Research Article

Egyptian Propolis Bioassay Guided Fractionation and GC/MS, HPLC Analysis of Highly Anti-acetylcholinesterase Sub-fractions

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ABSTRACT

Among the possible strategies for enhancing brain cholinergic activity, acetylcholinesterase inhibitors have been the most extensively used for the symptomatic treatment of Alzheimer's disease. The aim of this study was to investigate the potential anticholinesterase activity of propolis extract and its sub-fractions. Propolis 70% alcoholic extract, ether and ethyl acetate fractions showed acetylcholinesterase inhibitory activity (62.7, 63.7 and 77.7% respectively), ether sub-fractions (A) and (B) (55.7 and 52.7%) activity. Ethyl acetate sub-fractions 3 and 4 (C) showed very high significant acetylcholinesterase inhibitory activity (75.7 and 91%). HPLC analysis revealed; quercetin-7-methylether (191 µg/mg sub-fraction), galangin (76 µg), pinobanksin-3-acetate (53 µg), chrysin (39 µg) and naringenin (31µg) were significantly present in Sub-fraction (A). Dimethylallyl caffeate (78 µg) and phenylethyl caffeate (36 µg) were highly found in (B). Chrysin-7-methylether (63µg) and quercetin-3,7-dimethylether (53 µg) were with high concentrations in (C). GC/MS analyses: (A) was characterized by; flavonoids (38 %) - it is the only fraction showed the presence and very high percentage of myrecetin, pinobanksin and chrysin - , terpenes (12.7%), Phenolic acids (6.9%). (B) was characterized by the presence of phenolic acids esters (19.3%), twelve of them (18%) are -*cis*- and -*trans*- of caffeic acid esters. 3-methyl-2-butenyl-*cis*-caffeate, 3-methyl-3-butenyl-*trans*-caffeate and 3-methyl-2-butenyl-*trans*-caffeate represented (2.86, 3.13, 4.77%, respectively). 1-O-Octadecylglycerol (6.4%) is present only in (B). (C) was characterized by the presence of aliphatic acids (14.55%) and di-isooctyl phthalate (15.2%) a very major ester. It could be concluded that, Egyptian propolis bioassay guided fractionation on acetylcholinesterase enzyme revealed that; some sub-fractions are highly active inhibitors and some are inactive.

Keywords: Propolis, Bioassay Guided Fractionation, Acetylcholinesterase inhibitors, Chemical Composition, GC/MS and HPLC analysis.

INTRODUCTION

Acetylcholinesterase (AChE) is an acetylcholine hydrolase enzyme with esterase activity, localized in the synaptic gaps of the central and peripheral nervous system. It terminates nervous impulses by catalysing the hydrolysis of the neurotransmitter acetylcholine, a neurotransmitter of cholinergic system, which amongst other roles is involved in memory formation.[1]

Acetylcholinesterase inhibitors (AChEI) are used clinically to counteract various pathologies, including Alzheimer's disease[2] by enhancing cholinergic functions and increasing the amount of acetylcholine present in cholinergic synapses. To be effective inhibitors must reversibly bind to the active site of the enzyme as irreversible binding may lead to severe consequences, including death.

Synthetic AChE inhibitors such as physostigmine, tacrine and donepezil have been reported to have adverse effects, such as hepatotoxicity and gastrointestinal complaints.

Numerous natural products have been investigated for their potential to treat cognitive disorders and neurodegenerative diseases. It is known that honeybee propolis has been applied for centuries in traditional medicine as well as in diets and supplementary nutrition. Propolis and its polyphenolic/flavonoid compounds have been known to exhibit diverse biological activities.[3]

The main bioactive chemical compounds in propolis are reported to be phenolics acid, their esters, terpenes and flavonoids.[4-6] However, the chemical composition of propolis is qualitatively and quantitatively variable, depending on the available floral diversity, the bee species and the season of collection.[7,8] Because the diverse array and types of chemical components in propolis vary in size and polarity, the solvents used to extract the propolis play a key role in the bioactivities that are obtained in the crude extracts or subsequent fractions, due to the differential fractionation of components between different extracting solvents.[9]

The aim of this study was to evaluate Egyptian propolis bioassay guided fractionation on the inhibitory activity to acetylcholinesterase, with comparative correlation to chemical composition of these sub-fractions with GC/MS and HPLC analysis.

MATERIALS AND METHODS

Propolis extraction

Propolis (20 g) was cut into small pieces and extracted with distilled water (300 ml x 3) each for 2 hours at 85 °C

to give propolis water extract, the residue was extracted with 70% ethanol (300 ml x 3) under reflux conditions each for 2 h which gave (PEE 70%), this extract (5.5 g) was filtered and dried under reduced pressure, the dried residue was suspended in water and then partitioned successively in turn with ether and ethyl acetate.

Bioassay guided fractionation and characterization of the fractions

Ether fraction (2.7g) was subjected to Sephadex LH-20 column chromatography (10 x 1 cm) and stepwise gradient elution was carried out using a solvent system of decreasing polarity starting with 100% distilled water then water–methanol. Fractions of 10 ml were collected and investigated by TLC (silica gel DF_{245} Merck) using different spraying reagents, similar fractions were combined and concentrated to dryness under reduced pressure to obtain one main fraction (1.7g) it was fractionated again into many sub-fractions on column packed with silica gel (0.06-0.2mesh, Merck), stepwise elution with petroleum ether, (pet.ether –ethylacetate) was carried out.

