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Inhibitory effect of some natural and semisynthetic phenolic lipids upon acetylcholinesterase activity

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Abstract

The effect of phenolic lipids isolated from rye grains and cashew nut shell liquid (CNSL) from *Anacardium occidentale* and their semisynthetic derivatives on erythrocyte ghost's acetylcholinesterase activity was studied. It has been shown that all tested compounds decreased the enzymatic activity of acetylcholinesterase. This effect depends on the type of studied compounds. Three of them completely inhibit acetylcholinesterase activity at the micromolar concentration.

Keywords: Acetylcholinesterase; Erythrocyte ghosts; Phenolic lipids

1. Introduction

Acetylcholinesterase (AChE) plays an essential role in acetylcholine-mediated neurotransmission. It is present in cholinergic synapses in the central nervous system and in neuromuscular synapses where it rapidly hydrolyses acetylcholine. AChE is responsible for preventing re-excitation, after stimulated cells have recovered from a first action potential (Tougu, 2001). From a toxicological and a pharmacological point of view, AChE is a target for various cholinergic toxins, such as natural snake venom and plant glycoalkaloids, and also for therapeutically active compounds. There is growing evidence that AChE could participate in the pathological processes in Alzheimer's disease, such as β-amyloid formation or deposition (Benzi & Moretti, 1998). In this way, AChE inhibitors may modulate the processing of β-amyloid protein, thus reducing the deposition of β-amyloid itself (Nitsch, Slack, Wurtman, & Growdon, 1992).

Acetylcholinesterase has also been found on mammalian erythrocytes and in other organs, although the physiological function of AChE in these tissues is still unknown. Acetylcholinesterase in blood cells is biochemically identical with the enzyme, which occurs in neurons and reveals lower individual dispersion and also higher resistance towards external factors. AChE is located on the surface of erythrocytes, with the active site oriented towards the outside of the cell, whereas in the lipid bilayer it is anchored via a carboxy-terminal binding domain (Boschetti & Brodbeck, 1996). In previous studies, the correlation between AChE inhibition in blood and its inhibition in target tissues has been shown (Kale, Rathore, John, & Bhatnagar, 1999). It is known that numerous compounds, e.g., aliphatic ketones (Pereira, Adams, & Silva, 2004), hydrogen peroxide (Schallreuter, Elwary, Gibbons, Rokos, & Wood, 2004), cholesterol and triglycerides affect the activity of AChE (Alcantara et al., 2002).

Phenolic lipids constitute a heterogeneous group of natural lipids that includes simple phenols and polyphenols, as well as their derivatives. Among single-ring compounds those considered as lipids are found specifically in some plants and contain a catechol, resorcinol or hydroquinone moiety alk(en)ylated by a multicarbon chain (Kozubek & Tyman, 1999). Phenolic lipids used in our present study were isolated from cashew nut shell liquid (CNSL) from

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Anacardium occidentale (cardol, methylcardol, cardanol (alkylphenol) and anacardic acid) (Fig. 1) and rye grain (homologues C15:0, C21:0 and C25:0) (Fig. 2). From a chemical point of view, cardol, methylcardol and homologues from rye grain are resorcinolic lipids (AR) and cardanol and anacardic acid belong to alkylphenolic lipids (APh). The semisynthetic derivatives of phenolic lipids were synthesised on the base of hydrocardanol (alkylphenol). All of them have 15 carbon atoms in a saturated side chain; the modifications concern the phenol ring. Derivative A is an acid with a carboxylic group attached to the phenol *via* a carbon atom and hydroxylic residue. Derivative A with addition of a choline residue forms derivative B. Derivative C is enriched with a sugar moiety, whereas derivative D possesses an extra side chain with 16 carbon atoms and a carboxylic group at the *meta* position. Finally, derivative E has three saturated side chains: the two extra chains have 16 atoms of carbon (Fig. 3).

