛ كما أظهرت الدراسة على تواجد بعض المركبات التربينية لأول مرة فى عسل السدر.

كما أسفرت الدراسة عن وجود تباين فى الكفاءة المضادة للميكروبات وذلك تبعاً لنوع الميكروب الممرض حيث أظهرت المواد المستخلصة بالطريقة أ أعلى كفاءة مضادة للبكتريا بينما أسفرت المواد المستخلصة بالطريقة ب أعلى كفاءة مضادة للفطريات.