The ethyl acetate fraction (1.1 g) also was further subjected to column chromatography packed with silica gel (0.06-0.2mesh, Merck), elution was carried out with pet.ether, pet.ether –ethylacetate (9:0.5, 9:1, and 8:2), resulted in total four main sub-fractions.

Acetylcholinesterase (AChE) inhibitory activity

The AChE-inhibitory activity was performed followed the method previously described[10] with slight modification.[11] 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.

HPLC analysis of propolis Fractions

After extraction of the materials, the sub-fractions were 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.[12]

GC/MS analysis of propolis Fractions

Sample preparation for GC/MS analysis

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.[13]

GC/MS analyses

A Finnigan MAT SSQ 7000 mass spectrometer was coupled with a Varian 3400 gas chromatograph. DB-1 column, 30 m x 0.32 mm (internal diameter), was employed with helium as carrier gas (He pressure, 20 Mpa/cm^2), injector temperature, 310°C; GC temperature program, 85 - 310°C at 3 °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

Bioassay guided fractionation and characterization of the fractions

Ether fraction (2.7g) was subjected to Sephadex LH-20 and silica gel columns chromatography; resulted in total nine main sub-fractions. The ethyl acetate fraction (1.1 g) was subjected to silica gel column chromatography; resulted in total four main sub-fractions.

Acetylcholinesterase (AChE) inhibitory activity

The main purpose of this study was to investigate the Potential anticholinesterase effect of propolis extract and its sub-fractions. PEE 70% extract, ether, ethyl acetate fractions and their sub-fractions were investigated for their AChE inhibitory activity.

In the present study, PEE 70% extract, ether and ethyl acetate fractions caused significant increase in acetylcholinesterase inhibitory activity (62.7, 63.7 and 77.7% respectively, Figure 1).

In the ether nine sub-fractions; sub-fractions 1, 6, 7 showed no inhibitory activity. The sub-fractions 3, 5 and 9 had very low activity (29.3, 14.7, and 34.3 % respectively, Figure 1a). While, sub-fractions 4 and 8 showed high acetylcholinesterase inhibitory activity (55.7 and 52.7%

respectively) (Figure 1a), with IC_{50} (357± 0.003 and 377± 0.011 µg/ml, respectively).

The ethyl acetate sub-fractions 1 and 2 had no inhibitory activity, although the sub-fractions 3 and 4 showed very high significant acetylcholinesterase inhibitory activity (75.7 and 91% respectively, Figure 1b). It is very clear that the ethyl acetate sub-fraction 4 is the only one showed very high significant acetylcholinesterase inhibitory activity and with IC_{50} (250 ± 0.01µg/ml).

HPLC analysis of propolis

The three sub-fractions showed very high significant acetylcholinesterase inhibitory activity were selected for further HPLC and GC/MS analysis [ether sub-fractions; sub-fr. 4 = (A), sub-fr. 8 = (B) and ethyl acetate sub-fr. 4 = (C)].

Twenty one flavonoid compounds and two caffeic acid esters were quantitatively identified in propolis sub-fractions A,B and C. The flavonols quercetin-7-methylether (191.8 µg/mg sub-fraction), galangin (76.7 µg), the flavanone pinobanksin-3-acetate (53.53 µg), the flavone chrysin (39.0 µg) and the flavanones naringenin (31.7 µg) were significantly present in high concentrations in sub-fraction A. The caffeic acid esters dimethylallyl caffeate (78.1 µg) and Phenylethyl caffeate (36.6 µg), the flavone chrysin-7-methylether (15.4 µg/mg sub-fraction), were found in a high and moderate concentrations in (B). The flavone chrysin-7-methylether (63.4µg) and the flavanol quercetin-3,7-dimethylether (52.97 µg/mg sub-fraction), were also significantly present in high concentrations in (C). The other flavonoids were found in very minor concentrations in both samples **(**Table 1, Figure 2**)**.

GC/MS analyses

The GC/MS analyses of the [ether sub-fractions; sub-fr. 4 = (A), sub-fr. 8 = (B) and ethyl acetate sub-fr. 4 = (C)] which were highly active as acetylcholenesterase inhibitors, revealed the following:

Propolis sub-fraction A was characterized by the presence of**:**

Flavonoids (37.9%); is the only fraction showed the presence and very high percentage of myrecetin, pinobanksin and chrysin (**12.4, 10.5 and 9.1%** respectively), while galangin and pinobanksin acetate showed moderate presence (2.9 and 2.4 %), (Table 2, Figure 3).

Terpenes (12.7%): The only fraction contained three terpenes; Bicyclo[4.2.1]nona-2,4,7-triene,7-hydroxy and 3-Hydroxy-4[1'-(hydroxy cyclopentyl)- ethynyl]furan represent 12.5%.

Phenolic acids (6.9 %): it also showed the presence of phenolic acids, includes; cinnamic acid, p-methoxy-*cis*-cinnamic acid, p-methoxy-*trans*-cinnamic acid and m-methoxy cinnamic acid (0.6, 2, 4 and 0.3 % respectively); these cinnamic acids are present only in this fraction (Table 2).

