

# Potential multifunctional agents for dual therapy of age-related and associated diseases: Alzheimer's disease and Type 2 Diabetes Mellitus

Rohan Kumar and Leya Joykuty

American Heritage School, Plantation, Florida

## SUMMARY

Currently there is no cure for Alzheimer's Disease (AD) and there seems to be an age-related link between AD and Type 2 Diabetes Mellitus (DM) in terms of incidence, symptoms and causation. The objective of this experiment is to assess the biological potentials of methanol extracts and its derived fractions of four Ayurvedic plants *Buchania axillaris*, *Hemidesmus indicus*, *Pavetta indica* and *Ochna obtusa* to develop potent agents for dual therapy of both AD and DM. Plant extracts in different concentrations were used in five colorimetric assays: Cholinesterase (AChE, BuChE inhibition) assay, Glucosidase ( $\alpha$ -Glu) inhibition assay, an antioxidant activity assay, and MTT assay for cell viability and neuroprotective effects. It was found that that methanolic extract and its derived chloroform fraction of plants exhibited high inhibitory activity against AChE, BuChE,  $\alpha$ -Glc enzymes. The active chloroform fractions also showed high antioxidant potential and neuroprotective capacity against  $H_2O_2$  induced oxidative stress in human neuroblastoma cells. In all the assays, the samples were statistically different than the negative control meaning that the samples with the plant extracts were more effective than the controls without the treatment, while being as effective as the positive controls, which included current drugs used to treat the diseases individually.

## INTRODUCTION

Type 2 Diabetes Mellitus (DM) is an age-related metabolic disorder with complex etiology and affects 10% of the population across the world (1). Currently there are 366 million people with DM worldwide, and this is expected to worsen in the next 20 years and reach 552 million by 2030 (2). DM is characterized by cellular insulin resistance, chronic inflammation, and several metabolic abnormalities. It often leads to macro- and micro-vascular complications that accelerate aging and can damage several organ systems (3).

Alzheimer's disease (AD) is the most common form of dementia, accounting for 60–80% of all dementia cases (4). AD is an irreversible, progressive brain disorder that slowly destroys memory and thinking skills. AD affects 46.8 million people worldwide and this number is likely to double by 2030 due to lack of an effective cure (5). Many abnormal clumps (called amyloid beta ( $A\beta$ ) plaques) and tangled bundles of fibers (called neurofibrillary, or tau, tangles) are characteristics of Alzheimer patients' brains (5). While it remains unknown exactly what role plaques and tangles play

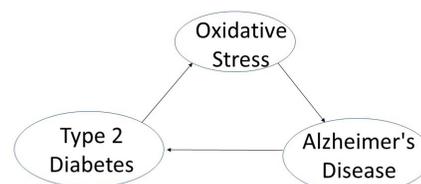
in AD, it is believed that they somehow play a critical role in blocking communication among nerve cells and disrupting processes that cells need to survive (4). This destruction of nerve cells causes memory failure, personality changes, problems carrying out daily activities, and other symptoms characteristic of AD (6).

According to many reports, DM is considered to be a chief risk factor for AD as it increases the incidence by almost two-fold (3). Early accumulation of  $A\beta$  is partially responsible for central nervous system insulin resistance and impaired insulin signaling. This leads to the onset of both diseases as it initiates brain injury via inflammatory and oxidative stress processes (3) (Figure 1).

The chronic elevation of serum glucose in DM is called hyperglycemia. Therapies designed to reverse the chronic hyperglycemia in DM in a noninvasive manner are mostly based on inhibition of intestinal absorption of sugar (7). Before carbohydrates are absorbed from food, they must be broken down into smaller sugar particles like glucose by enzymes in the small intestine. One of the enzymes involved in breaking down carbohydrates is called alpha glucosidase ( $\alpha$ -Glu). One of the therapeutic approaches to decrease postprandial hyperglycemia is the inhibition of carbohydrate hydrolyzing enzymes such as  $\alpha$ -glucosidases ( $\alpha$ -Glu), thereby delaying glucose digestion in the digestive tract. Alpha-glucosidase inhibitors thus may be able to prevent the development of diabetic symptoms (2).

Acetylcholinesterase also known as AChE or acetylhydrolase, is an enzyme that catalyzes the breakdown of acetylcholine (ACh), an important neurotransmitter. AChE is found mainly at neuromuscular junctions and in chemical synapses where it serves to terminate synaptic transmission by hydrolyzing ACh (8).

