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RESEARCH ARTICLE

Anti-amnesic Effect of Iridoids Fraction from *Pentas lanceolata* Leaves against D-Galactose Induced Brain Aging in Rats

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Abstract

In this work the anti-amnesic effect of the iridoids-rich fraction isolated from *Pentas lanceolata* (IFPL) leaves was investigated using D-galactose (D-gal) to artificially create aging in rats. Thirty-two rats were divided as follows: 1st group served as control; 2nd, 3rd and 4th rat groups were injected intraperitoneally with 100 mg/kg D-gal five days/ week for two months. The 2nd group was left untreated. While, 3rd and 4th groups were treated daily orally with IFPL (50 mg/kg b. wt.) and the reference drug donepezil (10 mg/kg b. wt.), respectively for one month. Behavioral tests, T-Maze and beam balance, showed deterioration in cognitive ability of aging rats. D-gal caused a remarkable decrease in oxidative biomarkers except lipid peroxides which revealed a significant increase. In addition, D-gal-treated rats showed a significant decrease in the levels of neurotransmitters, while, a significant increase in acetylcholine esterase (AChE) was recorded. Furthermore; caspase-3 levels were elevated in aged brains which were confirmed by immunohistochemical analysis. Histopathological alterations were observed in these rats. Treatment of these rats with IFPL exhibited improvement in behaviour, neurobehavioral changes, modulated oxidative stress as well as apoptotic markers which is confirmed by histopathological investigations.These findings provide scientific evidence that IFPL extract has antioxidant, anti-inflammatory, immunomodulatory, anti-apoptotic and anti-amnesic properties against D-gal induced brain aging.

Keywords: *D-galactose, Brain aging, Pentas lanceolata, Iridoids-rich fraction, anti-amnesic*

Introduction

Brain aging is one of the cardinal features of neurological disorders that induces progressive loss in physiological integrity [1, 2] and is manifested by impaired cognitive function and anxiety. These neurologic disorders are decrease in neurogenesis, mitochondrial impairment, reduced neurotransmitter levels, increased DNA mutation and lipid peroxidation, oxidative stress, beta amyloid (Aβ) overproduction and changes in brain structural connectivity [3, 6]. D-gal is a reducing nutrient that is considered as a model of accelerating aging. It is found abundantly in milk and to a lesser extent in fruits and vegetables. It induces mitochondrial dysfunction, apoptosis, deterioration of cognitive, neuroinflammation and neurodegeneration [7, 8]. Advanced glycation end products result due to reaction of this sugar with amino acids causing oxidative stress through non-enzymatic glycation reaction [9]. Rodents chronically injected with D-gal were reported to show these features of brain damage and altered gene expression in the brain [10, 12]. *Pentas lanceolata* (*P. lanceolata*) (Forssk), known as

Egyptian Star cluster, is a flowering plant, belonging to family Rubiaceae, and is used in the treatment of tropical diseases such as lymphadenitis, snake poisoning, abdominal cramps, and ascariasis [13, 14]. A decoction of the roots or leaves is used topically or taken orally to treat lymphadenitis or boils [13]. The alcohol flower extract showed wound healing improvement in rats [15]; while that of ethyl acetate roots exhibited antimicrobial activity [16], while, *n*-hexane leaves extract revealed analgesic activity [17]. Recently, the protective role of *Pentas lanceolata*-iridoids against genotoxicity and sperm defects have been reported [18]. In addition, both the ethyl acetate and *n*-butanol extracts of the plant were reported to be immunostimulants at both the humoral and cellular levels [19].

Iridoids and their glycosides constitute the major secondary metabolites in *P. lanceolata*, especially in the aerial parts [20, 22]. These fractions previously displayed a wide range of pharmacological activities such as cardiovascular, hepato protection, hypoglycemic, anti-mutagenic, antispasmodic, anti-tumor, antiviral, immunomodulation and purgative effects [23, 24]. Hence, the main goal of the present study is to explore the anti-amensic effects of iridoid-rich fraction extracted from the leaves of *P. lanceolata* against D-gal-induced brain aging which causes deterioration in many brain functions.

