ANTIMICROBIAL & ANTI-DIABETIC EFFECT OF TRITERPENE BARRINGOTOGENOL C GLYCOSIDES ISOLATED FROM AERIAL PART *OF MEDICAGOSATIVA L.*(ALFALFA) (FAMILY FABACEAE) CULTIVATED IN EGYPT.

BY

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ABSTRACT

Four triterpene barringotogenol C glycosidesnamely; 3-*O*-[α-L-rhamnopyranosyl (1-4)β-D-glucopyranosyl(1-4)-β-D-glucouronopyranoside]-21-*O*-α-L-rhamnopyranoside **barringotogenolC(I)**, 3-*O*-[α-L-rhamnopyranosyl(1-4) β-glucopyranosyl(1-4)β-D-galactopyranosyl]21-O-α-L-rhamnopyranoside**barringotogenolC(II)**,3-*O*-[β-D-glucopyranosyl(1-4) β-D-glucopyranosyl (1-4) β-D glucuronopyranoside] 21-*O*-α-L-rhamnopyranoside**barringotogenolC(III)**,3-*O*-[β-D-glucopyranosyl(1-6)β-D-glucopyranosyl]21-*O*-β-D-glucopyranoside**barringotogenol C(IV)**,Were isolated for the first time from the aerial part of *Medicago sativa L.*All compounds (**I-IV**) were identified by combination of spectroscopic methods (**ID & 2DNMR**) and compared with literature data**.**The extracts (Methanol & Dichloromethane) & isolated pure compounds were evaluated *in vitro* for their antimicrobial activity against *Streptococcus pneumoniae* (**RCMB010010**), *Staphylococcus aureus* (**RCMB010028**) (Gram+vestrain bacteria), *Pseudomonas aeuroginosa* (**RCMB010043**), *Escherichia coli* (**RCMB010052**) (Gram -vestrainbacteria) and Antifungal activity against *Aspergillus fumigatus* (**RCMB02568**) and *Candida albicans* (**RCMB05036**). Furthermore, theywere evaluated for their *in vitro* Anti-diabetic activity using α-Amylase inhibition method.

Introduction

Alfalfa is a perennial flowering forage herb plant in Family Fabaceae and botanically known as *Medicago sativa L.***(Benson1965)** (Alfalfa) *Medicago sativa L*. is a widely cultivated, environmentally tolerant forage crop**.**The Alfalfa plant is beneficial

to both humans & animals**.**The whole plant material contains many active constituents including steroidal saponins, sterols,coumarins, flavonoids, phenolic compounds isoflavones,coumesterolanalogues **(Duke *et al*;1985).***Medicago sativa L.* has received considerable attention due to itstheraputic potential as anti-inflammatory, reducing elevated cholesterol levels in blood, anti-oxidant activities, antimicrobial activities, anti-diabetic, anticancer **(Rathee*et al*;2009)**

Aim of the study

In this study, we describe the isolation of four compounds (**I-IV**) and their subsequent structural determination by spectroscopic analysis, in addition to the investigation of the Antimicrobial activity of barringotogenol C glycosides from (alfalfa) *Medicagosativa L.* against Gram+ve, -ve&fungi, also investigate the *in vitro* Anti-diabetic activity by α-amylase inhibition.

Materialsand Methods

General experimental procedures: The^{1}H and ^{13}CNMR measurements were obtained with Bruker Spectrometer operating at 850MHz (for^{1}H) and 213MHz (for ^{13}C) in DMSO-d6 solution and the chemical shifts were expressed in δ (ppm) with reference toTMS and coupling constant (*J*) in Hertz.Mass spectrometry (MS):AESI-MsDouble focusing sector field Finnigan MAT90 with ESI-II ion source for ESI-MS (Finnigan,Bremen and Germany) (The analysis was done in ICAS-institute for spectrochemistry, Dortmund and Germany) and HRESI-MS analysis was run on a LTQ-FT-MS spectrometer (ThermoElectron, Germany).Polyamide 6S (Riedel De HaenAG, Hannover) and Silica gel G(Merckgrade 60, mesh Merck) were used for open column chromatography.TLC was carried out on percoated silica gel 60 F254 (Merck) plates. Developed chromatogram was visualized by spraying with10-15% Ethanol/H2SO4 at 120°C).**Plant material**;Fresh (aerial part) of *Medicago sativa L.* (alfalfa) were collected from ISMAELIA governorate (November 2014). The identity of the plant was established by Prof. Dr Said Ahmed Mohamed Omar, Head Researcher of Plant Pathology Research Institute.The plant material after being dried then grounded &kept in tight closed container.

Extraction

The dry ground powder aerial part of *Medicago sativa L.* (alfalfa) (4Kg) was extracted with70% alcohol under reflux for several times. The total extract was dried under reduced pressure to give (650gm) and defatted with **dichloromethane** under reflux(175gm).The residue after defatting was suspended in least of water and absolute ethanol was added to precipitate inorganic salts and sugars then filter,the filtrate was evaporated under reduced pressure. The residue was applied on polyamide column eluted with H2O and decrease polarity by adding Methanol until 100% Methanol to obtain 7 collective fractions;Fraction2-7were successively separated on series of column silica gel G-60 by using Petroleum ether: ethyl acetate system to the offered four

compounds.Compound (**I**) 50mg, compound (**II**) 15mg, compound (**III**)20mg, compound (**IV**) 20mg.

