DOI:10.5138/09750185.2443

AR JOURNALS ADVANCED RESEARCH

International Journal of Phytomedicine
2020;12(4):107-118

RESEARCH ARTICLE

Prevention of β-amyloid-induced toxicity in adifferentiated neuronal (IMR32) cell line by Khaya grandifololia(Welw) C. DC.

Fils Armand Ella[1,2,3], Manjula Shantaram[2], Simon Ngamli Fewou[4], Frederic Nico Njayou[1], Sayali Chandrashekhar Deolankar[3], Prashant Kumar Modi[3] and Paul Fewou Moundipa[1*]

Abstract

Alzheimer's disease (AD) belongs to the class of neurodegenerative disorder and is biochemically characterized by amyloid-β (Aβ) plaques deposition, accumulation of neurofibrillary tangles (NFTs) accumulation and ultimately neuronal loss. Even though, the progress made in developing efficient AD therapy, there is no effective drug capable to stop and/or slow down AD progression. In the current article, we investigated the neuroprotective effect of Khaya grandifololia crude extract and fraction 2 against Aβ_{42}-induced cytotoxicity and hyperphosphorylation of tau protein in differentiated neuronal cells (IMR32). Reactive oxygen species production, apoptosis and mitochondrial dynamics and function, synaptic protein, and tau phosphorylation were evaluated using fluorescence microscopy and immunoblotting. Cell viability was assessed using the MTT assay. Findings revealed that exposure of differentiated IMR32 cells to Aβ_{42} alone induced the impairment of mitochondrial dynamics, decrease synaptic protein expression and increase hyperphosphorylation of tau protein (phospho tau181). In contrast, the presence of crude extract and KGf2 significantly inhibited the cleavage of Caspase-3 activation. In addition, the levels of synaptic proteins (Symptosomal associated protein 25 and Synaptosin) and superoxide dismutase were restored upon treatment with crude extract and fraction 2. Hyperphosphorylation of tau protein (Thr181) and ERK (Thr202/Tyr205) activities were also significantly reduced after treatment with crude extract and fraction 2. Our findings suggest that KG extract is a potential source for candidate drug against AD and may contribute to the development of efficient therapeutic strategy against AD.

Keywords: Alzheimer's disease; Khaya grandifololia; neuroprotection; tau protein; Amyloid β1-42.

Introduction

Alzheimer's disease (AD) belongs to the class of neurodegenerative disorder that affects the central nervous system and is biochemically characterized by amyloid-β (Aβ) plaques deposition, accumulation of neurofibrillary tangles (NFTs), and neuronal loss with a cognitive impairment as consequence [1]. It primarily affects elderly people over 65 years of age and account for 50-60% dementia cases [2, 3]. Approximately 47.5 million people worldwide are affected by AD and the number of affected people is expected to rise during the next 20 years [4, 5]. The pathological manifestations of AD and include extracellular amyloid beta deposits and intracellular tau protein abnormality characterized by hyperphosphorylated tau which eventually forms neurofibrillary tangles (NFTs) inside the cells. In addition, synaptic dysfunction has been observed in other regions of the brain [6]. Overall, clinical manifestations include progressive memory loss, deterioration of intellectual functions, decreased speech function, disorientation, and gait irregularities.

*Correspondence: pmoundipa@hotmail.com

[1]Department of Biochemistry, Faculty of Science, University of Yaounde 1, Yaounde, Cameroon

Full list of author information is available at the end of the article.

Received: 06 Aug 2020, Accepted:14 Dec 2020

The prevalence of disease in a specific in population is dependent on factors like incidence and survival. Therefore, AD prevalence rises exponentially with age, increasing markedly after 65 years of age. AD in people 65 years of age and older was about 6% [7]. Overall, approximately 47 million people worldwide were living with AD in 2015 [8]. Since AD has become a major public health because of an increasingly aging population. Epidemiologic studies indicate that the worldwide prevalence of AD will double by the year 2030 and triple by 2050. Regional studies indicate that 3.6 million people in Asia live with AD and in Europe, America and Africa 2.3, 1.2 and 0.5 million people are affected, respectively [9].

Current Medications used for AD treatment include acetylcholinesterase inhibitors (donepezil, tacrine and galantamine) for mild to moderate cases, and memantine, an NMDA (N-methylD-aspatarte)-receptor antagonist for the treatment of moderate to severe AD. These drugs seem to be able to produce modest symptomatic improvement in some patients [10].

However, no available medication appears to be able to cure or stop AD progression. Previous investigations using medicinal plants that include Punica granatum, Vitis vinifera, Olea europaea, Cinnamomum verum [11] have reported some anti-amyloidogenic effects in transgenic mice and also in an in vitro AD model [12]. In other investigations, medicinal plants extract have shown neuroprotective activities [13], therefore can be use or be helpful in the development of new drugs for Alzheimer's disease. For this purpose, we decided to screen Cameroonian medicinal plants used in traditional medicine practices for anti-AD activity. More specifically, Khaya grandifoliola (KG) is a plant used in west Cameroon traditional medicine to combat various diseases including neurological disorders.

In addition, numerous investigation have shown that KG possess various activities including anti-malarial, anti-bacterial, anti-ulcer, anti-anaemic, anti-inflammatory, anti-fungal, hypoglycemic, hypocholesterolemic activities [14, 15]. In addition, antioxidant, cytotoxic and hepato-protective effects have also been reported [16, 17]. The current study was undertaken to investigate the anti-amylodogenic activity of KG. To perform this task, we first generated AD model by exposing differentiated neuronal cells (IMR32) to amyloid beta peptide aggregates ($A\beta_{1-42}$). We found that IRM32 cells were sensitive to $A\beta$–induced toxicity and also tau hyperphosphorylation as observed in AD patients. Mitochondrial dysfunction was also observed when exposing IMR32 cells to $A\beta_{1-42}$. In contrast, prevention of key parameters of neuronal dysfunction in AD such as tau hyperphosphorylation, apoptosis, and mitochondrial dysfunction was observed in the presence of KG extract and fraction.

Moreover, restoration of the level of reactive oxygen (ROS) production and synaptic proteins (SNAP25 and SYP) was also observed when IMR32 cells were exposed to Aß presence of KG crude extract and its purified fraction. The present results indicate KG possesses a neuroprotective activity, therefore may be potentially considered in the process of the development of new drug against AD.

