BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902019000118083

Original Article

Acetylcholinesterase inhibition, antioxidant and identification of some chemical constituents of *Phyllanthus atropurpureus* cultivated in Egypt

Walid Elsayed Abdallah[1,*], Wael Mohamady Elsayed[1], Rasmia Ali Hassan[1], Naglaa Mohammed Nazif[1], Mohammed Ibrahim Ali[2], Khaled Abdelhady Abdelshafeek[1,3]

[1]Department of Phytochemistry, Pharmaceutical and Drug Industries Research Division, National Research Centre, Dokki, Giza, Egypt, [2]Department of Medicinal and Aromatic Plants Research, Pharmaceutical and Drug Industries Research Division, National Research Centre, Giza, Egypt, [3]Department of Chemistry, Faculty of Science, Elbaha University, KSA

Investigation of the lipid constituents of the aerial parts of *Phyllanthus atropurpureus* resulted in isolation and identification of the fatty acid mixture which consists of eight acids with linolenic acid as major and the unsaponifiable fraction that contain a series of hydrocabons, sterols, in addition to one triterpene (α-amyrin). The acetone insoluble fraction was found to contain two fatty alcohols and three n-hydrocabons in which the n-eicosane is the most abundant (44.16%). The flavonoidal constituents were isolated from ethyl acetate and butanol fractions which were identified as: luteolin-7-*O*-glucoside, kaempferol 3-*O*-(*p*-coumaroylglucoside), kaempferitrin, luteolin and kaempferol. Evaluation of different extracts as acetylcholinesterase inhibitors (AChI), established the chloroform fraction as a promising inhibitor of the enzyme. The antioxidant testing with DPPH radical revealed the potential of precipitate from MeOH extract as a radical scavenger.

Keywords: *Phyllanthus atropurpureus*. Euphorbiaceae. Flavonoids. Acetylcholineesterase inhibitors. Antioxidant.

INTRODUCTION

Phyllanthus (Euphorbiaceae) is a large genus and widely distributed in tropical and subtropical zones like tropical Africa, tropical America and Asia. This genus, consisting of more than 700 species. It has been employed as herbal drugs for a long time in China, India, Brazil and Southeast Asian countries. The most abundant species are used and have a beneficial role for the treatment of digestive, genitourinary, respiratory, skin diseases, antioxidant, antihepatotoxic, anticancer, antidiabetic, diuretic, anti-inflammatory to treat hepatitis B, hypertension, dropsy, sore throat, to cure jaundice, renal calculus and malaria (Mao *et al*., 2016). Also, *P. niruri* contain many phytochemical constituents like alkaloids, anthocyanins, flavonoids, phenolic acids, lignans and saponins. Combined saponin and alkaloid of the plant showed more potency and may offer an alternative therapeutic agent against bacterial infections (Ajibade, 2014; Kaur, Kaur, Sirhindi, 2017).

The chemical review on genus *Phyllanthus,* revealed the presence of sterols, and/ or terpenes, flavonoids (5,6,8,4′-tetrahydroxy isoflavone, robustaside A and 6′-(3″,4″- dihydroxycinnamoyl), arbutin, demethoxysudachitin, polyphenolic compounds and tannins, in addition to minor alkaloids (EL-Saywd, 2009; Sarg *et al.*, 2012).

Alzheimer's disease (AD) is the most common form of dementia and accounts for approximately 60% of all dementia cases. Till now there is no discovered medication that can slow or stop the neurodegeneration process of AD, where current treatment medicines can only control and improve the disease-associated symptoms (Finn, 2017). In AD, there is an excessive activity of acetylcholinesterase (AChE) enzyme that hydrolyze acetylcholine, a potential neurotransmitter in cholinergic neurons of the cerebral cortex part of the brain, leading to acetylcholine deficiency, choline accumulation and loss of memory (Quirion, 1993;

***Correspondence:** W. E. Abdallah. Department of Phytochemistry, Pharmaceutical and Drug Industries Research Division, National Research Centre, 33 El Buhouth St. (Former El Tahrir St.), 12622-Dokki, Giza, Egypt. Telephone: 00201223809338. E-mail: walsay2003@yahoo.com

Greenblatt *et al*., 1999). Therefore, current medicines for AD symptoms are essentially acetylcholinesterase inhibitors such as donepezil and galantamine that act by preventing the excessive AChE-catalyzed hydrolysis of acetylcholine and thereby improving cognitive symptoms (Ali *et al*., 2013). In addition, oxidative stress resulting from free radicals has been implicated in the pathogenesis of neurodegenerative diseases including AD (Omar *et al*., 1999; Mariani *et al*., 2005). Therefore, many antioxidant compounds have been very useful in neuroprotection against AD (Finn, 2017). There are many raw plant extracts/herbal formulations with immense uses as natural remedies in the treatment of AD and other neurodegenerative diseases. Many traditional natural medical systems have various brain tonics and memory enhancers (Perry *et al*., 1999).