Low levels of ACh are considered to play definitive roles in the pathophysiology of AD due to their dramatic effect on the cholinergic system. (9). According to the "cholinergic



**Figure 1: The Diabetes-Alzheimer's Cycle.** Diabetes Mellitus(DM) is a risk factor for Alzheimer's Disease, while amyloid beta plaques characteristic of AD increases insulin resistance leading to DM as well as oxidative stress leading to brain injury.

hypothesis”, AChE acts primarily as a regulatory enzyme at cholinergic synapses (9), while butyrylcholinesterase (BuChE), an enzyme closely related to AChE, serves as a co-regulator of cholinergic neurotransmission by hydrolyzing ACh (10). Concerning the cholinergic hypothesis, one rational and effective approach to treat AD’s symptoms is raising the ACh through inhibition of AChE, which is responsible for hydrolysis of ACh. Furthermore, BuChE, the second member of the cholinesterase family, seems to be involved in the hydrolysis of ACh during the last stages of the disease to compensate for the reduced levels of AChE (10). Moreover, AChE and BuChE are responsible for upregulating the expression of the amyloid precursor protein (APP) (10). Therefore, dual inhibition of AChE and BuChE could be effective in the management of AD symptoms.

Oxidative stress is one of the earliest events in the pathogenesis of both AD and DM (7). Oxidative stress is defined as the imbalance between the generation of reactive oxygen/nitrogen species (ROS/RNS) and the cell’s ability to neutralize them by the antioxidant defense. The ROS/RNS are capable of damaging and modifying several types of macromolecules within the cell, including DNA, RNA, lipids, and proteins. Oxidation of lipids could be deleterious since this has the potential to damage the cell membranes. Numerous studies have also suggested that oxidative stress and A $\beta$  protein are linked to each other (11). Therefore, the antioxidants that scavenge free radicals have proven to be a treatment option for AD and T2D (11).

Medicinal plants have always been recognized as an important source of secondary metabolites with various beneficial effects on human health. They are regarded as valuable material for the development of modern medicines, nutraceuticals, food supplements, pharmaceutical intermediates, and chemical entities for synthetic drugs. However, due to less effort towards examining extracts of plants, scientific information on medicinal properties of various plants is still scarce. Ayurveda is an ancient Indian medicinal system practiced from 2000 BC in which plants with medicinal properties are well documented (12). Based on traditional anti-inflammatory and antioxidant uses in Ayurveda (13), the following plants were selected for the study: *Buchania axillaris* (BA) (14), *Hemidesmus indicus* (HI) (15-17), *Pavetta indica* (PI) (18-19), and *Ochna obtusata* (OO) (20-21). Methanolic extractions of the above plants (BAM, HIM, PIM, OOM) and fractionations in water (BAW, HIW, PIW, OOW) and chloroform (BAC, HIC, PIC, OOC) of the above plants were used in the experiment.

The objective of the experiment was to explore an *in vitro* multipronged treatment option for both DM and AD that could be cost effective with minimal side effects. It was hypothesized that the methanolic extracts and fractionations of test plants (BA, HI, PI, OO) would show concentration dependent inhibitory activities against AChE, BuChE enzymes,  $\alpha$ -glucosidase enzymes while providing antioxidant and neuroprotective benefits. This experiment assessed

the biological potentials including anticholinesterase,  $\alpha$ -Glucosidase inhibition, antioxidant, and neuroprotective activity of methanol extracts and its derived fractions of *B. axillaris*, *H.indicus*, *P. indica*, and *O.obtusa* to develop potent agents for dual therapy of both AD and DM.

