*Egypt. J. Chem.* **Vol. 65**, No. 4 pp. 411 – 421 (2022)

**Egyptian Journal of Chemistry**

**http://ejchem.journals.ekb.eg/**

# Ameliorative Effect of Probiotic-Fermented Milk and Costus Extract in Alzheimer's Disease Model Induced by D-Galactose and Aluminum Chloride

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**Abstract**

Alzheimer's disease (AD) is a neurodegenerative disease associated with memory and cognitive dysfunction and is the main reason of dementia worldwide. The present research aimed to evaluate the protective effect of administration of probiotic-fermented milk and costus extract against AD in rats. Total phenolic content (TPC), total flavonoids (TF) and antioxidants activity (AA) of costus extracts were studied. The study included 40 rats divided into five groups. Group 1 and 2 served as normal control and AD control, respectively. Groups 3 and 4 were given daily oral dose of costus extract (100 and 200mg/KG RBW), while group 5 was given probiotic-fermented milk (1 ml/rat/day). AD was induced in rats by intraperitoneally (i.p.) injection of D-galactose and aluminium chloride for 21 days. Hippocampal gene expression of PI3K and Akt, biochemical, nutritional and histopathological studies were evaluated in all rats' groups. Microflora of fecal samples from cecum and colon were studied in group 1, 2 and 5. Costus root extract showed 98% AA and contains TPC and TF as 378.1 mg GAE/g and 275.3 mg/g extract, respectively. Induction of AD in rats led to reduction of mRNA level of PI3K and Akt in hippocampal of rats, also elevation of inflammatory and oxidative stress markers in association with significant elevation of acetylcholinesterase activity were observed. The results revealed relevant new visions towards the influences of probiotic-fermented milk and costus extract on cognitive function through reduction of neurofibrillary tangles formations, regulation of hippocampal gene expression of PI3K and Akt, inflammatory and oxidative stress markers. **Conclusion:** Administration of probiotic-fermented milk and costus extract have a promising effect on cognitive function through reduction of neurofibrillary tangles formation, regulation of hippocampal gene expression of PI3K and Akt, inflammatory and oxidative stress markers. The present results suggested that intake of probiotic-fermented milk had a beneficial effect on the composition of cecum and colon flora. Probiotic-fermented milk was the most effective for prevention of AD in the present study.

**Keywords:** Probiotic-fermented milk, costus roots extract, Alzheimer's disease, hippocampal gene expression, oxidative stress, neurofibrillary tangles.

## 1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease associated with memory and cognitive dysfunction in elderly, which the main reason of dementia worldwide. AD is a global health problem, AD patients will rise to 106.8 million by 2050 [1]. Numerous reasons can participate in AD, but the extreme threat is aging [2], degradation of anatomical pathways, oxidative stress and inflammation [3,4]. The formation of neurofibrillary tangles (NFTs) and senile plaques (amyloid-β) are the major histopathological changes in AD [5,6]. Nutritional intervention can play an important role in the management of oxidative stress and inflammation, which are leading causes of neurodegenerative diseases through presence of phytochemicals. Fermented dairy products and their components, including lactic-acid bacteria, peptides and fatty acids generated during fermentation, may protect against dementia or cognitive impairment [7-9]. Gut microbiota can have an impact in creating numerous neurotransmitters and neuromodulators, which affect gut–brain communication and brain function [10]. *Saussurea lappa* roots, Family Asteraceae (costus roots) is an important plant displayed multiple biological activities such as anticancer, anti-inflammatory, antioxidant, antiulcer, anti-diabetic,

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Receive Date: 27 September 2021, Revise Date: 08 October 2021, Accept Date: 19 October 2021

DOI: 10.21608/EJCHEM.2021.98261.4577

hepatoprotective, and immunomodulatory activities [11-13] as it is rich in triterpenoids, flavonoids, steroids and sesquiterpene lactones [14]. Previously several studies have shown that D-galactose was effective in induction of mimic natural aging in rat brain [15,16]. Aluminium was effective in induction of AD model in rats [17]. The results of previous studies of some authors of the present research encourage us for studying the effect of combination of D-galactose and aluminium in induction of AD in rats as both are neurotoxic. The present study postulated that administration of probiotic-fermented milk and costus extract may have a prophylactic effect towards AD rats through diminishing inflammation, oxidative stress and formations of neurofibrillary tangles. For achieving the objective of the present study total phenolic content, total flavonoids (TF) and antioxidants activity of costus extracts were studied.

## 2. Material and Methods

### 2.1. Raw materials and chemicals

Fresh cow's milk was obtained from the dairy department, Cairo Univ. and was used without standardization of the fat content. Costus root (*Saussurea lappa*, Family Asteraceae) was purchased from local markets, Cairo, Egypt. Chemicals and pure reagents were purchased from Sigma Chemical Companies (Sigma-Aldrich, St. Louis, MO, USA).