Acid Hydrolysis(I-IV)

Each compound (3-4 mg) was treated with 2N Hcl in 50% aqueous methanol for 2hr at 100°c.After evaporation of methanol cold aqueous hydrolysate was exhaustively extracted with ethyl acetate in separating funnel.Aglycones were identified in the ethyl acetate fraction by Co-PC alongside authentic aglycones or using different spectroscopic analysis. The aqueous phase was neutralized with 5% sodium bicarbonate and used for investigation of the sugar moieties on Co-PC against authentic samples using aniline hydrogen phthalate as a spray reagant.Which indicate the sugars to be rhamnose, glucuronic acid and glucose for compound **(I)**, rhamnose, glucose and galactose for compound (**II**),glucose, rhamnose and glucuronic acid for compound (**III**) and glucose for compound (**IV**).

Results and Discussion

Table (1): 1D ^{1}HNMR, DMSO-d_6-850,213MHZ,DEPT-135,213MHZ and 2D(HMQC, COSY,HMBC)NMR data of compound (I)

C no	^{1}HNMR[a]	^{13}CNMR/HMQC	DEPT[b]	COSY	HMBC[c]	
					$^{2}J_{CH}$	$^{3}J_{CH}$
1	1.09/1.18 (m)	41.2 (t)	CH2	H-1b,H_2-2 H-1a,H_2-2	C2,C10	C-25
2	1.31/1.45 (m)	25.5(t)	CH2	H-2b,H_2-1,H-3 H-2b,H3	C3,C1	C4, C10
3	3.58 (m)	90.1 (d)	CH	H_2-2,H-1a	C2,C4	-
4	-	36.9(s)	-	-	-	-
5	0.79 (d, *J* = 9.35Hz)	55.1 (d)	CH	H-6a	C-6	C-9,C-3
6	1.45/1.52 (m)	17.8 (t)	CH2	H-6b,H2-7,H5 H-6a,H5	C-7,C-5	C8,C10
7	1.31/1.67 (m)	32.5 (t)	CH2	H-7a,H_2-6 H-7b,H-6b	C-6,C-8	-
8	-	41.6 (s)	-	-	-	-
9	1.66 (m)	46.8 (d)	CH	H_2-11	C-8, C-10,C-11	C-12,C-25, C-26
10	-	35.8 (s)	-	-	-	-
11	2.00(m)	23.2 (t)	CH2	H-11b,H-9,H_{12} H-11a,H-9,H_{12}	C-9,C-12	C-13,C-8
12	5.17 (brs)	121.6 (d)	CH	H_2-11	C11, C13	C18,C19, C9,C14
13	-	144.1 (s)	-	-	-	-
14	-	42.8(s)	-	-	-	-
15	1.45 (m)	38.0 (t)	CH2	H-15a,b,H_2-16	C-14,C-16	-
16	5.06 (OH)	68.0 (d)	CH	H-16a,bH_2-15	C-15,C-17	C-18
17	-	43.0 (s)	-	-	-	-
18	3.08 (t)	44.5 (d)	CH	H_2-19	C-17	C-14
19	2.98 (d, *J* =8.5)	46.0 (t)	CH2	H-19a,b,H-18	C-18,C-20	-
20	-	35.8 (s)	-	-	-	-
21	3.85(d,*J* = 9.35Hz)	90.5 (d)	CH	H-22	C-22	C-19

22	5.15 (OH)	74.0 (d)	CH	H-21	C-21	C-16
23	1.29 (s)	22.3 (q)	CH3	-	C-4	C-3
24	0.75 (s)	15.3 (q)	CH3	-	C-4	C-3,C-5
25	0.88 (s)	14.2 (q)	CH3	-	C-10	-
26	0.85 (s)	15.4 (q)	CH3	-	C-8	C-7,C-10
27	1.79 (s)	22.1 (q)	CH3	-	C-14	C-13
28	3.21 (m)	59.8 (t)	CH2	-	C-17	-
29	1.06 (s)	28.3 (q)	CH3	-	C-20	C-21-Me-30
30	0.97 (s)	16.6 (q)	CH3	-	C-20	C21-Me-29
Glucuronic Acid						
1	4.76 (d,*J* =6.8Hz)	99.9 (d)	CH	-	C1-**Glu A**	C-3
2	-	72.3	CH	-	-	-
3	-	74.6 (d)	CH	-	-	-
4	-	77.4 (d)	CH	-	-	-
5	-	75.7	CH	-	-	-
6	-	170.4 (s)	-	-	-	-
Glucose						
1	4.80(d , *J* =6Hz)	100.1 (d)	CH	-	C1 (**glu**)	C-4(**Glu- A**)
2	-	72.4 (d)	CH	-	-	-
3	-	74.5 (d)	CH	-	-	-
4	-	76.6 (d)	CH	-	-	-
5	-	76.4(d)	CH	-	-	-
6		60.1 (q)	CH2	-	-	-
Rhamnose-I						
1	5.00 (brs)	100.3 (d)	CH	-	C-1 (**Rham-II*)***	C-21
2	-	70.5 (d)	CH	-	-	-
3	-	70.6(d)	CH	-	-	-
4	-	74.0 (d)	CH	-	-	-
5	-	69.3 (d)	CH	-	-	-
6	1.17 (d,*J* =6.8Hz)	17.9 (q)	CH3	-	-	-
Rhamnose-II						
1	4.94 (brs)	99.9 (d)	CH	-	C1 (**Rham-I)**	C4 (**glu**)
2	-	70.5 (d)	CH	-	-	-
3	-	70.6 (d)	CH	-	-	-
4	-	74.0 (d)	CH	-	-	-
5		68.7 (d)	-			
6	1.18 (d,*J* =7.6Hz)	17.9 (q)	CH3	-	-	-