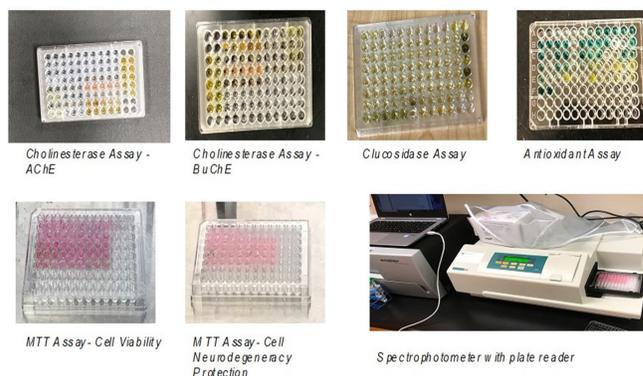
## RESULTS

In order to evaluate the plant extracts for their multifunctional potency against both Alzheimer’s Disease and Type 2 Diabetes, several invitro assays on relevant targets of AD and DM have been used. The methanolic extract and its derived chloroform fraction of the four plants screened (BA, HI, PI, OO) exhibited satisfactory inhibitory activity against AChE, BuChE,  $\alpha$ -Glc enzymes. They also showed high antioxidant potential and neuroprotective capacity against H<sub>2</sub>O<sub>2</sub> induced oxidative stress in neuronal cells.

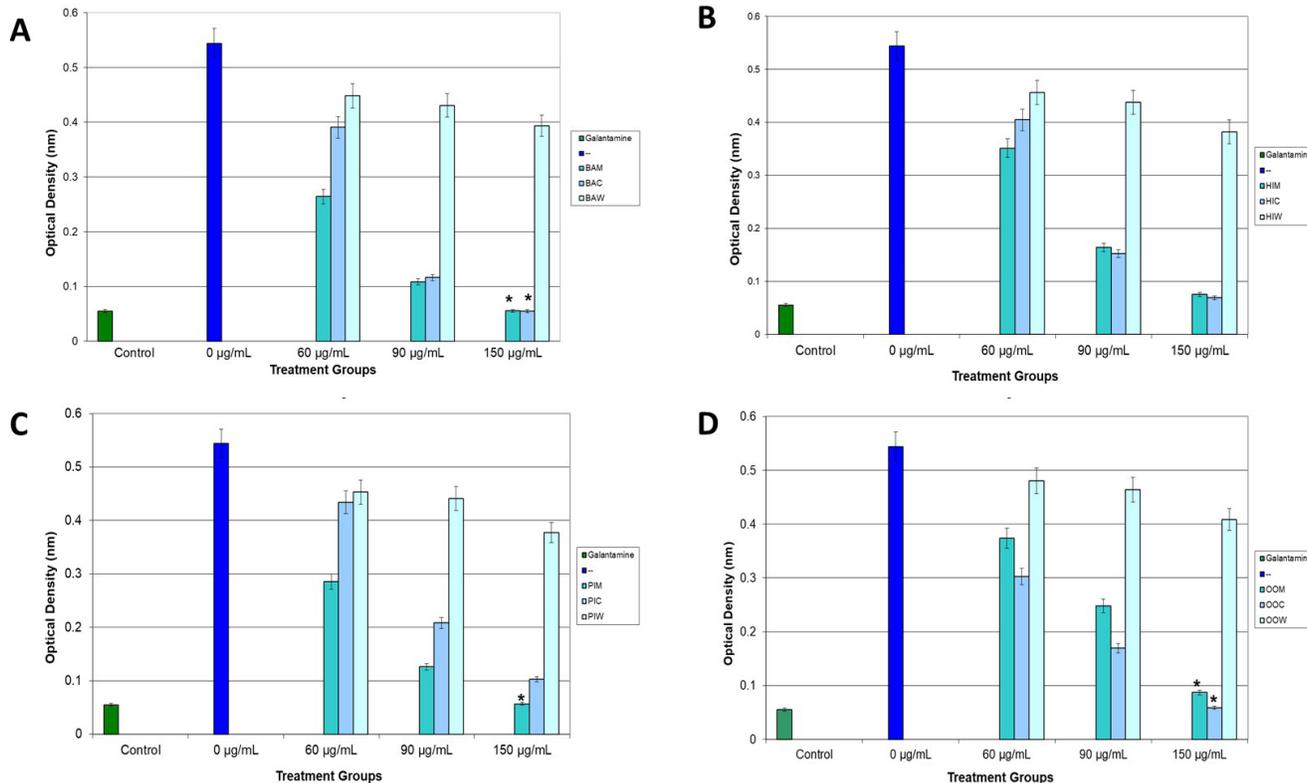
### The Effect of Plant Extracts on AChE and BuChE Inhibition

The methanolic extracts of four plants and their derived fractions were screened for their inhibitory activity against AChE and BuChE enzymes using Ellman’s colorimetric method (22). The cholinesterase Assay is based on an improved Ellman method, in which thiocholine produced by the action of AChE and BuChE forms a yellow color with 5,5’-dithiobis(2-nitrobenzoic acid). The intensity of the product color, measured at 412 nm, is proportionate to the enzyme activity in the sample.

