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LETTER TO THE EDITOR

STARRING ROLE OF ACETYLCHOLINESTERASE FROM MEDICINAL PLANTS ON LACTATE DEHYDROGENASE PRODUCTION IN CYTOTOXIC HEPATIC CELLS

UMM-E-HABIBA[1], KHALIL-UR-REHMAN[1], N. JAHAN[2] and M. SHAHID[1]

[1]Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan; [2]Department of Chemistry, University of Agriculture, Faisalabad, Pakistan

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To the Editor,

Acetylcholinesterase (AChE) is an enzyme that hydrolyzes the acetylcholine (ACh) in cholinergic synapse and converts it into acetate and choline. AChE also participates in cell differentiation and development of different tissues. Acetylcholine has two receptors, muscarinic and nicotinic that promote differentiation, proliferation, apoptosis and cytoskeleton organization of the cells during phases of cell cycle (1). Hyper production of acetylcholine intriguingly increases the Ca^{2+} influx and diverse activation of molecules such as Ras-mitogen-activated protein kinase, phosphatidylinositol 3-kinase-Akt, protein kinase C and c-Src through muscarinic and nicotinic receptors and resulting cell proliferation and apoptosis occur (2).

Due to exposure to pesticides and chemicals, acetylcholine level is increased in hepatic cells that ultimately induce more differentiation and proliferation of cells and, as a result, hepatocellular carcinoma occurs (3). Therefore, AChE enzyme is needed to breakdown the extra acetylcholine produced as a result of environmental insults like chemicals and toxins etc. The plants which show minimum inhibition of AChE enzyme are supposed to have more hepatoprotective activity. Lactate dehydrogenase (LDH) is a cytotoxicity marker of cells that is normally involved in glucose metabolism pathway. Liver is the major organ involved in glucose metabolism pathways like gluconeogenesis. When hepatic cells are treated with toxicants then they become swollen and necrotic and, as a result, more LDH is released from the hepatic cells (4). To achieve this objective, toxicological evaluation of selected medicinal plants was carried out through hemolytic and thrombolytic activities and then AChE inhibition activity of these plants was accomplished. Sibsequently, *in vitro* liver slice culture assay was performed to measure the level of LDH released from hepatic cells against hepatotoxicity induced by carbon tetrachloride.

MATERIALS AND METHODS

Collection and extractions of plants

The selected parts of plants were collected from the Faisalabad region and identified by the plant taxonomist Department of Botany, University of Agriculture Faisalabad, Pakistan as shown in Table I. The extraction of selected parts of plants was performed in methanol through maceration at 37°C and then lyophilized. After that each plant concentration 500 µg/mL was prepared from dry plant extracts for the study.

Key words: hepatoprotective; hemolysis; cytotoxicity; acetylcholinesterase; hepatocellular carcinoma

Corresponding Author:
Dr Umm-e-Habiba,
Clinico-Medical Biochemistry Lab,
Department of Biochemistry,
University of Agriculture,
Faisalabad, 38000, Pakistan
e-mail: habibaakram518@gmail.com

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Hemolytic activity

For hemolytic activity, fresh blood was taken and centrifuged at 2000 rpm for 3 min. Afterward the centrifugation pellet was taken and washed three times with chilled (4°C) phosphate buffer saline of pH 7.4. The washed red blood cells were suspended in 20 mL chilled phosphate buffer saline and kept on ice to perform hemolytic activity. In micro centrifuge tubes 20 µL of each plant sample and 180 µL of diluted blood cell suspension were taken and incubated at 37°C for 30 min with shaking (80 rev/min). After incubation these samples were immediately placed on ice for 5 min and then centrifuged at 3000 rpm for 5 min. After centrifugation 100 µL supernatant of each sample was taken and diluted with 900 µL of chilled phosphate buffer saline. The absorbance of these diluted samples were noted at 576 nm. Triton X-100 was used as a positive control and phosphate buffer saline as a negative control. Percentage hemolysis was calculated using the formula:

Hemolysis (%) = (Absorbance of sample-Absorbance of negative control/ Absorbance of positive control) × 100

Thrombolytic activity

For thrombolytic activity, 500 µL of blood was put into pre-weighed micro centrifuge tubes and incubated for 45 min at 37°C. After incubation, serum was removed and the tubes were weighed again with blood clots and used for thrombolytic activity. 100 µL of each plant sample was put into the blood clot-containing tubes and incubated at 37°C for 90 min. After incubation, dissolved clots were removed carefully from the tubes and weighed again. The difference in weight of the tubes before and after clot lysis was calculated and expressed in terms of % clot lysis. Distilled water and streptokinase were used respectively as negative and positive control.

Acetylcholineterase (AChE) inhibition assay

In vitro acetylcholinesterase inhibitory activity of medicinal plants was executed following the method described by Rahman and Choudhary (2001). Sodium phosphate (0.1 M) buffer of pH 7.8 was prepared first and then 10 mM solution of Ellman's Reagent (DTNB) and 14 mM solution of acetylthiocholine (ATCI) were perpared in sodium phosphate buffer and distill water, respectively. After preparing the solutions, 120 µL sodium phosphate buffer and 20 µL of each plant extract were put into a microplate and incubated at 25°C for 15 min. Subsequently, 10 µL of DTNB and 10 µL of ATCI solutions were added to the mixture and again incubated for 10 min and then absorbance of the colored product was taken at 412 nm by ELISA reader. Galantamine (10 µM) was used as a positive control. The percentage inhibition of AChE was calculated by the formula:

AChE Inhibition (%) = (1 – Abs. of sample/Abs. of control) × 100

In-vitro hepatoportective activity through liver slice culture assay

The hepatoprotective potential of selected medicinal plants was explored by liver slice culture model which was prepared after slight modification (6). For this purpose 20-22 liver slices from prepared liver slice culture model were put into capped glass tubes that contained 2 mL Krebs – Ringer – Hepes (KRH media). The liver slices were then incubated at 37°C for 30 min on a water bath shaker. After incubation, the KRH medium was replaced with fresh 2 mL KRH medium with 500µg/mL of each plant extract, and ascorbic acid (10 mM) was used as a reference standard. The liver slices with plant extratcs were incubated for 1 h at 37°C and then 1 mL of CCl_4 (40 mM) was added to each glass tube to induce heaptotoxcity and again incubated for 2 h on a water bath shaker at 37°C. In this period of 2 h, liver slices were aerated every 10 minutes with oxygen by removing the caps of the glass tubes. After incubation, culture medium was collected to measure the Latate dehydrogenase (LDH) level, which was the cytotoxic marker of liver injury. For the measurement of lactate dehydrogenase, commerical LDH cytotoxicity assay kit II Abcam (ab65393) was used. To calcutae the percentage cytotoxicity of LDH released from liver slices the formula used was:

Cytotoxicity (%) = [(Test sample Abs – Low control Abs)/(Low control Abs – High control Abs)] × 100

Here, "Test samples $_{Abs}$" showed incubated liver slices with both plant extract and 40 mM CCl_4, "High control $_{Abs}$" showed incubated liver slices with 40 mM CCl_4 only and "Low control $_{Abs}$" indicated incubated liver slices with KRH medium only.