(a) Assignments were based on **^{1}H-^{1}HCOSY,HMQC** experiments, coupling constants in Hertz are given in parentheses;(s): singlet (d): doublet m: multiplet . **(b)DEPT,** C-Multiplicities were established by DEPT experiments, s = c, d = CH, t = CH2,q = CH3.**(c)**The Correlations in **HMBC** have been shownfrom protons to carbo

Table(2): ^{1}HNMR&^{13}CNMR (DMSO-d6 -850,213MHZ) data of compoundsII,III& IV

C no	II		III		IV	
	^{1}HNMR	^{13}CNMR	^{1}HNMR	^{13}CNMR	^{1}HNMR	^{13}CNMR
1	-	39.9	-	41.6	-	40.1
2	-	25.6	-	25.9	-	25.9
3	3.58 (m)	90.1	3.15 (m)	90.5	3.17dd (11.9,3.4)	90.5
4	-	41.2	—	40.3	-	40.2
5	0.79 d (9.4)	55.3	0.79 d (9.3)	55.7	-	55.7
6	1.4/1.5 (m)	unresolved	-	18.3	-	18.3
7	-	32.6	-	33.0	-	33.0
8	-	41.6	-	42.1	-	41.6
9	-	46.8	-	46.5	-	47.3
10	-	38.1	-	37.4	-	38.5
11	-	25.0	-	25.4	-	25.4
12	5.17 (brs)	121.6	5.17 brs	122.0	5.17brs	122.0
13	-	144.1	-	144.5	-	144.5
14	-	43.0	-	43.5	-	43.2
15	-	35.8	1.66 d (8.5)	36.2	-	36.2
16	-	68.0	4.49 brs	69.1	4.47 brs	69.1
17	-	46.8	-	47.2	-	47.2
18	-	42.8	-	43.2	-	42.1
19	-	48.6	-	47.3	-	49.0
20	-	36.9	-	37.4	-	37.4
21	4.02 d (8.5)	90.5	3.91	90.9	3.96 d (9.4)	90.8
22	3.85 d (8.5)	74.0	3.85 d (9.5)	74.5	3.83d (9.4)	74.5
23	1.09 (s)	28.3	1.12 (s)	28.7	1.12 (s)	28.7
24	0.75 (s)	15.3	0.75 (s)	15.9	0.76 (s)	15.9
25	0.88 (s)	15.4	0.89 (s)	14.5	0.89 (s)	15.7
26	0.85 (s)	16.6	0.85 (s)	17.0	0.85 (s)	17.0
27	1.12 (s)	25.5	1.14 (s)	21.2	1.14 (s)	25.9
28	3.2 (m)	68.0	-	68.4	3.45 d (10.2)	68.4
29	1.06 (s)	30.2	1.06 (s)	30.6	1.06 (s)	30.6
30	0.97 (s)	20.3	0.97 (s)	20.5	0.97 (s)	18.3

C no	II		III		IV	
	^{1}HNMR	^{13}CNMR	^{1}HNMR	^{13}CNMR	^{1}HNMR	^{13}CNMR
Rhamnose-I			**Glucuronic Acid**		**Glucose-I**	
1	4.94	100.3	4.7 5d (6.8)	100.3	4.78 d (7.6)	100.3
2	-	70.6	-	72.8	-	72.7
3	-	70.5	-	76.1	-	77.1
4	-	74.6	-	**77.8**	-	71.0
5	-	68.7	-	77.4	-	76.2
6	1.66 d (6.9)	17.9	-	170.8		69.7
Rhamnose-II			**Rhamnose**		**Glucose-II**	
1	4.60	103.8	4.59	100.8	4.81 d (7.6)	100.5
2	-	70.6	-	70.9	-	72.7
3	-	70.5	-	71.0	-	76.8
4	-	74.6	-	75.0	-	71.0
5	-	69.3	-	69.1	-	75.5
6	0.94 (d, *J* =6.8Hz)	17.9	1.09 d (5.9)	18.3	-	60.2
Glucose			**Glucose-I**		**Glucose-III**	
1	4.75 (d, *J* =8.4Hz)	99.9	4.76 d (7.6)	100.4	4.76 d (7.6)	100.7
2		72.4	-	72.8	-	72.7
3	-	75.7	-	75.5	-	76.2
4	-	**77.5**	-	77.6	-	71.0
5	-	77.1	-	77.1	-	75.0
6	-	59.9	-	60.2	-	60.7
Galactose			**Glucose-II**			
1	4.81(d,*J* =8.5Hz)	100.1	4.81 d (7.6)	100.6		
2	-	72.3	-	72.7		
3	-	75.1	-	75.0		
4	-	**76.9**	-	71.0		
5	-	76.7	-	76.8		
6	-	60.1	-	60.5		

