Research Article

Bioactive Metabolites from Propolis Inhibit Superoxide Anion Radical, Acetylcholinesterase and Phosphodiesterase (PDE4)

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ABSTRACT

Cycloartane-triterpenes (cycloartenol, 3α-cycloartenol-26-oic acid and 3β-cycloartenol-26-oic acid) together with α-amyrin acetate and flavonoids (pinostrobin, tectochrysin and chrysin) were isolated from Egyptian propolis for the first time. Their antioxidant activity was evaluated with DPPH and superoxide anion radical ($O_2^{\cdot-}$). All compounds possessed both ($O_2^{\cdot-}$) scavenging as well as XOD inhibitory activity in the range of 50 – 75 %. With DPPH, only the flavonoids showed scavenging activity (48 – 83 %). Acetylcholinesterase (AChE) and phosphodiesterase type 4 (PDE4) inhibitors are currently considered as intracellular targets for treatment of Alzheimer's disease and Chronic obstructive pulmonary disease (COPD). 3β-cycloartenol-26-oic acid moderately inhibited AChE and PDE4 activities *in vitro* with IC_{50} values of 0.8±0.2 and 1.9±0.4 µM, respectively, while 3α-cycloartenol-26-oic acid inhibited AChE activity with an IC_{50} value of 2.1±0.1 µM. The flavonoids pinostrobin and chrysin reduced PDE4 activity by 43 and 40 %, respectively (10 µM) as well as moderately inhibited the growth of the HepG2 cell line, whereas chrysin reduced proliferation of NIH-3T3 cells at 50 µM. Therefore, our results with 3β- and 3α-cycloartenol-26-oic acids can contribute to further research on alternative drugs for the treatment of neurological and neurodegenerative diseases, as well as asthma and COPD.

Keywords: Propolis, Acetylcholinesterase (AChE), Phosphodiesterase (PDE4), Cycloartane triterpenes, Radical scavenging activity.

INTRODUCTION

Alzheimer's disease (AD) is characterized as a progressive neurodegenerative disorder and considered as prominent cause of dementia in the elderly. The treatment of AD is a clinical challenge. AD is estimated to account for 50-60% of dementia cases in persons over 65 years of age.[1] One important change observed in the brain of AD patients is a decrease in level of the neurotransmitter acetylcholine (ACh) by nearly 90%, which affects behavioral aspects and causes impairment in cognitive function.[2] Acetylcholinesterase hydrolyzes the neurotransmitter acetylcholine and is a major therapeutic target for the symptomatic treatment of Alzheimer's disease. Oxidative balance is emerging as an important issue in understanding the pathogenesis of Alzheimer's disease.[3] Examination of Alzheimer's disease brain has demonstrated a great deal of oxidative damage, associated with both hallmark pathologies (senile plaques and neurofibrillary tangles) as well as in normal appearing pyramidal neurons.

COPD is a major cause of morbidity and mortality and is currently the fourth most common cause of death in the world according to the World Health Organization (WHO). The WHO estimates that by 2020, COPD will be the third leading cause of death and the fifth leading cause of disability worldwide.[4]

The selective targeting of phosphodiesterases type 4 (PDE4) has been actively pursued as a novel therapeutic approach in the treatment of respiratory diseases associated with inflammatory processes, such as asthma and COPD. PDE4 represents the major class of PDEs expressed in human inflammatory cells and in particular in macrophages and neutrophils, the main cell types present in the lungs of COPD patients.[5] The ability of compounds to inhibit PDE4 enzymatic activity is correlated with their anti-inflammatory potency.[6] PDE4 catalyzes the cleavage of the second messenger cyclic adenosine monophosphate, (cAMP) to AMP, and plays a role in anti-inflammatory responses.[7]

Oxidative stress is an important feature in the pathogenesis of COPD. Targeting oxidative stress with antioxidants or boosting the endogenous levels of antioxidants is likely to be beneficial in the treatment of COPD.[8]

Bioflavonoids possess both antioxidant and anti-inflammatory properties and hence may influence chronic inflammatory diseases such as COPD.[9] Triterpenoids exhibited several types of pharmacological activities including improvements of some central nervous system disorders.[10]

Propolis is a resinous hive product collected by the bees, rich in bioflavonoids and terpenoid compounds.[11,12] Propolis has antioxidant[12], anti-inflammatory[13], antibacterial, antifungal[11], antiviral[12] and antitumor activities.[14]

The aim of the present study was to reveal further interesting bioactivities including the search for potential xanthine oxidase; acetylcholinesterase and phosphodiesterase type 4 inhibitors from Egyptian propolis.