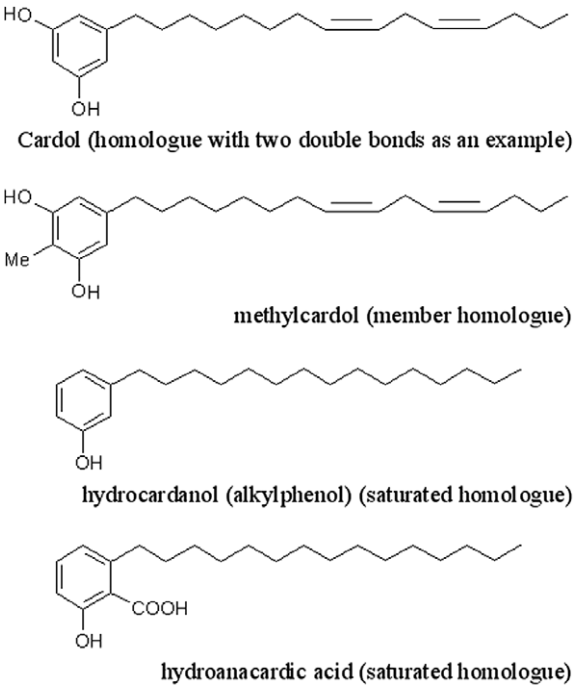

Fig. 1. The structures of resorcinolic and alkylphenolic lipids from *Anacardium occidentale*.

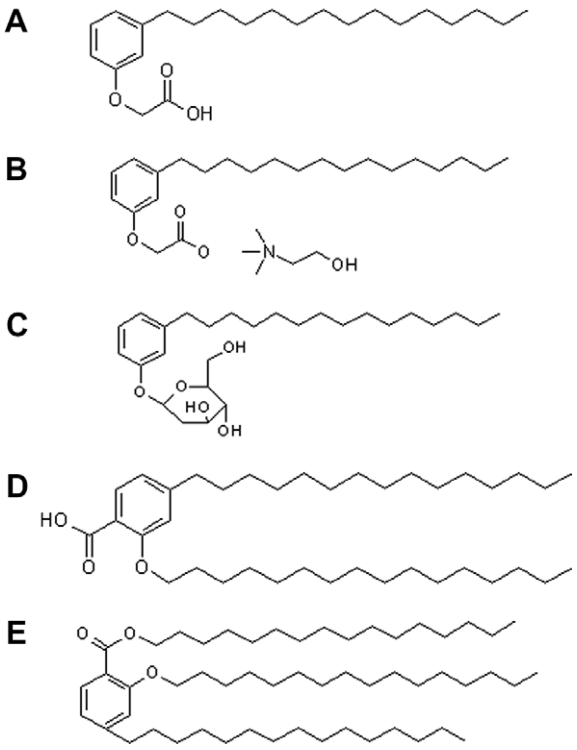

Fig. 3. The structures of the semisynthetic derivatives of phenolic lipid (cardanol).

CNSL (cashew nut shell liquid from *A. occidentale*) is a unique source of materials useful both for industrial technology, in semi-synthesis and for biological/pharmaceutical applications. Technical CNSL without separation is widely used in the production of friction dusts for the automobile industry and in certain polymeric/surface coating applications. Cardanol separated from technical CNSL has uses in semi-synthesis, e.g., in the formation of polyethoxylate surfactants, chelators for metals and for boron. Natural CNSL, and anacardic acid separated from it, have potential industrial applications in semi-synthesis and in biological studies. Cardol separated, either from technical or natural CNSL, also has potential use in semi-synthesis, and its homologues, more recently, as a potential biological marker (Kozubek & Tyman, 1999). From the biological point of view anacardic acid acts as a potent inhibitor of histone acetyltransferase, and also inhibits the activities

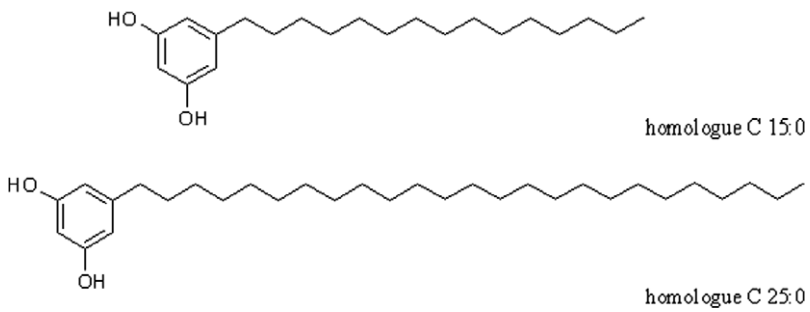

Fig. 2. The structures of resorcinolic lipids from rye grain (C15:0 and C25:0 as an example).

of prostaglandin synthase and lipoxygenase (Ha & Kubo, 2005). It also has anti-microbial property (Muroi & Kubo, 1996). Cardanol derivatives were found to show antibacterial, antifungal, antioxidant, and antitumour activities (Chen et al., 1998), without any appreciable mutagenic, carcinogenic, and cocarcinogenic activities (Kozubek & Tyman, 1999).