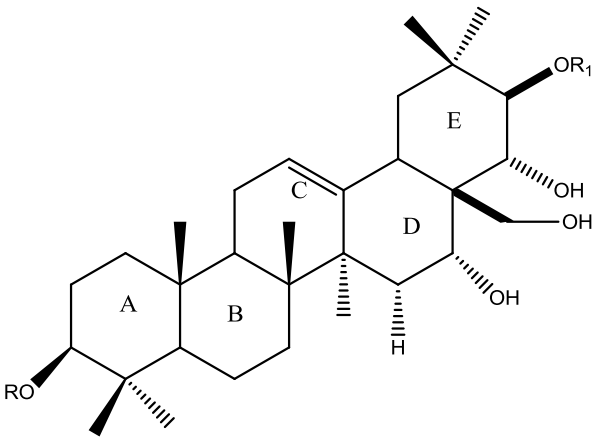
Discussion

Compound I;

Weight is50 mg,On acid hydrolysis Compound (**I**) gave glucose, rhamnose and glucuronic acid in the aqueous phase (Co-TLC& authentic sugar samples), the EI positive mass showed the fragmentation pattern of aglycone (sapogenin) ; which displayed prominent peaks at 234 (base peak), 219,201,187 and 176 due to molecular ion and rupture of ring **C** with other fragment at 161,147,133 and119 due to rupture of ring **B** supporting the proposed structure of the compound, The **^{13}CNMR** and **DEPT** spectra showed 30 carbon atoms attributed for aglycone part as seven methyl's at δC 22.3,15.3,14.2,15.4, 22.1,28.3 and 16.6; Characteristic for C23, 24, 25, 26, 27, 29 and 30 respectively, eight methylene at δ_C 41.2, 25.5, 17.8, 32.5, 23.2, 38.0, 46.0 and 59.8 assigned for C1,2,6,7,11,15,19 and 28 respectively, eight methin at 90.1, 55.1, 46.8, 121.6, 68.0, 44.5, 90.5 & 74.0 assigned for C3, 5, 9, 12, 16 , 18, 21 and 22 respectively. Six quaternary carbons at δ_C36.9,41.6,35.8, 42.8, 43.0 and 35.8 assigned for C4,8,10,14,17&20 respectively and one Olifenic carbon at144.1 assigned for C13. The**^{1}HNMR** spectrum exhibited also, seven singlets, 3H each of seven methyls at δ_H1.29,0.75,0.88,0.85,1.79,1.06 and 0.97 assigned for H-23,24,25,26,27,29 and 30 respectively and also showed a characteristic brs,^{1}H resonance at δ_H 5.17 assigned for H-12 Olifenic protons together with another brs, ^{1}H resonance at 5.06 for OH-16 and at 5.15 for OH-22, from the previousdata expected the sapogenin part as olean-12-ene substituted by OH groups at 3β, 16α, 21β, 22α stereoisomer which is known as **barringotogenol C** and by comparison with previously reported data **(Crublet M.L.*et al*;2002, Takao K.*et al*;1986)** confirm the aglycone part as barringotogenol C.The**^{1}HNMR** spectrum of compound**(I)** displayed four anomeric proton signals at 4.76 (d,*J* =6.8Hz), 4.80 (d,*J*=6Hz),5.00 brs and 4.94 brs and four anomeric carbon signals at δ = 99.9, 100.1,100.3 and 99.9 consequently compound (**I**)was assumed to possess four sugar units which were identified as glucoside, rhamnoside and glucouronic acid by comparison with authentic samples on TLC. The sugar linkages were determined by 2DNMR experiments. In the **HMBC** spectrum, long range correlation was observed between H-1 δ = 5.00 brs of rhamnose-II and C-21 (δ = 90.5) of the aglycone, H-1 (δ = 4.76, d,*J* = 6.8 Hz) of glucuronic acid and C-3(δ = 90.1) of the aglycone, H-1 of glucose (δ = 4.80, d, *J* =6Hz) and C-4 (δ = 77.4)of glucuronic acid, H-1(δ =4.94brs) of terminal rhamnose and C-4 of (δ = 76.6) of glucose. From **NMR** data each sugar is a pyranosyl with β configuration for glucosyl and glucuronic acid and α configuration for rhamnosyl. Therefore, the structure of compound (**I**) was assigned as 3-***O***-[α-L-rhamnopyranosyl (1-4)-β-D-glucopyranosyl (1-4)-β-D-glucouronopyranoside] 21-***O***-α-L-rhamnopyranosidebarringotogenolC.

CompoundsII, III, IV;

