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Cytotoxic, Anti-acetylcholinesterase, Antioxidant and Antimicrobial Activities of Sudanese Propolis with Correlation to its GC/MS and HPLC Analysis

Faten K. Abd El-Hady[1*], Ahmed M. A. Souleman[2], Ibrahim G. Ibrahim[3], Mohamed S. Abdel-Aziz[4], Zeinab A. El-Shahid[5], Eman A. Ali[6] and Mohamed S. A. Elsarrag[7]

[1,5,6]Chemistry of Natural Products Department, National Research Center, Egypt
[2]Department of Phytochemistry and Plant Systematic, National Research Center, Egypt
[3]Department of Crop Protection, Faculty of Agriculture Sinnar University, Sudan
[4]Department of Microbial Chemistry, National Research Center, Egypt
[7]Department of Crop Protection, Faculty of Agriculture Khartoum University, Sudan

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ABSTRACT

Propolis is a complex resinous honeybee product. It is reported to display diverse bioactivities, which depends on the geography, climate and floral sources. The present work aimed "for the first time" to evaluate the chemical composition and its correlation to some biological activities for three Sudanese propolis samples from different localities. Assessment of total phenolic and total flavonoid contents revealed that sample (C) showed the highest total phenolic, while sample (B) showed the highest total flavonoid contents. The highest free radical scavenging activity was obtained from sample (C). Sudanese propolis was investigated for the in-vitro cytotoxic activity against four human cancer cell lines; HCT116, MCF-7, HEPG2 and PC3. Sample (A) had a strong cytotoxic activity against MCF-7 and PC3-cell lines more than that of the drug doxorubicin. Sample (B) showed the highest significant acetylcholinesterase inhibitory activity than that of the drug (Distigmine bromide). Samples (A and B) showed strong antimicrobial activity against Staph. aureus, P. aeruginosa and C. albicans. Three food-related mycotoxin producer molds (A. niger, A. flavus and F. oxysporum) were resistant to all propolis extracts. All the three samples showed no α-glucosidase inhibitory activity. GC/MS analyses revealed the identification of 87 compounds; alkylresorcinols were solely present in Sample (A), penta- and hexahydroxy-flavans, phosphoric acid-2,3-dihydroxypropyl ester in Sample (B) and caffeoylquinic acid esters in Sample (C). Eighteen flavonoids were quantitatively identified by HPLC analysis in all propolis samples. Chrysin-7-methylether and 8-methoxykaempferol were significantly present in (A), while naringenin, and biochanin A in (B). There was minor presence for flavonoids in (C).

Key words: Sudanese Propolis, Antiacetylcholinesterase, Antioxidant, antimicrobial, Cytotoxic Activities, GC/MS and HPLC analysis.

INTRODUCTION

Propolis is the most natural antibiotic man has ever discovered 2000 years ago, with promising no side effects. It fights bacterial strains that have become resistant to synthetic antibiotics [1]. Propolis has been shown to have antimicrobial, antiviral, anti-inflammatory, antioxidant and immune stimulating effects [2-5]. The chemical composition of propolis is quite complicated. The main chemical classes found in propolis appeared to be the principal components responsible for the biological activities, include; flavonoids, aromatic acids, terpenic acids and

phenolic compounds [6]. The composition and biological activities depend on many different factors such as the geographical regions and plant source [7].

Oxidative stress is a set of intracellular or extracellular conditions that lead to the chemical or metabolic generation of reactive oxygen species (ROS), which can cause oxidative damage to essential cellular constituents, such as membrane lipids, mitochondria, proteins and DNA, which may cause cell death [8]. Furthermore, oxidative damage mediated by (ROS) is known to contribute to the aging process and the pathogenesis of cancer [9]. Free radicals, generated as by-products of normal cellular metabolism, have been implicated in the etiology of several diseases such as liver cirrhosis, atherosclerosis, cancer, Alzheimer's disease and diabetes. The compounds that have the ability to scavenge free radicals could play an important role in ameliorating these disease conditions [10]. Cancer is one of the main causes of death worldwide. In the following decades, the number of people with cancer will continue to increase, largely due to lifestyle, nutrition and environmental conditions in developed countries [11].
Alzheimer's disease is a progressive degenerative neurologic disorder resulting in impaired memory and behavior. Most treatment strategies have been based on the cholinergic hypothesis. Cholinergic neurotransmission is specially affected in patients with Alzheimer's disease. One of the most promising approaches for treating this disease is to enhance the acetylcholine level in brain using acetylcholinesterase inhibitors [10, 11].

Diabetes mellitus is a chronic metabolic disease with the highest rates of prevalence and mortality in both developed and developing countries. It has been reported to associate with oxidative damage. Prevention of oxidative damage with natural antioxidants and control of postprandial hyperglycemia, by inhibiting digestive enzymes such as α-glucosidase are two important diabetic prevention strategies [5, 12].

Information about Sudanese Propolis is still limited. So this study aimed "for the first time" to evaluate Chemical composition and some biological activities for Sudanese propolis, through investigating the chemical composition with GC/MS and HPLC analysis and studying the biological potentiality as cytotoxic, antioxidant, antimicrobial as well as the inhibitory activity against acetylcholenesterase and alpha-glucosidase enzymes.

MATERIALS AND METHODS

Collection of propolis samples

Locations and Sampling period

Three propolis samples were collected from different localities [Alrahad (A), Alfao (B) and Basonda (C)] within Gadarif state, Sudan.

Propolis samples were collected during August to April and were kept until processed. The samples were frozen, ground and homogenized prior to beginning extraction.