Material and Methods

Chemicals

$A\beta_{1-42}$ (rpeptide, Georgia, USA), 2',7'-Dichlorofluorescin diacetate (DCFH-DA), Hoescht 33342, propidium iodide (PI), anti-rabbit IgG- alkaline phosphatase conjugated secondary antibody were from Sigma Aldrich, St. Louis, MO, USA. Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) were obtained from Himedia Laboratories, India. Primary antibodies Cleaved Caspase-3 (1:500), pERK (1:500), SOD (1:1000), SNAP25 (1:1000), SYP (1:1000) from Cell Signaling, Beverly, MA; pT181-tau (1:1000) from Santa Cruz Biotechnology, Santa Cruz, CA; and β-actin (1:1000) from Sigma Aldrich, St. Louis, MO, USA.

Methods

Preparation of K haya grandifololia (KG) crude extract and fraction 2

The stem bark of KG was collected in July 2017 in Foumban in the West Region of Cameroon. The botanical identification of the plant was done at the Cameroon National Herbarium in Yaounde, where voucher specimens are kept under the reference number 23434 YA. The collected plant material was washed with distilled water, air-dried and powdered. A portion (200g) of the powder was extracted twice with 2L of ethanol/water 65/35 (v/v) at room temperature with regular agitations during 48 hours. The extracts were filtered through Whatman paper No.1, pooled, evaporated using a rotary evaporator and dried in an oven (HP-AD070) at 50°C and then subjected to silica gel 60/120 (particle size 40–63 μm) (MERCK, Vetec^{TM}, Germany) column chromatography by eluting with a gradient of polarity in the hexane/acetyl acetate (60/40 v/v); resulting in the collection of eight fractions. These eight fractions were analyzed using thin layer chromatography (TLC) and combined into 2 fractions labeled KGf1 and KGf2 based on the similarity of their TLC profiles.

For the biological assays, the crude extract and fraction KGf2 were reconstituted in DMSO at a concentration of 50 mg/mL.

Preparation of oligomeric Aβ stock solution

The 42–amino acid version of Aβ peptide (A$\beta_{42,}$ rpeptide, Georgia, USA) was dissolved in sterile 1% ammonium hydroxide, sonicated for 30 s, and incubated at 37°C for 5 days and was used at a final concentration of 1 μM as previously reported [18].

Cell culture and differentiation

Human Neuroblastoma cell line IMR32 was purchased from National Centre for Cell Science (NCCS, Pune, India). Cells were maintained in Dulbecco Modified Essential Medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum, a mixture of 1 % penicillin/streptomycin at 37 °C. Differentiation was induced by exposing undifferentiated IMR32 cells to retinoic acid (RA, 10 μM). Cell morphology was examined using an inverted microscope Primovert with digital camera Axiom 105 color (Zeiss, NY, USA) until cells were fully differentiated, which was after 8 to 10 days of culture.

Cell treatment

To determine the effect of the crude extract and fraction 2 on differentiated IMR32 cells physiology, cells were seeded at a density of 500,000 cells/well in 6-well plates and treated as follow: (1) untreated IMR32 cells; (2) IMR32 cells incubated with Aβ_{42} (1 μM) alone; (3) IMR32 cells treated with the crude extract and fraction 2 at 5 μg/mL and Aβ_{42} 1 μM; (4) IMR32 cells treated with the crude extract and fraction 2 alone at 5 μg/mL final concentration. Cells were incubated for 48 hr and the treatment was terminated by removing the culture medium. Cells were washed with PBS 1x before used for different assays.

Cytotoxicity assay

Test for cytotoxicty was done using 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, IMR32 cells were seeded in 96-well plates at a density of 3 000 cells/well and incubated with the plant extract and fractions at different concentrations (0; 2; 5; 10, 20; 30; 40 and 50 μg/mL) for 48 hr. Then the culture medium was replaced by fresh serum-free DMEM supplemented with 70 μL of MTT (0.5 mg/mL) solution and the cells were further incubated at 37 °C for 4 hr. The medium was discarded and 200 μL of a mixture of DMSO/Ethanol (1:1 V/V) was applied to the well to dissolve the formazan derived from the mitochondrial cleavage of the tetrazolium ring by live cells. The absorbance of the solubilized MTT formazan product was measured at 570 nm and 650 nm using a Multiskan microplate reader (Multiskan Sky, ThermoFisher, MA, USA).

Morphological analysis of IMR32 cells

IMR32 cells treated with KG plant extracts or fraction 2 was compared to untreated cells in order to determine the order to determine the morphological by the plant extracts and fraction 2. IMR32 cells were seeded in 12-well plates, exposed to increasing concentrations (0, 5, 15 and 25 μg/mL) of KG crude extract and fraction 2 for 48 hr. The change in cell morphology was determined by phase contrast microscopic analysis (Zeiss Axiovert 100; 20 X magnification) (Zeiss, NY, USA).

Determination of cell apoptosis

Differentiated IMR32 cells were seeded and treated with Aβ_{42} and 5 μg/mL of the extract and fraction 2 for 48 h at 37°C and 5% CO_2. After treatment, IMR32 cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min. After that, cells were washed again with PBS and stained with propidium Iodide (PI) (20 μg/mL) and Hoechst 33342 (5 μg/mL). Cells were incubated in dark for 30 min at room temperature. The incubated cells were washed again with PBS and analyzed under by fluorescent microscopy using cell imager ZOE^{TM} (BioRad, Portland, USA).

Determination of intracellular ROS generation

Differentiated IMR32 cells were treated with Aβ_{42} with or without KG crude extract and fraction 2 (5 μg/mL) for 48 hr. At the end of the incubation period, treated and untreated cells were incubated with 25 μM 2',7'–dichlorofluorescin diacetate (DCF-DA) at 37 °C in dark for 45 min followed by washing with PBS. The intracellular levels of Reactive Oxygen Species (ROS) generation were monitored by fluorescence detection of DCF that is proportional to the level of production of intracellular ROS. The fluorescence was captured by a fluorescent cell imager ZOE^{TM} (BioRad, Portland, USA) at an excitation and emission wavelength of 488 nm and 560 nm, respectively.