Propolis sub-fraction B was characterized by the presence of**:**

Phenolic acids esters (19.3%): are represented by fifteen esters; where twelve of them (18%) are -*cis*- and -*trans*- of caffeic acid esters; ethyl ester, 3-methyl-3-butenyl caffeate, 2-methyl-2-butenyl caffeate, 3-methyl-2-butenyl caffeate, benzyl caffeate, phenylethyl-*trans*- caffeate, methyl caffeate and butyl caffeate. 3-methyl-2-butenyl-*cis*-caffeate, 3-methyl-3-butenyl-*trans*-caffeate and 3-methyl-2-butenyl-*trans*-caffeate represented (**2.86, 3.13, 4.77%, respectively**), while *trans*-caffeic acid ethyl ester, 2-methyl-2-butenyl-*trans*-caffeate and Benzyl-*trans*-caffeate (1.5, 1.04, 1.69 **%, respectively**) (Table 2, Figure 4). The others are in minor presence; less than 1% (Table 2). Our results are in agreement with previous studies.[14]

1-O-Octadecylglycerol (6.4%) is present only in fraction B (Table 2, Figure 4).

Propolis fraction C was characterized by the presence of**:**

Aliphatic acids (14.55%): fourteen aliphatic acids were identified; 2-hydroxyl-Propanoic, Hexadecanoic, Octadecanoic, 2,3-dihydroxy-hexadecanoicacids (1.33, 1.96, 2.0, 2.3%, respectively), that is beside octadecanoic acid-2,3-dihydroxy propyl ester (1.19%). The other acids present in small percentage (Table 2).

The Phenolic compound; 4-hydroxy-phenyl ethanol **(7.6%)** (Table 2, Figure 5).

Phenolic acids (3%): thirteen acids were identified in small percent; benzoic, 4-hydroxy-benzoic, 3,4-dimethoxy benzoic, 3,4,5-trihydroxy-benzoic acids, besides 3,4-dimethoxy-cinnamic , caffeic, coumaric and -*cis*-, -*trans*- of ferulic and isoferulic acids. 3-Phenyl-3-hydroxypropanoic, 4- hydroxy-benzene-acetic and 4-hydroxy phenyl-acetic acids were also present.

Phenolic acid esters (16.4%): five esters were identified, di-isooctyl phthalate (15.2%) was present as a very major ester (Table 2, Figure 5), while Phthalic acid- butyl octyl, methyl caffeate, pentyl caffeate and 2-methyl-2-butenyl *trans* caffeate esters were identified in minor percent (Table 2). Butane, 2,3-diol (5%) was also identified.

DISCUSSION

Among the possible strategies for enhancing brain cholinergic activity, acetylcholinesterase inhibitors have been the most extensively used for the symptomatic treatment of AD.[15] The use of synthetic AchE inhibitors in AD patients is limited by their undesirable side effects such as the short half-life peripheral cholinergic side effects of physostigmine, and dose dependent hepatotoxicity of tacrine.[16] The identification of new natural products with fewer side effects in AD patients is required.

Table 1: Flavonoids assessed by HPLC of propolis sub-fractions (A), (B) and (C) (µg /mg sub-fraction)

No.	Name	Chemical name	RT	Sub-fraction (A)	Sub-fraction (B)	Sub-fraction (C)
		Flavones				
1	Luteolin	5,7,3',4'-tetrahydroxyflavone	24.53		0.22	0.10
2	Apigenin	5,7,4'-trihydroxyflavone	37.95		0.04	
3	Luteolin-3'-methylether	5,7,4'-trihydroxy-3'-methoxyflavone	42.06		0.26	4.60
4	Chrysin-7-methylether	5- hydroxy-7-methoxy flavone	61.91	**0.55**	15.37	63.43
5	Chrysin	5,7-dihydroxyflavone	64.18	**39.01**		
6	Acacetin	5,7- dihydroxy-4'-methoxy flavone	65.4		10.45	0.58
	Total			**39.56**	**26.34**	**68.71**
		Flavonols				
7	Myricetin	3,5,7,3',4',5'- hexahydroxyflavone	12.88	0.16		
8	Quercetin-3-methylether	5,7,3',4'-tetrahydroxy-3-methoxyflavone	29.33	0.02	0.08	0.25
9	Quercetin-3,7-dimethylether	5,3',4'-trihydroxy-3,7-dimethoxyflavone	34.6			**52.97**
10	8-Methoxykaempferol	3,5,7,4'- tetrahydroxy-8- methoxyflavone	37.58		0.77	
11	Kaempferol-3-methylether	5,7,4'- trihydroxy-3-methoxyflavone	44.46		0.08	
12	Quercetin-3,3'-dimethylether	5,7,4'-trihydroxy-3,3'-dimethoxyflavone	45.53			0.67
13	Quercetin-7-methylether	3,5,3',4'-tetrahydroxy-7-methoxyflavone	56.88	**191.80**		2.62
14	Galangin	3,5,7- trihydroxyflavone	64.83	**76.71**		
	Total			**268.69**	**0.93**	**56.51**
		Flavanones				
15	Eriodictyol	5,7,3',4'- tetrahydroxy flavanone	19.88	0.11		
16	Liquiriteginin	7,4'-dihydroxyflavanone	20.45			0.47
17	Naringenin	5,7,4'-Trihydroxyflavanone	33.18	**31.67**		0.27
18	Pinobanksin-3-acetate	5,7-dihydroxy-3-acetyloxyflavanone	33.26	**53.53**		
19	Hesperetin	5,7,3'- trihydroxy-4'-methoxyflavanone	39.1		0.29	0.40
20	Biochanin A	5,7-dihydroxy-4'-methoxyflavanone	65.15	2.21	0.03	
	Total			**87.52**	**0.32**	**1.14**
		Isoflavones				
21	Genistein	5,7,4'-trihydroxyisoflavone	35.8			0.56
	Total					0.56
		Caffeic acid esters				
22	Dimethylallylcaffeate	3-Methyl-2-butenyl *trans* caffeate	62.65	3.73	**78.10**	3.59
23	Phenylethylcaffeate	Phenylethyl-*trans*-caffeate	64.70		**36.57**	
	Total			**3.73**	**114.67**	**3.59**