The a glycone parts of each of compound **II,III & IV**expected as barringotogenol C in comparison with the previous reported data and data of compound **I** confirmed it but the glycone parts are differ for each one.**On complete acid hydrolysis ofcompound II** gave glucose, galactose, rhamnose in aqueous phase (Co-TLC& authentic sugar samples). Two β-anomeric protons were assigned at 4.75 d (8.4) and 4.81 d (8.5) beside with two anomeric α-type protons at 4.94brs and 4.60brs with Methyl proton at 1.66 d (6.9) & 0.94 d (6.8) together with four anomeric carbon resonance at 103.8, 100.3, 100.1 and 99.9.The most downfield carbon resonance at 103.8 expected for rhamnose moiety at C-21, as well as the downfield shift of C-4 of galactose to 76.9(Δ≈+7ppm) and C-4 of glucose at 77.5 (Δ≈+7ppm), from all the previous data & in comparison with compound (**I**)identification of compound (**II**)according to**(Crublet M.L.*et al*;2002)**as3-***O***-[α-L-rhamnopyranosyl(1-4)-β-glucopyranosyl(1-4)-β-D-galactopyranosyl]21-***O***-α-Lrhamnopyranoside**barringotogenol C**.**On complete acid hydrolysis Compound(III);**gave glucose, rhamnose, glucuronic acid in aqueous phase (CoTLC& authentic sugar samples).Three β anomeric protons were assigned at 4.75d (*J* = 7.6Hz), 4.79 d (*J* = 6.8Hz) and 4.81d (*J* = 7.6Hz) which were expected for a linear triglycosidic residue on C-3 due to the assignment of its ^{13}C-signal downfield at 90.5**(Crublet M.L.*et al*;2002).** The δ values of three anomeric carbons at 100.3, 100.4,100.6 assigned for glucuronic acid,glucoseI,glucoseII respectively, due to the downfield shiftof C-4 of glucuronic acid to77.8 (Δ≈+7ppm) and C-4 of glucose-I to 77.6 (Δ≈+7ppm), additionally the up field of α-anomeric proton were assigned at 4.59brs assigned for rhamnose moiety with 6-methyl group at 1.09d (*J* =5.9Hz)& the most downfield anomeric carbon at 100.8 and methyl rhamnose at 18.3, from the previous data compound (**III**)was defined as3-***O***-[β-D-glucopyranosyl (1-4)-β-D-glucopyranosyl (1-4) - β-D glucuronopyranoside] 21-***O***-α-L-rhamnopyranoside**barringotogenol C.On complete acid hydrolysis compound(IV)**gave glucose in aqueous phase, three β-anomeric protons were assigned at 4.81,4.78 and 4.76 each doublet with *J* value equal 7.6 Hz confirmed with three anomeric carbon resonance at 100.5, 100.3,100.7 characteristic for three glucose moieties, the most upfield proton & downfield carbon assigned for β-glucopyranosyl moiety at C-21 and the other two glucose units attached at C-3 aglycone the interglycosidic linkage of both two glucose units expected (1-6) due to the downfield shift of C-6 of glucose (glucose-II to 69.7 (Δ ≈ +7-8ppm). **(Crublet M.L.*et al*;2002)(Schmid TJ*et al*;2004)**,accordingly compound (**IV**) was defined as3-***O***-[β-D-glucopyranosyl(1-6)-β-D-glucopyranosyl]21-***O***-β-D-glucopyranoside **barringotogenol C**.



Fig(1) Structures of the isolated compound

	R	R_1
I	α-L-rhamnopyranosyl(1-4)-β-D-glucopyranosyl(1-4)-β-D-glucopyranoside	α-L-Rhamnose
II	α-L-rhamnopyranosyl(1-4)-β-glucopyranosyl(1-4)-β-D-galactopyranosyl	α-L-Rhamnose
III	β-D-glucopyranosyl (1-4)-β-D-glucopyranosyl (1-4) -β-D glucuronopyranoside	α-L-Rhamnose
IV	β-D-glucopyranosyl(1-6)-β-D-glucopyranosyl	β -D-Glucose

Biological Studies

A wide range of biological activities have been reported for alfalfa plant due to the structure diversity of its phytochemical constituents. In this genus, the aerial parts, roots, flowers, leaves, stem & sprout of the different *Medicago* species have been reported as potent anti-inflammatory,(it is well used against rheumatoid arthritis, rheumatism and gout) **(Chen L.2015)**, hepatoprotective**(Xie Z. *et al*; 2008& Al-DosariMS[1]. 2012).**Cancer chemoprotective therapy **(Rosenthal G.A. et al;2011)**, Antibacterial activity against bothgram negative & positive bacterial**(Sabrine K. et al;2015)**

Antimicrobial Susceptibility testing

Antimicrobial tested compound preparation.100 ul of tested compounds solution prepared by dissolving 10 mg of the isolated pure chemical compounds, methanol & dichloromethane extracts in 1ml (DMSO used as negative control)

Amphotericin B, Ampicillin and Ciprofloxacin were used as standards for Gram positive &Gram negative and Fungi respectively

Inoculum Preparation

Inoculum was prepared as saline suspension of isolated colonies selected from 24 hours at 37◦c for bacteria, 48 hours at 28◦c for fungi. The suspension is adjusted to match the 0.5 MCFarland turbidity standards using a vortex mixer and using saline as standard.

Cups plate Method

A lawn culture was produced in Müeller-Hinton agar plates. Six cubs were made on the petridishes and 100 ul aliquots of extracts were pipetted on. The plates were left for 1 hour at room temperature and then incubated at 37◦c for 48 hours. Plates were examined for inhibition zones of the growth of bacteria and fungi around the extracts. The averages of those zones were recorded in millimeters. The experiment was carried out in triplicate and average zone of inhibition ± SD were calculated **(SmâniaA.*et al*;1999).**

Minimum inhibitory concentration (MIC)

The broth microdilution method was used to determine MIC. All tests were performed in Mueller Hinton Broth supplemented with Tween 80 at a final concentration of 0.5% (v/v). Briefly, serial doubling dilutions of the compounds were prepared in a 96-well microliter plate ranged from 1000ug/ml to 0.24 ug/ml. To each well, 10 µl of indicator solution and 10 µl of Mueller Hinton Broth were added. Finally, 10 µl of bacterial suspension (10^6 CFU/ml) was added to each well to achieve a concentration of 10^4 CFU/ml. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The plates were prepared in triplicates, and then they were placed in an incubator at 37°C for 18–24 hours. The microorganism growth was indicated by turbidity. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth.

