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Original Article

CYTOTOXICITY STUDY OF ANTIDIABETIC PLANTS ON NEUROBLASTOMA CELLS CULTURED AT NORMAL AND HIGH GLUCOSE LEVEL

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ABSTRACT

Objective: In diabetes, chronic hyperglycemia causes damage (glucose toxicity) on some cells leading to micro and macro vascular complications. The aim of this study was to investigate the effect of antidiabetic plants extracts in high glucose concentration *in vitro*.

Methods: *Phyllanthus amarus* (whole plant), *Vitex doniana* (leaves), *Tectona grandis* (leaves and trunk bark) and *Plumeria alba* (roots) hydroalcoholic extract (at the concentrations of 6.25, 25, 75, 125, 250 and 500 µg/ml) were tested for their possible cytotoxicity using the 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on neuroblastoma cells lines in standard condition (extract alone) and high glucose concentration (extract+50 mM glucose).

Results: At concentrations of 6.25 and 25µg/ml, *T. grandis* bark and leaves and *P. amarus* induced a significant decrease (*p<0.01; p<0.001*) on cell viability as compared to controls. The decrease on cell viability was very pronounced in the presence of the extracts plus glucose 50 mM. *P. amarus* extract becomes increasingly toxic as the concentration of extract increased in the presence of glucose. With *P. amarus* at 125 µg/ml and glucose at 50 mM, there is no more viable cells in the medium. By contrast, *T. grandis* bark extract induced a significant reduction of the cytotoxicity in the presence of glucose compared to standard condition.

Conclusion: It appears that, only hydroalcoholic extract of *T. grandis* bark possesses neuroprotective activity in high glucose concentration.

Keywords: Glucose toxicity, Neuroblastoma cells, Plants extract.

INTRODUCTION

Diabetes mellitus is a chronic disease characterized by an elevated fasted blood glucose (fasted blood glucose exceeds 6.9 mmol/l) [1]. This chronic hyperglycemia causes damage (glucose toxicity) to some types of cells by production of advanced glycation end products (AGE), elevation of reactive oxygen species (ROS) production and abnormal stimulation of hemodynamic regulation systems (such as the renin-angiotensin system, RAS). The chronic hyperglycemia related to diabetes mellitus is a leading cause of micro vascular and macro vascular complications including retinopathy, nephropathy, and peripheral neuropathy and cerebro vascular diseases [2, 3].

The prevalence of diabetes is increasing. In 2000, the number of diabetic globally was estimated at 171 million [4]. Currently this number reached 385 million and the forecasts for 2035 are estimated at 592 million, corresponds to an increase of 55%. Diabetes is the direct cause of 5.1 million deaths and more than 80% of deaths occur in low and middle-income country [5].

In the management of chronic hyperglycemia in diabetics, it is important to reduce the risks of complications related to this disease. At present, several plants are used alone or in association to maintain blood glucose level at normal. Previous studies conducted in our laboratory have reported the antidiabetic activity *in vivo* of *Phyllanthus amarus* (Euphorbiaceae), *Tectona grandis* (Verbenaceae), *Plumeria alba* (Apocynaceae), *Bridelia ferruginea* (Euphorbiaceae) and *Waltheria indica* (Sterculiaceae) [6-10]. The present study was undertaken to evaluate the effect of *Phyllanthus amarus*, *Vitex doniana*, *Tectona grandis* and *Plumeria alba* hydroalcoholic extract on neuroblastoma cells lines in high glucose concentration, whether to know if the extracts are capable to protect neurons against glucose toxicity *in vitro* model.

MATERIALS AND METHODS

Plant material

Phyllanthus amarus (whole plant), *Vitex doniana* (leaves), *Tectona grandis* (leaves and trunk bark) and *Plumeria alba* (roots) were collected and each specimen were identified and kept in the herbarium of the Laboratory of Botany and Plant Ecology (Faculty of Science/University of Lome). Plants were dried and extracted with ethanol/water (50:50 v/v for *P. amarus*, *V. doniana*, *T. grandis* and 80/20 v/v for *P. alba*) as following. Twenty two hundred gram of each plant material was macerated during 72 h in ethanol/water solution. The crude extracts were filtered with Whatman paper and evaporated in vacuo at 40 °C using a rotary evaporator. The yield of preparations was respectively 13% (*P. amarus*), 13% (*V. doniana*), 11.34% (*P. alba*), 19.5% and 20% (*T. grandis* leaves and trunk bark).