Sample extraction [13]

Three grams of each Propolis sample was chopped into small pieces and extracted with 50 ml of 70% ethanol at room temperature (twice for 72 h). The ethanolic extract was evaporated under vacuum at 50 °C until dryness. The percentage of extracted matter was as follows: **Alrahad (A)** propolis 0.17 gm/dry weight, **Alfao (B)** propolis 0.24 gm/dry weight and **Basonda (C)** propolis 0.26 gm/dry weight.

Assessment of propolis total Phenolics [14]

The total phenolic content in propolis extract was measured using Folin-Ciocalteu reagent based on procedure described by Singleton *et al.* with some modifications. The experiment was carried out in triplicates. Gallic acid was used for constructing the standard curve (20 to 100 µg/ml; $y = 0.0058x - 0.0025$, $R^2 = 0.9979$) and the total phenolic content concentration in the extract was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of extract.

Assessment of propolis total flavonoid content [15]

Aluminium chloride colorimetric method was used for flavonoids determination. Quercetin was used for constructing the standard curve (20 to 100 µg/ml; $y = 0.007x - 0.012$, $R^2 = 0.999$) and the total flavonoid concentration in the propolis extract was expressed as milligrams of quercetin equivalent per gram of dry weight (mg QE/g) of extract. The experiment was carried out in triplicates.

DPPH Radical Scavenging Activity [16]
DPPH radical scavenging activity of propolis extracts was assessed according to a modified procedure of Matsushige *et al*. The % inhibition of DPPH was calculated as follows:

% Inhibition = [(Aco – At) / Aco] X 100
Where, Aco is absorbance of the control and At is absorbance of the sample.

Evaluation of cytotoxic activity [17]
Human MCF-7 (breast), HEPG2 (liver), PC3 (prostate) and HCT116 (colon) carcinoma cell lines were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.). Tested extracts dissolved in DMSO were added to the wells in triplicates for 72h. 0.5% DMSO was used as negative control, while 2 µM doxorubicin was used as positive control. The cytotoxic activity was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described by Mosmann. Lethal concentration of the sample which causes the death of 50% of cells (LC_{50}) in 48 hrs was calculated.

Evaluation of Acetylcholinesterase (AChE) inhibitory activity [18, 19]
The AChE-inhibitory activity was performed following the method previously described with slight modification. Electric-eel AChE (Sigma) was utilized; the enzymatic hydrolysis of acetylthiocholine was measured at a wavelength of 412 nm (15 min). All the reactions were performed in triplicate in 96-well micro-plate. Distigmine bromide was used as a standard and compared with all extracts. The AChE inhibitory activity was expressed as % inhibition and was calculated as follows:

% Inhibition = [(Aco – At) / Aco] X 100
Where, Aco is absorbance of the control and At is absorbance of the sample.

α-Glucosidase inhibition assay [20]
The α-glucosidase inhibitory activity was assessed by the standard method, with slight modifications. Absorbance readings (A) were recorded at 405 nm by micro-plate reader. All the reactions were performed in triplicate. Acarbose was used as a standard and compared with all extracts. The α-glucosidase inhibitory activity was expressed as % inhibition and was calculated as follows:

% Inhibition = [(Aco – At) / Aco] X 100
Where, Aco is absorbance of the control and At is absorbance of the sample.

Antimicrobial activity [21-23]
Agar plate method has been recognized to estimate the antimicrobial activities of different proplis samples. Two bacterial test microbes; *Staphylococcus aureus* (Gram positive) and *Pseudomonas aeruginosa* (Gram negative); one yeast test microbe *Candida albicans* and three plant pathogenic fungal test microbes, i.e. *Aspergillus niger*, *Aspergillus flavus and Fusarium oxysporum* were selected to evaluate the antimicrobial activities. The bacterial and yeast test microbes were grown on a nutrient agar medium (NA). On the other hand, the fungal test microbes were cultivated on Szapek-Dox agar medium. The culture of each test microbe was diluted by distilled water (sterilized) to 10^7- 10^8 colony forming units (CFU)/ml then 1ml of each was used to inoculate 1L-Erlenmeyer flask containing 250ml of solidified agar media [22]. These media were put onto previously sterilized Petri dishes (10 cm diameter having 25ml of solidified media). Filter paper discs (5 mm Ø, Whatman No.1 filter paper) loaded with 0.2mg of each extract. The discs were placed on the surface agar plates seeded with test microbes and incubated for 24 hrs. at the appropriate temperature of each test organism [23].

HPLC analysis of propolis samples [24]
The dried 70% alcoholic propolis extract, was then dissolved in MeOH. Both the mobile phase and the dissolved materials were filtered by a Millex-HX Nylon syringe filter (0.45 um, 25 mm; Millipore, Bedford, MA). The materials are subjected to chromatographic analysis with High-Performance liquid Chromatography (HPLC), Reverse phase with the following specifications; Shimadzu SCL-10Avp System controller. Dual pump shimadzu liquid chromatography (LC-10Avp), shimadzu degasser (DGU-14A), shimadzu UV-Vis detector (SPD-10Avp) and column: phenomenex RP-18 (UK; 250 x 4.00 mm, 5 micron). Elution was with water/formic acid (19:1 v/v; solvent A) and acetonitrile (solvent B), and the flow rate was 1 ml/min. Gradient elution started with 20% B, reaches 25% B at 25 min and 30% B at 35 min, and then the system became isocratic until 50 min, reaches 50% B at 60 min and

70% B at 67 min, at ambient temperature. The mobile phase solvents are HPLC grade and di-ionized H2O. The compounds were detected with a UV detector and the chromatograms were recorded at 340 and 290 nm for flavones and flavanones, respectively.