All the plant extracts and fractions evaluated at different concentrations (60, 90, and 150  $\mu$ g/mL) showed dose-dependent inhibitory activities against enzymes AChE and BuChE. Galantamine (brand names Razadyne, Reminyl, and others) is used for the treatment of cognitive decline in mild to moderate Alzheimer’s disease and various other memory impairments and is known for its acetylcholinesterase (AChE)-inhibiting properties and was thus used as the positive control



**Figure 2. Spectrophotometer and Colorimetry.** Most spectrophotoreaders apply a logarithmic function to the linear transmittance ratio to calculate the “absorbance” of the sample. This is proportional to the concentration of the chemical being measured. In the experiments conducted, there is an inverse relationship between the effectiveness of the extracts and the density reading (indicated by the lighter color) and a direct relationship between enzyme activity and the readings.



**Figure 3. AChE inhibition by the plant extracts.** Mean spectrophotometer readings for *A. B. axillaris* B. *H. indicus*, C. *P. indica* D. *O. obtusa* (Data are mean +/- SD (n=3) and asterisk indicate that there was no significant difference between the treatment and the Control at 95% confidence)

drug. The lower the spectrophotometer reading, the higher the inhibitory activity (the color developed due to the formation of the 5-thio-2-nitrobenzoate anion, indicating the inhibition activity—the lighter the color, the greater the inhibition of the enzyme). (Figure 2). BAM, HIM, and PIM extracts were potent in inhibiting AChE and BuChE enzymes (Figures 3 & 4). Among the fractions, BAC, HIC, and OOC showed higher activity than other fractions against AChE and BuChE enzymes. In the case of water fractions, only HIW displayed moderate activity against both AChE and BuChE. Overall, the most prominent AChE inhibition was recorded with BA, while HI was most active against BuChE. All plants showed reduced activity against AChE when compared to galantamine. Of the fractions, BAC, HIC, OOC, PIC, and PIW showed moderate inhibition on AChE activity (Figure 3). However, BAM, HIM, and HIC exhibited stronger inhibition against BuChE. BAC, HIC, HIW, PIC, and OOC showed significant inhibition on BuChE activity (student's t-test  $p < 0.05$ ) (Figure 4).

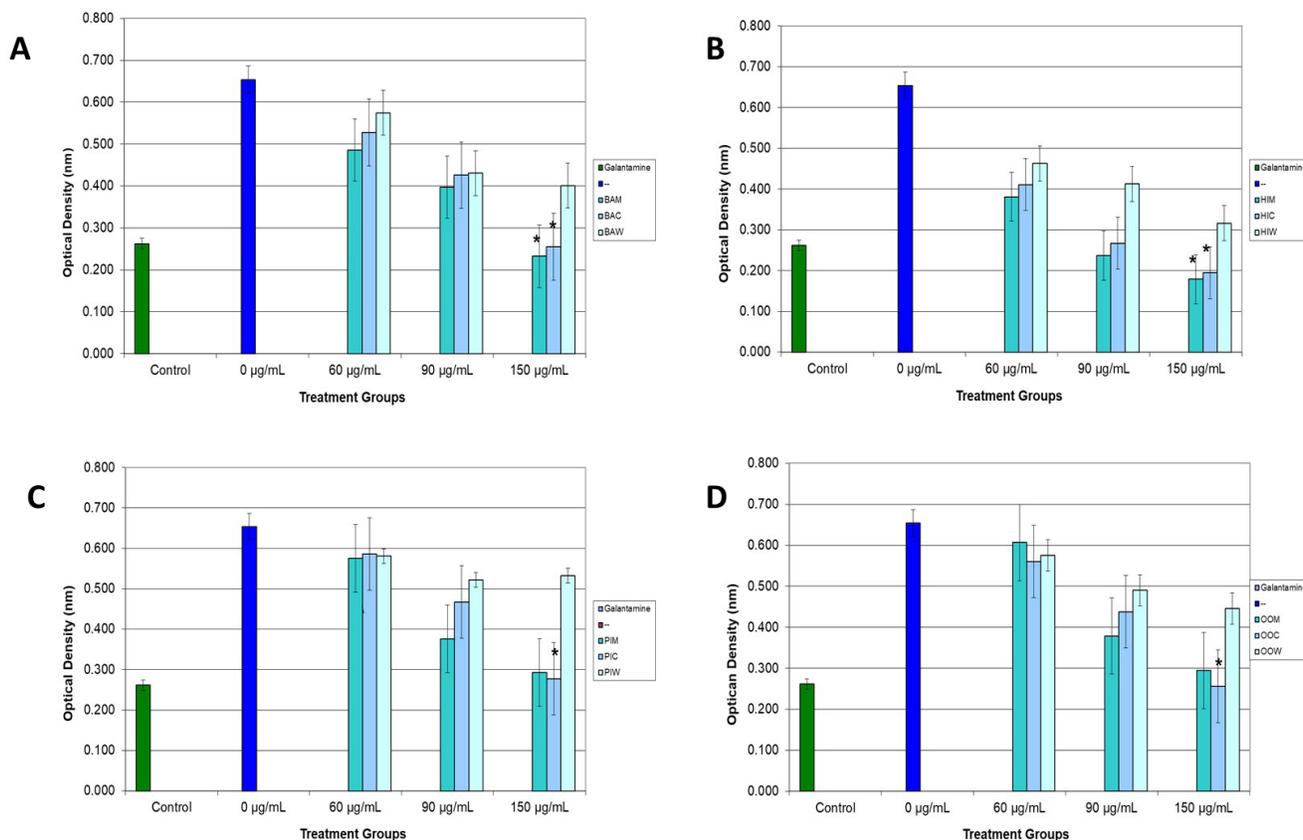
#### The Effect of Plant Extracts on $\alpha$ -Glucosidase Inhibition