### 2.2. Bacterial strains

All lactic acid strains provided from Dairy Department, National Research Centre. All strains *Bifidobacterium bifidum* NRRL B-41410, *Lactobacillus helveticus* CNRZ32, and *Lactobacillus casei* FEGY 9973 were activated by MRS broth medium and incubated at 37 °C for 24h under anaerobic condition.

### 2.3. Preparation of costus extract

The air-dried powdered of costus root were extracted successively in a continuous extraction apparatus (Soxhlet) until exhaustion with ethanol for preparation of crude ethanol extract. The solvent was completely removed by evaporation under reduced pressure at a temperature not exceeding 40°C. Crude extract of Indian costus roots were kept in deep-freeze till used.

### 2.4. Phenolic compounds and total flavonoids of costus crude ethanol extract

Total phenolics and total flavonoid contents were determined colorimetrically in costus roots crude ethanol extract using Folin-Ciocalteu reagent [18] and aluminium chloride method [19]. Total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g sample, while total flavonoid content was expressed as mg quercetin equivalent per g (QE). The results were expressed as Mean± SD.

### 2.5. Determination of total antioxidant activity of costus crude ethanol extract by ferric thiocyanate method (FTC).

Total antioxidant activity of costus crude ethanol extract was determined by applying ferric thiocyanate (FTC) method; the standard method as described by Kikuzaki and Nakatani [20] was used. A mixture of 4.0 mg extract in 4 ml absolute ethanol, 4.1 ml of 2.5% linolenic acid in absolute ethanol, 8.0 ml of 0.05M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in a vial with a screw cap and then placed in an oven at 40 °C in the dark. To 0.1 ml of this solution was added 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after addition of 0.1 ml of 0.02M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red color was measured at 500nm in the seventh day. BHT was used as positive controls while the mixture without extract sample was used as the negative control. The antioxidant activity of costus crude ethanol extract and BHT standard were carried out in triplicate. Calculation of antioxidant activity according to the following equation [21]:

Inhibition (%) = 100-[(A1-A0)×100]

where, A0 is the absorbance of the control and A1 is the absorbance of the sample.

### 2.6. Production of probiotic-fermented milk

Fresh cow's milk was heated to 80 °C for 15 min, and then rapidly cooled to 40 °C. Three probiotic bacteria: *Bifidobacterium bifidum*, *Lactobacillus helveticus* and *Lactobacillus casei* (1:1:1) were added at the level of 3% (w/v) served as mixed culture into the milk. The fermented milk was incubated at 37°C for 5 h. At the end of the fermentation pH reached 4.8 and incubation was ended. The resultant coagulates of probiotic-fermented milk were stirred, filled in 100 g glass bottles and then stored in the refrigerator ($5^{o}$C) for 7 days [22]. The probiotic-fermented milk was prepared weekly as previously mentioned in the production to save the counts of lactic acid strains at 8.00 log CFU/ml in oral dose.

### 2.7. Animals and experimental design

#### 2.7.1. Animals.

Male Sprague Dawley rats of 127.3 ± 5.685 g (mean ± SD) were obtained from the animal house of the National Research Centre, Cairo, Egypt. Animals were kept individually in stainless steel cages under standard laboratory conditions (23–25 °C, 12 h light/dark cycle) and with free access to diet and water. This study has been carried out as a part of internal project No12050203 in the National Research Centre, Cairo, Egypt. This project was approved by the Medical Research Ethics Committee, National Research Centre, Cairo, Egypt with approval number 19176, and followed the recommendations of the National Institutes of Health Guide for Care and Use of

Laboratory Animals (Publication No. 85-23, revised 1985).

#### 2.7.2. Animals' diet

Balanced diet (12% casein as a protein source, 10% corn oil, 10% sucrose, 58.5% maize starch, 5% fibre, 3.5% salt mixture, and 1% vitamin mixture), salt and vitamin mixtures were prepared in accordance with AIN-93 [23].