GC/MS analysis of propolis samples

Sample preparation for GC/MS analysis [25]

1.5 mg of the dried matter was prepared for chromatography by derivatization for 30 min at 80 °C with 20 µl pyridine + 30 µl N,O, bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and analyzed by GC/MS.

GC/MS analyses

A Finnigan MAT SSQ 7000 mass spectrometer was coupled with a Varian 3400 gas chromatograph. DB-5 column, 30 m x 0.32 mm (internal diameter) , was employed with helium as carrier gas (He pressure, 20 Mpa/cm^2), injector temperature, 310ºC; GC temperature program, 85 - 310ºC at 3 º C/ min (10 min. intial hold).The mass spectra were recorded in electron ionization (EI) mode at 70 eV. The scan repetition rate was 0.5 s over a mass range of 39 - 650 atomic mass units (amu).

Identification of compounds

The identification was accomplished using computer search user-generated reference libraries, incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the bases of its mass spectral fragmentation. Reference compounds were co-chromatographed when possible to confirm GC retention times.

RESULTS AND DISCUSSION

Propolis is a complex resinous honeybee product. It is reported to display diverse bioactivities, such as antimicrobial, anti-inflammatory and anti-tumor properties. The diversity of bioactive compounds depends on the geography and climate, since these factors affect the floral diversity [26].

Assessment of Propolis Total Phenolics

The amount of total Phenolics of 70% ethanol extract of 3 samples of Sudanese propolis from different localities [**Alrahad (A), Alfao (B), Basonda (C)]** within Gadarif state was evaluated. The highest level of phenolic contents recorded in Basonda sample (C) [17.33 mg/g GAE] followed by Alrahad (A) sample [16.6 mg/g GAE] and Alfao (B) sample [14.27mg/g GAE] (Figure 1).

Assessment of Propolis Total Flavonoid Content

The results showed that, the highest level of flavonoid contents was obtained from Alfao sample [4.27mg], followed by Alrahad sample [3.43mg] and Basonda sample [2.75mg/g QE] (Figure 2).

The levels of phenolic content in the 70% ethanolic extract for propolis from various Sudanese areas varied from 17.33±0.06 to 14.27±0.58 mg/g GAE. The variation was related to the flora surrounding the apiary, the geographical features, local climate and seasonal effects. The total flavonoid contents of Sudanese propolis range from 4.27±0.1 to 2.75±0.9 (mg/g QE). Previous studies showed that the flavonoid content varies even in raw propolis samples collected from the same geographical area [27].

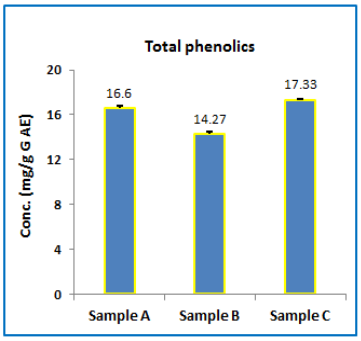

Figure 1:Total Phenolics in Propolis Samples (mg/g GAE)

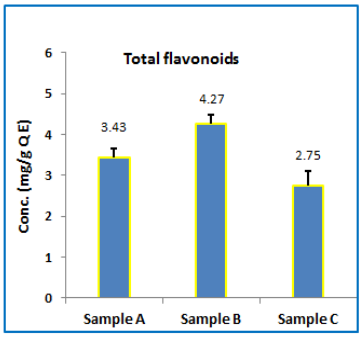

Figure 2:Total Flavonoids in Propolis Samples (mg/g QE)

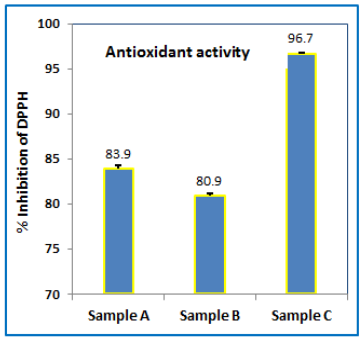

Figure 3: Free radical scavenging activity [DPPH] of propolis samples

GC/MS analyses of propolis samples

GC/MS analyses revealed the identification of **87** compounds**;** 55 compounds for sample (A), 52 compounds for sample (B) and 49 compounds for sample (C). Nitrogenous compounds, aliphatic acids/esters, phenolic compounds, alkylresorcinols, phenolic acids/esters, caffeoylquinic acid esters, sugars, terpenes and hydroxy-flavan compounds were identified (Table 1).

Compounds were solely present in Propolis sample (A): Alanine , proline, 5-oxo-proli- ne, 1H-indole-2,5-dihydroxy, 2-butenedioic acid (Z), ***alkylresorcinols;*** [1,3-dihydroxy-5-pentadecanylben- zene, 1,3-dihydr-oxy-5-hexadecylbenzene, 1,3-dihydroxy-5-heptadece- nylbenzene, 1,3-dihydroxy-5-heptadecanylbenzene, 1,3-dihydroxy-5-octadecenyl benze- ne], *cis*-caffeic acid, tetradecenyl caffeate, docosane-tetra-ene caffeate (*new to propolis*), 3,3-dimethylhexanal, 2-deoxy-erythropentono-1,4-lactone, L-gluconic acid lactone (Table 1).

Compounds were solely present in Propolis sample (B): n-Propionylglycine, di-isopropylthio- phosphin-amide, 2[(trimethylsilyl-methylamino)(methylthio)]methylene-1,3-indandione, 4-methyl-pentyl-pentan-oate, heptadecanoic acid, 9,12-octadecadienoic acid , Phosphoric acid-2,3-dihydr- oxypropyl ester, gluconic acid, Inositol (isomer), 3,5,7,3',4'-pentahydroxy-flavan[catechin /epi- catechin], 3,5,7,3',4',5'-hexahydroxy-flavan [gallocatechin/ epigallocatechin] (Table 1).