As mentioned earlier,  $\alpha$ -Glucosidase decomposes disaccharides into glucose, and increases blood glucose levels. Therefore, an increase in blood glucose can be suppressed by inhibiting the activity of  $\alpha$ -Glucosidase (Figure 5). To assess the antidiabetic potency of the plants, the extracts and derived fractions were tested for their  $\alpha$ -

Glucosidase inhibitory activity by invitro enzyme assay. In this assay  $\alpha$ -Glucosidase hydrolyzes the substrate mix to release the *p*-nitrophenol that can be measured colorimetrically (OD = 410 nm). Enzyme activity was measured by the quantity of *p*-nitrophenol released. The lower the quantity of *p*-nitrophenol released, the higher the inhibitory action against the glucosidase enzyme and higher the absorbance (Figure 2). All the plant extracts and fractions showed dose-dependent inhibitory action against  $\alpha$ -Glucosidase when compared to the sample without the extracts (Figure 6). Acarbose, a drug commonly used to lower blood sugar in patients with high blood sugar, was used as the positive control drug. All the plants displayed greater inhibition of  $\alpha$ -Glucosidase than the positive control drug acarbose with BAC, HIM, HIC, PIC, and OOC being the most active, HIW and PIW were moderately active, and BAW was the least active against the enzyme.

#### Radical Scavenging Activity of the Plant Extracts

The ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) assay is a widely accepted antioxidant assay to screen the total antioxidant power of fruits, vegetables, foods, and plants. In particular, it is recommended to be used for plant extracts because the long wavelength absorption maximum at 745nm eliminates color interference in plant extracts. In this assay, the ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate. Its reduction in



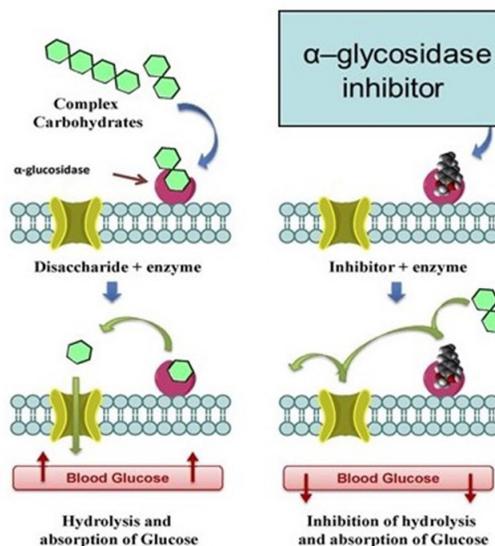
**Figure 4. BuChE inhibition by the plant extracts.** Mean spectrophotometer readings for A. *B. axillaris* B. *H. indicus*, C. *P. indica* D. *O. obtusa* (Data are mean +/- SD (n=3) and asterisk indicate that there was no significant difference between the treatment and the Control at 95% confidence)

the presence of hydrogen-donating antioxidants in chloroform fractions is measured spectrophotometrically. Trolox, a cell-permeable, water-soluble analogue of vitamin E, is used as a standard for measuring the antioxidant capacity of complex mixtures and is commonly used in biological or biochemical applications to reduce oxidative stress or damage. All the four plant extracts in the highest concentration in chloroform fractions had increased radical scavenging activity (RSA) compared to the control (Trolox), with the HIC and BAC being significantly effective (**Figure 7**).