#### 2.7.3. Induction of Alzheimer's disease in rats

After one week of adaptation the animals were divided into five groups (n = 8, each). Group one was served as normal control group (NC). Rats of group two were daily injected intraperitoneally (i.p.) with 150 mg/Kg BW of D-galactose/day and 10 mg/kg BW of $AlCl_3$/day for induction of Alzheimer disease in rats (AD control) according to the method of Bilgic *et al*. [24]. Rats of groups three and four were daily injected i.p. with the same dose of D-gal and $AlCl_3$ and administered orally with 100 (low dose) and 200 (high dose) mg/kg per day of costus crude ethanol extract, respectively. Rats of group five were daily injected i.p. with the same dose of D-gal and $AlCl_3$ and administered orally with 1 ml (8.00 log CFU/ml probiotic mixtures) per day of probiotic-fermented milk. The experiment lasted for three weeks during which all rats were fed on balanced diet, also body weight and food intake were recorded weekly. At the end of the study total food intake, body weight gain and food efficiency ratio were calculated. At the end of the study, rats were anesthetized using ether and the blood was withdrawn from all rats after an overnight fast. Rats were dissected and brain was immediately separated from each rat and weighed then part of each brain was immersed in 10% formaldehyde solution for histopathological examination [25]. Plasma malondialdehyde (MDA) as indicator of lipid peroxidation were determined according to Satoh [26]. Antioxidant enzymes; glutathione peroxidase (GPx) (SUNLOG, Cat No.SL1033Ra, ELISA kit), superoxide dismutase (SOD) (SUNLOG, Cat No.SL1341Ra, ELISA kit) and catalase (CAT) [27] were determined in all plasma samples. Plasma butyrylcholinesterase (BChE) activity was estimated according to the method of Vaisi-Raygani *et al*. [28], while acetylcholinesterase (AchE) was determined using ELISA kit (SUNLOG, Cat No. SL002Ra). C-reactive protein (CRP) was determined as inflammatory marker using ELISA kit （Catalogue # SL0202Ra Sunlong®） In addition, plasma was analyzed for the activities of aspartate transaminase (AST) and alanine transaminase (ALT) according to Reitman and Frankel [29].

#### 2.7.4. Hippocampal gene expression of PI3K and Akt in AD rats

Total RNA was isolated from rat`s hippocampus tissue with *PureLink® RNA Mini* Kit (ambion® Life technologies™) according to the manufacturer's instructions. RNA concentrations and purity were measured with a NanoDrop spectrophotometer. The cDNA was synthesized from 1.5 µg of total RNA in 20µl reaction with RevertAid first strand cDNA synthesis kit (Thermo Fisher®invitrogen™) according to the manufacturer's instructions.

Real-time PCR was performed with a Rotor-Gene®MDx instrument. The RT-PCR reaction mixture (25 µl) contained 1 µl template cDNA, 12.5µl the SYBR-Green®RTPCR master mix (Thermo Fisher®invitrogen™) and 0.25 µM of the primer pairs. Primers pairs sequence used for phosphatidylinositol-3 kinase (PI3K) and the serine/threonine protein kinase B (Akt) gene expression analysis was presented in Table (1). PCR reactions were performed using the following protocol: 50°C for 2 min then 95°C for 10 min, 45 cycles of 20 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C, melting curve program (60-95°C). PCR water was used instead of cDNA templates as a negative control. The relative expression of the target genes was calculated using $2^{-\Delta\Delta CT}$ method [30]; the target gene expression was normalized to the expression of the house-keeping gene GAPDH.

#### 2.7.5. Microbial analysis of rats' fecal samples from cecum and colon

At the end of the experiment, fecal samples from cecum and colon were collected from normal control, AD control and rats given daily oral dose of probiotic-fermented milk. The microbiological characteristics of these samples were detected by serial decimal dilution prepared in 9 ml sterile NaCl (0.85 %) as the following: total lactic acid strains counts were enumerated on MRS agar and the plates incubated at 37 oC for 72 h under anaerobic condition [33]. Total bacterial counts were aerobically incubated using plate count agar at 25°C for 48h [34]. Coliform groups were detected according to FDA [35] using Violet Red bile Agar (Difco) and the plates were incubated at 35 oC for 24 h. The Staphylococci sp. was detected by spreading 0.1 ml of enough dilution from each sample onto the surface of plate containing baired parker agar medium supplemented with egg yolk-potassium tellurite solution (Oxide). The plates were incubated at 37 oC for 48h [35]. The counts of Listeria sp. were detected using oxford agar base supplemented with listeria supplement (Oxide). The 0.1 ml of suitable dilution from each sample was spread on the surface of plates with medium and incubated at 35 oC for 48h [35].

#### 2.7.6. Statistical analysis

The results of animal experiments were expressed as the mean±SE and they were analyzed statistically

using one-way ANOVA followed by the Tukey multiple comparison test using the SPSS statistical program. Differences were considered significant at $p < 0.05$.