Chemicals

Roswell Park Memorial Institute medium (RPMI medium), fetal calf serum (FCS), Phosphate-Buffered Saline (PBS), trypsine-0.02%, Ethylenediaminetetraacetic acid (EDTA) mixture, 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Lyon, France). All other chemicals used were of analytical grade and from Sigma chemicals, France.

Cell lines and treatment

Neuro-2A cells (mouse neuroblastoma-like cell with functional *p53* gene) were routinely cultured at 37 °C in a humidified 5% CO2, 95% air atmosphere. They were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum, L-glutamine (8 mM), penicillin (100 UI/ml), and streptomycin (100 µg/ml).

The cells at sub-confluent stage were harvested from the flask by treatment with trypsin and an aliquot of cell suspension (10000 cells/200 µl/well) were transferred to each well of 96-well microplates. The cells were incubated and treated with plants extracts under standard and high glucose concentration condition. In standard condition, cell culture medium was removed after 24 h of incubation and replaced with RPMI medium containing extracts except control wells at different concentrations (6.25 µg/ml, 25 µg/ml, 75 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml) for 72 h. In high glucose concentration condition, cells were incubated for 72 h with different concentrations of extracts prepared with RPMI medium containing 50 mM of D-glucose. The positive control well has contained 50 mM of D-glucose without extract.

Cell viability tests

The cell viability tests were conducted according to the protocol of Creppy *et al.*, 2014 [11]. Briefly cell viability was determined using MTT colorimetric assay which is an indicator of mitochondrial activity. At the end of intoxication period, medium of each well was discarded and 100 µl of MTT solution (0.5 mg/ml in RPMI) was added to every wells. After two hours, 100 µl of Dimethyl sulfoxid (DMSO) solution was added to each well to dissolve the formed formazan crystals. Microplates were shaken gently for 10 min and the absorbance was read at 492 nm using a microplate reader (Labtech LT-4000 Plate reader). A minimum of 4 wells were used for each concentration.

RESULTS

Cell viability in high glucose concentration

As shown in fig. 1, there was a significant decrease (*p<0.001*) in neuro-2a (N2A) cell viability in the presence of glucose at a concentration of 50 mM as compared to controls. At 5 mM, glucose had no effect on cell viability.

Cell viability in the presence of extract and extract+glucose

At the concentration of 6.25 and 25 µg/ml, *T. grandis* bark (TGB) and leaves (TGL) and *P. amarus* (PA) induced a significant decrease (*p<0.01; p<0.001*) of cell viability as compared to controls (fig. 2 and 3). The decrease of cell viability was very pronounced in the presence of extracts plus glucose 50 mM (fig. 2 and 3). *V. doniana* (VD) and *P. alba* (PLA) extracts induced a decrease of cell viability in the presence of glucose (fig. 3B).

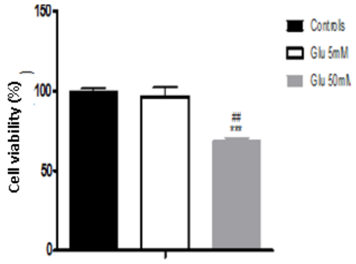

Fig. 1: Effect of D-glucose on viability of N2A cells cultured in the standard condition, The cells were cultivated for 72 h in RPMI medium (controls) and in RPMI glucose-enriched (Glu 5 mM and Glu 50 mM). Data are mean±SEM of three values. * *p*<0.001 versus controls. Glu 5 mM and Glu 50 mM: glucose at the concentration of 5 mM and 50 mM respectively**

The antiproliferative effects of the different extracts have been observed at concentrations of 75, 125, 250 and 500 µg/ml (fig. 3–6) in the presence or absence of glucose. However, high glucose concentration plus extracts has led to exacerbation of the antiproliferative effect of the plants. *P. amarus* extract becomes increasingly toxic as the concentration of extract increased in high glucose concentration condition. For PA125+Glu 50 mM, there is no more viable cells in the medium (fig. 5–7). By contrast, *T. grandis* bark extract induced a significant reduction of the cytotoxicity in the presence of glucose (fig. 4–7).