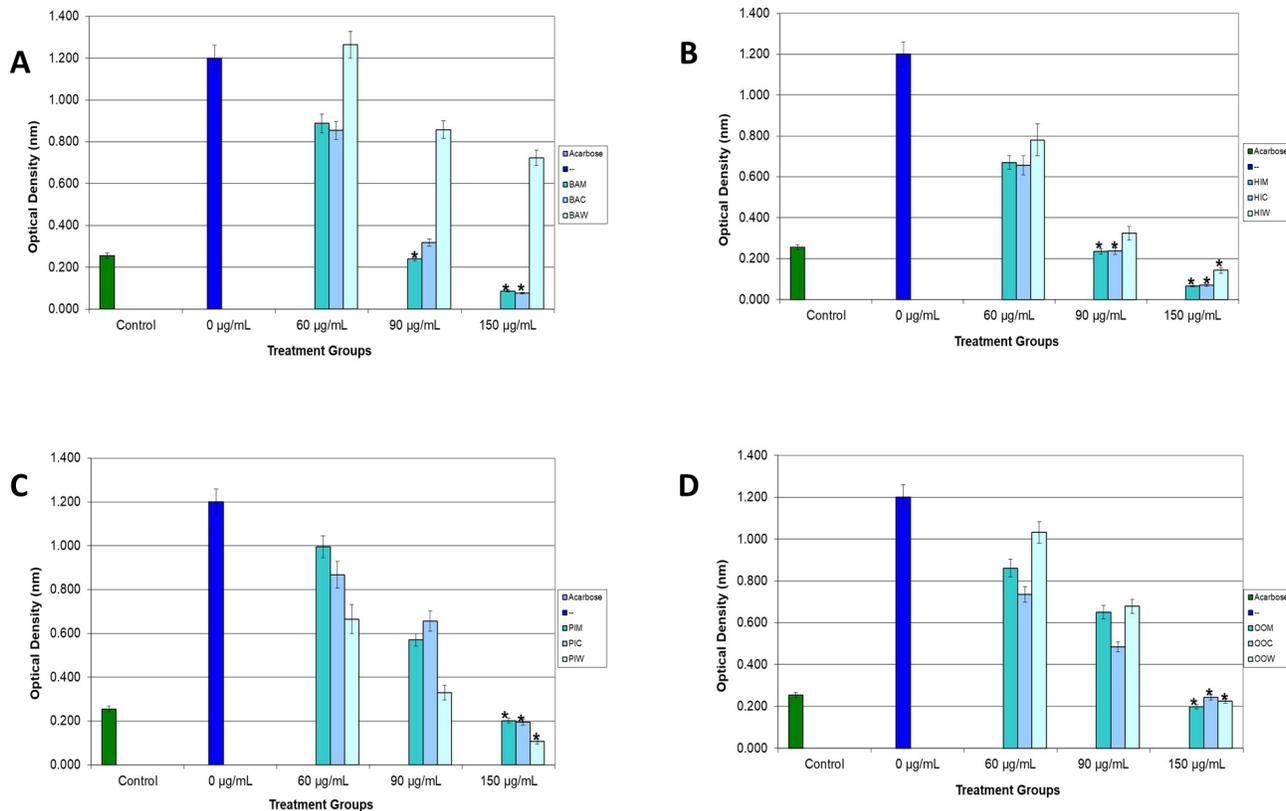
### Viability of Cells Treated with Extracts