Resorcinolic lipids are present, apart from in many higher plants, in bacteria, fungi, algae, and mosses (Kozubek & Tyman, 1999). They are most common in the bran of cereal grains, e.g., rye (*Secale cereale*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and millet (*Pennisetum thyphoides*) (Ross et al., 2003). Alkylresorcinols were reported to have antiparasitic, anticancer, antifungal, antimicrobial, and antioxidant effects. Their various biological activities suggest their involvement in the regulation of cell and organism metabolism (Wieringa, 1967; Kozubek & Tyman, 1999).

Alkylresorcinols contained in the diet have been found to be absorbed by rats, pigs (Ross et al., 2003) and humans (Linko, Juntunen, Mykkänen, & Adlercreutz, 2005), but little is known about their metabolism or the final/resulting metabolites. In experiments provided by Ross et al. deconjugated human urine after a wheatbran-based meal was shown to contain two alkylresorcinol metabolites, 3,5-dihydroxybenzoic acid and 3-(3,5-dihydroxyphenyl)-1-propanoic acid, as well as smaller amounts of unchanged alkylresorcinols, confirming the hypothesis that alkylresorcinols are metabolised in humans via ω-and β-oxidation of their alkyl chain (Ross, Aman, & Kamal-Eldin, 2004).

Attempting to find other AChE inhibitors of plant origin, which might be of potential pharmacological interest, three representative homologues of resorcinolic lipids from rye grain (C15:0, C21:0 and C25:0), four compounds isolated from *A. occidentale* (cardol, methylcardol, alkylphenol and anacardic acid) and five semisynthetic derivatives of hydrogenated alkylphenol were tested for their AChE inhibitory activity.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide and DTNB (5,5′-dithiobis(2-nitro)benzoic acid) were purchased from Sigma–Aldrich Poznan, Poland. All other reagents used in the experiments were of analytical grade.

2.2. Isolation of phenolic lipids

Homologues of alkylresorcinol from rye grain were isolated chromatographically, according to the procedure described previously (Kozubek, 1985).

Cardol, methylcardol, alkylphenol (cardanol) and anacardic acid were isolated chromatographically from cashew nut shell liquid from *A. occidentale,* according to the procedure described previously (Przeworska, Gubernator, & Kozubek, 2001).

The semisynthetic derivatives of cardanol were synthesised at the Pharmaceutical Research Institute in Warsaw (Poland).

The purity of the tested compounds was assessed by HPLC and was above 98%.

For all experiments 5 mM stock methanolic (or butanolic in case of derivatives D and E) solutions of all investigated compounds were used.

2.3. Preparation of erythrocytes

Erythrocytes were isolated from sheep blood collected from randomly chosen individuals at the Department of Epizootiology, Wroclaw University of Environmental and Life Sciences. Blood was collected into buffered dextrose (ACD solution) and erythrocytes were separated by centrifugation at 650*g* for 10 min and subsequently washed three times with 0.9% NaCl buffered with 10 mM Tris–HCl, pH 7.4. Washed erythrocytes were resuspended in the same buffer, and used to preparation of ghosts.

2.4. Preparation of erythrocytes ghosts

Erythrocytes ghosts were prepared from sheep erythrocytes, according to the procedure described previously (Dodge, Mitchel, & Hanakan, 1963).

2.5. Estimation of acetylcholinesterase activity

Acetylcholinesterase activity in isolated erythrocyte membranes was assayed by the method of Ellman, Courtney, Andres, and Featherstone (1961), in which acetylthiocholine is used as the substrate and the product, thiocholine, after reaction with DTNB forming a yellow anion, 5-thio-2-nitrobenzoic acid, as the indicator of enzymatic activity.

Erythrocyte ghosts suspension was diluted with 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0) to the final working reagent (50% haematocrit).