Compounds were solely present in Propolis sample (C): N-ethyl-N-vinyl acetamide, glycine, N-acetyl, 11,14-diphenyl-pyridazino [4',5':3,4]pyrrolo-[1,2f]phenanthridine (*new to propolis*), nonanoic acid, diethyl-2-ethyl-2-hydroxy malonate, glucaric acid, phenanth- rene-2,4-bis(1,1dimethylethyl)-5,7dimethyl, [2,2'-dihydroxychalcone], ***Caffeoyl-quinic acid esters;*** **[**4-caffeoyl quinic acid, 3-*cis*-caffeoyl quinic acid, 3-*trans*-caffeoyl quinic acid, 5-*cis*-caffeoyl quinic acid**]**, 2-furan-acetaldehyde-α,3,4,5tetrahydroxy (Table 1).

Compounds identified in the three samples : 2[(methylamino)(methylthio)]methylene-1,3-indandione (isomer), hydroxy-acetic acid, hexadecanoic acid, octadecenoic acid, octadecanoic acid, hexadecanoic acid-3-hydroxy-propyl ester, octadecanoic acid-2,3-hydroxy-propyl ester, 3,4,5-trihydroxy-benzoic acid, caffeic acid, 3-caffeoyl quinic acid (5.4 % in sample **C**), 2,2-dimethyl-3-oxa-5α-cholestane (Table 1).

Compounds identified in the samples (A) and **(B)**: N,N-diethylacetamide, N,N-diethyl (carbam- ate), 5-hydroxy-pipecolic acid, D-lactic acid, 2,3-dihydroxy butanedioic acid, dimethyl-2(1',4',9' ,10'-tetramethoxy-anthracen-2yl) ethylene]butanedioate (*new to propolis*), 2,2-dimethyl-3-oxa-5α-cholestane.

Sugars: the three samples were characterized by a special presence for twenty different types of sugars, from which**;** Erythritol, l-threonic acid, xylitol, d-pinitol, d-glucitol, inositol, glucose, gluconic acid isomer, inositol isomer were identified in the three samples from high to moderate concentrations **(**Table 1**)**.

HPLC analysis of of propolis samples

Eighteen flavonoid compounds were quantitatively identified in propolis samples **(A)**, **(B)** and **(C)**. Sample **(A)** contained significant high concentration of 8-methoxykaem- pferol (57mg/g propolis) and less amount of the

flavone chrysin-7-methylether (16mg/g propolis). The flavanones naringenin (64 mg/g propolis), and biochanin A (27 mg/g propolis), were found in high concentrations in sample **(B)**. Sample **(C)** flavonoids were present in very minor concentrations **(**Table 2**)**.

DPPH radical scavenging activity

The antioxidant activity was evaluated through scavenging the DPPH free radical. Basonda sample (C) had the highest free radical scavenging activity (96.7% Inhibition of DPPH). Alrahad **(A)** and Alfao **(B)** samples showed % Inhibition of 83.9 and 80.9 respectively, (Figure 3).

Table 1: Chemical composition of Sudanese propolis samples assessed by GC/MS analysis

N0	Compounds	RT	propolis (A)	propolis (B)	Propolis (C)
				TIC %*	
	Nitrogenous compounds				
1	n-Propionyl glycine	5.18	------	0.37	------
2	N,N-diethylacetamide	6.39	**0.41**	**0.08**	------
3	N-Ethyl-N-vinyl acetamide,	6.51	------	------	0.2
4	N,N-Diethyl(carbamate)	7.43	**0.48**	**0.08**	------
5	Alanine	9.99	0.19	------	------
6	Glycine, N-acetyl	12.59	------	------	0.73
7	Di-isopropyl-thiophosphinamide	12.28	------	0.1	------
8	1-Ethyl-4,7-dimethyl-5,6,8-trimethoxy-2(1H)quinolinone	14.47	------	------	0.39
9	Proline	20.32	0.52	------	------
10	5-Oxo-Proline	30.53	0.38	------	------
11	2[(Trimethylsilyl-methylamino)(methylthio)]methylene-1,3-indandione ^{t}	31.37	------	0.31	------
12	5-Hydroxy-pipecolic acid	34.05	**0.10**	**0.18**	------
13	1H-Indole-2,5-dihydroxy	44.29	0.1	------	------
14	2[(methylamino)(methylthio)]methylene-1,3-indandione ^{t} (isomer)	45.85	7.27	4.07	**25.5**
15	11,14-Diphenylpyridazino[4',5':3,4]pyrrolo[1,2f]phenanthridine ^{N}	47.46	------	------	0.61
	Aliphatic acids/esters				
16	D-lactic acid	8.35	**0.7**	**0.12**	------
17	Hydroxy-acetic acid,	8.98	0.44	0.05	0.11
18	Propanoic acid-2-hydroxy	9.54	0.12		0.24
19	4-Methylpentyl- pentanoate	10.10	------	0.07	------
20	Propanoic acid-3-hydroxy	12.56	o.11	------	0.07
21	Nonanoic acid	18.89	------	------	0.41
22	2-Butenedioic acid (Z)	20.89	0.08	------	------
23	Butanedioic acid	21.35	0.61	0.14	0.17
24	Propanoic acid-2,3-dihydroxy	22.57	0.42	0.47	0.12
25	2-Butenedioic acid	22.96	------	0.06	0.04
26	Nonanoic acid	23.26	------	------	0.05
27	2-Hydroxy Butanedioic acid,	29.73	2.08	0.78	0.56
28	Diethyl-2-ethyl-2-hydroxy malonate	29.85	------	------	0.16
29	2,3-Dihydroxy-butanedioic acid	36.02	**1.11**	**0.05**	------
30	Hexadecanoic acid	48.85	1.15	1.21	1.1
31	Glucaric acid (2,3,4,5-tetrahydroxy-hexanedioic acid)	49.82	-----	------	0.04
32	Heptadecanoic acis	51.85	------	0.05	0.06
33	9,12-octadecadienoic acid	53.68	------	0.1	------
34	Octadecenoic acid	53.91	0.77	0.42	1.1
35	Octadecanoic acid	54.60	0.33	2.16	1.26
36	Hexadecanoic acid-2,3-hydroxy-propyl ester	64.08	1.41	3.1	1.4
37	Octadecanoic acid-2,3-hydroxy-propyl ester	68.80	0.96	1.82	0.49
	Alkylresorcinols [1,3-dihydroxy-benzenes-5-alkyl]				
38	1,3-dihydroxy-5-pentadecanylbenzene	66.81	0.15	------	------
39	1,3-dihydroxy-5-hexadecylbenzene	70.56	0.09	------	------
40	1,3-dihydroxy-5-heptadecenylbenzene	71.12	0.79	0.12	------
41	1,3-dihydroxy-5-heptadecanylbenzene	71.23	0.1	------	------
42	1,3-dihydroxy-5-octadecenylbenzene	75.37	0.17	------	------
	Phenolic acids/ esters				
43	3,4,5-trihydroxy-benzoic acid ethyl ester	45.92	-----	0.03	0.02
44	3,4,5-trihydroxy-benzoic acid	46.79	0.25	0.29	0.25