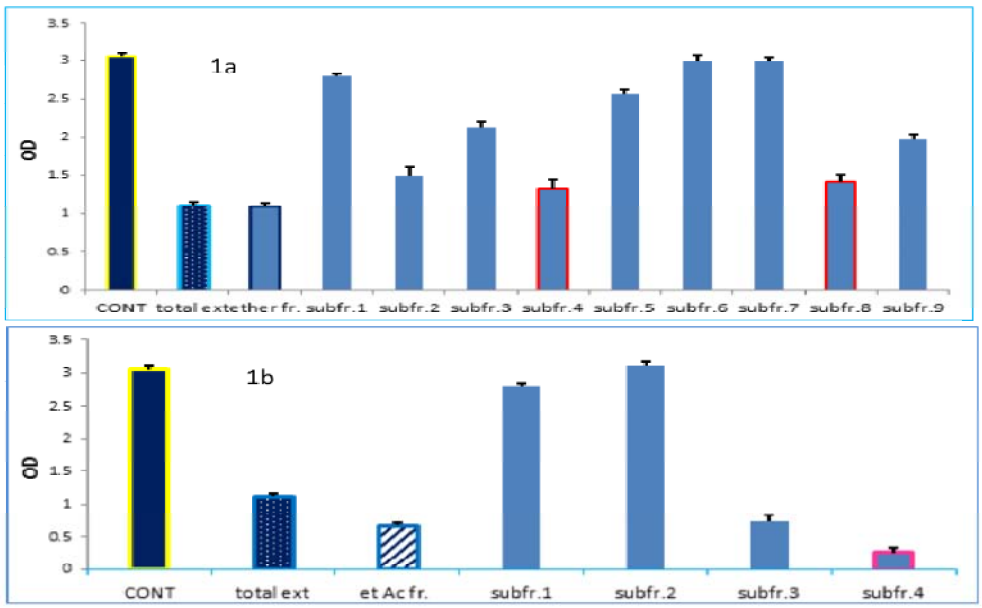

Figure 1: Bioassay guided fractionation of 70% PEE to show acetylcholinesterase inhibitory activity; Fig: 1a= ether, Fig.: 1b = ethylacetate fractions and their subfractions. Values are expressed as mean ± SD, n=3 (400 µg/ml)

Table 2: Chemical composition assessed by GC/MS of propolis sub-fractions (A), (B) and (C)

No.	Compounds	RT	* Sub-fraction A	* Sub-fraction B	* Sub-fraction C
	Aliphatic acids				
1	Butane, 2,3-diol	8.04		1.99	**4.97**
2	2- hydroxyl-Propanoic acid	8.23			**1.33**
3	Hydroxy acetic acid	8.83		0.2	0.1
4	Octanoic acid	18.68			0.07
5	Butanedioic acid,	21.41		0.79	0.13
6	Nonanoic acid	23.35			0.11
7	Dodecanoic acid	35.45			0.05
8	3-hydroxy-Capric acid	35.86			0.08
9	Tetradecanoic acid,	42.41			0.2
10	Hexadecanoic acid	49.04	0.76	1.36	**1.96**
11	9,12-Octadecadienoic acid	53.61			0.09
12	Octadecenoic acid,	53.95	0.05		
13	Octadecanoic acid,	54.82	0.76		**2.01**
14	Hexadecanoic acid -2,3-dihydroxy	64.09		1.32	**2.26**
15	Octadecanoic acid-2,3- dihydroxy propyl ester	68.67	0.82	0.88	**1.19**
	Total		**2.4**	**6.36**	**14.55**
	Phenolic compounds				
16	p-Hydroxy benzaldehyde	18.01	0.03		
17	Diphenyl ether	24.88		0.09	
18	Benzaldehyde-3-methoxy-4-hydroxy	30.28	0.04		
19	4-hydroxy-phenyl ethanol	34.18		0.27	**7.6**
	Total		**0.07**	**0.36**	**7.6**
	Phenolic acids				
20	Benzoic acid	17.57		0.2	0.36
21	Cinnamic acid	29.67	0.59		
22	3-Phenyl-3-hydroxypropanoic acid	33.55			0.14
23	4-hydroxy- Benzoic acid	34.48			0.18
24	Benzeneacetic acid-4- hydroxy	35.06			0.07
25	p-Methoxy *cis* cinnamic acid	36.97	**2.03**		
26	4-hydroxy phenylacetic acid	37.14			0.06
27	Benzoic acid, 3,4dimethoxy,	37.91		0.19	
28	p-Methoxy *trans* cinnamic acid	42.12	**4.00**		
29	m-Methoxy cinnamic acid	41.89	0.29	0.18	
30	*cis*-Isoferulic acid	45.04			0.09
31	*cis*-Ferulic acid	45.45			0.05
32	p-Coumaric acid	45.61			0.08
33	3,4,5-trihydroxy- Benzoic acid	46.65			0.34
34	3,4-Dimethoxy-cinnamic acid	48.49			0.32
35	*trans*-Isoferulic acid	49.89			0.96
36	*trans*-Ferulic acid	50.04			0.13
37	Caffeic acid	52.06			0.21
	Total		**6.91**	**0.57**	**3.0**
	Phenolic acids esters				
38	Benzoic acid, 3,4-dihydroxy-methyl ester	38.24		0.4	
39	Phthalic acid, butyl octyl ester	46.01	0.16		0.75
40	*cis*-Caffeic acid ethyl ester	46.1		0.36	
41	Methyl caffeate	47.71		0.13	0.15
42	*trans*-Caffeic acid ethyl ester	50.28		**1.56**	
43	4- hydroxy Benzeneacetic acid octadienyl ester[t]	51.15		0.36	
44	4- hydroxy Benzeneacetic acid hexanyl ester[t]	52.04		0.54	
45	Prenyl- hydrocaffeic acid ester	53.01	0.09		
46	3-methyl-3-butenyl *cis* caffeate	53.59	0.60	**0.97**	
47	2-methyl-2-butenyl *cis* caffeate	54.47	0.14		
48	3-methyl-2-butenyl *cis* caffeate	54.83	0.42	**2.86**	