The safety of the extract is absolutely crucial for a successful pharmaceutical formulation. In line with this, the possible toxic effects of active fractions BAC, HIC, PIC, and OOC in the human neuroblastoma SK-N-SH cells have been assayed with MTT assay. Neuroblastoma (NB) cell lines are transformed, neural crest-derived cells, capable of unlimited proliferation *in vitro*. These cell lines retain the ability of differentiation into neuronal cell types on treatment with various agents. This ability of NB cells to proliferate as well as to differentiate makes it an excellent *in vitro* system for various studies. NB cells are extensively used for testing neurotoxicity of putative drugs such as antimalarial or anticancer agents. They have been used to dissect the

relationships between proliferation, differentiation and apoptosis. This feature has been useful in understanding the pediatric cancer--neuroblastoma and for development of



**Figure 5: Plant extracts and hyperglycemia.** Plant extracts can help with hyperglycemia by inhibiting carbohydrate hydrolyzing enzymes, in this case  $\alpha$ -Glu (Picture courtesy: [www.slideshare.net/featured/category/health-medicine](http://www.slideshare.net/featured/category/health-medicine))



**Figure 6.  $\alpha$ -Glucosidase inhibition by the plant extracts:** Mean spectrophotometer readings for A. *B. axillaris* B. *H. indicus*, C. *P. indica* D. *O. obtusa* (Data are mean +/- SD (n=3) and asterisk indicate that there was no significant difference between the treatment and the Control at 95% confidence)

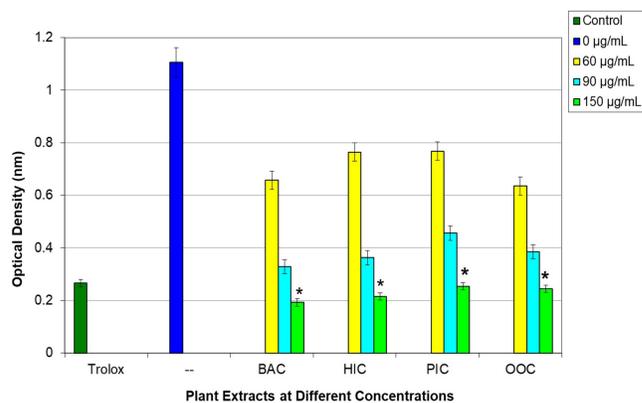
newer therapies. Since currently, we cannot look at amyloid plaques and tangles until autopsy, studying the effect of the extracts on the cells is a good starting point

We measured the percentage of viable cells in the presence or absence (control) of several concentration of *B.axillaris*, *H.indicus* , *P. indica*, and *O. obtusata*. All the extract concentrations were not significantly different than the control cells at 95% confidence level (Figure 8). Under the experimental conditions, BAC, HIC, PIC, and OOC, displayed increased cell viability in a concentration-dependent manner. Among the tested fractions, fraction OOC attenuated the cell toxicity significantly in a concentration-dependent manner.

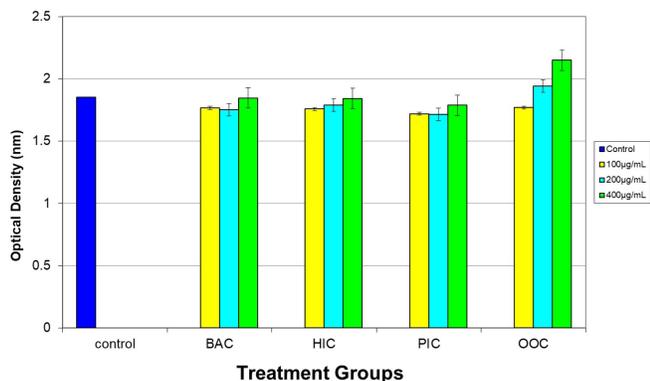
### Neuroprotective Capacities against H<sub>2</sub>O<sub>2</sub>-Induced Cell Death in SK-N-SH Cells

The neuroprotective effect of selected plant fractions against H<sub>2</sub>O<sub>2</sub>-induced oxidative injury in SK-N-SH cells was determined by pretreating the cells with different concentrations of plant extract for three hours before treatment with H<sub>2</sub>O<sub>2</sub>. 1 mM H<sub>2</sub>O<sub>2</sub> was added to induce oxidative stress in the cells (Figure 9) and the cell viability was measured by MTT colorimetry. The viability of SK-N-SH cells pretreated with 100, 200, or 400 µg of active fractions from BAC, HIC, PIC, and OOC for 24 hours before exposure to H<sub>2</sub>O<sub>2</sub> was significantly increased relative to control in a dose-dependent manner (Figure 10). When the neuroprotective effect induced

by fractions was compared with control (medium plus H<sub>2</sub>O<sub>2</sub>), the fraction HIC showed higher neuroprotectivity than the control at all concentrations. In fact, cells in the HIC fraction behaved almost similarly as the cells in medium only not exposed to H<sub>2</sub>O<sub>2</sub>. Fractions BAC, PIC, and OOC provided a higher neuroprotective profile to that of control at higher concentrations. This observation suggests that certain compounds present in fractions likely promoted cell survival or delayed the death of neurons when exposed to oxidative stress. Based on the results obtained, these fractions can be