MATERIALS AND METHODS

General

NMR spectra were measured on a Bruker DRX 500 spectrometer (*Bruker Biospin*, Rheinstetten, Germany). ^{1}H- and ^{13}C-NMR and HMBC spectra were measured using an inverse-detection probe (5 mm). Operating frequencies were 500.13 MHz for acquiring ^{1}H-NMR and 125.75 MHz for ^{13}C-NMR spectra. Samples were measured at 300 K in CD_3OD with TMS as an internal standard. Electrospray ionization mass spectra (ESIMS) (positive mode) were measured with a Hewlett Packard (Avondale, PA, USA). Silica gel (0.063 – 0.200 mm) and Sephadex LH-20 were used for column chromatography (CC). Silica gel F-254 was used for analytical thin layer chromatography.

Propolis

Egyptian propolis was collected from Gharbia province of east area of Nile Delta, Egypt. The sample was collected during March 2010.

Extraction and isolation

Propolis (1 kg) was cut into small pieces and extracted with distilled water (2 L x 3) each for 2 hours at 85 °C (PWE), the residue was extracted with 70% ethanol (2 L x 3) under reflux for 2 h (PEF 70%). The residue was again extracted with absolute ethanol (2 L x 3) under reflux condition for 2 h to give propolis ethanol fraction (PEF, 50 g) after evaporation. The PEF gave a higher yield than the two other extracts (10 g PWE and 8 g PEF 70%).The PEF (50 g) was subjected to Sephadex LH-20 column chromatography (100 x10 cm). Stepwise gradient elution was carried out using a solvent system of decreasing polarity starting with 100% water, water–methanol and then methanol-methylene chloride. Fractions of 500 ml were collected and investigated by TLC (silica gel DF 245). Chromatograms were visualized under UV light before and after exposure to 50% sulfuric acid in methanol. Similar fractions were combined and concentrated to dryness under reduced pressure to obtain four main fractions. These fractions were further purified on silica gel columns. Two terpenoid fractions were obtained, the 1st was eluted with n-hexane to give the triterpenoid **4** (α–amyrin acetate, 10 mg) and the 2nd eluted with toluene: ethyl acetate (9: 0.5) to give the triterpenoids **1** (3β-cycloartenol, 24 mg), **2** (3α-cycloartenol-26-oic acid, 20 mg**)** and **3** (3β-cycloartenol-26-oic acid, 18 mg). Two flavonoid fractions were eluted with petroleum ether: ethyl acetate (8:2) and petroleum ether: ethyl acetate (6:4) to give three flavonoids: **5** (pinostrobin, 12 mg), **6** (tectochrysin, 5 mg) and **7** (chrysin, 9 mg).

3β-Cycloartenol (1): ^{1}H-NMR (500 MHz, $CDCl_3$)showed a cyclopropyl methylene (δ 0.31 and 0.55 d, J = 4.1 Hz) of cycloartane-triterpene, methyl groups at δH 0.81-(s,3H-30), δH-0.86-(s, 3H-21) δH-0.88 (s, 3H-28), δH 0.96-(d, J = 6.3 Hz, 3H-29), and one olefinic proton at δH 5.12 (t, J = 7.0 Hz), signal of hydroxymethine proton at δH 3.3 (dd, J = 11.0, 3.0 Hz). ^{13}C-NMR (125 MHz, CDCl3) seven methyl groups at δ 14.01 (C-30) δ 17.64 (C-27), δ 18.04 (C-18), δ 18.21 (C-21), δ 19.31 (C-28), δ 25.44 (C-29), δ 25.72 (C-26), and double bond appeared at δ125.0 (C-24), δ130.9 (C-25) with hydroxymethine at δ 78.25 (C-3) and the characteristic signals for cyclopropane at δc 29.3(C–19). EIMS showed a molecular ion at m/z 426 suggested a molecular formula of $C_{30}H_{50}O$.