49	Butyl caffeate	55.31		0.3	
50	Pentyl caffeate	57.41			0.11
51	3-methyl-3-butenyl *trans* caffeate	58.35	0.68	**3.13**	
52	2-methyl-2-butenyl *trans* caffeate	59.38	0.17	**1.04**	0.17
53	3-methyl-2-butenyl *trans* caffeate	60.02	0.58	**4.77**	
54	Benzyl-*cis*-caffeate	62.14		**0.69**	
55	Di-isooctyl phthalate	62.68	1.89		**15.2**
56	Benzyl-trans-caffeate	67.31		**1.69**	
57	Phenylethyl-*trans*- caffeate	69.09		**0.49**	
	Total		**4.73**	**19.3**	**16.4**
	Terpenes				
58	Bicyclo[4.2.1]nona-2,4,7-triene,7-hydroxy	33.94	**6.5**		
59	3-Hydroxy-4[1'(hydroxy cyclopentyl)ethynyl]furan	33.94	**6.0**		
60	Eudesmol	46.34	0.17		
	Total		**12.67**	**----**	**----**
	Flavonoids				
61	Pinobanksin	64.66	**10.5**		
62	Pinobanksin acetate	66.55	**2.4**		
63	Chrysin	68.06	**9.1**		
64	Galangin	68.27	**2.9**		
65	Myrecetin	70.77	**12.4**		
66	5,7,3',4'-Tetramethoxy-quercetin	70.92	0.6		
	Total		**37.9**	**-----**	**----**
	Others				
67	Diethylene glycol,	18.03		0.35	
68	Glycerol	20.0		1.34	0.58
69	Oleanitrile	49.84	0.04		
70	Docosane	53.39			0.31
71	Tricosane	56.03			0.58
72	Tetracosane	58.75			0.86
73	Pentacosane	61.37			0.82
74	1-O-Octadecylglycerol	66.83		**6.42**	
	Total		**0.04**	**8.11**	**3.15**

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 from mass spectra

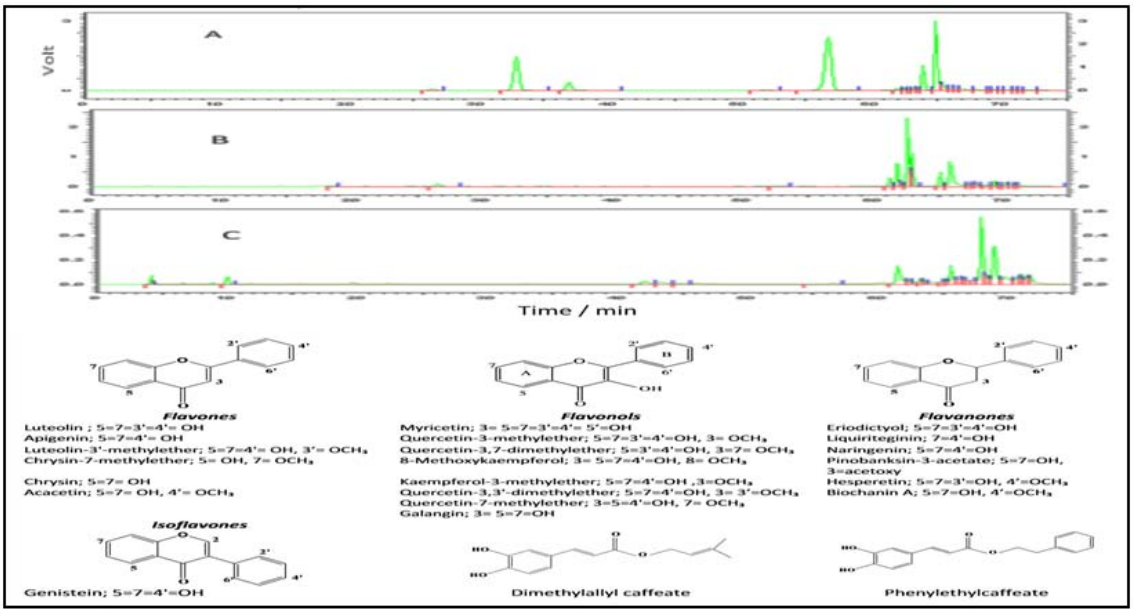

Figure 2: HPLC chromatograms of propolis sub-fractions (A), (B), (C) and structures of the identified compounds