Materials and Methods

Chemicals

D-gal was purchased from Bio Basic TNC., Canada. Donepezil was purchased from Sigma Chemical Company (USA). Antioxidant Kits were purchased from Biodiagnostic Company, Cairo Egypt. ELISA kits used for acetylcholine (Ach) and acetylcholinesterase (AchE) were purchased from BioVision Co, California (USA). ELISA kits used for determination of tau, phospho-tau and amyloid-β were purchased Innogenetics NV, Gent, Belgium.

Plant Material

P. lanceolata leaves (Forssk.) Deflers L. were collected from El-Orman Botanical Garden, Giza, Egypt, during October 2017 and were identified and authenticated by a specialized botanist. A herbarium specimen (No. 13-P.2) was deposited at the herbarium of N.R.C., Egypt.

Preparation of iridoid-rich Fraction (IFPL)

This was carried out using water: ethanol mixture (1:1 w/w) (75 ml of solvent). Fifteen g of calcium carbonate were added to the mixture which was allowed to boil under reflux for 30 minutes. The mixture was filtered and decanted and the resultant extract was isolated on a chromatography column filled with activated aluminum oxide (6 g). The filtrate was evaporated to dryness (Rotary Vacuum Evaporator Laborota- 4011, Heidolph Co., Germany) at 40°C.

Experimental Design

Animals

Thirty-two week old Wistar male rats (100-120 g) were used. They were maintained under standard laboratory conditions (temperature 25±2°C), relative humidity 55±5% and a 12 h/12 h light/dark cycle. All animals were fed with standard pellets and water *ad libitum*. The experimental animals were given proper care and handling according to the institutional animal ethics committee of the National Research Centre, Egypt (Approval number, 19039).

Animals were randomly divided into four groups of eight animals each. Group 1: Control group. Groups 2 -4: Animals were injected intraperitoneally with 100 mg/kg D-gal; five days/ week) for two consecutive months [25], then the following method was applied: Group 2: served as positive control.

Group 3: treated orally with a daily dose of IFPL (50 mg/kg), for 30 consecutive days [26]. Group 4: daily administered with standard drug (donepezil), 10 mg/kg for 30 consecutive days [27].

Behavioral Assessment

Assessment of Cognitive Abilities and Motor Coordination

T-maze is locally constructed at N.R.C. Cognitive ability and impairment of spatial memory of rats was evaluated after chronic D-gal administration (two months) at the end of treatment period [28]. Motor ability was assessed using the beam balance test as previously described [29].

Serum Preparation and Brain Tissue Homogenate Collection

After 24 hours of behavioral tests, blood was

collected in clean dry test tube by puncture of the sublingual vein and serum was separated by centrifugation at 4000 rpm for 15 minutes and kept at -80°C. Then, rats were sacrificed under slight diethyl ether anesthesia and the whole brain rapidly dissected, washed with isotonic saline and dried.

A portion of brain was weighed, homogenized (1:10 w/v) in ice-cold phosphate buffered saline, centrifuged at 5000rpm for 15 minutes and the supernatant stored at -80°C. Another portion of the brain was fixed in 10% formalin for histological and immunochemical investigations.

Biochemical Determinations

Determination of Antioxidant Parameters

All animal groups were subjected to determine the non-enzymatic, glutathione reduced (GSH), malondialdehyde (MDA) and total antioxidant capacity (TAC), and enzymatic antioxidants; superoxide dismutase (SOD), and catalase (CAT), in brain tissues by using standard diagnostic kits according to manufacturer instructions.

Determination of Neurotransmitter Parameters

Acetylcholine (Ach) and acetyl cholinesterase (AchE) were measured as previously described [30]. Adrenaline (AD), noradrenaline (NA), dopamine (DA) and serotonin (5-HT) were determined using HPLC according to the method of Giday *et al* [13]. Tau, Phospho-tau and Amyloid-ß concentrations were assayed using ELISA kits according to manufacturer instructions.

Histopathological Examination

After fixation in formalin, brain specimens from all animals were washed, dehydrated, cleared and embedded in paraffin. Paraffin blocks were cut at 4-5µ thickness, stained with hematoxylin and eosin and examined under a light microscope (Olympus BX50, Japan) [31]. Histopathological damage in the cerebral cortex and hippocampus were graded from (0-4) as follow: (0) indicated no changes; (1) indicated percentage area affected (<10%);

(2) indicated percentage area affected (20-30%); (3) indicated percentage area affected (40-60%) and (4) indicated percentage area affected (>60%) [32].