Statistical analysis

Hemlytic and thrombolytic activites of plants were expressed as mean±SD. The results of AChE inhibition

Table I. *Details of selected medicinal plants with their parts used.*

Botanical name	Family	Common name	Selected parts of plants	Voucher number
Ficus religiosa	Moraceae	Peepul tree	Leaves	804-2-17
Cassia fistula	Fabaceae	Amaltas	Leaves	804-3-17
Ziziphus jujuba	Rhamnaceae	Unaab	Fruit	804-5-17
Phyllanthus emblica	Phyllanthaceae	Aamla	Fruit	804-6-17
Ocimum basilicum	Lamiaceae	Niazboo	Seeds	804-8-17

Table II. *Percentage hemolysis and clot lysis of medicinal plants.*

Medicinal plants	Hemolysis (%)	Clot lysis (%)
F. religiosa	14±1.5*	4±1.33*
C. fistula	12±0.56*	3±5.73*
Z. jujuba	23±0.31*	9±0.38*
P. emblica	16±1.06*	10±3.82*
O. basilicum	30±0.45*	12±0.57*
Negative control	1.8±0.24*	2.5±0.58 *
Positive control	83±1.83	99±14.56

** indicates significant difference with positive control*

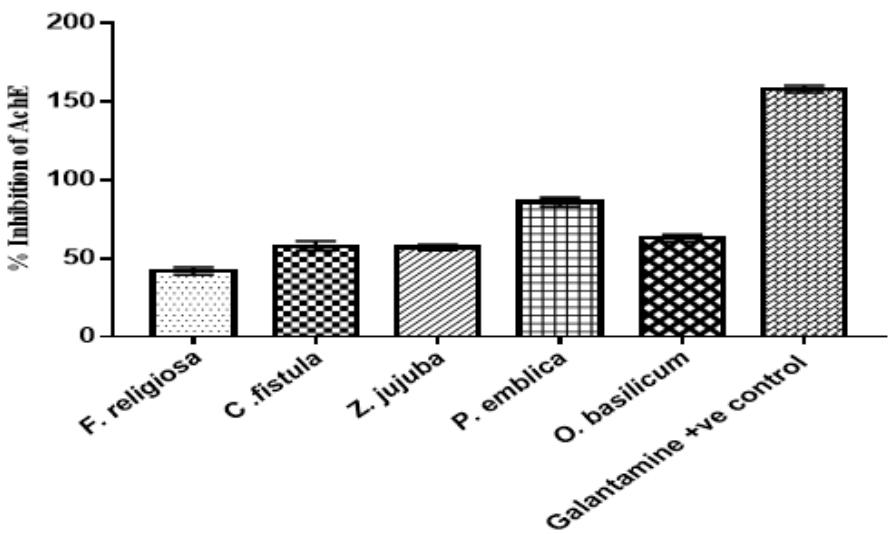

Fig. 1. *Percentage inhibition of AChE activity in medicinal plants.*

assay and liver slice culture assay were analyzed through One-way ANOVA Tukey's multiple comparison test by using Graph pad prism 7.

RESULTS

Among all the studied plants, *C. fistula* showed minimum percentage of hemolysis 12±0.56 and clot lysis 3±5.73 as compared to positive control that exhibited maximum parentage of hemolysis (83±1.83) and clot lysis (99±14.56). On the other hand, in *O. basilicum* high percentages of hemolysis and clot lysis were observed 30±0.45 and 12±0.57, respectively as described in Table II. *In vitro* AChE activity was expressed in terms of percentage inhibition of acetylcholinesterase and plants that showed minimum percentage inhibition of enzyme were considered best in terms of hepatoprotective potential. It was observed from graphical presentation (Fig. 1) that *F. religiosa* showed significantly ($p<0.05$) low percentage inhibition 42±2.4 of AChE, while in *P. emblica* highest percentage inhibition 86±2.8 of AChE was observed as compared to positive control galantamine (95±2.3).