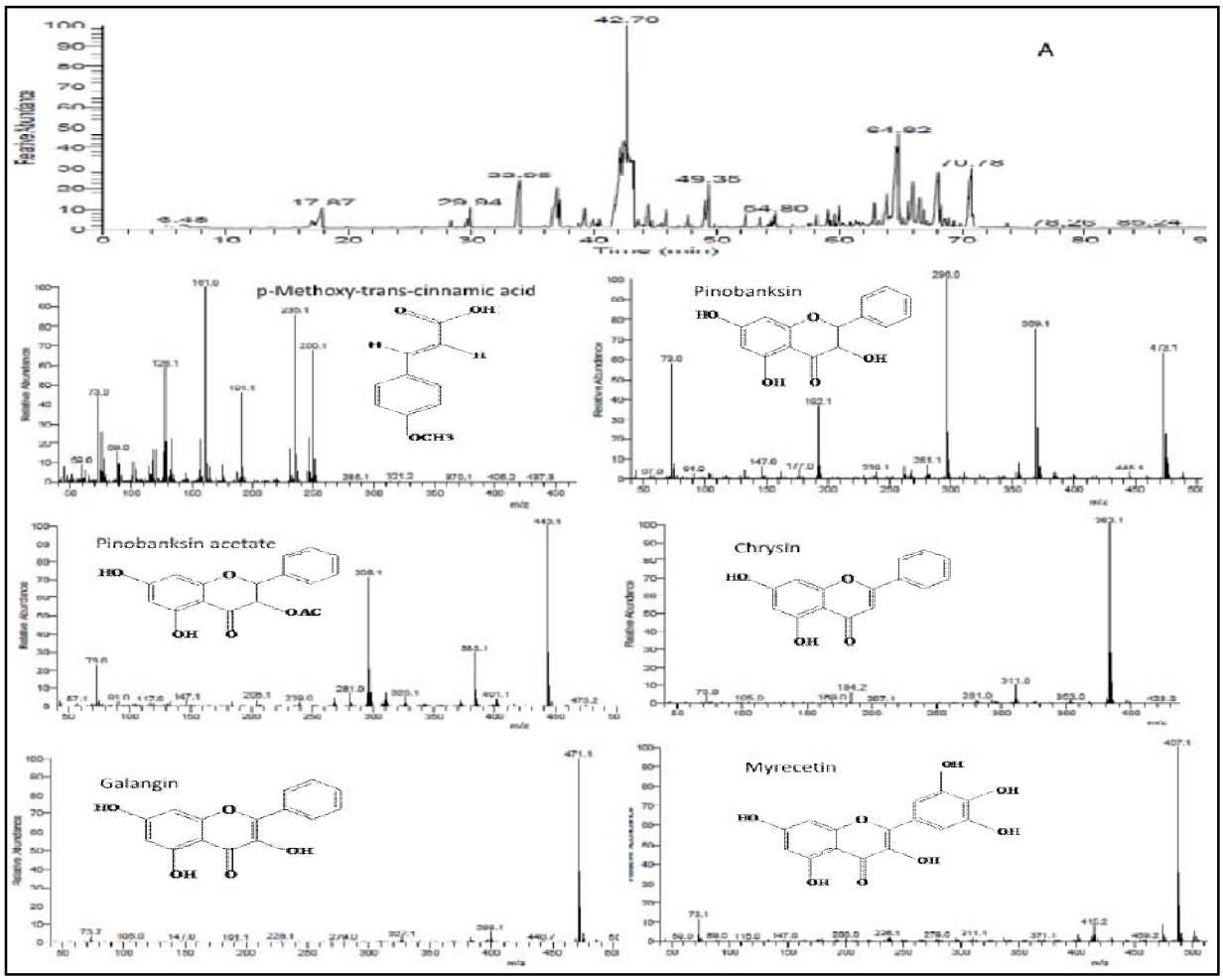

Figure 3: GC/MS Chromatogram of propolis sub-fraction (A) and mass spectra of prominent peaks