45	*cis*-Caffeic acid	47.34	0.06	------	------
46	Ethyl caffeate	50.17	0.32	----	0.34
47	Caffeic acid	51.81	1.17	0.08	0.28
48	Tetradecenyl caffeate	74.33	0.11	------	------
49	Docosane-tetra-ene caffeate [N]	76.72	0.39	------	------
	Caffeoyl quinic acid esters				
50	Quinic acid	43.99	5.74	2.22	8.57
51	4-Caffeoyl quinic acid ,	69.16	------	------	0.23
52	3-*cis*-Caffeoyl quinic acid	73.70	------	------	0.1
53	3-*trans*-Caffeoyl quinic acid	74.29	------	------	0.25
54	5-*cis*-Caffeoyl quinic acid	76.68	------	------	0.26
55	5-*trans* Caffeoyl Quinic acid	76.96	1.2	0.06	**5.35**
	others				
56	3,3-Dimethylhexanal	10.11	0.35	------	------
57	Ethyl phosphate	17.10	0.37	0.16	0.08
58	Phosphoric acid	19.80	**1.85**	**10.32**	3.26
59	Glycerol	20.03	**0.73**	**1.09**	
60	2-Furan-acetaldehyde-α,3,4,5-tetrahydroxy	40.01	------	------	0.09
61	Phosphoric acid, 2,3-dihydroxypropyl ester [N]	40.62	------	0.47	------
62	dimethyl -2(1',4',9',10'-tetramethoxy-anthracen-2yl) ethylene] butanedioate [N]	51.49	**1.42**	**2.9**	-------
	Sugars				
63	2-Deoxy-erythro-pentono-1,4-lactone	30.10	0.10	------	------
64	Erythritol	30.73	0.94	0.55	0.2
65	L-Threonic acid	32.20	0.55	3.21	0.22
66	Erythropentulose	33.61	------	**0.08**	**0.12**
67	Xylitol	39.11	0.88	0.3	0.09
68	Ribonic acid	41.14	------	1.4	------
69	Fructose	42.78	------	10.13	------
70	D-Pinitol	43.17	0.4	1.67	0.28
71	D-Psicose	45.03	------	------	0.77
72	Glucose	45.11	**0.6**	**0.75**	
73	L-Gluconic acid lactone	45.27	0.32	------	------
74	Saccharo-1,4-lactone	45.30	------	------	0.11
75	d-Glucitol	46.14	**16.63**	3.63	0.75
76	Inositol	47.17	0.11	0.67	0.45
77	Gluconic acid	47.47	------	0.13	------
78	Inositol (isomer)	48.04	------	0.29	------
79	glucose	48.33	0.62	------	------
80	Gluconic acid isomer	48.63	1.04	2.17	0.68
81	Inositol (isomer)	50.93	0.26	0.13	0.43
82	Sucrose	65.24	**0.6**	**1.3**	------
	Tetracyclic triterpenes				
83	2,2-Dimethyl-3-oxa-5α-cholestane (isomer)	56.40	0.37	0.18	0.08
84	2,2-Dimethyl-3-oxa-5α-cholestane	61.77	**0.24**	**0.12**	------
	Flavonoid compounds				
85	1,3-Bis[2(345ydroxyl)phenyl]2-propen-1-one [2,2'-dihydroxychalcone]	60.00	------	------	0.15
86	3,5,7,3',4'-pentahydroxy-flavan [catechin /epicatechin]	71.70	------	**0.9**	0.13
87	3,5,7,3',4',5'-Hexahydroxy-flavan [gallocatechin /epigallocatechin]]	72.65	------	0.07	------

RT=retention time., TIC =The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation. t= tentatively identified, N= new to propolis*

Table 2: Flavonoids assessed by HPLC for Sudanese propolis samples (Conc. mg/g propolis)