To 3 ml of 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0) 50 μl of suspension of erythrocyte ghosts and DTNB with Na_2CO_3 were added (their final concentrations in the sample were 320 and 450 μmol/l, respectively). Subsequently, microlitre amounts of investigated compounds (concentration range from 10 to 100 μM) were added. After 5 min of preincubation with ghosts acetylcholine iodide was added to give a final concentration of 0.476 μM.

The time course of acetylthiocholine iodide hydrolysis was recorded spectrophotometrically (Shimadzu UV-2401 PC, UV–Vis recording spectrophotometer; Shimadzu, Kyoto, Japan) at 37 °C. The changes of absorbance at 412 nm of the samples were recorded continuously for 10 min.

Rate of the reaction (V) was calculated as follows

$$V = \frac{\Delta A/\min}{1.36 \times 10,000}\,[\mathrm{mol/l/min}]$$

where ΔA is the increase of absorbance in the sample in 10^{-1} min at 37 °C.

Enzyme activity modulated by studied compounds was calculated as a percentage, compared to the control reaction. The influence of solvent was considered in the control and blank samples.

3. Results and discussion

Alkylphenolic lipids (cardanol and anacardic acid) from CNSL (Fig. 1), resorcinolic lipids from rye grain (C15:0, C21:0 and C25:0 homologues) (Fig. 2), alkylresorcinols (cardol and methylcardol) (Fig. 1) and semisynthetic derivatives of alkylphenol (Fig. 3) were tested for their *in vitro* AChE inhibitory activity.

The results are expressed as graphs (Figs. 4–6) and IC_{50} values summarised in Table 1. These results suggest that the observed AChE inhibitory effect seems to be related to some structural characteristic among the different "hydrophilic head" types and length of alk(en)yl chains present in the molecule.

At the micromolar range of concentration all studied natural compounds inhibit sheep erythrocyte ghost acetylcholinesterase activity. The order of inhibitory potency of resorcinolic lipids from rye grain was as follows: C25:0 > C21:0 > C15:0 (Fig. 4). All tested compounds isolated from *A. occidentale* affect the AChE activity (Fig. 5). In the presence of all types of lipids from *A. occidentale* a decrease of AChE activity was observed. The most active of all tested natural compounds was cardol, which exhibits an inhibitory activity similar to that of merulinic acid isolated from *Merulius tremellosus* (heptadecenylresorcinolic acid, resorcinolic acid) (Stasiuk, Jaromin, & Kozubek, 2004). Both, merulinic acid and cardol were found to be almost twice as potent as anacardic acid from *A. occidentale* and three times higher than two homologues from rye grain.

The semisynthetic derivatives of cardanol with modification of the phenol ring (e.g., sugar moiety addition or supplementary side chain) were found to have similar inhibitory potency to natural alkylphenol. It suggests that modification only in length of side chain could have more pronounced effect in inhibitory activity of these molecules, as it is observed for homologues of resorcinolic lipids with sidechains of different lengths.

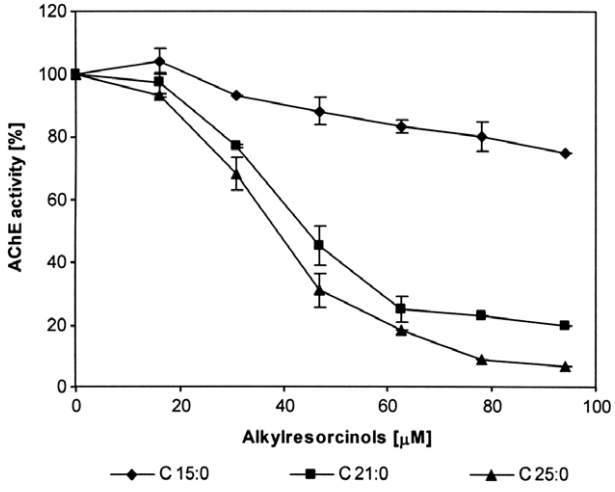

Fig. 4. Inhibitory effect of resorcinolic lipids from rye grains on acetylcholinesterase activity. Data are given as means ± SD of three individual determinations, each performed in triplicate (♦ – C 15:0, ■ – C 21:0, ▲ – C 25:0).