Protein extraction and Western blot

Human neuroblastoma IMR32 cells were cultured in T25 flasks to 80% confluency. Cells were seeded into 6-well plates at a density of 5.0 $\times$ 10^5 cells/well and incubated in the presence of retinoic acid (RA) for 8-10 days. Differentiated cells were further cultured with and without KG extract and/or fraction 2 (5 μg/mL) in the presence of Aβ_{42} peptide for 48 hr. Cells were then harvested and rinsed with ice-cold PBS and incubated with 150 μL of lysis buffer (50mM Tris-HCl buffer pH 7.2; 2% SDS) containing a cocktail of protease inhibitor (Thermo Scientific Ltd, MA, USA) on ice and incubated for 30 min. Cell homogenate was then obtained by using a cell scraped and sonicated. The protein extracts were obtained by centrifugation at

14000 × g for 10 min at 4°C. Supernatant (total cell protein) was collected and protein concentration determined using Bicinchoninic Acid (BCA) assay kit (Thermo Scientific, MA, USA). Thirty (30) microgram of total protein extract was separated on 12% SDS–PAGE and blotted onto nitrocellulose membrane (Bio-Rad, Portland, USA). The membranes were incubated in 5% skimed milk for 2 hours under agitation at room temperature. The membranes were further incubated with the appropriate primary antibodies [(cleaved Caspase-3, SOD, SNAP25, SYP (Cell Signaling, Beverly, MA), β-actin (Sigma Aldrich, MA, USA)] overnight at 4°C. For pERK and pTau (Santa Cruz Biotechnology, Santa Cruz, CA); membranes were blocked in BSA before incubation with the appropriate primary antibody. Membranes were washed with PBS three times for 10 min and further incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature under agitation. Proteins were visualized using chemiluminescence reagents (ThermoFisher Scientific, MA, USA). Relative protein level was determined with normalization to β-actin signal by densitometry using ImageJ software.

Statistical analysis

Results of all analysis are presented as means ± standard deviation (SD) (n = 3). Band intensities of fluorescence images and western blots were quantified using ImageJ and expressed as relative values to the controls. Multiple comparisons were analyzed by one-way ANOVA followed by Tukey's test. Statistical analyses were performed using GraphPad prism version 5.0. Differences were considered significant at $p < 0.05$.

Results

Prior to determine whether KG and fraction 2 prevent the β-amyloid-induced toxicity in a human neuroblastoma (IMR32), KG crude extract and fraction 2 were first tested for intrinsic cytotoxicity. Undifferentiated IMR32 cells were incubated with various concentrations of KG crude extract and fraction 2. The cellular viability was estimated by the MTT assay. Results indicate that KG crude extract and fractions 2 alone had no toxic effect on differentiated IMR32 cells till 25 μg/mL (supplemental figure 1). Our results indicates that lowest concentration of KG crude extract and fraction 2, which is not cytotoxic to differentiated IMR32 cells was 5 μg/mL (Supplemental figure 2) and used for subsequent experiments.

Protective effect of KG extract and fraction 2 on Aβ_{42} -induced reactive oxygen species (ROS) production and apoptosis.

Effects of KG crude extract and fraction 2 on Aβ_{42}-induced ROS production in cultured differentiated IMR32 cells are shown in figure 1. Treatment of IMR32 cells for 48 hours using Aβ_{42} (1 μM) significantly increased the production of intracellular ROS. Co-treatment of IMR32 cells with KG crude extract and fraction 2 with Aβ_{42} significant reduced the Aβ induced intracellular ROS production.

In figure 2, treatment of IMR32 cells with KG crude extract or fraction 2 alone significantly reduced the intracellular ROS level, indicating that KG protect cells against Aβ-induced ROS production.

.

The finding that the intracellular levels of reactive oxygen species is controlled through the activities of SOD enzymes, prompted us to investigate whether KG crude extract and fraction 2 induces the affects ROS level through SOD enzymes activity. Therefore, IMR32 cells were treated with Aβ_{42} in the presence and absence of KG crude extract or fraction 2, and later total proteins were extracted from cells and were probed for SOD by immunoblotting revealing. A significant decrease SOD protein expression in Aβ_{42}-treated cells compared to control (figure 3A-D). However, the presence of KG crude extract and fraction 2 during Aβ_{42} treatment of IMR32 restores the level of SOD protein expression ($P<0.001$).

It is shown that elevated ROS levels are also associated with oxidative damage, increased deposition of Aβ_{42}, the formation of senile plaques and cell death. We assessed whether the KG crude extract and fraction 2 protect cells from Aβ_{42} -induced apoptosis.

The extract and fractions of KG Inhibit Cleaved Caspase-3 in Aβ_{1-42} treated IMR32 cells

The effects of crude extract and fraction 2 of KG on cleaved Caspase-3 expression were evaluated by western blot. As shown in figure 3E-H, there was a significant up-regulation of caspase-3 in Aβ-treated cells compared to control cells ($P<0.001$). However, treatment of these cells with crude extract and fraction 2 of KG inhibit the cleavage of cleaved Caspase-3, confirming therefore KG possesses an anti-apoptotic activity.

KG extract and fraction 2 protect synaptic integrity against Aβ_{42}-induced synaptotoxicity

To assess synaptic integrity after Aβ_{1-42} treatment, we quantified the expression of presynaptic vesicle membrane proteins synaptosomal-associated protein 25 (SNAP-25) and synapto-

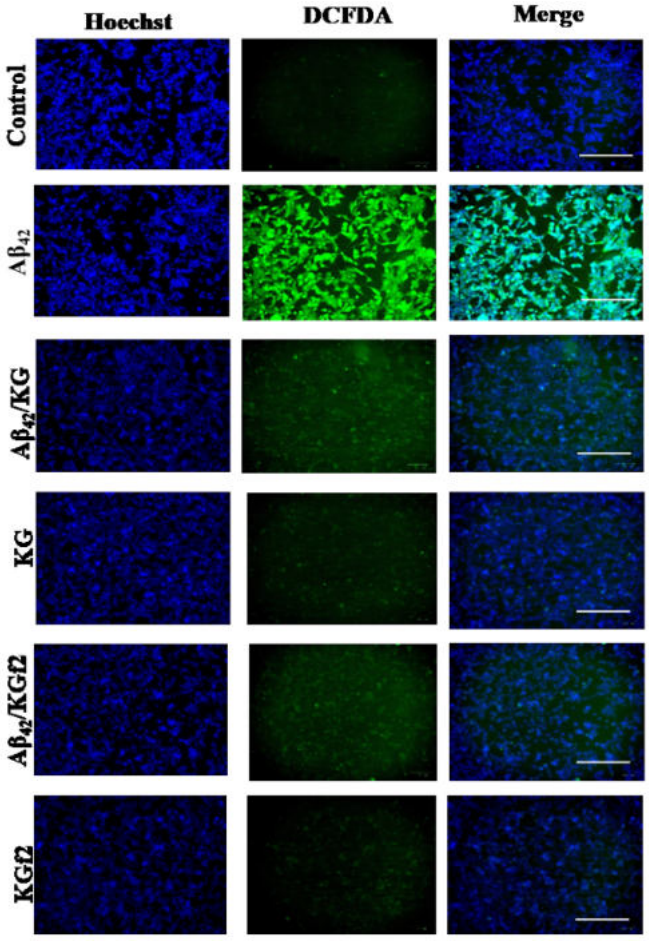

Figure 1 Effect of extract and fraction of KG on ROS level in Aβ_{42} induced IMR32 cells. ROS: reactive oxygen species; KG: Khaya grandifololia. Aβ_{42}: Amyloid beta 1-42. Scale bars, 100 μm.