Immunohistochemical Analysis for Caspase-3 Expression

Brain sections were incubated with primary antibodies against caspase-3 (1:100 dilutions) (Santa Cruz Biotechnology Inc., Dallas, TX, USA). The immune reaction was visualized using diaminobenzidine tetrachloride (DAB, Sigma Chemical Co., St. Louis, MO, USA).Quantification of caspase-3 apoptotic protein was estimated by measuring the area % expression from 5 randomly chosen fields in each section and averaged using image analysis software (Image J, version 1.46a, NIH, Bethesda, MD, USA) [33].

Statistical Analysis

Statistical comparison between groups was performed using SPSS version 9.05 (USA). Significant difference was analyzed by one way analysis of variance (ANOVA) followed by Co-state computer program. $P \leq 0.05$ was considered significant.

Results and Discussion

Dementia is a disease that affects elderly people worldwide and results defects in their memory, abilities, behavior and reduced brain cognitive function [34]. The number of people affected by dementia will progressively increase and all individuals are liable to face these brain disorders [35].

Susceptibility and vulnerability to disease in aging is a biological phenomenon characterized by reduced physiological capacity and ability to environmental stimuli, changes in biochemical composition [36] and results in cell oxidative damage defined by the imbalance of the rate of reactive oxygen species production and degradation induced by the antioxidant defense system [37].

Pentas genus, family Rubiaceae, is a plant that has high content of radical scavengers including the simple phenolic compounds, quinones, and iridoids [24]. Iridoids are monoterpenoid compounds that have many beneficial effects *e.g* antioxidant, anti-inflammatory, immunomodulatory properties which protect cells and fine molecules like DNA from damaging effect of free radicals and enhancing the antioxidant defense mechanism [38].

Many iridoids such as asperuloside, tudoside, 13R-*epi*-gaertneroside, 13R-*epi*-

poxygaertneroside, uenfoside, ixoside, griselinoside, 6α, 7β-epoxysplendoside, 13R-methoxy-*epi*-gaertneroside were previously isolated from *P. lanceolata* [39]. Previously, iridoids asperuloside and asperulosidic acid were reported to possess an anti-inflammatory activity *via* inhibition of inflammatory cytokines and mediators [40] and shown potent antioxidant properties. Monotropein, a natural iridoid glycoside decreased soluble receptor activator of NF-κB ligand (sRANKL) and interleukins level [41].

Another iridoid glucoside, catalpol, isolated from the root of *Rehmannia glutinosa*, was reported to protect various cells from damage [42]. D-gal is present in many types of food and its normal concentration in blood is less than 10 mg/dl [43].However, high intake may cause aging effects in several organs, mainly brain generating excess free radicals and thereby may induce mitochondrial dysfunction, oxidative stress, inflammation and apoptosis [44, 45].

Numerous behavioral tests have been designed to assess extent of brain aging. In our study, both T-Maze beam balance tests showed that D-gal caused significant deterioration in brain cognitive functions, as shown in Table 1. However, treatment of rats with donepezil or IFPL extract resulted in an improvement in behavioral status as represented by an improved motor coordination and improved cognition, as shown in Table 2.

These data are in agreement with previously obtained results which demonstrated that $AlCl_3$-neurointoxicated rats took more time to catch food in T-maze, compared to control rats denoting deteriorated neuro-cognitive function [46, 47]. The disruption in antioxidant defense mechanism and ROS excessive generation are considered as the main causes of mitochondrial dysfunction induced intracellular damage.