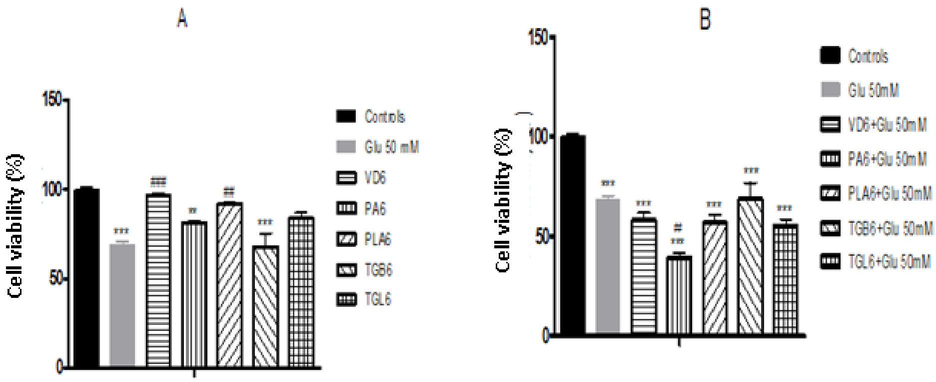

Fig. 2: Effect of different extracts on viability of N2A cells in the presence of extracts at 6.25 µg/ml (A) and in the presence of extracts at 6.25 µg/ml+D-glucose at 50 mM (B)
Data are mean±SEM of three values. * *p*<0.001 versus controls. #p<0.05; ## p<0.01; ### p<0.001 versus glucose 50 mM**

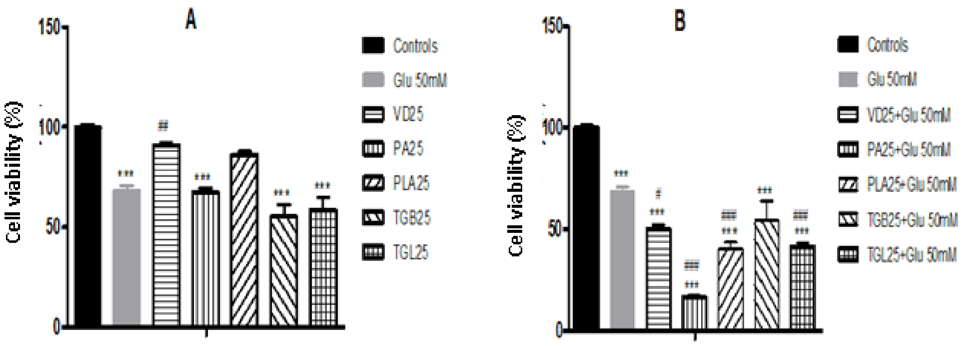

Fig. 3: Effect of different extracts on viability of N2A cells in the presence of extracts at 25 µg/ml (A) and in the presence of extracts at 25 µg/ml+D-glucose at 50 mM (B)
Data are mean±SEM of three values. * *p*<0.001 versus controls. #p<0.05; ## p<0.01; ### p<0.001 versus glucose 50 mM**

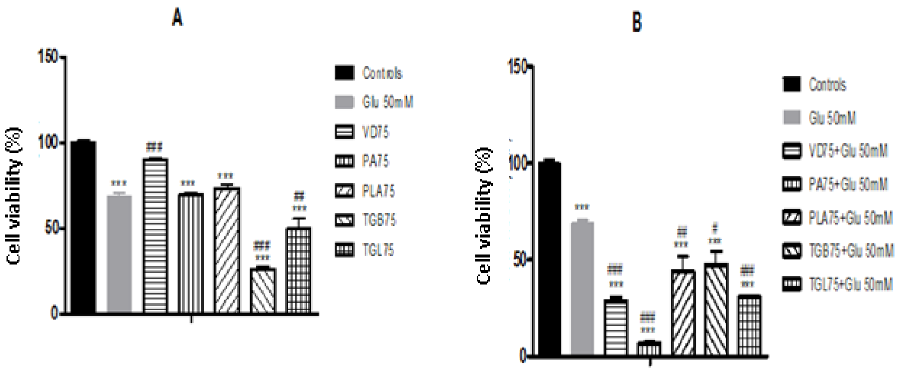

Fig. 4: Effect of different extracts on viability of N2A cells in the presence of extracts at 75 µg/ml (A) and in the presence of extracts at 75 µg/ml+D-glucose at 50 mM (B)
Data are mean±SEM of three values. * *p*<0.001 versus controls. #p<0.05; ## p<0.01; ### p<0.001 versus glucose 50 mM**