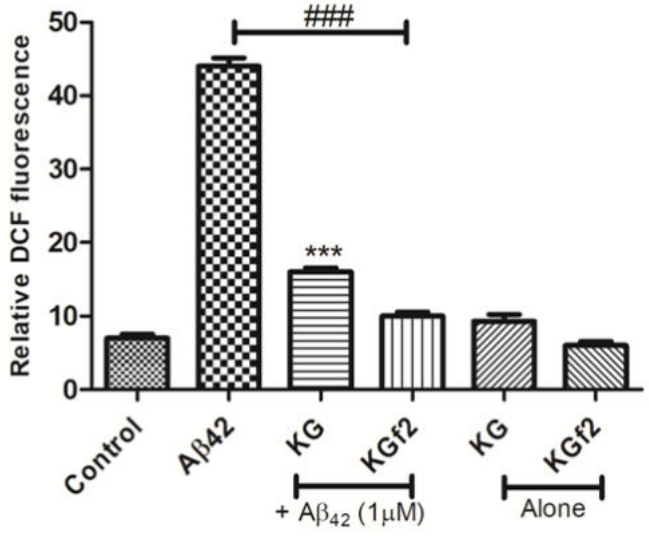

Figure 2 Quantification of ROS level inAβ_{42} induced IMR32 cells. ROS: reactive oxygen species; KG: Khaya grandifololia. Aβ_{42}: Amyloid beta 1-42. ***P<0.001 vs control; $^{\#\#\#}$P<0.001 vs Aβ_{42} group

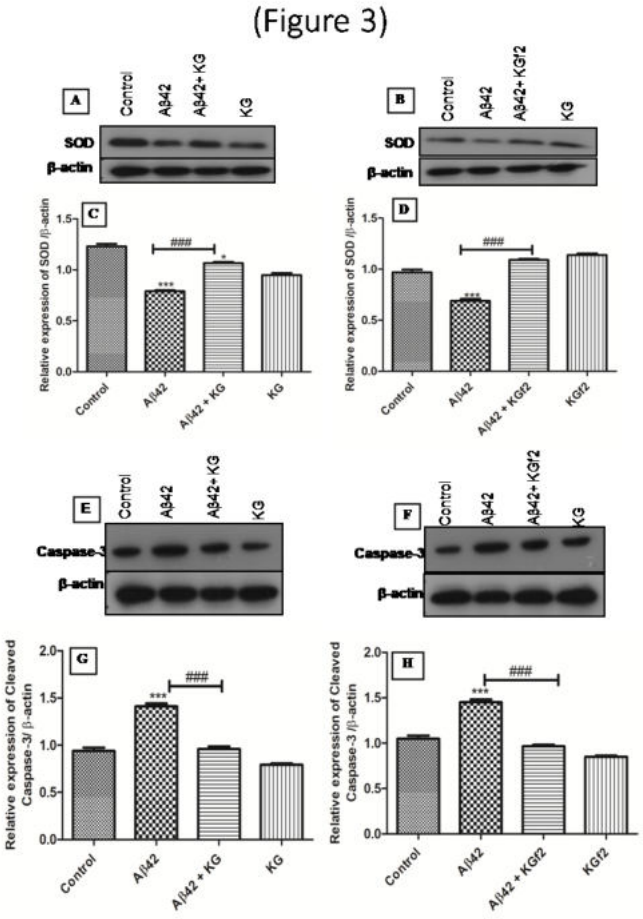

Figure 3 Effect of extract and fraction of KG on apoptosis induced by Aβ_{42} in IMR32 cells. (A, B) Effect of crude extract and fraction 2 of KG on SOD activation, respectively. (C, D) Densitometry analysis of blots, respectively, for crude extract and fraction 2. (E, F) Effect of crude extract and fraction 2 of KG on inhibition of cleavage of Caspase-3, respectively. (G, H) Densitometry analysis of blots, respectively, for crude extract and fraction 2. *P<0.05; ***P<0.001 vs control; $^{\#\#\#}$P<0.001 vs Aβ_{42} group. Lane1: control; Lane2: Aβ_{42}; Lane3: Aβ_{42} + KG or Aβ_{42} + KGf2; Lane4: KG or KGf2. KG: Khaya grandifoliola, KGf2: fraction 2 of KG. Aβ_{42}: Amyloid beta 1-42.

physin (SYP). Cells were treated without or with Aβ_{42} (1 μM), or co-treated with Aβ_{42} (1 μM) and crude extract and fraction 2 of KG (5 μg/mL) for 48 h. After treatment, total proteins were extracted from cells and investigated for SNAP25 and SYP respectively. A western blot analysis showed a significant reduction of SNAP25 and SYP levels in Aβ_{1-42}-treated IMR32 cells compared to control, indicating a synaptic dysfunction (Fig. 4AB and Fig. 4EF). However, when IMR32 cells were treated with Aβ_{42} in the presence of KG crude extract and fraction 2, the expression level of SNAP-25 (Fig. 4AD) and SYP (Fig. 4EH) were restored (P<0.001).

Extract and fraction 2 of KG inhibit hyperphosphorylation of tau protein in Aβ_{1-42} -treated IMR32 cells

To determine whether KG crude extract and fraction 2 protect tau protein phosphorylation, cells were treated without or with Aβ_{42} (1 μM), or co-treated with Aβ_{42} (1 μM) and crude extract and fraction 2 of KG (5 μg/mL) for 48 h. After treatment, total proteins were extracted from cells and the lysates were probed for p-tau. As presented in figure 5, compared with the control group, the level of tau phosphorylation at Thr181 residue was significantly increased in the Aβ_{42} treated group (P<0.001). Conversely, the levels of tau phosphorylation in the extract and fraction KGf2 of KG groups were much lower than in the model group (P<0.05 and P<0.001) respectively. The levels of tau phos-