**Table 1. Primers used for real-time PCR amplifications**

| Target genes | Sequences | Ref |
|---|---|---|
| PI3K | FW (5′-AGCTGGTCTTCGTTTCCTGA-3′)<br>RW(5′- GAAACTTTTTCCCACCACGA-3′) | Li *et al*. [31] |
| Akt | FW (5′-ACTCATTCCAGACCCACGAC-3′)<br>RW(5′- CCGGTACACCACGTTCTT-3′) | Li *et al*. [31] |
| GAPDH | FW (5′- GTATTGGGCGCCTGGTCACC-3′)<br>RW(5′- CGCTCCTGGAAGATGGTGATGG-3′) | Khan *et al*. [32] |

**Table 2. Nutritional parameters of normal and different AD groups.**

| | NC | AD control | Low dose-extract | High dose-extract | Probiotic-fermented milk |
|---|---|---|---|---|---|
| **Initial body weight (g)** | **$127.5^{a}$ ±2.153** | **$127.5^{a}$ ±2.034** | **$127.1^{a}$ ±2.55** | **$127^{a}$ ±1.487** | **$127.4^{a}$ ±2.226** |
| **Final body weight (g)** | **$160.4^{b}$ ±2.839** | **$143.4^{a}$ ±2.877** | **$146.1^{a}$ ±2.012** | **$149.4^{a}$ ±2.810** | **$159.1^{b}$ ±3.854** |
| **Body weight gain (g)** | **$32.9^{b}$ ±3.118** | **$15.9^{a}$ ±1.756** | **$19.0^{a}$ ±2.29** | **$22.4^{a}$ ±2.226** | **$31.8^{b}$ ±4.339** |
| **Brain weight (g)** | **$0.983^{a}$ ±0.019** | **$0.986^{a}$ ±0.032** | **$1.3^{b}$ ±0.109** | **$1.5^{c}$ ±0.025** | **$0.975^{a}$ ±0.018** |
| **Total food intake (g)** | **$309.4^{b}$ ±11.50** | **$258.8^{a}$ ±16.93** | **$280.0^{ab}$ ±8.844** | **$286.3^{ab}$ ±14.74** | **$293.8^{ab}$ ±9.983** |
| **Food efficiency ratio (g)** | **$0.108^{b}$ ±0.011** | **$0.064^{a}$ ±0.009** | **$0.067^{a}$ ±0.007** | **$0.079^{ab}$ ±0.009** | **$0.108^{b}$ ±0.015** |

Data are expressed as mean ± SE

Values with different superscript letters in the same raw are significantly different at $p<0.05$ levels.

## 3. Results

### 3.1. Phytochemicals and antioxidant activity of costus root crude ethanol extract.

Costus root crude ethanol extract contains total phenolic and total flavonoids as 378.1±5.64 mg GAE/g extract and 275.3±1.57 mg/g extract, respectively. Costus crude ethanol extract and BHT showed antioxidant activity in ferric thiocyanate compared with normal group whereas administration of different treatments improved this reduction significantly. AD rats recorded the lowest body weight gain and total food intake, while groups given

### 3.3. Biochemical changes of different experimental groups.

AD group showed significant elevation in BChE and AChE plasma levels, respectively (Table 3). Elevation in acetyl cholinesterase (AChE) associated injection of D-Gal and $AlCl_3$ is a valuable indicator of AD disease in rats. It was obvious that the injection with D-Gal and $AlCl_3$ mediated oxidative stress which observed in the present study via the elevation of MDA in the plasma as indicator of lipid peroxidation (Table 3) in association with significant reduction in plasma antioxidant enzymes CAT, GPx and SOD in AD group in comparison with normal control. In the present study CRP as an inflammatory marker showed significant elevation in AD group

method, through inhibiting the linoleic acid oxidation. Costus root crude ethanol extract and BHT showed inhibition activity by 98% and 96%, respectively.

### 3.2. Nutritional parameters of different experimental groups

Data in Table 2 showed that significant reduction in the final body weight was noticed in AD group different oral doses showed significant improvement in body weight gain. Probiotic-fermented milk was the most promising treatment in improving nutritional parameters

(Table 3) compared to normal group. Oral administration of crude ethanol extract of costus roots or probiotic-fermented milk exhibited significant improvement in the antioxidant status (reduction of MDA and elevation of CAT, GPx and SOD) in association with reduction in CRP as an inflammatory marker compared with AD control. Also AChE reduced significantly in all treated groups, probiotic-fermented milk was the superior. As shown in table 3; AST and ALT as indicator to liver function were significantly increased in the rats of AD group compared with other rats groups. On the other hand, the studied extract and probiotic-fermented milk ameliorate the elevations in liver function. Probiotic-fermented milk was the most

promising treatment in improving all biochemical changes.

### 3.4. Hippocampal gene expression of PI3K and Akt in AD rats.

Hippocampal gene expression of PI3K and Akt were detected by RT-PCR. The mRNA level of PI3K and Akt were significantly decreased ($p< 0.01$ and $p< 0.05$) in AD-control rats compared with normal-control rats (Fig 1). Treatment with high dose of crude ethanol extract of costus was significantly up-regulated the expression of PI3K and Akt by 95% and 80%, respectively. The mRNA levels of PI3K and Akt were elevated by low dose of crude ethanol extract of costus extract treatment, but the levels did not reach the significant levels compared to AD-control rats. Moreover, the superior treatment among all treatments was probiotic-fermented milk. The gene expression of PI3K and Akt were significantly up-regulated ($p< 0.001$) with 31 and 8.5-fold-change, respectively.