In the present study, some chemical constituents such as lipids and some phenolics were isolated and identified in addition to the acetylcholinesterase inhibitors and antioxidant activities of some extracts of *P. atropurpureus* were evaluated.

MATERIAL AND METHODS

Plant Material

The aerial parts of *P. atropurpureus* was collected in June 2015 from botanical garden, Faculty of Agriculture, Cairo University and kindly identified by Dr. Mohammed Elgebaly at Phytochemistry and Plant Systematic Dept., National Research Center (NRC). Voucher specimen was deposited at the herbarium of the NRC (Herbarium specimen number: 11501).

Extraction of lipid and phenolic constituents

About 1.5 kg of the air dried powdered plant material of *P. atropurpureus* were extracted with hexane in a Soxhlet for two days**.** The hexane extract was passed over Fuller's earth to remove the colored pigments and evaporated *in vacuo* to give a yellowish residue (6.5 g), which was dissolved in hot acetone to afford two fractions, acetone insoluble fraction which was filtered and analyzed by Gas chromatography-Mass spectrometry (GC/MS) and acetone soluble fraction which subjected to saponification process to afford the unsaponifiable materials and fatty acid methyl esters fraction which were identified using Gas liquid chromatography (GLC) (Gören *et al*., 2003). The defatted plant material (1400 g) was divided into two portions (700 g each), the first portion was extracted with methanol: chloroform mixture (50:50) for two days, filtered and the precipitated material during the concentration was separated and washed with chloroform to afford a dark brown precipitate and chloroform/methanol extract. The second portion was macerated in aqueous methanol (70%) for five days to produce a brownish extract, concentrated up to 700 mL which increased to 1000 mL with hot distilled water, the precipitated material was filtered off and the filtrate was partitioned with successive portions of chloroform, ethyl acetate and butanol. The butanol fraction was applied into a Polyamide column (5x75) eluted with 100% water, water/methanol gradients and decreasing the polarity by increasing the amount of methanol up to methanol 100%. The different fractions which contain the main compounds were further purified using small columns of Polyamide and Sephadex LH-20 to afford three compounds (PA-I, PA-II and PA- III). The ethyl acetate fraction (about1.5 g) was dissolved in methanol and subjected to preparative paper chromatography, developed with AcOH (20%). Two main bands were localized, cut off and eluted with hot methanol to afford two main compounds which were further purified by passing through Sephadex LH-20 column eluted with methnol (95%) to afford compounds PA-IV and PA-V.

Gas liquid chromatography (GLC) analysis of unsaponifiable matters

The GLC analyses were carried out using the GC instrument, Varian model 3700. The GC was equipped with capillary column (AG-Bp-70). Polysilphenylene-siloxane, 60 m length, 320 µmL internal diameter, 0.25 µmL film thickness. Analyses were carried out using the following temperature program: 70 °C, rising at 4 °C/min to 270 °C and the injector and detector were held at 280 °C and 290 °C, respectively. Flow rates of N_2 and H_2 30 mL/min and Air 300 mL/min.

Gas liquid chromatography (GLC) analysis of fatty acid methyl esters (FAMEs)

The GLC analyses were carried out using the GC instrument, Varian model 3700. The GC was equipped with capillary column (AG-Bp-70). Polysilphenylene-siloxane, 60 m length, 320 µmL internal diameter, 0.25 µmL film thickness. Analyses were carried out using the following temperature program: 70 °C, rising at 4 °C/in to 190 °C and the injector and detector were held at 240 °C and 280 °C, respectively. Flow rates of N_2 and H_2 30 mL/min, Air 300 mL/min.

AChE Inhibitory activity

The AChE inhibitory activity of each extract was

tested using 96-well micro-plate assay based on previously published methods (Ingkaninan *et al*., 2000; Rhee *et al*., 2001) with minor modifications. Each extract (25 µL of 10× of final concentrations in DMSO) was dispensed in duplicates onto 96 well microplate and mixed with 200 µl of Ellman's mixture [final concentrations of 10 mM Tris–HCl, pH 8.0, 0.1% bovine serum albumin (BSA, fraction V), 1.5 mM acetylthiocholine iodide (ATCI, Carbosynth, UK) and 150 µM 5,5'- dithio-bis-(2-nitrobenzoic acid), DTNB, Sigma-Aldrich, Germany]. The control wells contained the extract vehicle (DMSO) instead of the extract. The reaction was started with the addition of AChE enzyme solution (0.02 U/mL, type VS –from electric eel, Sigma-Aldrich, Germany). Non-enzymatic autohydrolysis of the substrate was corrected by replacing the enzyme with 25 µl of enzyme buffer (50 mM Tris–HCl, pH 8, 0.1% BSA) in duplicate wells. The enzymatic activity was monitored kinetically at 405 nm in a microplate reader (Zenyth 200, Biochrom® Anthos, Cambridge, UK), every 20s intervals for 20 cycles. The enzyme rate was calculated from the slope of the curve of absorbance change vs. time. Neostigmine methyl sulphate (0-31.25 µg/mL) was used as reference AChE inhibitor. As screening strategy, final concentration of 50 µg/mL from each extract was examined and the average % inhibition was calculated relative to the enzyme rate of the vehicle control wells according to the equation:

$$\%\ ACHE\ Inhibition = \frac{\left(\begin{matrix}mean\ slopes\ of\ the\\ vehicle\ control\end{matrix}\right) - \left(\begin{matrix}mean\ slopes\ of\\ the\ sample\end{matrix}\right)}{\left(\begin{matrix}mean\ slopes\ of\ the\\ vehicle\ control\end{matrix}\right)}$$