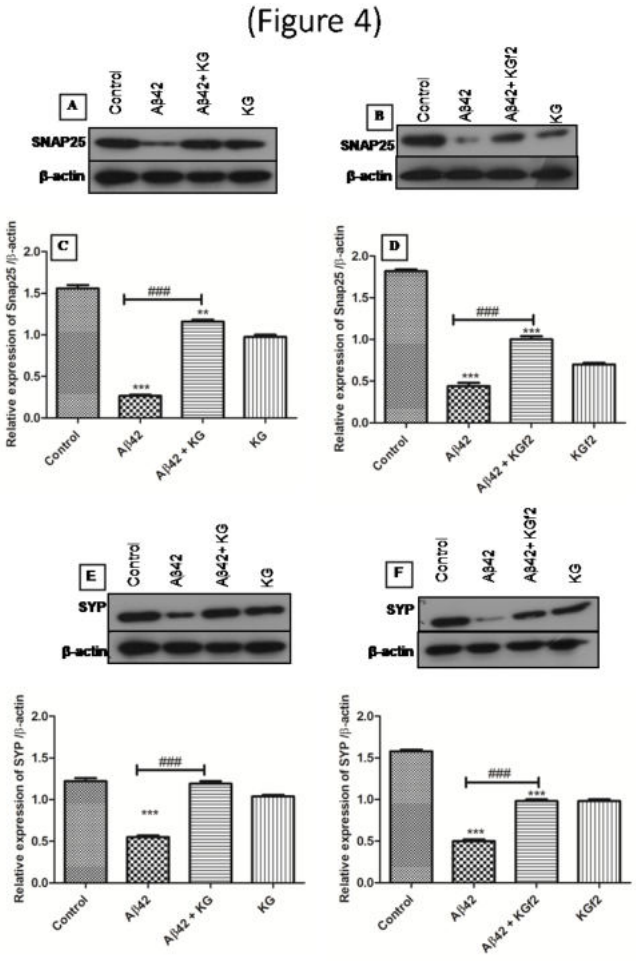

Figure 4 Crude extract and fraction 2 of KG inhibited Aβ_{42}-induced synaptotoxicity. (A, B) Effect of crude extract and fraction 2 of KG on Snap25 activation, respectively. (C, D) Densitometry analysis of blots, respectively, for crude extract and fraction 2. (E, F) Effect of crude extract and fraction 2 of KG on SYP activation, respectively. (G, H) Densitometry analysis of blots, respectively, for crude extract and fraction 2. ***P<0.001, **P<0.01 vs control; ###P<0.001 vs Aβ_{42} group. Lane1: control; Lane2: Aβ_{42}; Lane3: Aβ_{42} + KG or Aβ_{42} + KGf2; Lane4: KG or KGf2. KG: Khaya grandifoliola, KGf2: fraction 2 of KG. Aβ_{42}: Amyloid beta 1-42.

phorylation were most significantly decreased following treatment with 5 μg/mL of extract and fraction KGf2.

Inhibition of ERK activity by crude extract and fraction 2 of KG in IMR32 cells exposed to Aβ_{42}

The dysregulation of the ERK signaling pathway, which affects tau hyperphosphorylation has been associated with the Aβ_{1-42} model of AD. The increased level of phosphorylated ERK at Thr202/Tyr204 is a reliable marker of the severity of AD. To investigate whether KG crude extract and fraction 2 prevents Aβ_{42}induced ERK hyperphosphorylation, Western blotting using a pERK antibody specific for the detection of p-ERK (Thr202/Tyr204) was performed. Differentiated IMR32 cells were treated with Aβ_{42} for 48 h and we found that Aβ_{42} significantly increased the levels of pERK. As shown in Figure 6, phosphorylation levels of ERK were significantly elevated, indicating that increased kinase activities might have contributed to tau hyperphosphorylation following Aβ_{42} insult which was reduced upon KG crude extract and its purified fraction (P<0.001).

Discussion

The current investigation was undertaken to investigate whether the *Khaya grandifololia* (KG) extracts prevent the differentiated neuronal cell line (IMR32) from the β-amyloid induced toxicity. Herein, KG crude extract or fraction 2 showed a preventive activity against Aβ–induced cytotoxicity in IMR32 cells. More

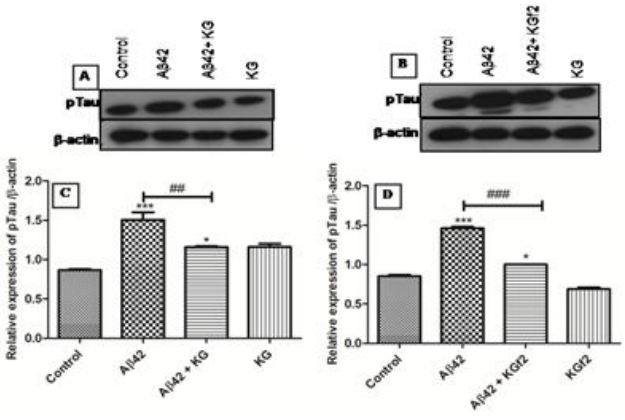

Figure 5 Extract and fraction of KG inhibited tau hyperphosphorylation induced by Aβ_{42} in IMR32 cells. (A, B) Effect of crude extract and fraction 2 of KG on pTau inhibition, respectively. (C, D) Densitometry analysis of blots, respectively, for crude extract and fraction 2. ***P<0.001, *P<0.05 vs control; ###P<0.001, ##P<0.01 vs Aβ_{42} group. Lane1: control; Lane2: Aβ_{42}; Lane3: Aβ_{42} + KG or Aβ_{42} + KGf2; Lane4: KG or KGf2. KG: Khaya grandifoliola, KGf2: fraction 2 of KG. Aβ_{42}: Amyloid beta 1-42.

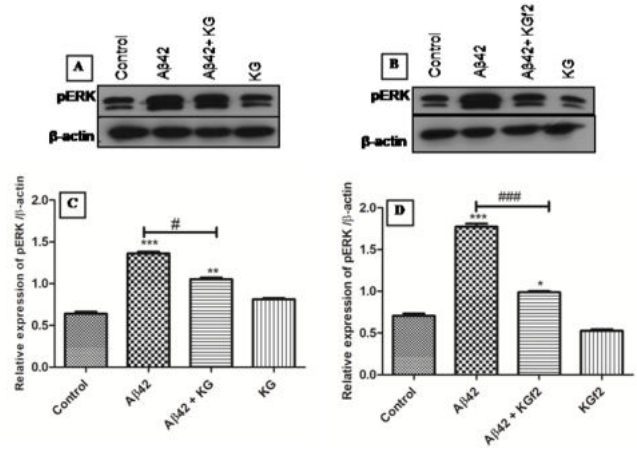

Figure 6 pERK inhibition in response to Aβ_{42} in IMR32 cells by extract and fraction of KG. (A, B) Effect of crude extract and fraction 2 of KG on pERK inhibition, respectively. (C, D) Densitometry analysis of blots, respectively, for crude extract and fraction 2. ***P<0.001; **P<0.01 vs control; ###P<0.001 vs Aβ_{42} group. Lane1: control; Lane2: Aβ_{42}; Lane3: Aβ_{42} + KG or Aβ_{42} + KGf2; Lane4: KG or KGf2. KG: Khaya grandifoliola, KGf2: fraction 2 of KG. Aβ_{42}: Amyloid beta 1-42.