No.	Name	Chemical name	RT	propolis (A)	propolis (B)	Propolis (C)
Flavones						
1	Luteolin	5,7,3',4'-tetrahydroxyflavone	24.53	1.6	0.004	-----
2	Luteolin-3'-methylether	5,7,4'-trihydroxy-3'-methoxy flavone	42.06	2.4	0.004	0.78
3	Chrysin-7-methylether	5- hydroxy-7-methoxy flavone	61.91	**16**	-----	3.5
4	Acacetin	5,7- dihydroxy-4'-methoxy flavone	65.4	1	0.10	0.413
	Flavonols					
5	Quercetin-3-methylether	5,7,3',4'-tetrahydroxy-3-methoxy flavone	29.33	-----	0.02	-----
6	Quercetin-3,7-dimethylether	5,3',4'-trihydroxy-3,7-dimethoxyflavone	34.6	-----	0.28	-----
7	Quercetin-7-3'-dimethylether	3,5,4'-trihydroxy-7,3'-dimethoxy flavone	66.13	**6.5**	0.30	**1.0**
8	8-Methoxy-kaempferol	3,5,7,4'- tetrahydroxy-8- methoxy-flavone	37.58	**57**	0.24	-----
9	Kaempferol-3-methylether	5,7,4'- trihydroxy-3-methoxy flavone	44.46	0.34	0.02	-----
10	kaempferol	3,5,7,4'-tetrahydroxyflavone	41.32	-----	0.004	-----
11	Quercetin-3,3'- dimethyl ether	5,7,4'-trihydroxy-3,3'-dimethoxyflavone	45.53	-----	0.02	-----
12	Quercetin-7-methylether (Rhamnetin)	3,5,3',4'-tetrahydroxy-7-methoxy flavone	56.88	**1.8**	-----	-----
13	Galangin-7-methylether	3,5-dihydroxy-7-methoxy flavone	72.84	**1.4**	-----	-----
Flavanones						
14	Naringenin	5,7,4'-Trihydroxyflavanone	33.18	-----	**64**	-----
15	Biochanin A	5,7-dihydroxy-4'-methoxy flavanone	65.15	0.06	**27**	0.91
16	6-prenyl-pinocembrin	5,7-dihydroxy-6-pentenyl-flavanone	73.55	0.33	**0.71**	0.54
		Isoflavones		-----	-----	-----
17	Genistein	5,7,4'-trihydroxy isoflavone	35.8	-----	2.3	2.2
18	Formonontin	7-hydroxy-4'- methoxy isoflavones	51.45	-----	-----	3.6

Different phenolic components present in aqueous and methanolic extracts, as for example, flavonoids, may have contributed to these results. It was mentioned that flavonoids and phenolics compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties [11]. The antioxidant activity of propolis can be related to its content of phenolic compounds, as well as its period of collection [28].

Evaluation of cytotoxic activity of propolis samples

Sudanese propolis was investigated for the in-vitro cytotoxic activity against four human cancer cell lines; HCT116 (colon), MCF-7 (breast), HEPG2 (liver) and PC3 (prostate).

In HCT116-cell line: Sample (A) showed high LC_{50} (51.4 µg/ml) compared to the drug (doxorubicin, 37µg/ml), sample (B) showed moderate LC_{50} (46 µg/ml), while sample (C) had no cytotoxic activity (Figure 4).

In MCF-7-cell line: Sample (A) revealed a strong cytotoxic activity, with lower LC_{50} (16.3 µg/ml) than that of the drug (doxorubicin, 26 µg/ml). Samples (B & C) showed moderate LC_{50} of (42.6 and 33.9 µg/ml, respectively) (Figure 4).

In HePG2-cell line: all the samples showed high LC_{50} of (60, 59 & 57µg/ml, respectively) higher than that of the drug (doxorubicin, 21µg/ml) (Figure 4).

In PC3-cell line: Sample (A) showed the highest cytotoxic effect, with lower LC_{50} (11 µg/ml) than that of the drug (doxorubicin, 23 µg/ml). Both samples (B) and (C) had high LC_{50} (57 & 60 µg/ml, respectively) (Figure 4).

Several reports have shown the cytotoxic effects of propolis from different origins in several cancer cell lines. The ethanolic extract of Brazilian propolis showed potent cytotoxicity against prostate [29] and MCF7cancer cell lines [30]. The inhibitory effects against the proliferation of colony potential of HCT116 cell with Iraqi propolis was reported [31]. The further analysis of the three propolis samples with GC/MS and HPLC revealed the presence of several bioactive compounds that were reported to display cytotoxic activities, where**;** quercetin-7-methylether (Rhamnetin) showed a moderate antiproliferative activity against MCF-7 [32]. Kampferol induced apoptosis in MCF-7 cells at a concentration of 50 µM [33]. Caffeic acid was found to have anticancer potential and has also been shown to affect DNA methylation [34].