Microwell assay of DPPH radical scavenging

Extracts were prepared in DMSO as 10x stocks from each test concentration and briefly sonicated when necessary in an ultrasonic water bath. In a preliminary screen, extracts that produced radical scavenging activities ≥ 50% at 100 µg/mL were taken for further concentration-response testing to determine the EC_{50} (concentration of the extract producing 50% scavenging of the DPPH). Quercetin dihydrate was used as reference antioxidant. The method used in the present study was based on previously published methods (Braca *et al*., 2001; Nara *et al*., 2006). The extract stock solutions (20 µL/well) were pipetted in triplicate onto 96-well plates (flat-bottomed, Grene Bio-one, Germany). The assay was started with the addition of DPPH reagent (0.004% wt/v in methanol, 180 µl /well). Appropriate blanks were prepared using the solvent only in addition to the same amount of DPPH reagent to get rid of any inherent solvent activity. Negative controls were run in parallel to correct for sample color by OD subtraction. The plate was incubated in dark for 30 min at room temperature and then absorbances were read on a Zenyth 200 microplate reader (Biochrom® Anthos, Cambridge, UK).

The percentage of antioxidant activity (% AA) was calculated using the following equation:

$$\%\ DPPH\ Scavening = 100 - \left[100 \times \frac{\left(\begin{matrix}color\ corrected\ OD\\ of\ test\ sample\end{matrix}\right)}{\left(\begin{matrix}color\ corrected\ OD\\ of\ vehicle\ control\end{matrix}\right)}\right]$$

EC_{50} values (concentrations that scavenge 50% of the DPPH in the vehicle control) were derived using non-linear regression analysis of the concentration-% DPPH scavenging plot on GraphPad Prism® V6.0 (San Diego, USA). Data were presented as means ± S.E of three experiments.

RESULTS AND DISCUSSION

Identification of lipoidal matter

The lipid constituents of the aerial parts of *P. atropurpureus* were extracted with hexane (Table I-III) and fractionated to acetone insoluble fraction, unsaponifiable matter and fatty acid methyl esters. The acetone insoluble fraction was analyzed using Gas chromatography-Mass spectrometry (GC/MS) (Table I) and it was found to contain fatty alcohols and hydrocarbons with n-eicosane is the main hydrocarbon while octadecanol is the main fatty alcohol.

The data in Table II proved that the fatty acid fraction was found to consist of eight acids, three of them were unsaturated (73.38%) in which linolenic acid the main one (48.19%) and five acids are saturated (26.62%) with palmitic acid as major (17.90%). These findings were in agreement with that reported by Rao, Sino (1992), where they found that linolenic acid was the major component in *P. calycinus*.

The Gas liquid chromatography (GLC) analysis of the unsaponifiable fraction (Table III) revealed the presence of n-hydrocarbons mixture (66.76%) in which n-heptacosane (C_{27}) is the main (28.81%), a sterol fraction (26.10%) contain three components [campasterol, stigmasterol (14.22%) and β-sitosterol] and α-amyrin (4.45%) as a triterpene. These data were in agreement with that reported by Subraya, Satyanarayana, Yamini (2013), where they isolated β-sitosterol, stigmasterol and α-amyrin acetate from aerial part of *P. lawii*.

TABLE I - GC/MS analysis of acetone insoluble fraction of *Phyllanthus atropurpureus*

Peak No.	R_t (min.)	%	Mol. formula	Mol. Wt.	Compounds
1	20.92	9.54	$C_{16}H_{34}O$	242	hexadecanol
2	25.57	15.87	$C_{18}H_{38}O$	270	octdecanol
3	30.50	2.50	$C_{20}H_{42}$	282	9-methylnonadecane
4	32.60	44.16	$C_{20}H_{42}$	282	eicosane
5	37.67	9.67	$C_{27}H_{46}O$	386	cholesterol
6	40.57	15.50	$C_{25}H_{52}$	352	pentacosane
7	-----	2.76	-----	-----	unknowns