specifically, KG extracts prevented apoptosis by blocking the intracellular ROS production and inhibition of caspase-3 upon exposure of IRM32 to Aβ_{42}. Moreover, we also observed a significant protection of the synaptic integrity and inhibition of tau hyperphosphorylation by KG extract upon exposure of IRM32 cell to Aβ_{42}. Beside the prevention of Aβ_{42}–induced cytotoxicity by KG extracts observed in the current study, it has already shown that KG extract possesses potent activity against acetylcholine esterase, one of the characteristics of Alzheimer disease (AD) pathology and responsible for most AD symptoms including memory decline and cognition [19]. The findings highlighted in our study demonstrate that KG extracts may be considered as potential therapeutic source in a strategy of new drug development against AD.

It is shown that Aβ_{42} dyshomeostasis is one of the main factors in AD pathogenesis [20], and this view point is expanded towards a multi-target approach to achieve better therapeutic effects. Aβ is associated with oxidative stress in neuronal cells and the exposure of differentiated neuronal IMR32 cells to Aβ_{42} peptides resulted in elevated levels of intracellular reactive oxygen species (ROS) and significantly decreased SOD activity. The consequence is the mitochondrial dysfunction due to a change in the mitochondrial membrane permeability [21]. Thus,

the activity of antioxidant enzymes SOD is often considered a biomarker of the resultant antioxidative response. In this study, using the fluorescent activity as an indicator of the ROS production, DCFH-DA staining demonstrates that the level of ROS in Aβ_{42} induced IMR32 cells were remarkably higher than in the control group. Treatment with KG crude extract and fraction 2 significantly reduced the level of ROS. Concerning antioxidant status, the SOD activity from IMR32 cells treated with Aβ were much lower than the control, but it was significantly reversed following the treatment by crude extract and fraction 2 of KG.

The oxidative stress induced by Aβ creates a microenvironment that promotes the onset of apoptosis [22]. In this study, cell apoptosis was investigated using the PI/Hoescht staining method. When IMR32 cells were exposed to Aβ_{42}, apoptotic features were obviously observed after PI and Hoescht double staining, total apoptotic rate was increased, which could be partially abrogated by the plant extract treatment. In order to verify the effect of KG on this process, the caspase activity was also measured. Caspases are serine-aspartyl proteases that are involved in the initiation and execution of apoptosis [23]. Caspase-3 is essential for the implementation of the final step of apoptosis and acts as an executioner [24]. Our results showed that the level of cleaved Caspase-3 was higher in the Aβ_{42}-treated cells compared to the control group. Treatment with extract and fractions of KG ameliorated the Aß42-induced upregulation of cleavage of Caspase-3 and significantly decreased the level compared with Aβ_{42} treatment alone. The crude extract and fraction 2 of KG decreased expression of cleaved caspase-3 and inhibits neuronal apoptosis in IMR32 cells. These results agreed with the data from the above cytotoxicity assay, suggesting that the ability of extract and fractions KG to attenuate Aβ_{42}-induced IMR32 cell death was mediated by its anti-apoptotic activity [25].

Taking the mitochondrial apoptosis and oxidative stress results together, crude extract and fraction 2 of KG may inhibit mitochondrial apoptosis-related oxidative stress.

Many studies showed that alterations in synaptic proteins expression are major contributors that precede neuronal loss in AD [26]. Aβ accumulation cause synaptic toxicity. Synaptophysin (SYP) and synaptosomal-associated protein 25 (SNAP-25) levels are decreased in the brain of AD patients and Aβ-induced toxicity in cells. The increase in the synaptic proteins expression could be considered as an early option for treatment of AD [27]. The results from our studies demonstrated that Aβ induced an impaired synaptic structure and also significantly reduce the expression of synaptic proteins that include SNAP25 and SYP. However, crude extract and fraction 2 of KG could obviously improve the expressions of synaptic related proteins which are significantly increased after treatment.

These data suggest that the inhibition AD by KG may be mediated by the restoration of the expression of the synaptic proteins.

More recent studies have found a better correlation between tau and memory impairment in AD [28]. Tau hyperphosphorylation is a key event and important initial step in the degradation of neurons occurring in the pathogenesis of AD and because the microtubule structure of the neuron is destroyed after Tau abnormally phosphorylated [29]. In AD brains, hyperphosphorylation of tau protein occurred at several Ser or Thr phosphorylated sites, including Thr181, Thr205, Ser202, Ser396, Ser404 [30]. The destabilization of the cytoskeletal dynamic, apoptosis and ultimately memory dysfunction is caused by tau proteins hyperphosphorylation. It is obviously is an important task for researcher to develop drugs that specifically target tau protein hyperphosphorylation in neuronal cells exposed to Aβ [30]. In the present study, Aβ was used to construct the tau hyperphosphorylation model, treating IMR32 cells with 1 μM oligomeric peptide Aβ_{42}. After 48 hr, a cell model of tau hyperphosphorylation was successfully established and the expression of Tau (pT181) was significantly increased. The level of tau phosphorylation at the site mentioned was reduced, following treatment of the cells with crude extract and fraction 2 of KG at 5 μg/mL.

Therefore, compound of KG inhibited Aβ-induced hyperphosphorylation of tau. Moreover, extracellular signal regulated kinase (ERK) is a kinase family involved in gene expression, mitosis, metabolism, apoptosis, proliferation, differentiation and movement. It can also affect tau phosphorylation in AD pathology models [31]. Even though the mitogen-activated kinase (MEK–ERK) pathway is important for neuronal survival, neuronal death due to neurotoxic agents can be the consequence of the aberrant activation of this pathway [18]. Our results showed that crude extract and fraction 2 of KG inhibited the expression of ERK showing that KG attenuates hyperphosphorylation of Tau by inhibition of ERK.

AD's drug discovery targeting the amyloid precursor proteins (APP) and Aβ have so far not generated a successful disease modifying candidate for this devastating pathology. Attention to tau protein was almost completely left behind, even though it forms aggregates in AD. Tangles are later manifestations of tau pathology and soluble phosphorylated tau is the species responsible for neurodegenerative damage [28]. A large number of herbal extracts, fractions, phytochemicals and herbal formulations may possess anti-AD properties via their antioxidant, anti-inflammatory, anti-apoptotic and anti-acetylcholinesterase activ-

ities, antiglycation, anti-β-amyloid aggregation, resulting in a reduction in Aβ-induced toxicity [32].