So, the use of antioxidants is regarded as a useful therapy for ROS-induced brain damage [12]. After administration of D-gal, MDA increased while GSH, SOD, CAT and TAC enzyme activities decreased (Table 3) which agrees with the oxidative stress and associated disorders caused by D-gal injection in rodents [48-50]. Treatment with IFPL extract showed its potent antioxidant activities as evidenced by increasing the levels of antioxidant defense system GSH, CAT, SOD, TAC as well as reducing MDA in brain tissues; Table 3. It has been previously reported that iridoids, flavones and monoterpenoids are powerful therapeutic targets in Alzheimer's brain [51]. Previous reports have shown that D-gal accelerated brain aging and induced many aspects neurodegeneration. D-gal is transported to brain through blood brain barrier (BBB). Oversupply of D-gal accelerates the time course of aging markers and causes deterioration in cognition function, oxidative stress and reduction in respiratory chain enzymes and mitochondrial dysfunction in different brain regions which are significant in aging process [52, 54].

Interestingly, D-gal activates both intrinsic and extrinsic pathways of apoptosis [5] and increases inflammatory markers [10]. Our results suggest that D-gal model of aging results in a significant decrease in Ach accompanied by a significant increase in activity AChE activity (Table 4), while, AD, NA, DA and 5-HT recorded significant reduction in D-gal aging rats as compared to control rats (Table 5). On the other hand, D-gal model of aging increased the levels of tau, P-tau and amyloid ß proteins compared to control (TA (Table 6).

The oral administration IFPL ameliorated and restored the dysfunction of various neurotransmitters more or less near to the normal values. The iridoides glycosides appear to offer as neuro-modulators, particularly in Alzheimer`s disease, due to their aglycones and glycosylated constructed forms [51].

In our study, cerebral cortex of D-gal rats showed numerous neuropathologic alterations described as shrunken necrosis neurons associated with neurofibrillary tangles and neuronophagia of degenerated neurons as well as thickening with hyalinosis of the wall of cerebral blood vessel (Fig. 1b, 1c, 1d).

While, hippocampus of D-gal rats showed atrophy, pyknosis, shrunken and necrosis of pyramidal neurons with appearance of flame shaped neurofibrillary tangles (Fig. 2b). Moreover, IFPL extract treated significantly decreased the damage of cerebral cortex and hippocampus in our studied brain tissues (Fig. 1f & Fig. 2d). Immunohistochemistry expression of caspase-3 showed immune-reactive cells in the cerebral cortices of D-gal

rats (Fig. 7). Meanwhile, moderate expression was noticed from treated groups with IFPL extract (Table 7).

Table 1: Effect of IFPL by using T- maze test post D-gal induction

Groups	Baseline	Induction (two months)	Treatment (one month)
Control	12.04±1.03a	13.17±1.26a	12.30±1.32a
D-gal (% change)	16.46±0.85b (+36.71%)	30.17±1.54c (+129.08%)	-
IFPL (% of improvement)	-	-	18.53±1.57d (94.63%)
Donepezil drug (% of improvement)	-	-	19.62±1.93d (85.77%)

Data were expressed in seconds as mean ±SD (n=8). Groups with similar letter are not significantly different; while, those with different letter are significantly different at $P \leq 0.05$

Table 2: Effect of IFPL by using the beam balance test post D-gal induction

Groups	Baseline	Induction (Two months)	Treatment (one month)
Control	9.54±0.63a	11.17±1.26a	10.30±1.32a
D-gal (% change)	7.46±0.85b (-21.80%)	1.57±0.54c (-85.94%)	-
IFPL (% of improvement)	-	-	8.53±0.57a (67.57%)
Donepezil drug (% of improvement)	-	-	9.02±0.63a (72.33%)

Data were expressed in seconds as mean ±SD (n=8). Groups with similar letter are not significantly different; while, those with different letter are significantly different at $P \leq 0.05$

Table 3: Effect of IFPL on some antioxidant enzymes activity post D-gal induction