### 3.5. Effect of probiotic-fermented milk on gut microbiota

The count of total lactic acid bacteria in the fermented milk samples were evaluated by MRS agar medium during the experimental period and recorded at 8.29, 8.36 and 8.21 log CFU/ml at the first, second and the third week, respectively. The microbial counts of fecal samples from cecum and colon of normal, AD group and group of rats given oral dose of probiotic-fermented milk are observed in Table (4). The counts of lactic acid strains were increased in the rats' samples that given oral dose of probiotic-fermented milk than normal and AD groups. The counts of lactic acid strains were recorded 7.51 and 6.64 log CFU/g in cecum and colon, respectively for rats given oral dose of probiotic-fermented milk, but was recorded 6.55 and 6.03 log CFU/g in cecum and colon for normal groups and recorded 5.92 and 5.82 log CFU/g, respectively in cecum and colon for AD group. So, our results indicated higher in the lactic acid strains counts around 1.00 and 0.60 log cycles in cecum and colon, respectively for the group given oral dose of probiotic-fermented milk than other groups. Moreover, our results did not indicate significant difference in the lactic acid strains counts in normal and AD rats' groups. On the other hand, *coliforms* count was decreased in the rats given oral dose of probiotic-fermented milk than other groups (normal and AD). The *coliform* counts were recorded in cecum as 2.66, 2.81 and 1.90 log CFU/g, respectively for normal, AD and probiotic-fermented milk groups. Also, *coliforms* count was recorded in colon as 2.83, 3.21 and 1.84 log CFU/g respectively for normal, AD and rats given oral dose of probiotic-fermented milk groups.

The highly count of *coliforms* were recorded in normal and AD groups without significant differences. The same trend of results was observed for the total bacterial counts, which the lessened total bacterial counts enumerated in group given oral dose of probiotic-fermented milk, but the highest counts enumerated for normal and AD groups without significant differences. The total bacterial counts diminished around 1.40 and 1.80 log cycles in cecum and colon, respectively for group given oral dose of probiotic-fermented milk than other groups. Also, the counts of *Staphylococci* sp. were reduced in group given oral dose of probiotic-fermented milk than normal and AD groups. This reduction was detected around 2.50 and 2.00 log cycles in cecum and colon respectively for probiotic-fermented milk group than other groups (normal and AD groups). Moreover, the *Listeria* sp. counts were detected more in normal and

**Table 3. Biochemical parameters of different experimental groups.**

| | NC | AD Control | Low dose-extract | High dose-extract | Probiotic-fermented milk |
|---|---|---|---|---|---|
| AchE (ng/ml) | $1.19^{a}$ ±0.064 | $2.84^{c}$ ±0.152 | $1.96^{b}$ ±0.157 | $1.75^{b}$ ±0.145 | $1.26^{a}$ ±0.088 |
| BhChE (U/L) | $230.13^{a}$ ±8.00 | $419.25^{d}$ ±7.25 | $247.25^{a}$ ±11.75 | $290.38^{b}$ ±7.63 | $373.4^{c}$ ±4.13 |
| MDA (nmol/ml) | $2.59^{a}$ ±0.04 | $6.34^{d}$ ±0.2 | $3.86^{c}$ ±0.06 | $3.38^{b}$ ±0.11 | $2.82^{a}$ ±0.07 |
| CAT (U/l) | $591.0^{c}$ ±6.589 | $452.1^{a}$ ±10.356 | $537.8^{b}$ ±6.529 | $577.8^{c}$ ±5.233 | $585.8^{c}$ ±11.168 |
| GPx (U/ml) | $47.6^{c}$ ±0.962 | $31.8^{a}$ ±1.097 | $40.3^{b}$ ±1.097 | $43.0^{b}$ ±1.647 | $46.5^{c}$ ±1.052 |
| SOD (U/ml) | $11.36^{d}$ ±0.29 | $3.93^{a}$ ±0.27 | $8.89^{b}$ ±0.33 | $9.28^{b}$ ±0.30 | $10.26^{c}$ ±0.35 |
| CRP (ng/ml) | $2.96^{a}$ ±0.11 | $8.7^{e}$ ±0.39 | $6.64^{d}$ ±0.25 | $4.86^{c}$ ±0.26 | $3.79^{b}$ ±0.17 |
| AST (U/L) | $43.1^{a}$ ±4.36 | $71.5^{b}$ ±4.84 | $51.5^{a}$ ±3.18 | $48.8^{a}$ ±3.18 | $46.9^{a}$ ±1.69 |
| ALT (U/L) | $15.0^{a}$ ±1.22 | $19.4^{b}$ ±1.58 | $16.6^{ab}$ ±1.66 | $15.6^{ab}$ ±1.16 | $14.5^{a}$ ±0.94 |

Data are expressed as mean ± SE

AchE: acetylcholinesterase, BhChE: butyrylcholinesterase, MDA: malondialdehyde, CAT: catalase, GPx: glutathione peroxidase, SOD: superoxide dismutase, CRP: C-reactive protein, AST: aspartate transaminase, ALT: alanine transaminase.