In addition, the compounds with properties such as mitochondrial function protection, antiinflammation and anti-oxidant activities may also provide the protective effect against neurodegeneration. However, potential beneficial effects of polyphenols, flavonoids in AD may play a role in downstream targets such as tau phosphorylation [33].

Conclusion

In summary, crude extract and fraction 2 of KG protected IMR32 cells from neuronal cell death induced by amyloid β by inducing apoptosis, and it significantly attenuated the Aβ_{42}-induced hyperphosphorylation of tau at Thr181 through the inhibition of phosphorylation of ERK. These results indicate that the extract and fraction 2 of KG inhibited mitochondrial apoptosis, synqptic toxicity and hyperphosphorylation of tau induced by Aβ. Taken together, the results presented here increase our knowledge about the neuroprotective effect of extract of KG, and suggest this plant as a candidate for the development of phytotherapeutic agents for Alzheimer's disease and other tau pathology-related neuronal degenerative diseases.

Abbreviations

AD: Alzheimer's disease; Aβ: Amyloid beta; BCA: Bicinchoninic acid assay; CNS: Central nervous system; DCF-DA: 2',7'-dichlorofluorescein diacetate; DMEM: Dulbecco's modified eagle medium; DMSO: Dimethylsulfoxide; ERK: Extracellular signal regulated kinase; FBS: Fetal bovine serum; KG: Khaya grandifololia; KGf2: fraction 2 de KG; NFTs: Neurofibrillary tangles; PI: Propidium iodide; pT181: PhosphoTau at Thr 181; RA: Retinoid acid; ROS: Reactive oxygen species; SNAP25: Symptosomal associated protein 25; SOD: Superoxide dismutase; SYP: Synaptosin; TLC; Thin layer chromatography.

Acknowledgements

The first author of this article is grateful for the financial support rendered by the Department of Science and Technology (DST) of the Government of India; and the Federation of Indian Chambers of Commerce and Industry (FICCI) with the Research Training Fellowship for Developing Countries Scientists (RTF-DCS) (No.DCS/2018/000007).

Conflict of interest

The authors have declared no conflicts of interest.

Author contributions

FAE, SNF, FNN, and PFM defined the research subject and the aims, designed the experiments. MS, PMK, and PFM provided facilities to perform the work. FAE, SCD, PMK performed the experiments. FAE, SNF, FNN, MS, PMK and PFM analyzed the data and wrote the paper.

All the authors read and approved the final version of this manuscript.

Author details

[1]Department of Biochemistry, Faculty of Science, University of Yaounde 1, Yaounde, Cameroon. [2]Department of Studies & Research in Biochemistry, Post Graduate Centre, Mangalore University, Chikka Aluvara571232, Karnataka, Kodagu, India. [3]Yenepoya Research Centre, Yenepoya (Deemed to be University), Mangalore575018, Karnataka, India. [4]Faculty of Health Science, Université des Montagnes, Bangangté, Cameroon.

References

[1] Li K, Wei Q, Liu FF, Hu F, Xie A, Zhu LQ, et al. Synaptic Dysfunction in Alzheimer's disease: Aβ, Tau, and Epigenetic Alterations. MolNeurobiol. 2017;55(4):3021–3032.

[2] Francis PT, Palmer AM, Snape M, Wilcock GK. The cholinergic hypothesis of Alzheimer's disease: a review of progress. Journal of Neurology, Neurosurgery & Psychiatry. 1999;66(2):137–147. Available from: https://dx.doi.org/10.1136/jnnp.66.2.137.

[3] Ma WW, Tao Y, Wang YY, Peng IF. Effects of Gardenia jasminoides extracts on cognition and innate immune response in an adult Drosophila model of Alzheimer's disease. Chin J Nat Medicines. 2017;15(12):899–904.

[4] Zhifeng Z, Han J, Zhang J, Xiao Q, Hu J, Chen L. Pharmacological activities, mechanisms of action, and safety of salidroside in the central nervous system. Drug Des Dev Ther. 2018;12:1479–1489.

[5] Poirier Y, Grimm A, Schmitt K, Eckert A. Link between the unfolded protein response and dysregulation of mitochondrial bioenergetics in Alzheimer's disease. Cellular and Molecular Life Sciences. 2019;76(7):1419–1431. Available from: https://dx.doi.org/10.1007/s00018-019-03009-4.

[6] Xia W, Li DW, Xiang L, Chang JJ, Xia ZL, Han EJ. Neuroprotective effects of an aqueous extract of Futokadsura stem in an Aβ-Induced Alzheimer's disease-like rat model. Chin J Physiol. 2015;58(2):104–113.

[7] Burns A, Iliffe S. Alzheimer's disease. BMJ. 2009;338(feb05 1):b158–b158. Available from: https://dx.doi.org/10.1136/bmj.b158.

[8] Mroczko B, Groblewska M, Litman-Zawadzka A, Kornhuber J, Lewczuk P. Amyloid β oligomers (AβOs) in Alzheimer's disease. Journal of Neural Transmission. 2018;125(2):177–191. Available from: https://dx.doi.org/

10.1007/s00702-017-1820-x.

[9] Kuca K, Soukup O, Maresova P, Korabecny J, Nepovimova E, Klimova B, et al. Current Approaches Against Alzheimer's Disease in Clinical Trials. Journal of the Brazilian Chemical Society. 2016;27(4):641–649. Available from: https://dx.doi.org/10.5935/0103-5053.20160048.

[10] Clark CM, Karlawish JHT. Alzheimer Disease: Current Concepts and Emerging Diagnostic and Therapeutic Strategies. Annals of Internal Medicine. 2003;138(5):400–400. Available from: https://dx.doi.org/10.7326/0003-4819-138-5-200303040-00010.

[11] Andrade S, Ramalho MJ, Loureiro JA, do Carmo Pereira M. Natural Compounds for Alzheimer's Disease Therapy: A Systematic Review of Preclinical and Clinical Studies. International Journal of Molecular Sciences. 2019;20(9):2313–2313. Available from: https://dx.doi.org/10.3390/ijms20092313.

[12] Wang L, Jin GF, Yu HH, Lu XH, Zou ZH, Liang JQ. Protective effects of tenuifolin isolated from Polygala tenuifolia Willd roots on neuronal apoptosis and learning and memory deficits in mice with Alzheimer's disease. Food Funct. 2019;10:7453–7460.