TABLE II - GLC analysis of FAME fraction of *Phyllanthus atropurpureus*

Peak No.	R_t (min.)	Rel. %	Mol. formula	Mol. Wt.	Compounds
1	9.47	1.20	$C_{12}H_{24}O_2$	200	Lauric acid , n-$C_{12:0}$
2	10.47	1.93	$C_{13}H_{26}O_2$	214	Traidecanoic acid , n-$C_{13:0}$
3	11.95	3.61	$C_{14}H_{28}O_2$	228	Myristic acid , n-$C_{14:0}$
4	14.77	17.90	$C_{16}H_{32}O_2$	256	Palmetic acid , n-$C_{16:0}$
5	17.85	1.98	$C_{18}H_{36}O_2$	284	Stearic acid , n-$C_{18:0}$
6	18.35	7.89	$C_{18}H_{34}O_2$	282	Oleic acid , n-$C_{18:1}$
7	19.36	17.30	$C_{18}H_{32}O_2$	280	Linoleic acid, n-$C_{18:2}$
8	20.62	48.19	$C_{18}H_{30}O_2$	278	Linolenic acid, n-$C_{18:3}$

TABLE III - GLC analysis of the unsaponifiable fraction of *Phyllanthus atropurpureus*

Peak No.	$R_{t\ (min)}$	%	Compounds
1	11.4	21.58	n- tetradecane (C_{14})
2	12.5	0.9	n-pentadecane (C_{15})
3	15.2	0.9	n-heptadecane (C_{17})
4	18.7	0.9	n-cosane (C_{20})
5	19.8	1.22	n-hencosane (C_{21})
6	20.8	1.67	n-dodacosane (C_{22})
7	21.8	2.22	n-triacosane (C_{23})
8	22.8	3.11	n-tetracosane (C_{24})
9	23.4	2.34	n-pentacosane (C_{25})
10	24.6	1.33	n-hexacosane (C_{26})
11	25.8	28.81	n-heptacosane (C_{27})
12	28.6	1.78	n-nonacosane (C_{29})
13	31.9	7.45	Campasterol
14	32.5	14.22	Stigmasterol
15	33.4	4.45	β-sitosterol
16	36.4	4.45	α-amyrin

Identification of flavonoidal components

The flavonoidal constituents were isolated from the butanol and ethyl acetate fractions using different columns chromatography as follow:

Compound PA-I

Luteolin-7-O-glucoside: It was obtained as yellowish powder, appeared as a brown spot turned yellow on spraying with $AlCl_3$ under UV light and it's behavior on paper chromatography proved it's glycosidic nature. The UV spectra in methanol and shifts reagents showed that it is a flavone type with occupation at C-7, with an ortho-dihydroxy system in ring B. UV spectrum of the aglycone of $NaOAc/H_3BO_3$ relative to methanol spectrum confirmed the presence of a free OH group at C-3′ and C-4′. The presence of free OH group at C-5 is confirmed by the bathochroic shift of band-1 with $AlCl_3$ relative to methanol. The absence of free OH group at C-7 was confirmed through NaOAc spectrum (Mabry, Markham, Thomas, 1970). The EI-MS displayed M^+ at m/z = 448 which fit to the formula $C_{21}H_{20}O_{11}$. Another important fragments at m/z = 420 and 286 are due to M^+- CO and hexose moiety respectively. The ^1H NMR (400 MHz ,

DMSO) gave signals at: 7.56 (1H, *dd*, *J*=7.6, 1.842, H-6'), 6.93 (*d*, *J*=2.5, H-2'), 6.92 (1H, *d*, *J*=7.5, H-5'), 6.24 (*s*, H-3), 6.16 (*d*, *J*=2.0 Hz, H-6), 6.28 (*d*, *J*=2.0 Hz, H-8), 5.45 (1H, *d*, *J*=7.5, H-1"), 3.35-2.75 (as complex *m*, rest of glucose protons). The acid hydrolysis proved the presence of glucose as a sugar and luteolin as an aglycone. And the UV spectra of the aglycone proved the presence of the glucose moiety at C-7. So, the structure of compound **PA-I** could be elucidated as *luteolin 7-O-glucoside* (Giannasi, 1988; Harborne, Baxter, 1999; Saxena, Aprajita, 2008).