Parameters	Groups			
	Control	D-gal	IFPL	Donepezil drug
GSH (µg/mg protein)	2962.20±102.80^{a}	1254.55±30.98^{b}	2351.39±70.76^{c}	2053.75±54.90^{d}
% of change	----	- 57.65	-20.62	-30.67
% of improvement	----	----	37.30	26.95
MDA (µg/mg protein)	36.62±3.99^{e}	68.03±3.87^{f}	50.00±3.76^{g}	53.1±4.10^{g}
% of change	----	+85.77	+36.54	+45.00
% of improvement	----	----	49.21	40.77
SOD (µmol/mg protein)	1780±60.02^{h}	858±20. 10^{i}	1462.67±65.10^{j}	1653.33±43.90^{h}
% of change	----	-51.80	-17.83	-7.12
% of improvement	----	----	33.97	44.68
CAT (U/g tissue)	204.1±10.90^{k}	68.15±3.85^{l}	139.31±5.90^{m}	157.77±11.90^{n}
% of change	----	-66.61	-31.74	-22.70
% of improvement	----	----	33.97	43.91
TAC (U/g tissue)	1.96±0.030^{o}	1.02±0.21^{p}	1.88±0.45^{o}	1.91±0.33^{o}
% of change	----	-47.96	-4.08	-2.55
% of improvement	----	----	33.97	43.91

Data were expressed as means ± SD (n=8). Groups with similar letters are not significantly different; while, those with different Letters are significantly different at $P \leq 0.05$.GSH: glutathione; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; TAC: total antioxidant capacity

Table 4: Effect of IFPL on the levels of Ach and AchE activity post D-gal induction

Parameters	Groups			
	Control	D-gal	IFPL	Donepezil drug
Ach (nmol/g tissue)	56.50±3.22[a]	19.15±0.56[b]	35.50±2.33[c]	39.80±3.34[c]
%change	-	-66.11	-37.17	-29.56
%improvement	-	-	28.94	36.55
AchE (µmol/g tissue)	3280.50±319.10[d]	9377.50±868.93[e]	5271.02±488.02[f]	5178.03±298.76[f]
%change	-	+185.86	+60.68	+57.84
%improvement	-	-	125.18	128.01

Data were expressed as means ± SD (n=8). Groups with similar letters are not significantly different; while, those with different letters are significantly different at $P \leq 0.05$. Ach: Acetylcholine; AchE: Acetylcholine esterase

Table 5: Effect of IFPL on the levels of AD, NA, DA and 5-HT post D-gal induction

Parameters	Groups			
	Control	D-gal	IFPL	Donepezil drug
AD (ng/gm tissue)	322.50±24.76 [a]	162.50±12.11 [b]	270.50±25.64[c]	265.50±24.75[c]
%change	-	-49.61	-16.12	-17.67
%improvement	-	-	32.12	31.94
NA (ng/g tissue)	204.05±19.35 [d]	113.20±9.65 [e]	162.60±14.65[f]	167.00±14.88 [f]
%change	-	-44.52	-20.31	-18.16
%improvement	-	-	24.21	26.37
DA(ng/g tissue)	68.50± 5.87[g]	26.50±1.88[h]	48.50±2.10[i]	47.30±2.68[i]
%change	-	-61.31	-29.20	-31.95
%improvement	-	-	32.12	30.36
5-HT(ng/g tissue)	89.30±6.15[j]	47.21±2.72[k]	66.40±4.80[l]	67.50±3.32[l]
%change	-	-47.13	-25.64	-24.41
%improvement	-	-	21.50	22.72

Data were expressed as means ± SD (n=8). Groups with similar letters are not significantly different; while those with different Letters are significantly different at $P \leq 0.05$. AD: adrenaline, NA: Noradrenaline, DA: Dopamine, 5-HT: Serotonin

Table 6: Effect of IFPL extracton the levels of Tau, P-Tau and amyloid-β proteins post D-gal induction

Parameters	Groups			
	Control	D-gal	IFPL	Donepezil drug
Tau protein (ng/l)	295.30± 24.90[a]	1040.02±112.09[b]	422.50±39.12[c]	378.01±34.90[d]
%change	-	+252.19	+43.07	+28.01
%improvement	-	-	129.52	224.18
P- tau protein (pg/ml)	568.50±42.90[e]	1585.30±140.90[f]	840.00±64.87[g]	810.50±70.87[g]
%change	-	+178.86	+47.76	+42.57
%improvement	-	-	131.09	136.29
Amyloid β (g/l)	665.00±39.10[h]	1350.40±126.86 [i]	870.00±62.13[j]	845.10±77.32[j]
%change	-	+103.07	+30.83	+27.08
%improvement	-	-	72.24	75.98

Data were expressed as means ± SD (n=8). Groups with similar letters are not significantly different; while those with different letters are significantly different at $P \leq 0.05$