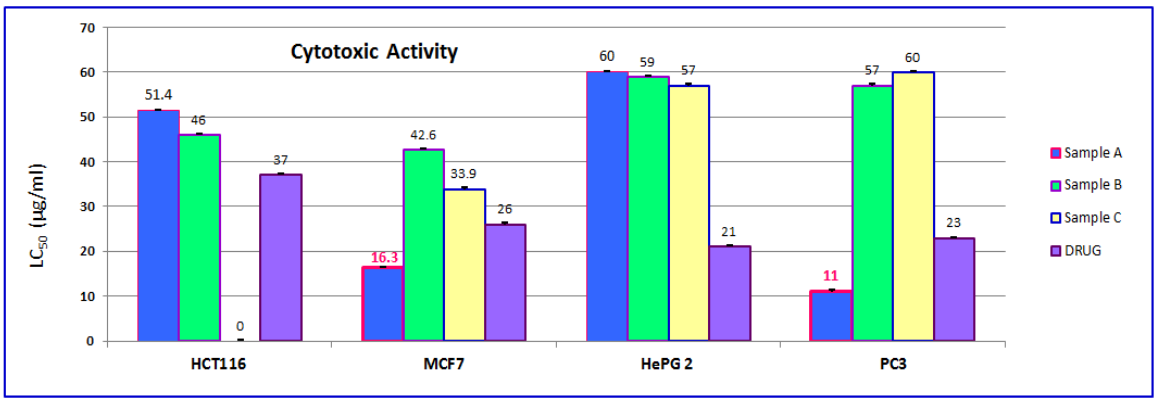

Figure 4: Cytotoxic Activity of propolis samples against Human cancer cell lines; HCT116 (colon), MCF-7 (breast), HEPG2 (liver) and PC3 (prostate) expressed as LC_{50}

Alkylresorcinols isolated from wheat bran exerted high cytotoxic activity; four of them with strong inhibitory properties against the growth of PC3 cells, including 5-heptade- cylresorcinol (IC_{50}=22.5 µg/ml), 5-(16-heneicosenyl) resorcinol (*trans*) (IC_{50}=13.7 µg/ml), 5-(14-nonadecenyl)resorcinol (*trans*) (IC_{50} = 42.2 µg/ml) and 5-(2-oxotrico- sanyl)resorcinol (IC_{50}=10.9µg/ml). This research suggested that ***alkylresorcinols*** are important for the cancer preventive activity of wheat bran [35].

Acetylcholinesterase (AChE) inhibitory activity

The AChE inhibitory activity of three Sudanese propolis samples was studied. Sample (B) showed the highest significant acetylcholinesterase inhibitory activity (91.7%, Figure 5) comparing to that of the drug (Distigmine bromide, 72.4%). Sample (A) showed moderate inhibitory activity (60%), while sample (C) had the lowest activity (25.5%, Figure 5).

Acetylcholinesterase (AChE) inhibitors from natural resources are gaining an interest as new approach to treat the cognitive symptoms of Alzheimer disease (AD). The current study showed that the Sudanese propolis had a role in alleviating AD symptoms through the inhibition of the enzyme AChE. The tested propolis samples from different localities showed a variable inhibitory activity and that is in agreement with previous work, where the inhibitory activity of propolis is different from locality to another and proved that the high activity of propolis is due to its high content of several classes of compounds that is known to possess high activity against the enzyme such as flavonoids, phenolic acids and their esters [11, 36].

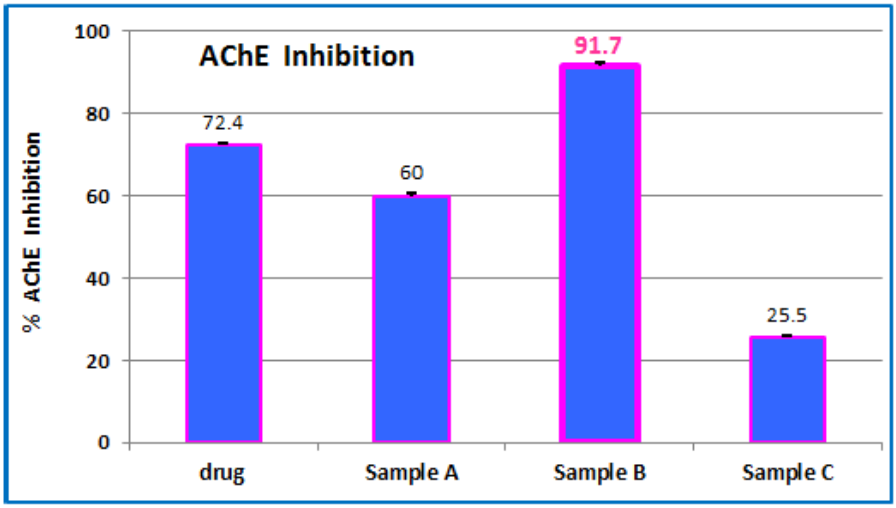

Figure 5: % Inhibition of the acetylcholinesterase activity by propolis samples. Values are expressed as mean ± SD, n =3 (200 µg/ml for all extracts and drug).

The further analysis of propolis samples with GC/MS and HPLC, revealed the presence of several bioactive compounds that are reported to display acetylcholinesterase inhibitory activity. Öztürk reported that, the best (AChE) inhibitory activity was found for 9,12-octadecadienoic acid and 9-octadecenoic acid as 0.267±0.05 mg/mL and 0.127±0.03 mg/mL, respectively while, hexadecanoic and octadecanoic acids are more than 4mg/mL [37]. It was supposed that gallic acid, catechin, and epicatechin have various antiamnesic effects in neurodegenerative diseases such as Alzheimer's disease (AD) [38]. It was reported that, (+)-catechin inhibited (AChE) more effectively than (-)-epicatechin [39]. **Naringenin** inhibited AChE activity in a dose-dependent manner. Naringenin, when administered to mice at 4.5 mg/kg body weight, significantly ameliorated scopolamine-induced amnesia. These results suggest that naringenin may be a useful chemopreventive agent against Alzheimer's disease [40]. Biochanin-A (BCA), a potent phytoconstituent, has been previously used as an antitumour, a dopaminergic neuron protective agent, an antioxidant, and anticholinergic activities [41]. Genistein (65.7%) exerted a moderate inhibition on BChE [42].

α-glucosidase inhibitory activity

All the three Sudanese propolis samples showed no α-glucosidase inhibitory activity if compared to drug (acarbose) (Figure 6).