Compound PA-II

Kaempferol 3-O-(p-coumaroylglucoside): The compound was isolated from the butanol fraction as a yellowish powder and it is a glycosidic in nature through its chromatographic behavior in different solvent system (R_f 0.77 in BAW and 0.34 in 15% AcOH). It is a flavonol type of kaempferol structure occupied at C-3 where it's UV spectrum in MeOH gave band-I at 359, also the spectrum of the NaOMe reagent proved the presence of a free OH groups at C-4'. The C-7 contain a free OH due to the bathochromic shift in band-II relative to methanol in NaOAc spectrum. The EI MS showed a molecular ion peak at *m/z* 594 and a fragmentation of 285 [kaempferol-H] due to the loss of a coumaroyl-glucoside moiety (-308), in addition to fragment ions at 447 [M-coumaroyl] and at *m/z* 307 [M-kaempferol]. The ^{1}H-nmr spectrum displayed signals at δ in ppm at 7.9 (2H, *d*, *J*=8.7 Hz, H-2',6'), 6.92 (2H, *d*, *J*=8.95 Hz, H-3',5'), 6.1 (1H, *d*, *J*=2.1 Hz, H-6) and 6.15 (1H, *d*, *J*=1.75 Hz, H-8). The *p*-coumaric acid at 7.5 (2H, *d*, *J*=8.8 Hz, H-2'',6''), 6.7 (2H, *d*, *J*=8.9 Hz, H-3'',5''), 6.75 (1H, *d*, *J*=10.7 Hz, H-7') and 5.69 (1H, *d*, *J*=11.2 Hz, H-8'), while the anomeric proton of the glucose moiety appeared at 5.2 as (1H, *d*, *J*=6.7 Hz). The acid hydrolysis revealed the presence of keampferol as an aglycone with glucose and *p*-coumaric acid with aid of paper chromatography and authentic samples. All these data were agree with that reported and supported the identification of compound **PA-II** as kaempferol 3-*O*-(*p*-coumaroylglucoside) (Felipe *et al*., 2014).

Compound PA-III

Kaempferitrin: The compound was isolated as a dark yellow powder and it is a glycosidic in nature where it's R_f is 0.68 in AcOH (15%) and a flavonol type structure where it displayed band-I at 350 nm with free OH group at C-4' where band-I was shifted to 415 nm with increasing in intensity. The absence of an ortho dihydroxy system was confirmed through both $AlCl_3$/HCl spectrum where there is no hypsochromic shift in band-I relative to $AlCl_3$, and no bathochromic shift in band -1 in NaOAc/H_3BO_3 spectrum relative to methanol, also no free OH groups at C-3 and C-7. No bathochromic shift in band –II in NaOAc spectrum relative to methanol spectrum which indicates no free OH group at C-7. The ^{1}H-nmr (DMSO) gave signals at δ in ppm at 7.9 (2H, *d*, *J*=8.7 Hz, H-2', 6'), 6.95 (2H, *d*, *J*=8.3 Hz, H-3', 5'), 6.81 (1H, *d*, *J*=2.35 Hz, H 8), 6.52 (1H, *d*, *J*=2.35 Hz, H-6), the anomeric protons of two sugar moieties at 5.54 (1H, *d*, *J*=1.3 Hz, H-1'''), 5.42 (1H, *d*, *J*=1.3 Hz, H-1''), while the two methyl protons at 1.05 (3H, *d*, *J*=6.32 Hz, H-6'''), 0.83 (3H, *d*, *J*=6.32 Hz, H-6''), finally complex multiplet signals at 3.69-4.31 for the rest of the sugar protons. The mass spectrum exhibited the molecular ion peak M^+ at *m/z* =579 which correspond to the molecular formula $C_{27}H_{30}O_{14}$. Other peaks at 433 and 286 confirmed the presence of two deoxy hexose moieties. The acid hydrolysis proved the presence of kaempferol as an aglycone which confirmed through it's UV and only rhamnose as sugar which were confirmed through the comparison with authentic samples in different solvents. Thus all the chromatographic and spectroscopic measurements confirmed the structure of compound PA-III as *kaempferitrin* (kaempferol-3,7-*O*-dirhamnoside) (Gohara, Elmazar, 1997).

Compound PA-IV and Compound PA-V

These two known compounds were isolated from the ethyl acetate fraction and were identified as luteolin and kaempferol respectively by comparison of their spectral data with the literature (Giannasi, 1988; Harborne, Baxter, 1999; Saxena, Aprajita, 2008). According to the latest review about *Phyllanthus* genus reported by Mao *et al*. (2016), luteolin-7-*O*-glucoside, kaempferol 3-*O*-(*p*-coumaroylglucoside) and *kaempferitrin* (kaempferol-3,7-*O*-dirhamnoside) were isolated from this species for the first time while the other isolated flavonoids were isolated from other species like *P. niruri, P. virgatus* and *P. singampattiana*.

Acetylcholinesterase inhibition

The screening of the effect of *P. atropurpureus* extracts and fractions against AChE enzyme activity is presented in (Figure 1). The results revealed that the chloroform fraction produced the highest inhibition of the enzyme activity among other tested samples, recording 82.5% inhibition at 50 µg/mL. Weak inhibition was produced by the rest of samples as shown in Figure 1. To determine the IC_{50} of the chloroform extract, dose-response experiment was conducted and revealed a concentration-dependent inhibition of AChE enzyme. Non-linear regression analysis of the concentration- % inhibition plot

revealed the IC_{50} value to be 13.7 µg/mL (Figure 2). The IC_{50} for the reference AChE inhibitor was calculated as 3.3 µg/mL (Figure 3). The obtained activity of the chloroform extract could be attributed to it's alkaloidal contents which are known to cause AChE inhibition (Elgorashi, Stafford, Van Staden, 2004; Halldorsdottir, Jaroszewski, Olafsdottir, 2010; Kulhankova *et al*., 2013; Dong *et al*., 2015; Benamar *et al*., 2016). Kaur, Kaur, Sirhindi (2017) stated that *P. niruri* contain alkaloids of neuro-pharmacological activity.