Table 7: Histopathological lesions score in cerebral cortex and hippocampus of all experimental groups

Histopathological lesions	Cerebral cortex			
	control	D-Gal	Donepezil drug	IFPL
Necrosis of neurons	0	4	2	2
Neurofibrillary tangles	0	3	1	2

Neuronophagia	0	3	1	1
Thickening in the wall of cerebral blood vessel	0	3	0	0
Hippocampus				
Necrosis of pyramidal neurons	0	4	1	2
Neurofibrillary tangles	0	3	1	2

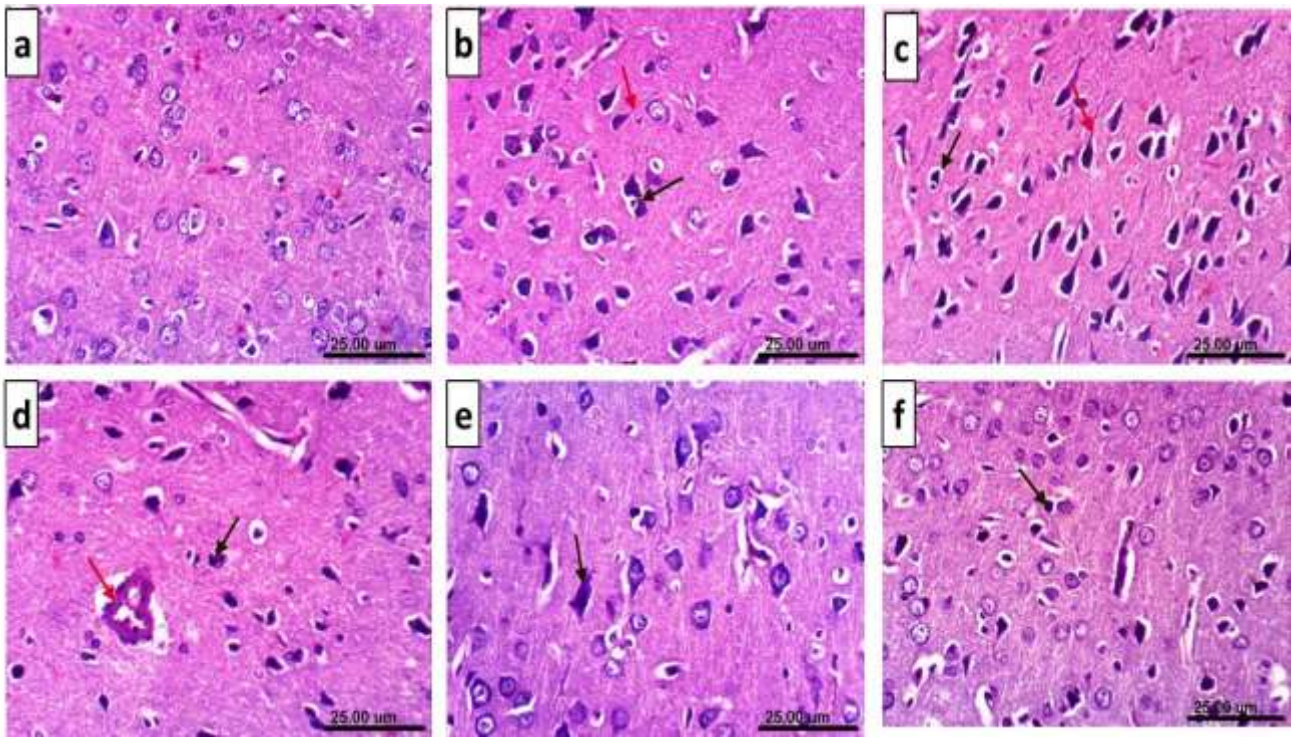


Fig. 1: Histological H&E stained sections of cerebral cortex: a) control rat showing the normal histological structure. b) D-gal treated rat showing shrunken and degenerated neurons (black arrow) associated with neurofibrillary tangles (red arrow). c) D-gal treated rat showing shrunken necrosed neurons associated with neurofibrillary tangles (red arrow) and neuronophagia of degenerated neurons (black arrow). d) D-gal treated rat showing degeneration of neurons (black arrow) and thickening with hyalinosis of the wall of cerebral blood vessel (red arrow). e) Donepezil treated rats showing shrunken and necrosis of some neurons (black arrow). f) IFPL treated rats showing shrunken and necrosis of some neurons (black arrow). (Scale bar 25 um)