3α-Cycloartenol-26-oic acid (2): The MS spectrometry of compound **2** showed a molecular ion peak at m/z 456 with 30 mass unit differences from compound **1**. The comparison of ^{1}H-NMR and ^{13}C-NMR spectra of compound **2** with compound **1** showed a close similarity except for downfield shift for the olefinic double bond by δΔ 1.53 and δΔ 17.10 in the proton and carbon respectively suggested withdrawing neighbor group. From the mass difference with compound **1** and the additional signal in ^{13}C-NMR at δ 168.8 for compound **2** assuming the presence of carboxylic group. The position of the carboxyl group was determined by HMBC correlation of the 3H-27(δ 1.74) with C-25, C-24 and C-27which indicates to the carboxylic group at C-26 position. The above finding confirmed the proposed structure is 3α-Cycloartenol-26-oic acid.[15,16]

3β-Cycloartenol-26-oic acid (3): The EI/ MS of compound **3**, exhibited the molecular ion peak at m/z 456 suggesting the similarity of compound **2**. The Comparison of ^{1}H-NMR and ^{13}C-NMR spectra of compound **3** with **2** showed a complete agreement except for the configuration of hydroxyl group at C-3 position. This was deduced from upfield shift in ^{1}H-NMR H-3 by δΔ 0.13 while downfield shift of C-30 at ^{13} C- NMR by δΔ 6.30. The above Data confirmed the proposed structure as 3-βCycloartenol-26-oic acid.[15,16]

Alpha-amyrin acetate (4): ^{1}H-NMR (500 MHz, CDCl3) δ 0.73-(s, 3H-28), δ 0.77(s, 3H-24), δ 0.82-(s, 3H-29), δ 0.91-(s, 3H-30), δ 0.97-(s, 3H-23), δ 1.05-(s, 3H-26), δ 1.06-(s, 3H-25), δ 1.20-(s, 3H-27), δ 1.99-(s, Ac), one olefinic proton observed at δ 5.05 (1H, t, J = 4.0 Hz, H-12), and oxymethine at δ 4.51(1H, dd, J = 5.1; 11.2 Hz, H-3).^{13}C NMR (125 MHz, CDCl3) confirmed the presence of nine methyl group at δ 16.75-(C-25), δ 16.88-(C-24) δ 17.51-(C-26), δ 18.26(C-29), δ 21.32(C-30) δ 23.24-(C-27), δ 28.08-(C-28), δ 28.08-(C-23), and the double bond at δ 124.33 (C-12), δ 139.64 (C-13) with oxymethine at δ 80.90 (C–3), and presence of a signal at δ171.00 suggest an acetyl group.

Pinostrobin (5): Electrospray ionization mass spectrometry (ESI-MS) m/z 271 $[M+H]^+$, ^{1}H-NMR (DMSO-d6) exhibited a flavonoid pattern and showed signals at δ 12.1(1H, s, 5-OH),7.53 (2H,br.d, J = 7.5, ,H-2`,H-6`), δ 7.56 (3H, m, H-3`,H-4`, H-5`) ,δ 6.12 (1H ,d , J = 2.7 Hz, H-6), δ 6.16 (1H,d, J = 2.7 Hz, H-8), δ5.63 (1H, dd, J =12.8,2.7, Hz, H-2), δ3.8 (3H, s,7-OMe), δ 3.06 (1H, dd, J =15.1,12.8 Hz, H-3a), δ2.85 (1H, dd, J=14.6, 2.7 Hz, H-3b).$^{13}$C-NMR : δ196.5 (C-4), δ 167.4 (C-7), δ163.18 (C-9), δ162.63 (C-5), δ 138.5 (C-1`), δ 128.5 (C-4`), δ 128.6 (C-5`,C-3`), δ 126.63 (C-6`,C-2`), δ 102.6 (C-10), δ 94.75 (C-8),

δ 93.87(C-6), δ 78.54(C-2), δ 55.92 (C-7-OMe), δ 42.11(C-3).

Tectochrysin (6): ESI-MS m/z 269 $[M+H]^+$.^{1}H-NMR (DMSO-d6): δ 12.8 (1H ,s,5-OH) , δ8.1(2H ,d, J=7, H-2`,H-6`), δ 7.59 (3H, m, H-3`,H-4`,H-5`) , δ7.07(1H, s, H-3), signal at δ 6.84 (1H,d , J = 2 Hz, H-8), δ 6.4 (1H ,d , J =2.5 Hz, H-6), δ3.9(3H , s,7-OMe) ; $^{13}$C-NMR : δ182.1 (C-4), δ165.36 (C-2), δ163.46 (C-7), δ161.17 (C-5), δ157.38 (C-9), δ 132.16 (C-4`), δ 130.58 (C-1`), δ 129.15 (C-3`, C-5`), δ 126.46 (C-2`, C-6`), δ 105.35 (C-3), δ 104.93 (C-10), δ 98.17 (C-6), δ 92.83 (C-8), δ 56.11 (C-7-OMe).