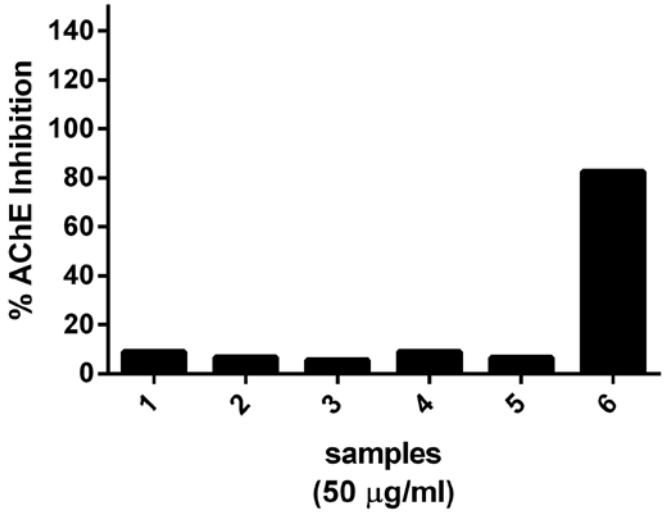

FIGURE 1 - Effect of of *P. atropurpureus* extracts and fractions on AChE enzyme activity. Sample key is 1= Ppt. from 70% MeOH extract, 2= BuOH fraction, 3= Water fraction, 4= Mother liquor of 70% MeOH extract, 5=70% MeOH extract, 6= $CHCl_3$ fraction.

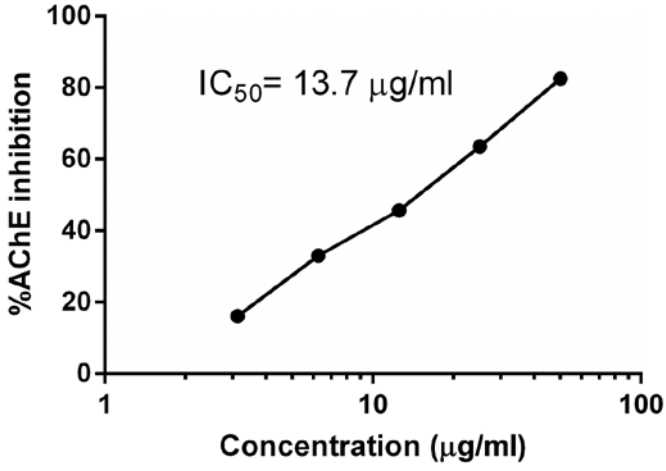

FIGURE 2 - Concentration-dependent effect of *P. atropurpureus* chloroform fraction on AChE enzyme Inhibition. IC_{50} of the inhibition was derived using non-linear regression analysis on GraphPad Prism V6.0.

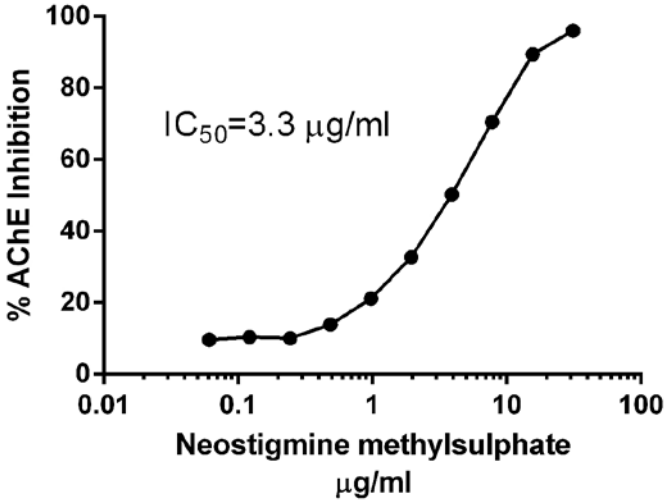

FIGURE 3 - Concentration-dependent effect of the reference AChE inhibitor neostigmine methyl sulphate on AChE enzyme Inhibition. IC_{50} of the inhibition was derived using non-linear regression analysis on GraphPad Prism V6.0.