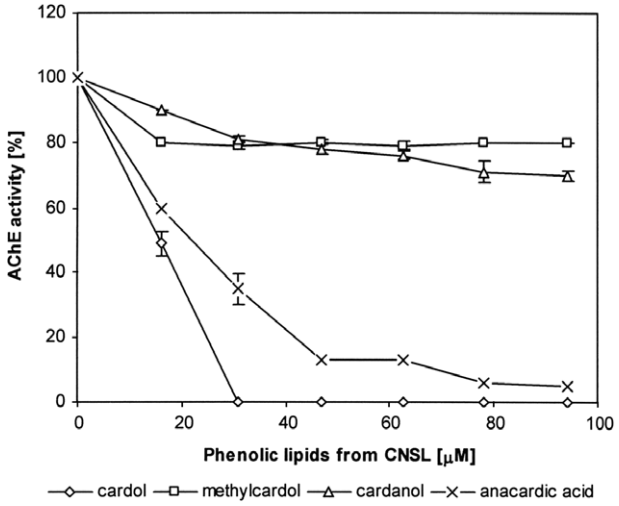

Fig. 5. Inhibitory effect of phenolic lipids (resorcinols and alkylphenols) from CNSL on acetylcholinesterase activity. Data are given as means ± SD of three individual determinations, each performed in triplicate (◇ – cardol, □ – methylcardol, △– cardanol, × – anacardic acid).

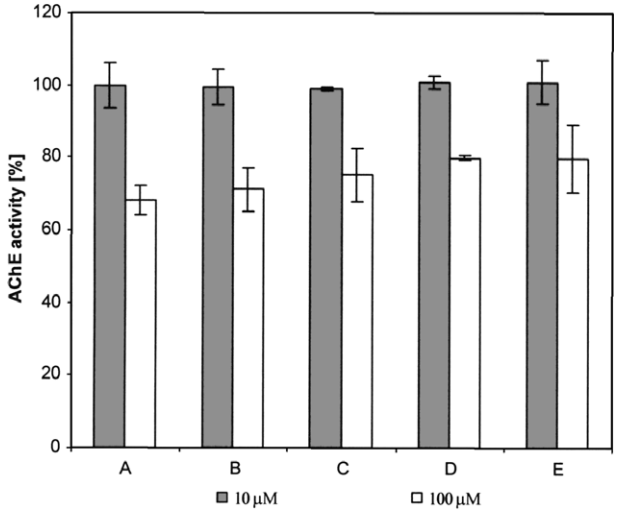

Fig. 6. The effect of semisynthetic derivatives of phenolic lipid (cardanol) on acetylcholinesterase activity (minimum and maximum used concentrations). Data are given as means ± SD of three individual determinations, each performed in triplicate.

Table 1
IC_{50} values of studied compounds affecting acetylcholinesterase activity

Studied compound	IC_{50}
AR C15:O	ND*
AR C21:O	44.5 μM ± 1.8
AR C25:O	38.5 μM ± 0.9
Cardol	15.5 μM ± 0.7
Methylcardol	ND*
Cardanol	ND*
Anacardic acid	22 μM ± 1.1
A	ND*
B	ND*
C	ND
D	ND*
E	ND*

Data are given as means ± SD of three individual determinations each performed in triplicate.
* ND, even at highest concentration tested only a part of AChE activity was inhibited (20–32%).

It is known that the changes in activity of AChE may result from direct binding of xenobiotic to enzyme, modifications of erythrocyte membrane, changes in electric charge of membrane layer and increase of reactive oxygen species (ROS), e.g., H_2O_2 (Bukowska & Hutnik, 2006). Previous studies showed that acute and chronic phenol intoxication induced changes in the AChE activity in carp (*Cyprinus caprio*) brain. These changes were provoked by oxidative stress but not by phenol intoxication (Kozlovskaya & Martemyanov, 1991). On the other hand it was found that no statistically significant changes in membrane erythrocytes' AChE activity, related to dose of phenol and catechol were observed (Bukowska & Hutnik, 2006). It may suggest that studied phenolic lipids act on AChE activity by the alteration of the distribution and the mobility of membrane phospholipids (fluidity of the bilayer). It is known that AChE activity is modulated by membrane hydrophobic environment and depends on membrane fluidity and surface charges (Tougu & Kesvatera, 2001). The greater membrane rigidity (caused by regular arrangement of phospholipid molecules, e.g., at the temperature below main phase transition temperature of phospholipids bilayer or in the case of imperfect mixing of variety kinds of phospholipids, when phase-separated domains with different packing of phospholipids molecules can coexist in lipid bilayer) induces increasing enzyme affinity towards the substrate (Piasecka, Leyko, Krajewska, & Bryszewska, 2000).