Chrysin (7): ESI-MS m/z 255$[M+H]^+$.^{1}H-NMR (DMSO-d6): δ 12.84 (s, OH-5), δ 8.09 (d, J = 7 Hz, H-2′; H-6′), δ 7.59 (m, H-3′, H-4′, H-5′), δ 7.04 (s, H-3), δ 6.86 (d, J = 1.5, H-8), δ 6.46 (d, J = 1.5, H-6). ^{13}C-NMR: δ 181.9 (C-4), 164.5 (C-2), 163.1 (C-7), 161.4 (C-5), 157.4 (C-9), 132.0 (C-4′), 130.7 (C-1′), 129.1 (C-3′, C-5′), 126.4 (C-2′, C-6′), 105.2 (C-3), 104.0 (C-10), 99.0 (C-6), 94.1 (C-8).

DPPH radical scavenging activity

DPPH radical scavenging activity of compounds (1-7) was analyzed according to a modified procedure of Matsushige*et al.*[17] 1 ml of methanol solution for each compound (50µg/ml) was added to 1 ml of methanol solution of DPPH (60µM). The prepared solutions were mixed and left for 30 min at room temperature. The optical density was measured at 520 nm using a spectrophotometer (UV-1650PC Shimadzu, Japan). Mean of three measurements for each compound was calculated.

Superoxide anion scavenging activity.

Superoxide anion scavenging activity was determined according to a modified method of Matsushige*et al.*[17] Reaction mixtures containing 1.4 mL of 50 mM Na_2CO_3 (pH 10.2), 100 µL of 3 mM xanthine, 100 µL of 3 mM EDTA, 100 µL of BSA (1.5 mg/mL), 100 µL of 75 mM Nitro blue tetrazonium, and 50 µL of each compound (50µg/ml) were preincubated at 30 °C for 10 min, and 50 µL of xanthine oxidase (0.3 unit/mL) was added. After incubation at 30 °C for 20 min, 200 µL of 6 mM $CuCl_2$ was added to stop the reactions and the absorbance was measured at 560 nm.

Acetylcholinesterase (AChE) inhibition assay

AChE activity was measured by adapting the colorimetric assay described by Ellman*et al.*[18] for a microplate test system. As positive control the reversible and specific inhibitor huperzine at a concentration of 0.1 µM was used. The determined IC_{50} value of huperzinewas0.12 µM.

Phosphodiesterase (PDE4) inhibition assay

The activity of phosphodiesterase PDE4 was measured using the PDE Light HTS cAMPphosphodiesterase kit (catalogue number LT07-600, *Lonza*, Rockland, USA) according to the manufacturer's instructions. For the inhibition of PDE4 1µM and 10 µM of the competitive and reversible inhibitor rolipram were applied as positive control. The determined IC_{50} value of rolipram was 0.75 µM. The calculation of the IC_{50} values of the enzyme inhibitory activities was performed by using the software Graph Pad Prism 5 (Graph Pad software, La Jolla, USA).

Cytotoxic assays (the CellTiter-Blue® Cell Viability Assay)