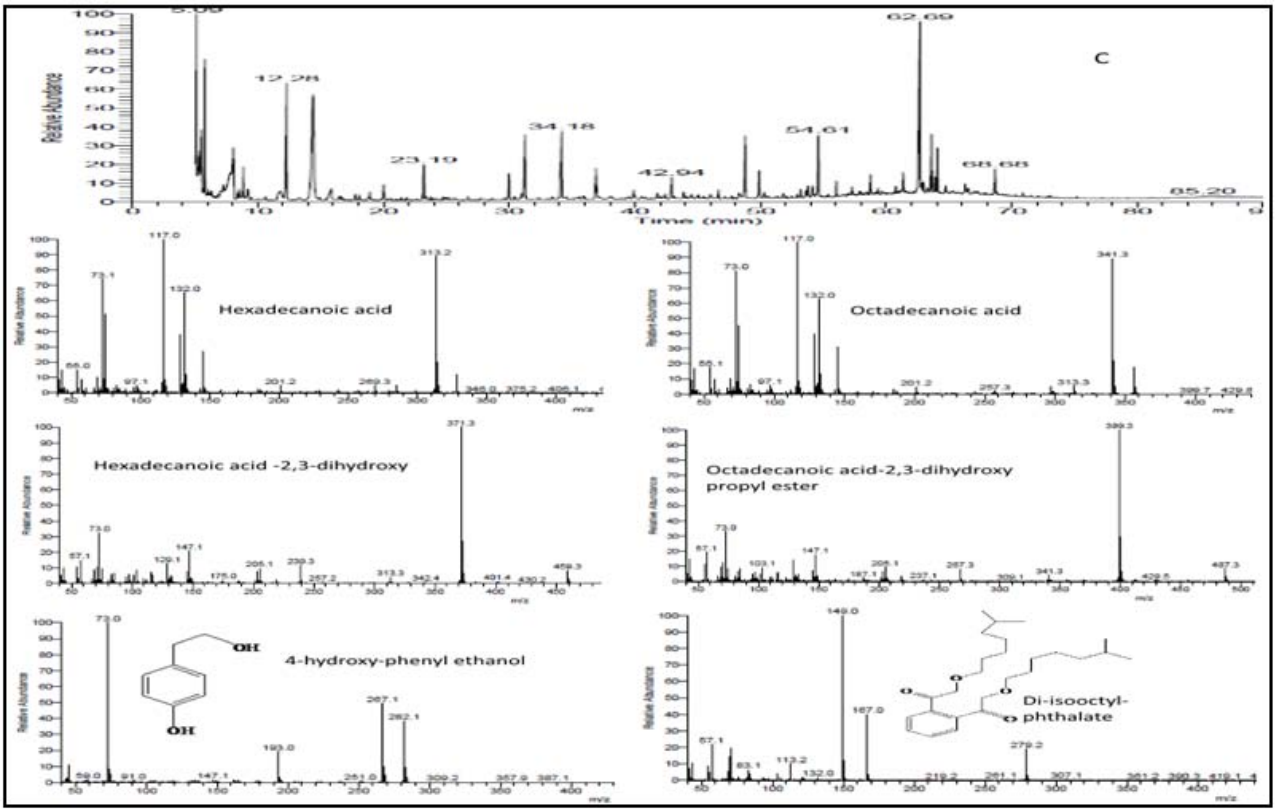

Figure 5: GC/MS Chromatogram of propolis sub-fraction (C) and mass spectra of prominent peaks

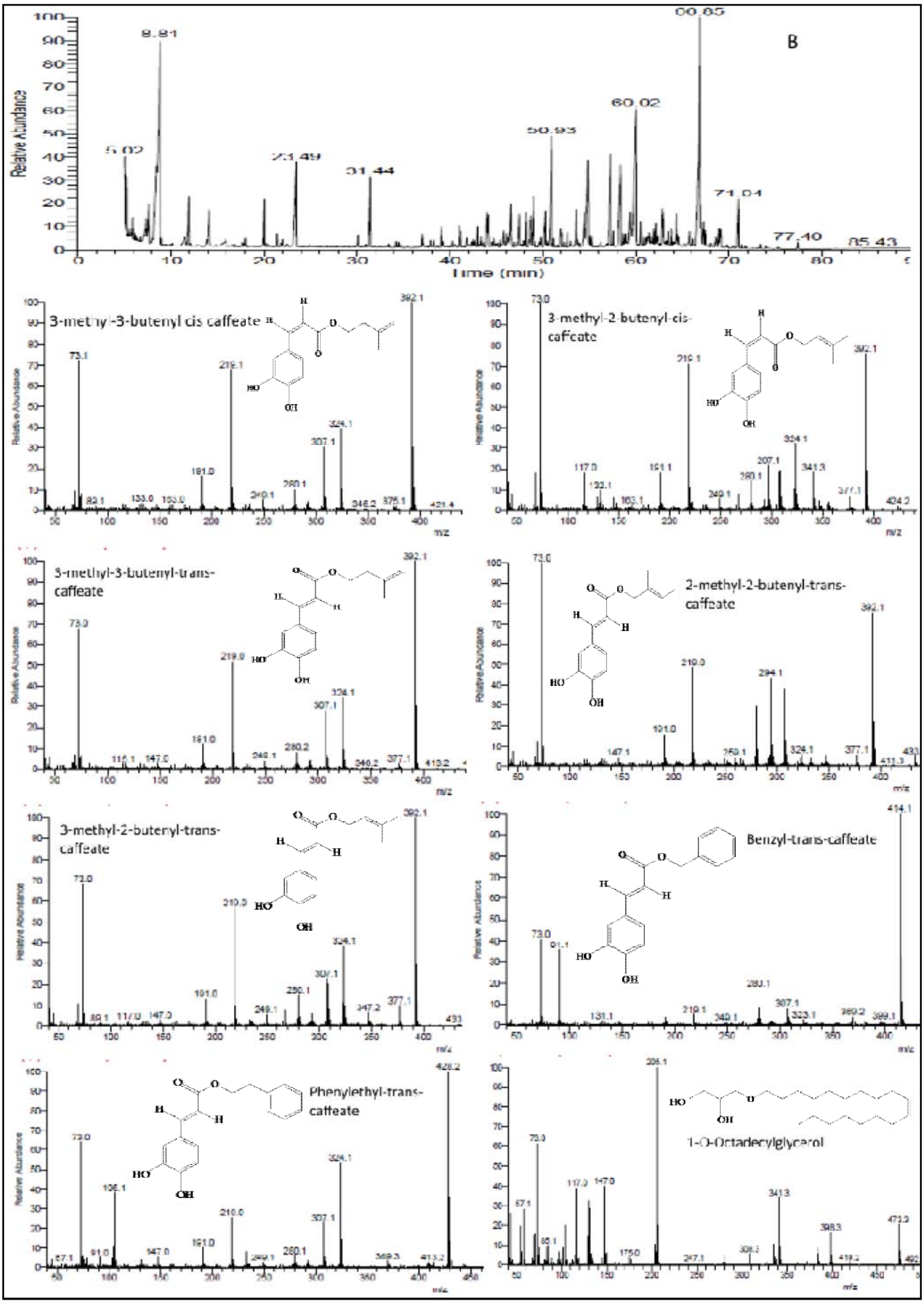

Figure 4: GC/MS Chromatogram of propolis sub-fraction (B) and mass spectra of prominent peaks