After determination of AChE activity of selected medicinal plants, their hepatoprotective potential was evaluated through liver slice culture assay. In this assay, release of lactate dehydrogenase was measured in terms of percentage cytotoxicity against hepatotoxicant CCl_4. Among all the studied plants *F. religiosa* possessed (14±1.32) maximum significant ($p<0.05$) hepatoprotective potential in terms of least percentage cytotoxicity as compared to the high control group (72±3.40) that received only CCl_4 intoxication. Whereas *F. religiosa* exhibited non-significant ($p>0.05$) hepatoprotective potential in comparison with the standard control group (17±1.87) that received ascorbic acid with hepatotoxicant CCl_4. On the other hand, comparison of *P. emblica* and the standard group showed significant difference 31±1.088 and 17±1.87, respectively, in percentage cytotoxicity that indicated minimum hepatoprotective potential of this plant (Fig 2). Overall comparison of percentage inhibition of AChE and cytotoxicity among all the studied plants indicated that *Ficus religiosa* possessed more hepatoprotective potential due to minimum inhibition of AChE.

DISCUSSION

Owing to the increase of resistance against pathogenic microbes and existing antibiotics, the demand for natural herbal remedies is increasing rapidly for the treatment of several diseases (7). Since ancient times, medicinal plants have been shown to possess remarkable potential against numerous diseases and are a highly valuable source of pharmaceutical drugs. However, before using medicinal plants for any treatment it is necessary to find out their effects on cells. Therefore, in the present study, hemolytic and thrombolytic activities of five different plants were

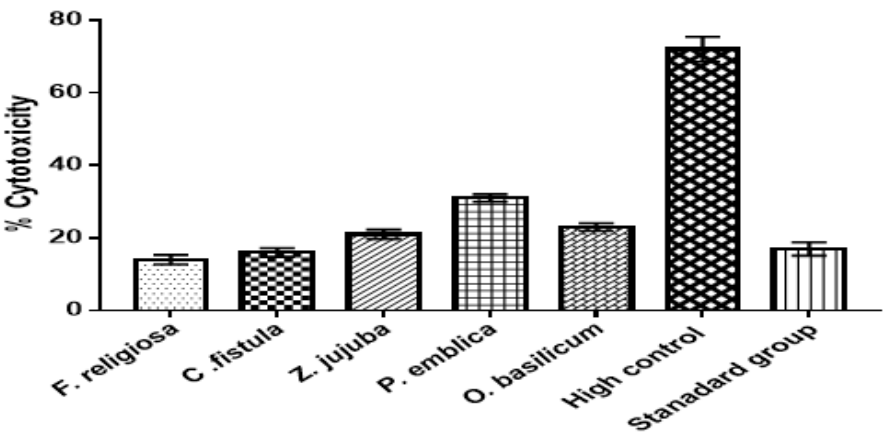

Fig. 2. *Trend of percentage cytotoxicity of medicinal plants in terms of LDH released*

carried out on human erythrocytes to find out which plants showed minimum toxic effects and which were considered best and safe.

The natural antioxidants of medicinal plants reduced oxidative stress that increased due to imbalance of reactive metabolites that ultimately disrupted the homeostasis of the body (8). In case of oxidative damage, necrosis and proliferation of the hepatic cells more lactate dehydrogenase is produced that eventually increases cytotoxicity (9). On the other side a certain level of neurotransmitter acetylcholine controlled by AChE plays a crucial role in cell survival, differentiation and proliferation (10). A variable amount of AChE is present in hepatic cells and in the absence of this enzyme more acetylcholine is accumulated and increased cell proliferation and differentiation by downregulation of p27 and cyclin proteins. The hyper production of acetylcholine induced hepatotoxicity and ultimately hepatocellular carcinoma (11, 12).

The obtained results of the present study indicated that *F. religiosa* has more AChE activity and hepatoprotective potential in terms of least percentage cytotoxicity due to minimum LDH release as compared to the other plants studied. The outcomes of the present research are in agreement with the study of Perez-Aguilar et al. (2015) in which they found that AChE activity decreased in cancer cells due to hyper production of acetylcholine that induced more proliferation and differentiation of the cells.

Therefore it was concluded that medicinal plants which have high AChE activity possess more hepatoprotective potential due to a decrease in LDH and are ultimately beneficial to combat against liver diseases which are prone to be carcinogenic.

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