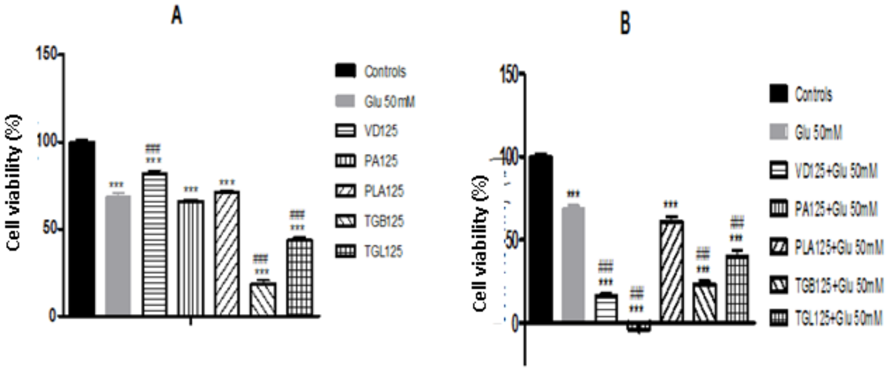

Fig. 5: Effect of different extracts on viability of N2A cells in the presence of extracts at 125 µg/ml (A) and in the presence of extracts at 125 µg/ml+D-glucose at 50 mM (B)
Data are mean±SEM of three values. * *p*<0.001 versus controls. #p<0.05; ## p<0.01; ### p<0.001 versus glucose 50 mM**

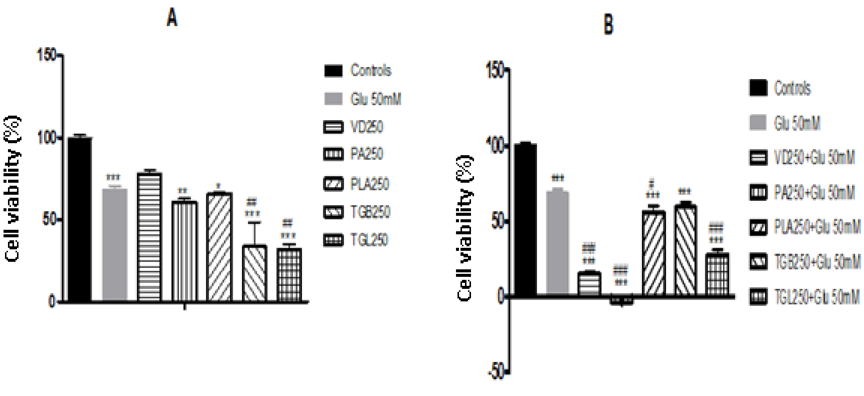

Fig. 6: Effect of different extracts on viability of N2A cells in the presence of extracts at 250 µg/ml (A) and in the presence of extracts at 250 µg/ml+D-glucose at 50 mM (B)
Data are mean±SEM of three values. * *p*<0.001 versus controls. #p<0.05; ## p<0.01; ### p<0.001 versus glucose 50 mM**

DISCUSSION

In diabetes, chronic hyperglycemia causes damage (glucose toxicity) to some types of cells leading to micro vascular and macro vascular complications including retinopathy, nephropathy and neuropathy. The present study aimed to evaluate *in vitro*, the effect of antidiabetic plants extracts on cell viability (especially in neurons) in high glucose concentration. Cells line used in this study was neuroblastoma cells line (N2A) which is often used to investigate a variety of neuronal responses including neurotoxicity [12]. The cells were cultured in high glucose concentration. Glucose is a sugar that plays an important role in energy production in biological systems. *In vitro*, glucose was added to all cell culture media and the amount in cell culture formulations ranges from 5.5 mM to 55 mM. Many classical media are supplemented with approximately 5.5 mM glucose which approximates normal blood sugar levels *in vivo*.

For providing an *in vitro* diabetes model with regard that diabetes complications appear after a long time of high-glucose concentrations, above 10 mM were considered in cell culture medium [13, 14]. The result of this study has shown that exposure (72 h) to high glucose concentration (50 mM) lead to significant reduction (31.17 % p<0.001) of cell viability compared to untreated cells (control). 5 mM of glucose did not induce toxicity to cells. High glucose concentration increases the effects of stress on cells. Studies have reported that high glucose treatment of cells leads to the overproduction of reactive oxygen species (ROS) that precedes cells apoptosis. High glucose concentration inhibits the activity of glutathione peroxidase which is responsible for elimination of H_2O_2 in the cytoplasm; therefore inhibition of synthesis or depletion of endogenous cellular antioxidant defenses by hyperglycemia would facilitate additional stress to cause apoptosis [15, 16]. In addition, the work of Tchounwou *et al.*, 2014 [17] had shown that D-glucose causes DNA damage in MCF-7 cells in a dose-dependent manner by inducing cytotoxic, genotoxic, and apoptotic effects.