Values with different superscript letters in the same raw are significantly different at $p<0.05$ levels.

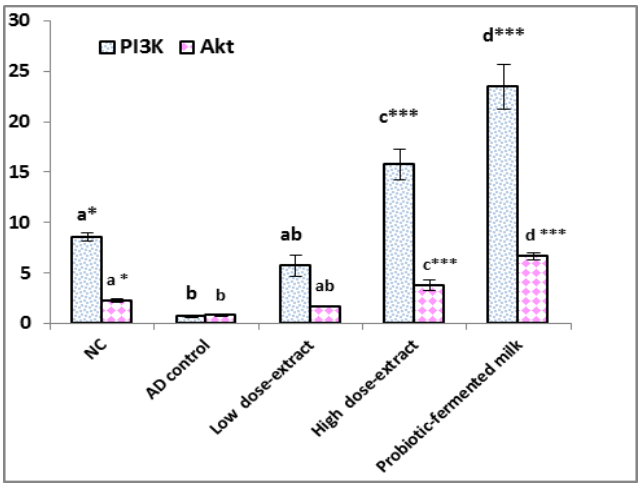


**Fig 1. The mRNA expression of PI3K and Akt genes in hippocampus of different experimental rats.**
PI3K: phosphatidylinositol-3 kinase gene, Akt: serine/threonine protein kinase B gene.
*The mRNA expression PI3K and Akt is normalized with house-keeping gene GAPDH, values are representing as means± SD, the same letter in each column are not significantly different and different letter are significantly different at the level of 0.05 probability levels.

AD groups than group given oral dose of probiotic-fermented milk. The *Listeria* sp. counts were detected at count 2.37, 2.66 and 1.24 log CFU/g in cecum and at 2.16, 2.66 and 1.18 log CFU/g in colon, which the count reduced around 1.30 and 1.00 in cecum and colon for group given oral dose of probiotic-fermented milk than other groups (normal and AD).

**Table 4. Microbial content of rats' cecum and colon of normal, Alzheimer disease and probiotic-fermented milk groups.**

| | NC | AD control | Probiotic-fermented milk |
|---|---|---|---|
| **Counts in cecum** | | | |
| *Lactic acid bacteria* | $6.55^{b}$±0.21 | $5.92^{a}$±0.13 | $7.51^{c}$±0.23 |
| *Coliform* group | $2.66^{b}$±0.14 | $2.81^{b}$±0.16 | $1.90^{a}$±0.22 |
| Total bacterial counts | $6.65^{b}$±0.18 | $6.68^{b}$±0.09 | $5.26^{a}$±0.15 |
| *Staphylococci* counts | $3.05^{b}$±0.18 | $2.96^{b}$±0.13 | $0.78^{a}$±0.35 |
| *Listeria* counts | $2.37^{b}$±0.17 | $2.66^{b}$±0.11 | $1.24^{a}$±0.41 |
| **Counts in colon** | | | |
| *Lactic acid bacteria* | $6.03^{a}$±0.13 | $5.82^{b}$±0.21 | $6.64^{a}$±0.65 |
| *Coliform* group | $2.83^{b}$±0.09 | $3.21^{b}$±0.21 | $1.84^{a}$±0.15 |
| Total bacterial counts | $6.43^{b}$±0.14 | $6.58^{b}$±0.16 | $4.78^{a}$±0.11 |
| *Staphylococci* counts | $3.29^{b}$±0.30 | $2.92^{b}$±0.14 | $1.30^{a}$±0.42 |
| *Listeria* counts | $2.16^{b}$±0.14 | $2.66^{b}$±0.18 | $1.18^{a}$±0.38 |

Data are expressed as mean ± SE
Values with different superscript letters in the same raw are significantly different at $p<0.05$ levels.

### 3.6. Histopathological examination of brain tissue

Results of histopathological examination of brain tissues of different experimental groups are shown in Fig. 2. Brain of normal rat group showed normal neurons in the hippocampus and absences of any neurofibrillary tangles in the cerebrum (Fig. 2 a). Brain tissues of AD rats group showed neurofibrillary tangles (Fig. 2b) and many neurofibrillary tangles hippocampal pyramidal neurons (Fig 2 c) also the tissue showed haemorrhage in the cerebrum (Fig 2 d). Oral administration of crude ethanol extract of costus in low (Fig 2 f) and high (Fig 2 g) doses showed

moderate neurofibrillary tangles in the hippocampus and the pyramidal neurons in the cerebrum. The severity of these neurofibrillary tangles was lower in high dose of extract compared with low dose of extract. Administration of probiotic-fermented milk showed necrosis and degeneration of the neurons, perineuronal edema and neurophagia (Fig 2 h) and mild number of neurofibrillary tangles in the hippocampus (Fig 2 i). Probiotic-fermented milk was the promising treatment in reduction of the presence of neurofibrillary tangles.