Antioxidant activity

The prescreen of the effect of *P. atropurpureus* extracts and fractions on the scavenging of DPPH radical revealed the activity of three fractions, namely the precipitate from MeOH extract, BuOH fraction and water fraction. Concentration-response experiments were conducted to reveal the EC_{50} values (Figure 4 and Table IV). The obtained EC_{50} revealed the most potent

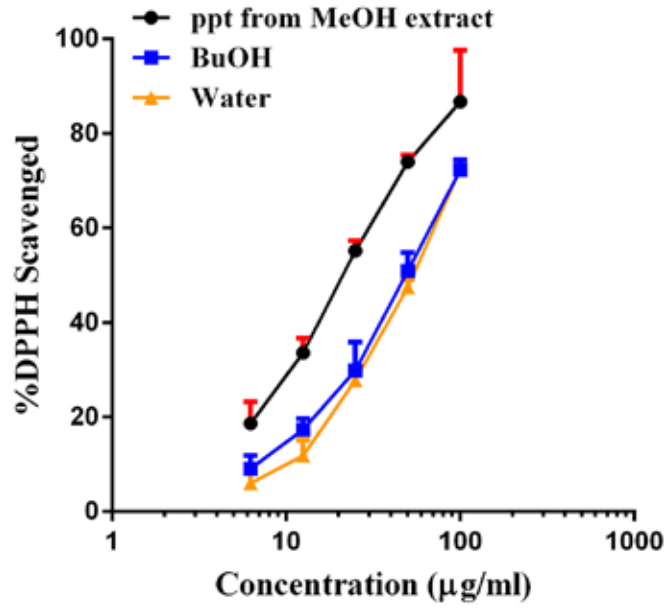

FIGURE 4 - Concentration-response plots of DPPH radical scavenging by different extracts from *P. atropurpureus*. Samples were tested for DPPH radical scavenging as described in the methods section. Data are means of three experiments each run in triplicate.

TABLE IV - EC_{50} values of different extracts and fractions of *P. atropurpureus* as analyzed with non-linear regression

Extract	EC_{50} (µg/mL)*
Ppt. from 70% MeOH extract	21.4±0.2
BuOH fraction	47.5±2.0
Water fraction	51.5±0.3
M.L. of 70% MeOH	>100
70% MeOH extract	>100
$CHCl_3$ fraction	>100
Quercetin dihydrate	2.5±0.04

*Data are means ±S.E of three experiments.

extracts to be the precipitate from MeOH extract, recording EC_{50} value of 21.4 µg/mL which may be attributed to it's phenolic content (Kaur, Kaur, Sirhindi, 2017). In addition The BuOH and water fractions exhibited similar pattern of concentration-response recording EC_{50} values of 47.5 and 51.5 µg/mL, respectively. Further investigation is needed to isolate the active constituents of the precipitate from MeOH extract through activity-guided isolation.

CONCLUSION

This study resulted in isolation and identification of lipid constituents (fatty alcohols, fatty acids and unsaponifiable matters), and the flavonoidal compounds (aglycone and glycosides). Evaluation of different extracts and fractions as acetylcholineesterase inhibitors proved that the chloroform fraction is the most active one, while the precipitate from MeOH extract exhibited the highest antioxidant activity.

CONFLICT OF INTEREST

All authors declare no conflicts of interest.

ACKNOWLEDGMENTS

The Authors thank Dr. Ahmed Ragab Hamed (Biology Unit, Central Laboratory of the Pharmaceutical and Drug Industries Research Division, National Research Centre) for performing the anti-acetylcholinesterase and antioxidant evaluation assays. We also thank the National Research Centre for support.

REFERENCES

Ajibade VA. Comparison of antibacterial activity of crude alkaloid and saponin extract from *Phyllanthus niruri*. Pak J Scient Indust Res Series B: Biol Sci. 2014;57(1):41-5.

Ali SK, Hamed AR, Soltan MM, Hegazy UM, Elgorashi EE, El-Garf IA, Hussein AA. *In-vitro* evaluation of selected Egyptian traditional herbal medicines for treatment of Alzheimer disease. BMC Comp Alter Med. 2013;13(121):1-10.

Benamar H, Tomassini L, Venditti A, Marouf A, Bennaceur M, Nicoletti M. Pyrrolizidine alkaloids from *Solenanthus Lanatus* Dc. with acetylcholinesterase inhibitory activity. Nat Prod Res. 2016;30(22):2567-74.

Braca A, De Tommasi N, Di Bari L, Pizza C, Politi M, Morelli I. Antioxidant principles from *Bauhinia tarapotensis*. J Nat Prod. 2001;64(7):892-5.

Dong JW, Cai L, Fang YS, Xiao H, Li ZJ, Ding ZT. Proaporphine and aporphine alkaloids with acetylcholinesterase inhibitory activity from *Stephania epigaea*. *Fitoterapia*. 2015;104:102-7.

Elgorashi EE, Stafford GI, Van Staden J. Acetylcholinesterase enzyme inhibitory effects of amaryllidaceae alkaloids. Planta Med. 2004;70(3):260-262.

El-Saywd MA. A pharmacognostical study of *Phyllanthus* family Euphorbiaceae cultivated in Egypt. [Thesis] Zagazig: Pharmacognosy Department, Faculty of Pharmacy, Zagazig University; 2009. 239p.