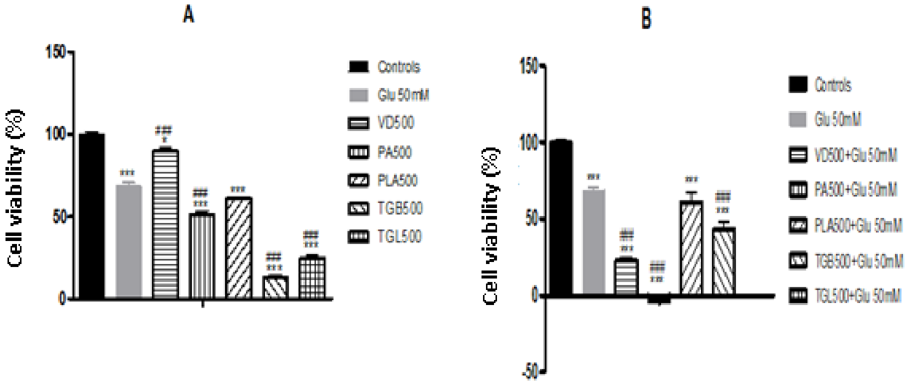

Fig. 7: Effect of different extracts on viability of N2A cells in the presence of extracts at 500 µg/ml (A) and in the presence of extracts at 500 µg/ml+D-glucose at 50 mM (B)

Data are mean±SEM of three values. * *p*<0.001 versus controls. #p<0.05; ## p<0.01; ### p<0.001 versus glucose 50 mM**

At all concentrations, hydroalcoholic extracts of *V. doniana*, *P. amarus*, *P. alba* and *T. grandis* leaves and bark induce in standard condition (single treatment) an inhibition on cell viability. A comparison between the extracts has shown that the hydroalcoholic extract of *T. grandis* bark have induced more reduction of cell viability. At concentrations of 75, 125, 250 and 500 µg/ml, *T. grandis* bark induced a reduction of cell viability respectively 37.5%; 27.2%; 48.8% and 19.4%. Several studies in the literature have demonstrated the antiproliferative activity of medicinal plants extracts *in vitro* [16, 18, 19].

Generally, plants extracts exhibit their antiproliferative effect *in vitro* through their chemical composition and the relative content of the active compounds in the extract. The polyphenols group is heavily involved in this inhibition of cell growth. *In vitro*, these phytochemical constituents can protect against high glucose induced oxidative stress but can also become pro-oxidants by generating free radicals [20-23]. Preliminary studies in our laboratory and elsewhere have already shown the presence of total phenols, flavonoids and tannins in the alcoholic extracts of *V. doniana* [24], *P. amarus* [7], *P. alba* [25] and *T. grandis* leaves and bark [6].

In the presence of extract plus 50 mM glucose, it has been observed in contrast to those which were expected, a potentiating of antiproliferative effect of extracts. With *P. amarus* at 25 µg/ml, the inhibition of cell viability decreased from 67.5% in standard condition to 16.25% in high glucose concentration condition in concentration-dependent manner. Above 75 µg/ml, the cytotoxicity effect of hydro alcoholic extract of *P. amarus* in high glucose concentration was total. By comparing, the effect of *T. grandis* bark extracts in standard condition to high glucose concentration condition, it was observed that, *T. grandis* bark becomes less cytotoxic (at all concentrations tested) in the presence of glucose.

This could be due as mentioned above to the antioxidant capacity of *T. grandis* bark extract. Indeed, *T. grandis* bark extract reduce the lipoperoxidation induced by 2,2'-Azobis 2 Amidinopropane Dihydrochloride (AAPH) *in vitro* [6] and inhibit the oxidative stress in diabetic rats [26].

CONCLUSION

It appears from this study, that the hydro alcoholic extracts of *P. amarus*, *V. doniana*, *P. alba* and *T. grandis* bark and leaves have an antiproliferative activity *in vitro* on neuronal cells N2A in standard condition (after 72 h of incubation) and in high glucose concentration condition. The action of these extracts is potentiated by the presence of high concentration glucose in the medium. Only the extract of *T. grandis* bark has exercised the neuroprotective effect. Further studies are needed to determine the causes of the antiproliferative and neuroprotective effects of these extracts.

CONFLICT OF INTERESTS

Declared None

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