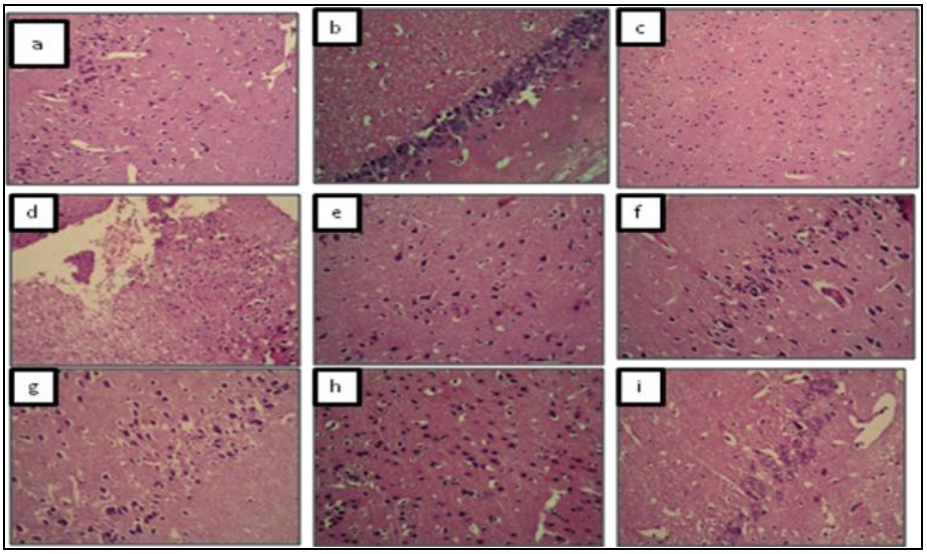


**Fig. 2. Histopatological of rat's brain tissue of different experimental groups**

Figure (2 a): Brain of normal rat group showed normal neurons in the hippocampus and absences of any neurofibrillary tangles (H & E stain, x200).

Fig. (2b): Brain of AD rat group showing neurofibrillary tangles in the cerebrum (H & E stain, x200).

Fig. (2c): Brain of AD rat group showed many neurofibrillary tangles hippocampal pyramidal neurons (H & E stain, x200).

Fig. (2d): Brain of AD rat group showed haemorrhage in the cerebrum (H & E stain, x400).

Fig. (1e): Brain of rats given low dose of extract showed moderate neurofibrillary tangles in the cerebrum (H & E stain, x200).

Fig. (2 f): Brain of rats given low dose of extract showed moderate neurofibrillary tangles in the hippocampus (H & E stain, x400).

Fig. (2g): Brain of rats given high dose of extract showed neurofibrillary tangles pyramidal neurons in the cerebrum (H & E stain, x400).

Fig. (2h): Brain of rat given probiotic-fermented milk showed necrosis and degeneration of the neurons, per-neuronal edema and neurophagia (H & E stain, x400).

Fig. (2i): Brain of rat given probiotic-fermented milk showed necrosis and degeneration of the neurons and mild number of neurofibrillary tangles in the hippocampus (H & E stain, x400).

## 4. Discussion

In the present study AD was induced in rats through i.p injection by D-Gal and $AlCl_3$ for 21 days. In a previous study conducted by some of the authors of the present research [16] D-Gal was effective in induction of brain aging in rats by i.p. injection of D-Gal for 6 weeks. Aluminium was successful in induction of AD model in rats by some of the authors in the present research [17,36,37]. These results encourage us for studying the effect of D-Gal and $AlCl_3$ combination in induction of AD in rats as both of them are neurotoxic. $AlCl_3$ and D-gal was preferred based on previous reports [5,38], which noted that this combination induce pathologies like Alzheimer's disease such as memory dysfunction, neuronal loss, increased acetyl cholinesterase activity, and tauopathy in Wistar albino rats. In the present study significant elevation of BChE and AChE levels was observed in AD group. AChE elevation is a valuable indicator of loss cholinergic neurons in the brain due to its activity in breaking down Ach in the synaptic cleft [39]. Both BChE and AChE play an important role in AD pathogenesis owing to their role in reducing acetylcholine transmitter, which is essential for memory [40]. The present results are in accordance with the results of Chiroma *et al*. [5]. Induction of AD in rats leads to elevation of oxidative stress as observed in the present study through elevation of MDA and reduction of antioxidant enzymes (CAT, SOD and GPx) in association with elevation of CRP as inflammatory marker. It was reported previously that administration of D-gal or $AlCl_3$ alone or in combination increased oxidative stress and inflammation dramatically [5,16,36,41]. Administration of low and high dose of costus and probiotic-fermented milk improved both of the oxidative stress and the elevated AChE and BChE significantly. This improvement may be due to the activity of costus as antioxidant as observed in the present research and in previous research by Sadik *et al*. [13]. The high content of total phenolic