The sensitivity of the cell lines HepG2 and NIH-3T3 (Mouse embryonic fibroblast cell line) to the isolated compounds was evaluated by monitoring the metabolic activity using the CellTiter-Blue® Cell Viability Assay (*Promega*, Mannheim, Germany). The human hepatocellular carcinoma cell line HepG2 was obtained from the German Collection of Microorganisms and Cell Cultures (*DSMZ*, Braunschweig, Germany). The mouse fibroblast cell line was kindly provided by G. Rimbach (University of Kiel, Germany). HepG2 and NIH-3T3 cells were maintained in RPMI 1640 medium. Media were supplemented with 10% foetal bovine serum (*Promocell*, Heidelberg, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (*Invitrogen*, Carlsbad, CA, USA). The cultures were maintained at 37°C under a humidified atmosphere and 5% CO_2. The cell lines were transferred twice every 3 or 4 days and the culture medium changed 48 hours after transfer. For experimental procedures, cells were seeded in 96-well plates at concentrations of 10,000 cells (HepG2) and 7,500 cells (NIH-3T3) per well. After 24 h incubation the medium was removed and 100 µl of the test sample adjusted to final concentrations of 10 and 50 µM by diluting in growth medium were added to the cells. Each sample was prepared in triplicate. 25 µM Tamoxifen, as a standard therapeutic drug, was applied as positive control. Following compound addition, plates were cultured for 24 h at 37°C. Afterwards, the assay was performed according to the manufacturer's instructions. Cells were incubated for 2 h at 37°C. Fluorescence was measured using the microplate reader Infinite® M200 (*Tecan*, Männedorf, Switzerland) at excitation 560 nm and emission 590 nm.

RESULTS

Structure determination of the isolated compounds

Based on 1D, 2D-NMR spectral data and mass spectrometry compound **1** was identified as 3β-cycloartenol.[19] The structures of compounds **2** and **3** were established by comparison of their spectroscopic data with compound **1**. The MS of **2** and **3** exhibited to m/z at 456 with 30 mass unit difference from compound **1**. The analysis of ^{1}H and ^{13}C spectroscopic data confirmed the structure of compounds **2** and **3** as 3α-cycloartenol-26-oic acid and 3β-cycloartenol-26-oic acid respectively and their data are in accordance with the reported literature.[15,16] The MS and ^{13}C-data of compound **4** are in agreement with α-amyrin acetate structure.[19] The flavonoid compounds **5**, **6** and **7** were identified by UV, MS and NMR spectroscopic data as pinostrobin, tectochrysin and chrysin respectively[20], (Fig. 1).

The DPPH free radical scavenging activity

Tectochrysin and chrysin showed inhibition of **83** % and 74 % respectively, while pinostrobin showed moderate inhibition of 48 % (Fig. 2)**.** The triterpenoid compounds (1-4) showed no activity.

Scavenging ability for superoxide anion radical

The free radical scavenging activity on superoxide anion radical generated by an enzymatic method (X-XOD system) was evaluated. Compounds **2** and **3** were the most active compounds (68 and 75 % scavenging activity at a concentration of 50 µg/ml, while the other isolated compounds revealed moderate antioxidant activity of 50 - 59 % (Fig. 2).

Acetylcholinesterase (AChE) inhibition

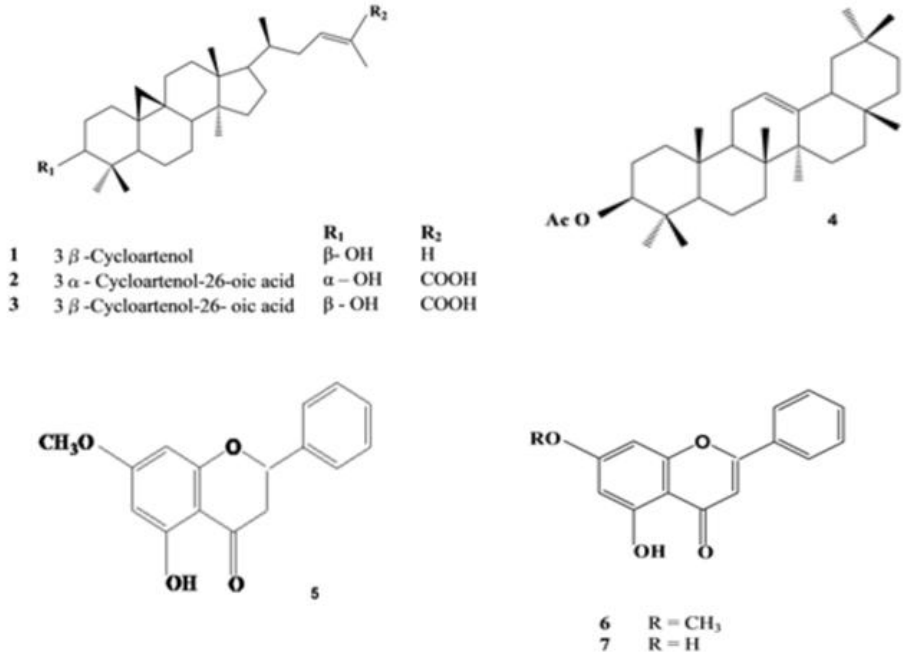
Inhibitory activity of the compounds isolated from Egyptian propolis on the recombinant enzyme AChE was analyzed. All flavonoid compounds as well as α-amyrin acetate (compounds **4-7**) showed no inhibitory activity of AChE, while the cycloartane-triterpenoid compounds (compounds **1-3**) inhibited acetylcholinesterase (AChE) in the range of 60 – 92 %, at a concentration of 10 µM (Fig. 3). Compound **3**exhibited the highest activity with an IC_{50} = 0.8 ±0.2µM.