Felipe DF, Brambilla LZ, Porto C, Pilau EJ, Cortez DA. Phytochemical analysis of *Pfaffia glomerata* inflorescences by LC-ESI-MS/MS. Molecules. 2014;19(10):15720-34.

Finn LA. Current medications for the treatment of Alzheimer's disease: acetylcholinesterase inhibitors and NMDA receptor antagonist A2-Adejare, Adeboye(ed.). In: Adejare A, editor. Drug discovery approaches for the treatment of neurodegenerative disorders. Amsterdam: Academic Press; 2017. chap.4.

Giannasi DE. Flavonoids and evolution in the dicotyledons. In: Harborne JB, editor. The flavonoids: advances in research since 1980. London: Chapman and Hall; 1988. p.479-504.

Gohara AA, Elmazar MM. Isolation of hypotensive flavonoids from *Chenopodium* species growing in Egypt. Phytother Res. 1997;11(8):564-7.

Gören AC, Bilsel G, Altun M, Satil F, Dirmenci T. Fatty acid composition of seeds of *Satureja thymbra* and *S. cuneifolia*. Z Naturforsch C. 2003;58(7/8):502-4.

Greenblatt HM, Kryger G, Lewis T, Silman I, Sussman JL. Structure of acetylcholinesterase complexed with (-)-galanthamine at 2.3 A resolution. FEBS Lett. 1999;463(3):321-6.

Halldorsdottir ES, Jaroszewski JW, Olafsdottir ES. Acetylcholinesterase inhibitory activity of Lycopodane-Type alkaloids from the Icelandic *Lycopodium annotinum* ssp. *alpestre*. Phytochemistry. 2010;71(2/3):149-57.

Harborne JB, Baxter H. The handbook of natural flavonoids. Chichester: John Wiley; 1999.

Ingkaninan K, De Best CM, Van Der Heijden R, Hofte AJ, Karabatak B, Irth H, *et al*. High-performance liquid chromatography with on-line coupled UV, mass spectrometric and biochemical detection for identification of acetylcholinesterase inhibitors from natural products. J Chromatog A. 2000;872(1/2):61-73.

Kaur N, Kaur B, Sirhindi G. Phytochemistry and pharmacology of *Phyllanthus niruri* L.: a review. Phytother Res. 2017;31(7):980-1004.

Kulhankova A, Cahlikova L, Novak Z, Macakova K, Kunes J, Opletal L. Alkaloids from *Zephyranthes robusta* Baker and their acetylcholinesterase- and butyrylcholinesterase-inhibitory activity. Chemist Biodivers. 2013;10(6):1120-7.

Mabry TJ, Markham KR, Thomas MB. The systematic identification of flavonoids. Berlin: Springer-Verlag; 1970.

Mao X, Wu, LF, Guo HL, Chen WJ, Cui YP, Qi Q, *et al*. The genus *Phyllanthus*: an ethanopharmacological, phytochemical, and pharmacological review. Evidence-Based Complementary Altern Med. 2016;2016(art 7584952):1-36.

Mariani E, Polidori MC, Cherubini A, Mecocci P. Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. J Chromatograph B: Anal Technol Biomed Life Sci. 2005;827(1):65-75.

Nara K, Miyoshi T, Honma T, Koga H. Antioxidative activity of bound-form phenolics in potato peel. Biosci, Biotechnol, Biochem. 2006;70(6):1489-91.

Omar RA, Chyan YJ, Andorn, AC, Poeggeler B, Robakis NK, Pappolla MA. Increased expression but reduced activity of antioxidant enzymes in Alzheimer's disease. J Alzheimer's Dis. 1999;1(3):139-45.

Perry EK, Pickering AT, Wang WW, Houghton PJ, Perry NS. Medicinal plants and Alzheimer's disease: from ethnobotany to phytotherapy. J Pharm Pharmacol. 1999;51(5):527-34.

Quirion R. Cholinergic markers in Alzheimer's disease and the auto-regulation of acetylcholine release. J Psychiatr Neurosci. 1993;18(5):226-34.

Rao KS, Sino D. Fatty acid composition of 20 lesser-known Western Australian seed oils. J Sci Food Agricult. 1992;58(4):585-7.

Rhee IK, Van De Meent M, Ingkaninan K, Verpoorte R. Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. J Chromatograph A. 2001;915(1/2):217-23.

Sarg T, Abdel-Ghani A, Zayed R, El-Sayed M. Bioactive compounds from *Phyllanthus atropurpureus*. J Nat Prod. 2012;5:10-20.

Saxena VK, Aprajita G. Isolation and study of the flavone glycoside; Luteolin-7-O-β-D-Glucopyranoside from the seeds of the *Capparis decidua* (Forsk). Int J Chem Sci. 2008;6(1):7-10.

Subraya CK, Satyanarayana D, Yamini, S. Isolation and characterization of steroids and a triterpenoid from *Phyllanthus lawii*. Pharma Chem. 2013;5(5):63-7.

Received for publication on 31st January 2018
Accepted for publication on 12th June 2018