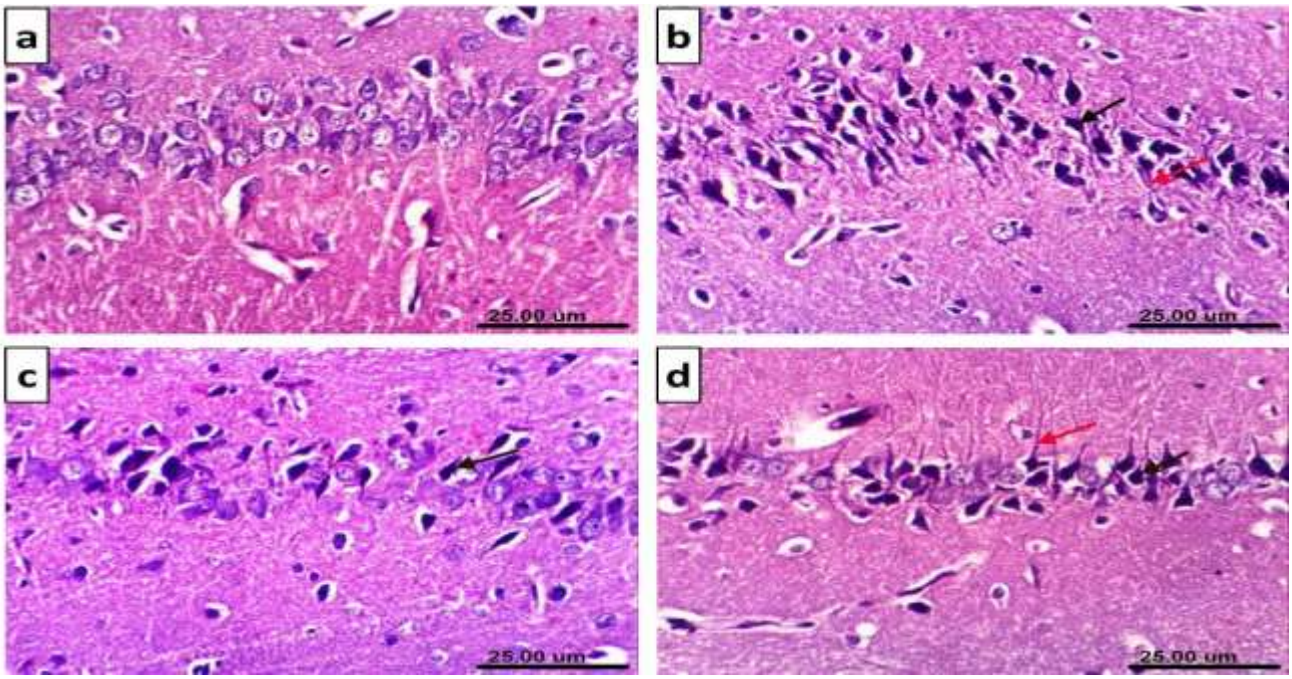
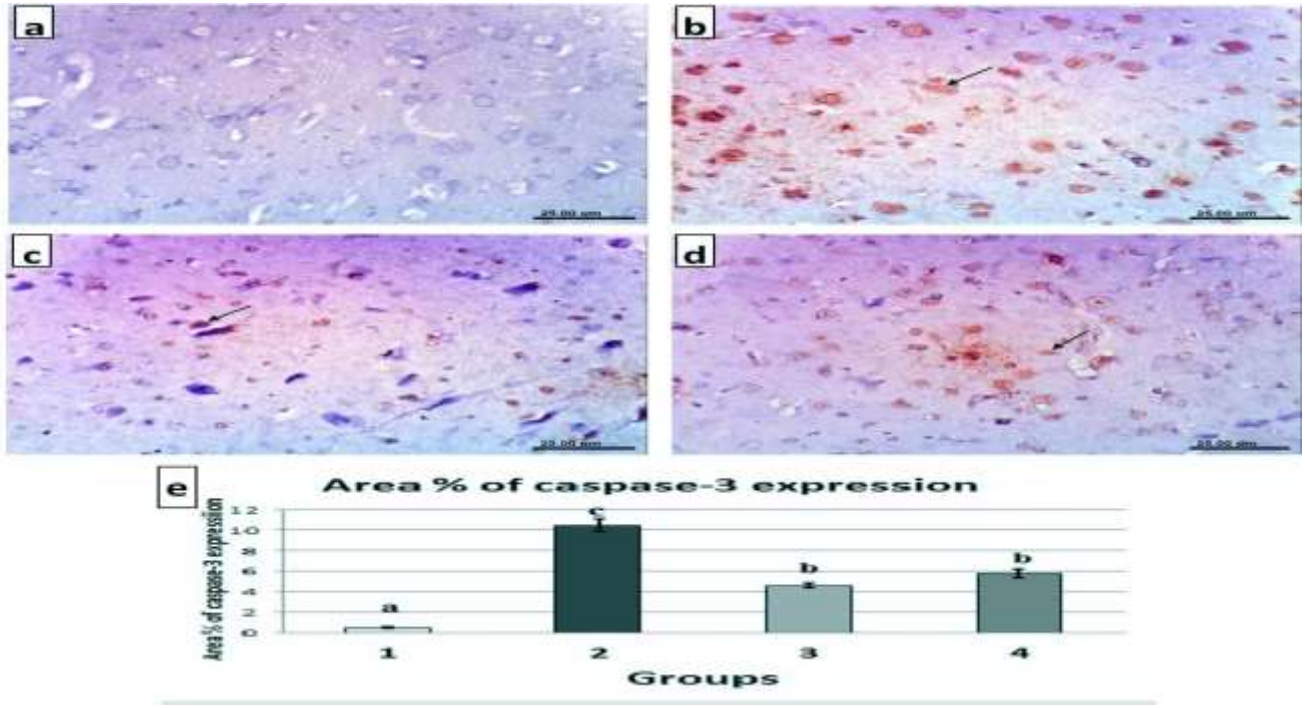