compounds and total flavonoids observed in the present study and the content of triterpenoids [14] are responsible for the antioxidant and anti-inflammatory activities of costus extract. Reduction of oxidative stress and inflammation is very important in management of AD as its lead to brain aging which is a leading cause of dementia and impairment in cognitive function. The improvement in oxidative stress in the present study are in accordance with previous studies [4,42-44] which suggested that oxidative stress and inflammation elevation are an important approach in evaluation of new therapeutic strategies in AD. Also, it was reported previously that administration of dietary sources of antioxidants, including probiotics are important strategy for neuroprotection [42,45-47]. Fermented dairy products and their components, including lactic-acid bacteria, peptides and fatty acids generated during fermentation, may protect against dementia or cognitive impairment [7-9]. Musa et al. [45] reported that administration of Lactobacillus increased the activity of antioxidant enzymes in brain tissue. The reduction in the inflammatory mediator CRP in the present study due to probiotic administration may be attributed to the metabolites secrete by probiotic and its activity as immuno-modulator [9,48].

Our results found that administration of lactic acid strains that recognized as probiotics have ability to modify gut microflora in rats, which able to reduce the pathogens counts and colonized in colon with sufficient counts, which the probiotic strains were produced different acids like (lactic acid, citric acid and acetic acid), hydrogen peroxide and bacteriocins that have antimicrobial activity against pathogens [49-52]. Also, these probiotic bacteria have appositive effect in the intestinal and all bodies' organs functions in rats. Angelucci *et al.* [53] established that the role of gut microbiota in Alzheimer's disease is well, the induce modifications in the gut microbiota with the use of pre-, pro-, or antibiotics able to give therapeutic effects. Also, Nimgampalle and Kuna [54] indicated that *Lactobacillus plantarum* MTCC1325 have anti-Alzheimer properties against D-Galactose induced Alzheimer's disease in albino rats. Ohsawa *et al*. [55] suggested that Calpis sour milk whey with *Lactobacillus helveticus* may be useful for the prevention of neurodegenerative disorders, such as Alzheimer's disease.

The PI3K/ Akt pathway play important role in cell proliferation, growth and survival. Akt is a signaling downstream of PI3K, the stimulation of Akt affects different substrates that are vital in cellular signaling [56]. Neurofibrillary tangles and amyloid-β accumulation in Alzheimer brain causes inhibition of PI3K/ Akt pathway. Suppression of PI3K/ Akt pathway leads to promote cell apoptosis and induce neuron death and oxidative stress activation [57,58]. Activation of PI3K/ Akt pathway may be one of the new therapeutic targets for treatment of Alzheimer. In the current study, treatments with high dose extract and probiotic-fermented milk activate PI3K/ Akt pathway. Gut microbiota play an important role in Akt signalling regulation. The probiotic *L. rhamnosus* releases several peptides including p75 and p40 that act through the Akt and PI3K pathways to induce growth and cellular proliferation [59]. *B. breve* binds to immune cells and activates important downstream pathways through the TLR-2 receptor including PI3K and GSK3β [60].

Histological results from this study showed marked degeneration of neurofibrillary tangles in the hippocampus of AD control group which confirmed the AD. The present results are in accordance with previous studies [61,62]. Administration of low or high dose of costus or probiotic-fermented milk improved the formation of neurofibrillary tangles, with the priority of probiotic-fermented milk. Hippocampus is the first area in the brain to be affected in AD patients [63]. Hippocampus is highly populated with monoaminergic, cholinergic and glutamatergic axonal terminals and abnormalities in neurotransmitters released by these terminals are regarded to be closely associated with AD [64].

## 5. Conclusion

Administration of probiotic-fermented milk and costus extract have promising effect on cognitive function through reduction of neurofibrillary tangles formation, regulation of hippocampal gene expression of PI3K and Akt, inflammatory and oxidative stress markers. The present results suggested that intake of probiotic-fermented milk had a beneficial effect on the composition of cecum and colon flora. Probiotic-fermented milk was the most effective for prevention of AD in the present study.

## 6. Financial Support

The research was fully funded and supported by the National Research Centre through the research project No. 12050203.

## 7. Acknowledgments

The authors acknowledge the NRC, Egypt for funding this research through the research project No. 12050203.

## 8. Conflicts of Interest

The authors declare no conflict of interest.

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