[13] Ebrahimi A, Schluesener H. Natural polyphenols against neurodegenerative disorders: Potentials and pitfalls. Ageing Research Reviews. 2012;11(2):329–345. Available from: https://dx.doi.org/10.1016/j.arr.2012.01.006.

[14] Ijarotimi SO, Agbedahunsi JM, Onyeji CO, Co A. Chemotherapeutic interaction between Khaya grandifoliola (Welw) CDC stem bark extract and two anti-malarial drugs in mice. Afr J Tradit Complement Altern Med. 2010;7(4):370–376.

[15] Hashem F, Aboutabl E, Souda SE, Moharam M, Mammoun A, Shabana M. Desmutagenic and antimutagenic potential of phenolics from Khaya grandifoliola (C.DC.), Meliaceae. Egyptian Pharmaceutical Journal. 2013;12(2):148–148. Available from: https://dx.doi.org/10.4103/1687-4315.124018.

[16] Njayou FN, Amougou AM, Tsayem RF, Manjia JN, Rudraiah S, Bradley B, et al. Antioxidant fractions of Khaya grandifoliola C.DC. and Entada africana Guill. et Perr. induce nuclear translocation of Nrf2 in HC-04 cells. Cell Stress and Chaperones. 2015;20(6):991–1000. Available from: https://dx.doi.org/10.1007/s12192-015-0628-6.

[17] Kouam AF, Yuan F, Njayou FN, He H, Tsayem RF, Oladejo BO, et al. Induction of Mkp-1 and Nuclear Translocation of Nrf2 by Limonoids from Khaya grandifoliola C.DC Protect L-02 Hepatocytes against Acetaminophen-Induced Hepatotoxicity. Frontiers in Pharmacology. 2017;8:653–653. Available from: https://dx.doi.org/10.3389/fphar.2017.00653.

[18] Modi PK, Komaravelli N, Singh N, Sharma P. Interplay between MEK-ERK signaling, cyclin D1, and cyclin-dependent kinase 5 regulates cell cycle reentry and apoptosis of neurons. Molecular Biology of the Cell. 2012;23(18):3722–3730. Available from: https://dx.doi.org/10.1091/mbc.e12-02-0125.

[19] Jahn H. Memory loss in Alzheimer's disease. Dialogues Clin Neurosci. 2013;15(4):445–454.

[20] Forner S, Baglietto-Vargas D, Martini AC, Trujillo-Estrada L, LaFerla FM. Synaptic Impairment in Alzheimer's Disease: A Dysregulated Symphony. Trends in Neurosciences. 2017;40(6):347–357. Available from: https://dx.doi.org/10.1016/j.tins.2017.04.002.

[21] Ali T, Yoon GH, Shah SA, Lee HY, Mo K. Osmotin attenuates amyloid beta induced memory impairment, tau phosphorylation and neurodegeneration in the mouse hippocampus. Scientific Reports. 2015;5:11708–11708.

[22] Kim A, Nam YJ, Lee MS, Shin YK, Sohn DS, Lee CS. Apigenin reduces proteasome inhibition-induced neuronal apoptosis by suppressing the cell death process. Neurochem Res. 2016;41(11):2969–2980.

[23] Zhuang J, Li Y, Chi Y. Role of p38 MAPK activation and mitochondrial cytochrome-c release in allicin-induced apoptosis in SK-N-SH cells. Anti-Cancer Drugs. 2016;27:312–317.

[24] Wang Z, Lin D, Zhang L, Liu W, Tan H, Ma J. Penehyclidine hydrochloride prevents anoxia/reoxygenation injury and induces H9c2 cardiomyocyte apoptosis via a mitochondrial pathway. Eur J Pharmacol. 2017;797:115–123.

[25] Chen Z, Tao S, Li X, Zeng X, Zhang M, Yao Q. Anagliptin protects neuronal cells against endogenous amyloid β (Aβ)-induced cytotoxicity and apoptosis. Artif Cell Nanomed B. 2019;47(1):2213–2220.

[26] Reddy PH, Manczak M, Yin X, Grady MC, Mitchell A, Kandimalla R, et al. Protective effects of a natural product, curcumin, against amyloid β induced mitochondrial and synaptic toxicities in Alzheimer's disease. Journal of Investigative Medicine. 2016;64(8):1220–1234. Available from: https://dx.doi.org/10.1136/jim-2016-000240.

[27] Daneshmand P, Saliminejad K, Shasaltaneh MD, Riazi GH, Nazari R, Azimzadeh P, et al. Neuroprotective effects of herbal extract (Rosa canina, Tanacetum vulgare and Urtica dioica) on rat model of sporadic Alzheimer's disease. Avicenna J Med Biotech. 2016;8(3):120–125.

[28] Adwan L, Subaiea GM, Basha R, Zawia NH. Tolfenamic acid reduces tau and CDK5 levels: implications for dementia and tauopathies. Journal of Neurochemistry.

2015;133(2):266–272. Available from: https://dx.doi.org/10.1111/jnc.12960.

[29] Cao F, Wang Y, Peng B, Zhang X, Zhang D, Xu L. Effects of celastrol on Tau hyperphosphorylation and expression of HSF-1 and HSP70 in SH-SY5Y neuroblastoma cells induced by amyloid-ß peptides. Biotechnol Appl Biochem. 2018;65(3):390–396.

[30] Yao Y, Wang Y, Chen Y, Yang J. Osthole decreases tau protein phosphorylation via PI3K/AKT/GSK-3β signaling pathway in Alzheimer's disease. Life Sci. 2019;15(217):16–24.

[31] Jhang KA, Park JS, Kim HS, Chong YH. Resveratrol ameliorates tau hyperphosphorylation at Ser396 site and oxidative damage in rat hippocampal slices exposed to vanadate: Implication of ERK1/2 and GSK-3β signaling cascades. J Agric Food Chem. 2017;64(44):9626–9634.

[32] Sereia AL, de Oliveira MT, Baranoski A, Marques LLM, Ribeiro FM, Isolani RG, et al. In vitro evaluation of the protective effects of plant extracts against amyloid-beta peptide-induced toxicity in human neuroblastoma SH-SY5Y cells. PLOS ONE. 2019;14(2):e0212089–e0212089. Available from: https://dx.doi.org/10.1371/journal.pone.0212089.

[33] Baptista FI, Henriques AG, Silva AMS, Wiltfang J, da Cruz e Silva OAB. Flavonoids as Therapeutic Compounds Targeting Key Proteins Involved in Alzheimer's Disease. ACS Chemical Neuroscience. 2014;5(2):83–92. Available from: https://dx.doi.org/10.1021/cn400213r.