Bee products especially propolis based medicines have long been used to treat the different ailments in human being. Propolis different extracts are reported to have effect on nerve and memory function.[17]

The acetylcholinesterase enzyme is a protein with 501 amino acid residues having an ellipsoid shape. The active site is located in a deep and narrow gorge of 20 °A that becomes larger in the bottom, where the catalytic site is located. The active site has two sub-sites, the esteratic (ES), comprising the catalytic site and the anionic (AS).[18] The anionic sub-site is the site known to bind the choline moiety. The quaternary moiety of choline seems to bind through interactions with electrons from the aromatic residues along the gorge.[19] Besides these two sub-sites, the active site also contains another location known as Peripheral Anionic Site (PAS), at the entrance of the gorge, which comprises another set of aromatic residues This site is where the inhibitor molecules are "selected" to modulate the catalytic activity of the enzyme[20] and several studies have revealed that binding to the PAS sub-site could change the conformation of the active site, thus inhibiting enzyme activity.[21]

The administration of cholinesterase inhibitors is based on the cholinergic hypothesis which means that the loss of cholinergic function in the central nervous system is associated with the cognitive decline in AD and this loss leads to a gradual loss of functional neurons in the brain of elderly people.[22] Interestingly, AChE inhibitors have been observed to affect the "amyloid cascade".[23] However, AChE may also be involved in the formation of Aβ-peptides accelerating its formation and it appears that the PAS of the enzyme is crucial for this activity.[24]

There are only few studies that define the molecular mechanisms of acetylcholinesterase inhibition by the polyphenols. Furthermore, studies with choline derivatives of caffeic acid have revealed that the presence of a methyl group increases the inhibitory activity[25], as one of the methoxy groups orientates towards the PAS sub-site. Flavonoid orientation inside the enzyme gorge also looks similar to that presented by donepezil (an AChE inhibitor). Flavonoids have an aromatic ring (B-ring) which contains –OH groups that establish bonds with the PAS of the enzyme. Flavonoids appear not to induce modifications in the ternary structure of the enzyme, meaning that the inhibition process occurs due to the blockage of the entrance to the active site. Quercetin has been found to establish several hydrogen bonds with the amino acid residues at the anionic sub-site. It seems that phenolic compounds having structural motifs similar to caffeic acid are capable of fitting into the gorge of the active site of AChE by positioning the aromatic ring into the PAS sub-site.[21] Flavonoids that possess a free OH-group at C3 position are more inhibitory compared to their C3 –OH glycosylated counterparts and those having no C3 –OH group, such as luteolin and apigenin. Our data is in agreement with the above mentioned data (Figure 1, Table 1,2).

Szwajgier mentioned that various structural differences among the tested phenolics were considered as potential factors leading to the differences in their anticholinesterase activity. He also stated that the combination of the A-ring A5-OH, A6-OH and A7-OH groups provides strong inhibition of AChE activity however presence of A5-OH and A7-OH only show less inhibitory effect as if the compound contain three OH groups together.[26] Stronger inhibitory activity of AChE depends also on (B4'-OH) B-ring hydroxyl groups but not the B3'-OH and B5'-OH. Szwajgier also related the activity of phenolic acids to their structure; he mentioned that the presence of -OH and -OCH3 groups elevated anti-ChE activity, as shown by the fact that cinnamic acid, exerted no inhibitory activity, while most of its derivatives were active. The presence of a -OH group in the para position elevated anti-AChE activity in comparison to a -OH group in the ortho or meta position shown by the fact that p-coumaric acid and p-OH-benzoic acid with elevated anti-AChE activity than o-coumaric acid m-OH-benzoic acid respectively. The presence of -OH group in the meta-position (caffeic acid) elevated the inhibitory activity in comparison to the presence of -OCH3 group (ferulic acid). Noticed that a single substitution in the phenol ring in the para position (as in p-OH-benzoic acid or p-coumaric acid) was especially beneficial for inhibitory activity.[26] Szwajgier and Borowiec found that; among the phenolic acids, p-coumaric acid had the largest share in the anticholinesterase activity, than ferulic acid. Our obtained data are in agreement with this result.[27]

Miyazawa estimated the anti-AChE activity of 17 monoterpenoid compounds (hydrocarbons, alcohols and ketones) with a p-menthane skeleton. The highest anti-AChE activity was exhibited by α-terpinene. Terpene ketones were more efficient inhibitors than terpene alcohols, which showed an identical inhibitory activity to terpene hydrocarbon compounds. (+)-p-Menth-1-ene and α-terpinene were equally strong as inhibitors as terpene ketones. Data obtained (tab.1 and tab.2) is in agreement with these findings.[16]

CONCLUSION

This is the first time to study Egyptian propolis bioassay guided fractionation on acetylcholinesterase enzyme with comparative correlation to variable chemical composition by GC/MS and HPLC analysis. Some sub-fractions are highly active than crude extract or original fractions. Other sub-fractions are inactive. These results encourage us to select highly active sub-fractions for further *in-vivo* studies which could play an important role in bio-prospecting a new drug for AD.

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