Phosphodiesterase (PDE4) inhibition

The isolated compounds were evaluated toward the enzyme PDE4. Compounds **1**, **4** and **6** showed no inhibitory activity of PDE4, while compounds **3, 5** and **7** showed significant inhibition of PDE4 in the range of 40 – 75 %, at a concentration of 10 µM (Fig. 3). Compound**3** had the highest activity with an IC_{50} value of1.9±0.4 µM.

Evaluation of potential cytotoxicity

The flavonoids pinostrobin and chrysin showed moderate anti-proliferative activity against HepG2 cell line (at 50 µM). Chrysin showed a significant activity than that of pinostrobin (46 %, Fig. 4), while the terpenoid compounds showed no activity. Finally, the flavonoid chrysin is the only compound showed weak growth inhibitory activity against the NIH-3T3 cell line (31%, at 50 µM) (Fig. 4).



		R_1	R_2
1	3 β -Cycloartenol	β- OH	H
2	3 α - Cycloartenol-26-oic acid	α – OH	COOH
3	3 β -Cycloartenol-26- oic acid	β - OH	COOH

Figure 1: Chemical structures of the isolated compounds from Egyptian propolis.

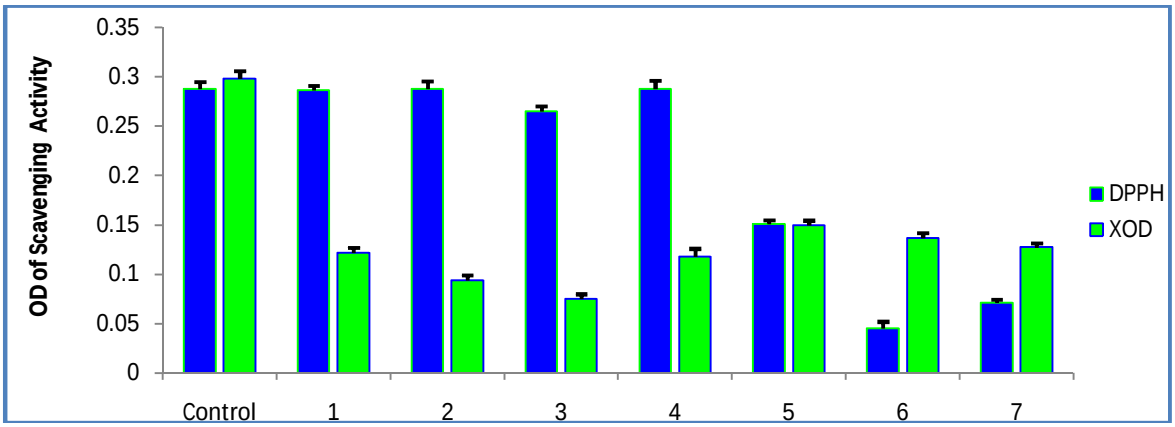


Figure 2: Free radical scavenging activity of propolis compounds (1-7) in the DPPH radical and the xanthine- XOD assays. Values are expressed as mean ± SD, n = 3 at a concentration of (50 µg/ml for all tested compounds).