Table(3):

Mean zone of inhibition in mm ± Standard deviation produced on a range of pathogenic microorganisms using (10 mg/ml) concentration of tested samples.

Sample / Tested microorganisms	*Methanol extract*	*chloroform extract*	*I*	*II*	*III*	*IV*	Standard drug
Fungi							**Amphotericin B**
Aspergillus fumigatus	**17.3 ± 0.58**	**12.1 ± 1.2**	**16.9 ± 0.63**	**13.3 ± 1.3**	**16.1± 0.63**	NA	**23.7± 1.2**
Candida albicans	**15.2± 1.2**	**10.3± 0.58**	**19.1± 0.58**	**15.2± 2.1**	**18.3 ± 0.36**	NA	**25.4± 0.58**
Gram Positive Bacteria:							***Ampicillin***
Streptococcus pneumoniae	**19.2 ± 1.6**	**11.3 ± 1.5**	**19.3 ± 1.5**	**17.4 ± 1.5**	**18.9 ± 0.67**	NA	**23.8± 1.2**
Staphylococcus aureus	**16.5 ± 0.63**	**10.6 ± 0.63**	**17.9 ± 1.2**	**14.6± 1.2**	**17.3 ± 0.56**	NA	**27.4± 0.72**
Gram negative Bacteria:							***Ciprofloxacin***
Pseudomonas aeruginosa	**13.3± 0.23**	**9.2 ± 0.58**	**16.8± 1.3**	**15.4 ± 0.53**	**16.2± 0.72**	NA	**20.6± 1.2**
Escherichia coli	**15.3± 1.5**	**12.1± 1.5**	**19.9± 0.72**	**19.3± 0.72**	**19.3± 1.5**	NA	**23.4± 0.63**

Table(4): **Antimicrobial Activity as MICS (µg / ml) of tested samples against tested microorganisms**

Sample / **Tested microorganisms**	***Methanol extract***	***chloroform extract***	***I***	***II***	***III***	***IV***	**Standard drug**
	Minimum inhibitory concentration (µg/ml)						
FUNGI							**Amphotericin B**
Aspergillus fumigatus	**15.63**	**125**	**31.25**	**62.5**	**31.25**	NA	**0.98**
Candida albicans	**62.5**	**250**	**7.81**	**62.5**	**7.81**	NA	**0.49**
Gram Positive Bacteria:							***Ampicillin***
Streptococcus pneumoniae	**3.9**	**250**	**3.9**	**15.63**	**3.9**	NA	**0.98**
Staphylococcus aureus	**31.25**	**250**	**15.63**	**31.25**	**15.63**	NA	**0.49**
Gram negative Bacteria:							***Ciprofloxacin***
Pseudomonas aeruginosa	**62.5**	**250**	**31.25**	**31.25**	**31.25**	NA	**1.95**
Escherichia coli	**31.25**	**125**	**3.9**	**3.9**	**3.9**	NA	**0.49**

Discussion of Antimicrobial Activity

Methanolic extract showed the highest antimicrobial activity while chloroform extract showed moderate activity against test organism as in Table (3). We completed the work of separation and purification bioguided. Hence we have made Antimicrobial test of the four isolated triterpene saponin compounds from the methanolic extract of alfalfa aerial part. The Antimicrobial activities of isolated compounds were evaluated against tested organism, the results showed that compound (**IV**) had no Antimicrobial activity against microorganisms assayed, on the other hand compound (**I**), (**II**) and (**III**) showed an interesting antimicrobial activity for all tested strains as in**Table (3)**.The current study illustrated in **Table (4)** that dichloromethan extract has the maximum inhibition. While compound (**II**), revealed minimum inhibitory concentration against *Candida albicans, Staph aureus and E coli.*

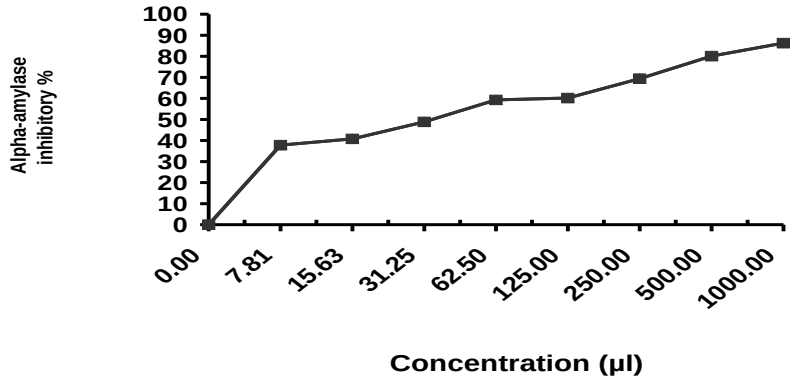
In vitro Anti-Diabetic Assay

α-Amylase inhibition method

A total of 500 µl of test samples and acarbose (1000- 7.81µg/ml) were added to 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution and were incubated at 25°C for 10 min. After these, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of 3, 5 dinitro-salicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. Control represent 100% enzyme activity and were conducted in similar way by replacing extracts with water. All determinations were carried out in triplicate manner and values & expressed as the mean ± SD. The IC50 value was defined as the concentration of sample to inhibit 50% of α- Amylase under the assay conditions**.**