Fig. 2: Histological H&E stained sections from CA1 region of hippocampus: a) control rat showing the normal histological structure. b) D-gal treated rat showing atrophy, pyknosis, shrunken and necrosis of pyramidal neurons (back arrow) with appearance of flame shaped neurofibrillary tangles (red arrow). c) Donepezil treated rat showing shrunken, pyknosis and necrosis of some pyramidal neurons (black arrow). d) IFPL treated rat showing shrunken and necrosis of some neurons (black arrow) associated with appearance of flame shaped neurofibrillary tangles (red arrow). (Scale bar 25 um)



Group1: control; Group2: D-gal; Group3: Donepezil; Group3: IFPL

Fig. 3: Immunostaining for caspase-3 protein in cerebral cortex tissue sections of: a) control rats showing no caspase-3 immune-reactive cells. b) D-gal rats showing strong positive immune reaction (arrow). c) Donepezil treated rats showing moderate immune reaction (arrow). d) IFPL treated rats showing moderate positive immune reaction (arrow). (Scale bar 25 um). e) Immunostaining area (%) of caspase-3 expression. Data shown as mean ±SE; error bars show the variations of determinations in terms of standard error. One−way analysis of variance was used for data analysis, mean values with unlike superscript letters were significantly different ($P \leq 0.05$)

Conclusion

Aging is a phenomenon that all living organisms inevitably face that may be caused by increased level of D-gal in blood and induce brain disorders. Iridoids-rich fraction of *P. lanceolata* (IFPL) was effective in reversing D-gal-induced brain aging in rats, by ameliorating brain functions, cognitive abilities and attenuating the functional outcome. This is revealed in improving behavioral tests, inflammatory, antioxidant and apoptotic markers and documented by immuno-histochemical and pathological examination. The possible mechanisms underlying the therapeutic effect of iridoids are potentially associated with their aglycones and glycosylated constructed forms. These compounds may serve in the future as therapeutic agents for reducing aging disorders and improving quality of life of elderly people.

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