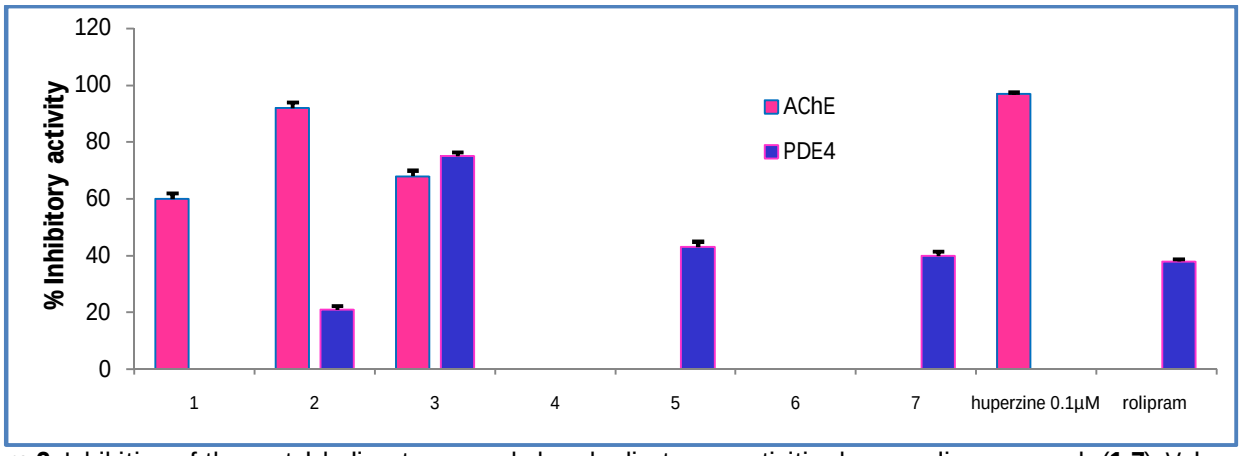


Figure 3: Inhibition of the acetylcholinesterase and phosphodiesterase activities by propolis compounds (**1-7**). Values are expressed as mean ±SD, n = 3 at a concentration of 10 µM including the positive controls rolipram (10 µM) and huperzine (0.1 µM).

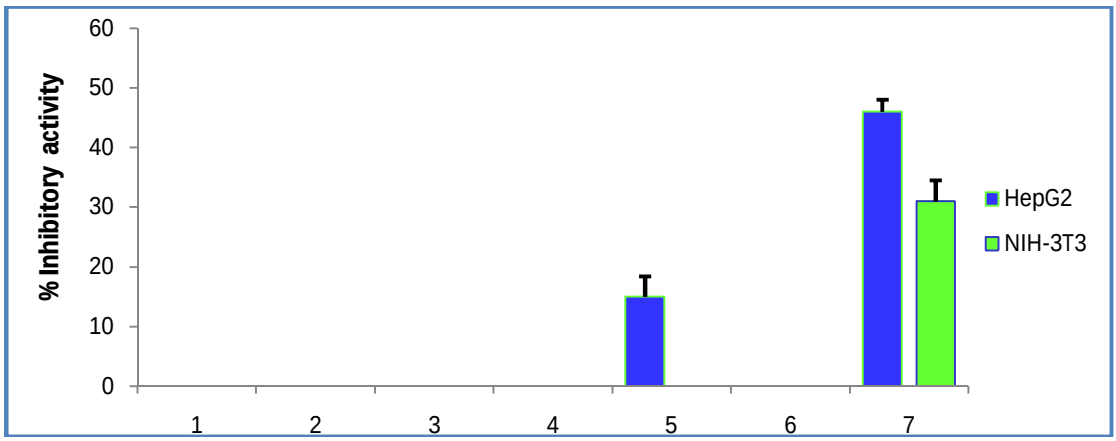


Figure 4: Cytotoxic activity of propolis isolated compounds (1-7) against HepG2 and NIH-3T3 cell lines). Values are expressed as mean ±SD, n = 3 at a concentration of 50 µM.

DISCUSSION

AChE inhibitors have received considerable attention as alternatives in treatment of Alzheimer's disease. PDE4 inhibitors appear to confer benefit in improving lung function. The damage caused by reactive oxygen species is considered a contributing factor to several diseases including AD[3] and COPD.[8] Bioflavonoids possess both antioxidant and anti-inflammatory properties and hence may influence chronic inflammatory diseases such as COPD.[9]

Work on bioactive compounds from Egyptian propolis has led to the isolation and structure elucidation of several exciting phytoconstituents having significant AChE and PDE4 inhibitory activity, along with mild anti-proliferative effect against HepG2 and NIH-3T3 cell lines (Figs. 3, 4).

In this study, the isolated flavonoids and triterpenoids were evaluated for free radical scavenging activity by DPPH and xanthin-xanthin oxidase assays. With DPPH, only the flavonoids showed highly significant scavenging activity (48 – 83 %). All the isolated triterpenoids and flavonoids showed significant superoxide anion ($O_2^{\bullet -}$) scavenging activity within the range of 50 – 75 %.