It was found that the activity of AChE depends on the electric charge of the membrane layer (Frenkel, Roelofsen, Brodbeck, van Deenen, & Ott, 1980). The increase of the electric charge of the membrane layer induces a rise of enzyme affinity towards the substrate. It was shown that incubation of erythrocytes with metmyoglobin and ferrylmyoglobin (with positive electric charge) affected non-specifically the increase of AChE activity (Sztiller & Puchala, 2003). The effect of tested phenolic lipids on AChE activity may be a result of both structural changes in the whole membrane or its partial or complete solubilisation and changes in the electric charge of the membrane layer. These interactions may involve not only the effect of the polar (hydrophilic) region of the bilayer modifier but also the effect of the hydrophobic alk(en)yl side chain. It is known that studied compounds show a high affinity for the lipid bilayer as well as for biological membranes. The incorporation of homologues into liposomal and biological membranes induces the increase of their permeability for small nonelectrolytes and cations (Kozubek & Demel, 1980). This increase in the permeability of membranes may result in the haemolysis of the cells (Stasiuk & Kozubek, 1997).

Our previous study showed that tested compounds induce increased permeability of liposomal vesicles. This effect was shown to be dependent on the composition both of the liposomal bilayer and the chemical structure of the lipid molecule (unpublished data). It is possible that tested compounds may act on erythrocyte ghost membrane and solubilise it. In this condition of microenvironment AChE may lose part of its enzymatic activity.

Some studied compounds have a chain of 15 carbon atoms in their molecules. These are resorcinolic lipids: C15:0 from rye grain with saturated chain, and cardol and methylcardol from *A. occidentale;* and alkylphenolic lipids: cardanol and anacardic acid, which are fractions of chromatographically separated CNSL containing three homologues, with one, two and three double bonds in the hydrocarbon chain, with the same kind of phenol ring. The semisynthetic derivatives of cardanol have also 15 carbon atoms in the sidechain, with modifications present only on the phenol ring.

Cardol completely inhibits AChE activity at a concentration of 31 μM ($IC_{50} = 15.5$ μM). Anacardic acid shows this effect at higher concentration, over 100 μM ($IC_{50} = 22$ μM), but C15:0 and methylcardol, as well as cardanol and its derivatives do not exhibit the ability to completely inhibit AChE activity and IC_{50} values for these compounds were impossible to determine.

The capacity of compounds to penetrate cell membrane depends on their lipophilicity (log *P*). Resorcinolic lipids exhibit strong amphiphilic character with values of octanol/water partition coefficient (log $P_{o/w}$) over 7.4 (Kozubek, 1995). It was suggested that the increase in the length of aliphatic chain of a phenol directly increases its lipophilicity (Moridani, Siraki, & O'Brien, 2003). It explains the order of inhibitory potency of resorcinolic lipids homologues from rye grain from the longest to the shortest. On the other hand the strong effect of cardol (a mixture of mono-, di- and tri-unsaturated C_{15} AR) suggests also the importance of side chain unsaturation in the inhibitory potency of phenolic lipids.

Our previous studies showed that these compounds also affect the surface charge and local membrane pH. It was studied by changes of fluorescence intensity of the membrane probe fluorescein-PE (unpublished data) both at low and high buffered ionic strength. Resorcinolic lipids isolated from rye grain have a neutral electric charge at pH near 7.0, but anacardic acid has a negatively charged

substituent (carboxylic group). This polar substituent seems to be responsible for the partial neutralisation of positive charge of erythrocyte ghost membranes (Stasiuk et al., 2004). This interaction may play the role in the modulation of AChE activity. The modification of hydrocardanol molecules at their polar head did not result in significant alteration of their inhibitory activity upon acetylcholinesterase. The most promising derivative contained a carboxylic moiety attached to the phenol ring but was lacking the –OH residue. Thus, the proper "balance" between the hydrophilic part of the molecule and the length and unsaturation of the sidechain seems to determine its ability for affecting the action of the enzyme. On the other hand, these derivatives may be considered as safe modifiers of liposomal drug carriers, as they would not significantly affect the properties of the membrane enzymes of the target cells.

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