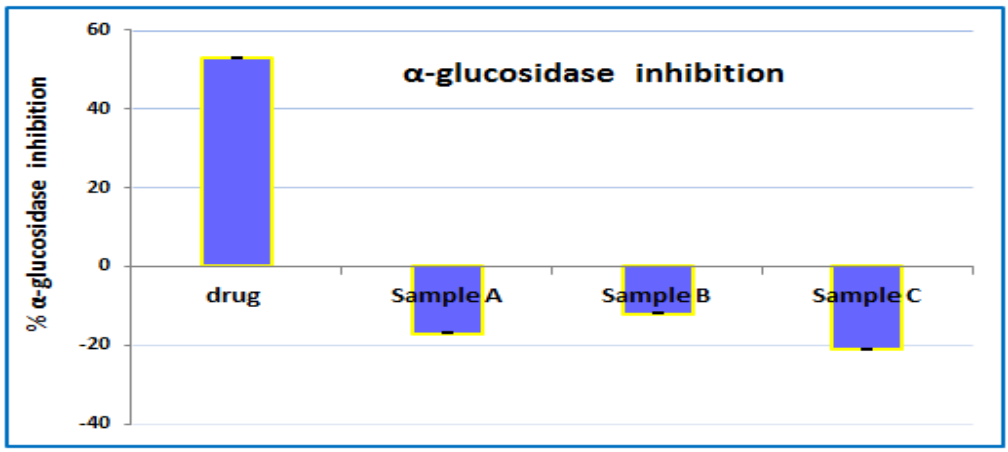

Figure 6: % Inhibition of the α-Glucosidase activity by propolis samples. Values are expressed as mean ± SD, n =3(200 µg/ml for all extracts and drug)

Antimicrobial activity

Antimicrobial activity of different propolis samples on human pathogens was investigated**.** The Sudanese propolis showed antimicrobial activity against Gram positive bacteria *(Staph. aureus*), Gram negative bacteria *(P.aeruginosa)* and yeast- like fungus *(C. albicans*).

In *Staph.aureus*: Samples (A and B) showed strong antibacterial activity with zone inhibition (11.5±0.71, 13±1.4 mm, respectively), while sample (C) showed moderate activity (7.5±0.707 mm) (Figure 7).

In *P. aeruginosa*: Samples (A and B) had strong antibacterial activity with zone inhibition (14±1.4, 15.5±0.71mm, respectively), while sample (C) showed moderate activity (7.5±0.71mm) (Figure 7).

In *C. albicans*: Samples (A and B) showed a strong antifungal activity with zone inhibition (13.5±0.71mm, for both of them), while sample (C) showed a moderate activity (8.5±0.71 mm) (Figure 7).

The three food-related mycotoxin producer molds, (*A. niger*, *A. flavus* and *F. oxysporum*) were resistant to all propolis extracts (Figure 7).

The results obtained are in agreement with previous studies; where it was reported that the growth of the *S.aureus*, an oral pathogen, was inhibited by the 70% ethanol extract of propolis from various regions in Egypt [43, 44].

Kosalec *et al.* reported a strong antimicrobial activity of propolis products against *C. albicans* and *S. aureus* with similar inhibition zones [45].

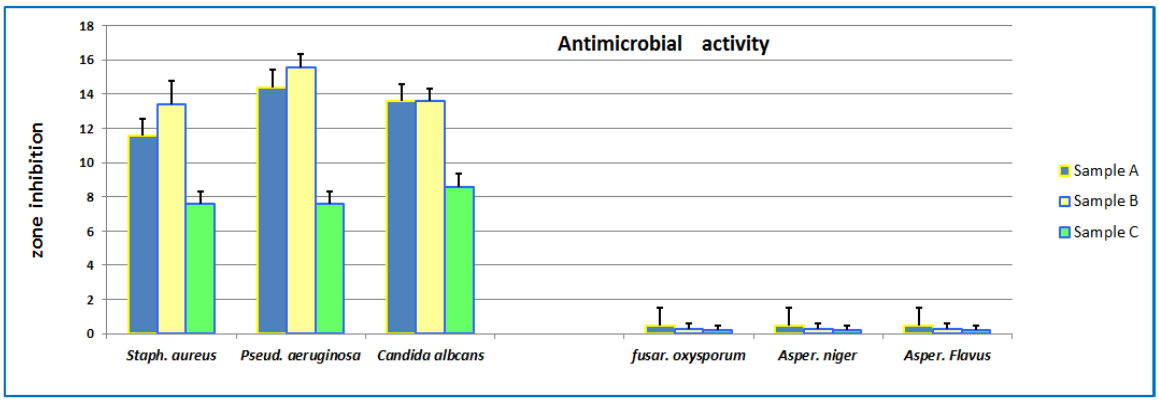

Figure 7: Antimicrobial Activity for Sudanese propolis samples. Values are expressed as mean of zone inhibition ± SD, n = 3 (200 µg/disc for all tested extracts).

CONCLUSION

That is the first time to evaluate Chemical composition and biological activities for **Sudanese propolis**. Sample (A) revealed a strong cytotoxic activity against MCF-7 and PC3-cell lines more than that of the drug doxorubicin. Sample (B) showed the highest significant acetylcholinesterase inhibitory activity (91.7%) than that of the drug (Distigmine bromide). Samples (A and B) showed strong antimicrobial activity against *Staph. aureus, P. aeruginosa and C. albicans.* Our study provides (for the first time) primary evidence suggesting that Sudanese propolis in further in-vivo studies could play an important role as acetylcholinesterase inhibitor, cytotoxic, antimicrobial and antioxidant activities.

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