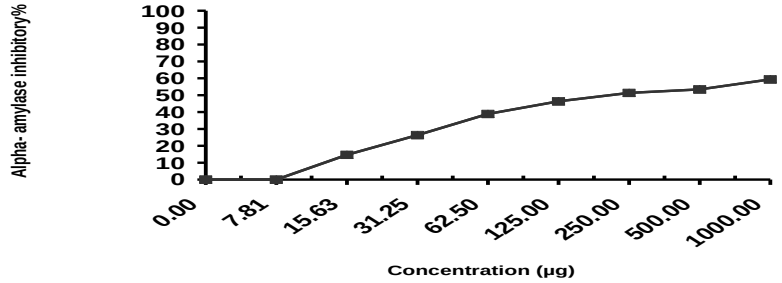
Sample conc. (µg)	Mean of Alpha-amylase inhibitory%	S.D.
1000	86.32	0.63
500	80.14	0.58
250	69.37	1.2
125	60.17	0.63
62.5	59.31	1.5
31.25	48.84	1.2
15.63	40.75	1.5
7.81	37.81	1.2
IC_{50}	34.71	

Fig (2): Anti-diabetic activity of acarbose (standard drug)



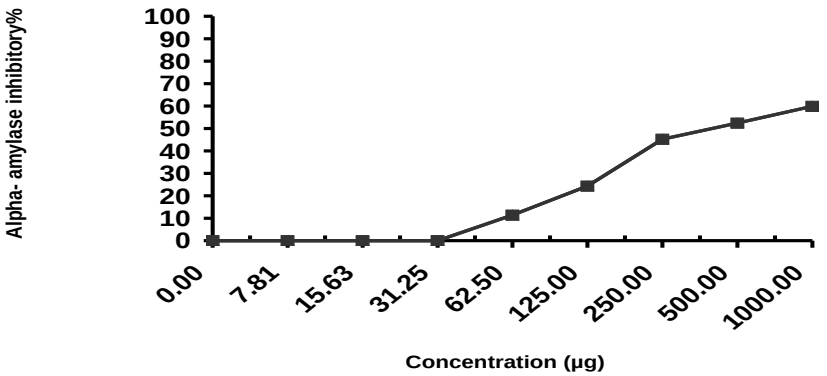
Sample conc. (µg)	Mean of Alpha-amylase inhibitory%	S.D.
1000	59.32	1.2
500	53.42	2.5
250	51.34	1.2
125	46.35	1.2
62.5	38.89	0.63
31.25	26.34	0.58
15.63	14.65	1.5
7.81	0	0
IC_{50}	216.4	

Fig (3): Anti-diabetic activity of Methanolic extract



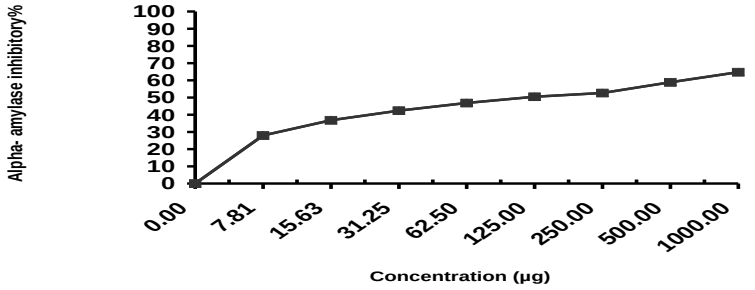
Sample conc. (µg)	Mean of Alpha-amylase inhibitory%	S.D.
1000	59.88	1.5
500	52.43	1.3
250	45.21	0.72
125	24.32	2.1
62.5	11.42	1.2
31.25	0	0
15.63	0	0
7.81	0	0
IC_{50}	415.9	

Fig (4): Anti-diabetic activity of dichloromethane extract



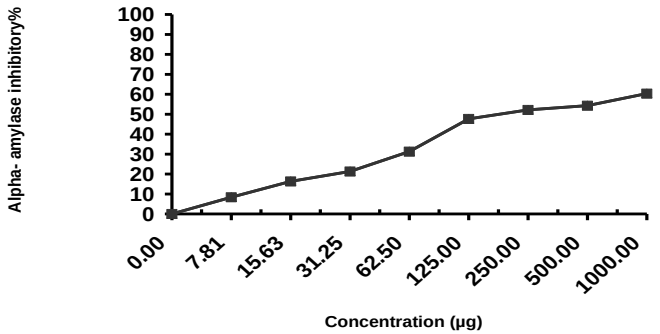
Sample conc. (µg)	Mean of Alpha-amylase inhibitory%	S.D.
1000	64.75	1.2
500	58.89	0.58
250	52.66	0.63
125	50.47	2.1
62.5	46.81	0.58
31.25	42.35	1.3
15.63	36.81	2.5
7.81	27.94	0.72
IC_{50}	116.9	

Fig (5): Anti-diabetic activity of Sample(I)