Superoxide anion is the first generated reactive oxygen species (ROS) after oxygen enters living cells. It was once considered to be highly deleterious to cell functions and aging. Superoxide anion and uncoupling proteins are linked to Alzheimer's disease in mitochondria. Simultaneous disorders of superoxide and uncoupling proteins create the conditions for neuronal oxidative damages. On the one hand, sustained oxidative damage causes neuronal apoptosis and eventually, accumulated neuronal apoptosis, leading to exacerbations of Alzheimer's disease.[21] It was also demonstrated that inhibition of superoxide anion production in human neutrophils by isopedicinflavanone is associated with an elevation of cellular cyclic adenosine 3,5′-monophosphate (cAMP) and activation of protein kinase A (PKA) through its inhibition of cAMP-specific PDE.[22]

In this context, a previous study showed that the treatment with the antioxidants vitamins E and C significantly decreased the action of AChE activity in hippocampus of female adult rats.[23] This demonstrates the high potential of the cycloartane-triterpenoid compounds (**1-3),** which significantly inhibited the action of AChE besides their significant antioxidant activity. In another study, moderate inhibitory activity of AChE by cycloartenol was found.[10]Thetriterpenoid3α-cycloartenol-

26-oic acid **(2)** and 3β-cycloartenol-26-oic acid **(3)** showed the highest significant inhibitionto AChE activity with IC_{50} values of 2.1±0.1 and 0.8±0.2μM and antioxidant activity 68 and 75% at concentration of 50 μg/ml, respectively. 3β-cycloartenol**(1)** showed moderate inhibition to AChE with an IC_{50} value of 3.6±0.1 μM as well as antioxidant activity of 59% (Figs. 2, 3).

On the other hand, some of the isolated flavonoids and triterpenoids showed PDE4 significant inhibitory activities; compound **(3)** (3β-cycloartenol-26-oic acid) and the flavonoids **(5)** and **(7)** (pinostrobin and chrysin) by (75, 43 and 40 %, respectively at 10 μM). Our results are in agreement with some previous studies, which found that luteolin flavone non-selectively and competitively inhibited PDE4[24] and biochanin A has the potential for treating allergic asthma and COPD.[25] Also the flavonoid dioclein possesses significant antioxidant and PDE4 inhibitory activity.[26] The triterpenes, betulinic and sericic acids, exhibited mild inhibitory activity on the isolated PDE4 isozyme.[27] Ursolic acid inhibited PDE4 activity with an IC_{50} of 51.21μM.[28]

PDE4 inhibitors act by increasing intracellular concentrations of cAMP, which has a broad range of anti-inflammatory effects on various key effector cells involved in asthma and COPD. Raising cAMP in neutrophils inhibits superoxide anion ($O_2^{\bullet-}$) production.[29] It was suggested that PDE4 inhibitors decreased N-Formyl-Methionine-Leucine-Phenylalanine (fMLP)-induced ($O_2^{\bullet-}$) release in bronchoalveolar lavage (BAL) cells enriched in neutrophils but not in macrophages, through p44/42(MAPK) activation by a cAMP- and a PKA-independent mechanism.[30]

The above mentioned data support our results, as it is clear that all the compounds had superoxide anion ($O_2^{\bullet-}$) scavenging activity (Fig. 2), but some of them showed inhibitory activity of PDE4 (Fig. 3), which could mean that not all the compounds have the potential to be developed into useful new therapeutic agent for treating neutrophilic inflammatory diseases. Also, it is clear that the pure isolated compounds had mild anti-proliferative effect.

CONCLUSION

It is the first time to reveal that3β-cycloartenol-26-oic acid (**3**) strongly inhibited acetylcholinesterase and phosphodiesterase activities with IC_{50} values of 0.8±0.2 and 1.9±0.4 μM respectively, while 3α-cycloartenol-26-oic acid (**2**) showed the highest inhibition of AChE activity with an IC_{50} value of 2.1±0.1 μM. Both compounds exhibited additional antioxidant activity; beside they mildly inhibit proliferation of the tested cell lines. These results encourage further studies to proof their possible application as alternative drugs for the treatment of neurological and neurodegenerative diseases, asthma and COPD.

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