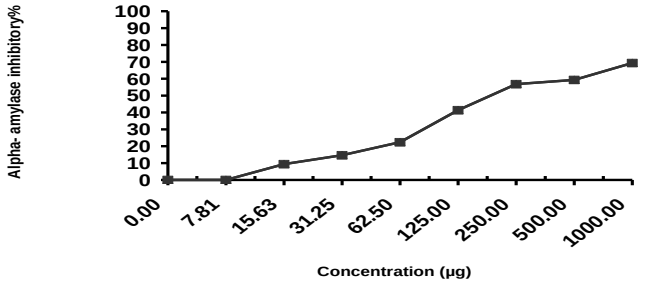
Sample conc. (µg)	Mean of Alpha-amylase inhibitory%	S.D.
1000	60.35	1.5
500	54.32	2.1
250	52.14	0.72
125	47.63	0.63
62.5	31.25	1.2
31.25	21.31	0.72
15.63	16.35	1.2
7.81	8.34	1.5
IC_{50}	190.7	

Fig(6):Anti-diabetic activity of Sample (II)



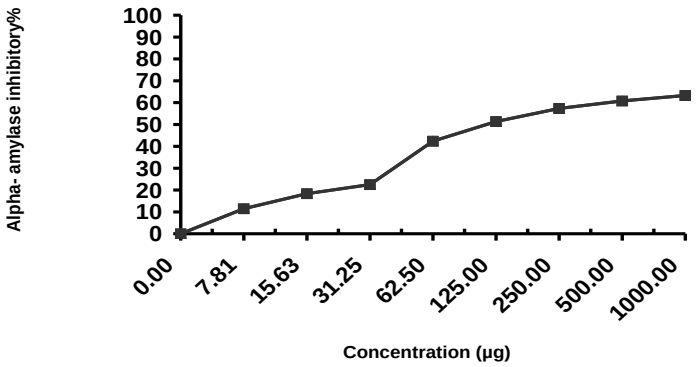
Sample conc. (µg)	Mean of Alpha-amylase inhibitory%	S.D.
1000	69.32	1.4
500	59.31	1.8
250	56.81	1.5
125	41.37	0.72
62.5	22.35	1.3
31.25	14.68	0.72
15.63	9.35	1.2
7.81	0	0
IC_{50}	194.9	

Fig(7):Anti-diabetic activity of Sample (III)



Sample conc. (µg)	Mean of Alpha-amylase inhibitory%	S.D.
1000	63.25	2.1
500	60.75	1.2
250	57.32	0.72
125	51.34	0.58
62.5	42.35	1.2
31.25	22.47	1.5
15.63	18.35	2.1
7.81	11.48	1.2
IC_{50}	115.7	

Fig(8):Anti-diabetic activity of Sample (IV)



Discussion

In accordance with the previous studies and examination, the Anti-Diabetic activity of aerial part extracts of alfalfa was tested against α-amylase enzyme **Fig (2-8)**.In general methanolic extract exhibited promising inhibition percentage in comparison with dichloromethane extract under these screening conditions. The current study illustrated that, the most promising Anti-Diabetic effect was compound (**IV**), which had the least IC_{50} .

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تأثير البرينجوتوجينول –س جليكوزيدز المفصوله من الجزء الهوائى لنبات الفصفصه (الفا الفا) (العائله البقوليه) المزروعة في مصر كمضادات للميكروبات بانواعها وكمضادات للسكري

للسادة الدكاترة

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ملخص البحث

أربعة مركبات برينجوتوجينول- س في صوره جليكوزيدز:

3-*O*-[α-L-rhamnopyranosyl (1-4) β-D- glucopyranosyl (1-4) β-D-glucouronopyranoside] 21-*O*-α-L-rhamnopyranoside **barringotogenol C (I)**, 3-*O*-[α-L-rhamnopyranosyl (1-4) β-glucopyranosyl (1-4) β-D-galactopyranosyl] 21-O-α-L-rhamnopyranoside **barringotogenol C (II)**, 3-*O*-[β-D-glucopyranosyl (1-4) β-D-glucopyranosyl (1-4) β-D glucuronopyranoside] 21-*O*-α-L-rhamnopyranoside **barringtogenol C (III)**, 3-*O*-[β-D-glucopyranosyl (1-6) β-D-glucopyranosyl] 21-*O*-β-D-glucopyranoside **barringtogenol C (IV)**، تم فصلها لأول مرة من الجزء الهوائى لنبات الفصفصه . تم تعريف جميع المركبات الاربعه المفصوله عن طريق الرنين المغناطيسي (1D&2D) ومقارنتها بما سبق نشره من البرينجوتوجينول-س جليكوزيدز. كما تم دراسه تاثير المركبات السابق ذكرها مع مستخلصات الميثانول و الكلورفورم كمضاد للميكروبات ضد *Streptococcus pneumoniae* (RCMB010010)، *Staphylococcus aureus*(RCMB010028) (بكتيريا موجبه الجرام)، *Pseudomonas aeuroginosa* (RCMB010043)، *Escherichia coli*(RCMB010052)بكتيريا سالبه الجرام والنشاط المضاد للفطر ضد Aspergillus fumigatus (RCMB02568)و *Candida albicans* (RCMB05036)،كما تم تقييمهم لنشاط المضاد لمرض السكر في المختبر باستخدام طريقة تثبيط ألفا-أميلاز.