**Figure 7. Radical scavenging activity of plant extracts.** Data are mean spectrophotometric readings +/- SD (n=3) and asterisk indicate that there was no significant difference between the treatment and the Control at 95% confidence.



**Figure 8. Figure 8: Cell viability of plant extract treated cells.** Data are mean spectrophotometer readings +/- SD (n=3) and asterisk indicate that there was no significant difference between the treatment and the Control at 95% confidence.

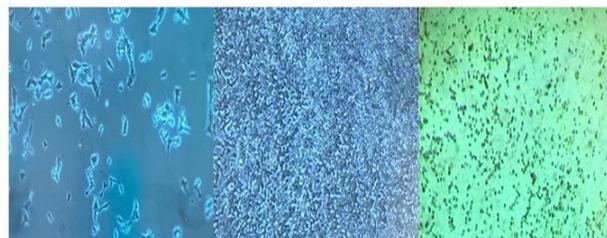
considered as potential oxidative suppressors.

In conclusion, the present study demonstrates the *in vitro* potential of *B. axillaris*, *H. indicus*, *P. indica*, and *O. obtusata* as multifunctional therapeutic remedies for the treatment of AD and DM.

## DISCUSSION

The objective of this experiment was to assess the biological potential of methanol extracts and the derived fractions of *B. axillaris* (BA), *H. indicus* (HI), *P. indica* (PI), and *O. obtusa* (OO) to help with both DM and AD with minimal side effects. This experiment was an attempt to use plants that were suggested to have some medicinal properties to combat age-related problems, like diabetes and Alzheimer's, by targeting the three areas of concern: high blood sugar, the depletion of ACh, and oxidative stress. In this study, the crude methanolic extracts of the plants were fractionated using polar and non-polar solvents to obtain phytoconstituent rich biologically active fractions. Methanol is commonly used for extraction of bioactive compounds as methanol is an amphiphilic compound and has a polarity index of 5:1. This means that methanol is widely used, mainly because many plant compounds dissolve in it with great freedom. It also easily evaporates so it can be separated from the extract. But for compounds that are strictly hydrophobic, a mixture of methanol and chloroform, or chloroform alone, was used for extraction of bioactive compounds. Subsequently, biological evaluations of extracts and fractions against various targets related to AD and DM suggest BA, HI, PI, and OO could serve as multifunctional agents for dual therapy.

Upholding ACh levels by reducing its metabolism in the synaptic cleft by inhibition of ChEs is beneficial for improvement in memory and cognitive dysfunction. Therefore, dual inhibition of AChE and BuChE of cholinergic neurotransmission is continuously referred to as the "gold standard" therapeutic strategy for the management of AD (10). The superior dual inhibitory potential of BAC, HIC, PIC, and OOC on AChE and BuChE in Cholinesterase assay indicates

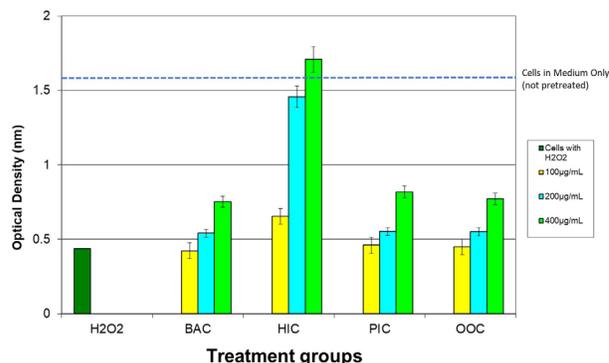


**Figure 9. H<sub>2</sub>O<sub>2</sub> induced oxidative stress on cells.** Photographs of SK N SH cells in culture. Left panel is initial culture, middle panel is cells at 80% confluence, right panel is cells after H<sub>2</sub>O<sub>2</sub> induced cell injury.

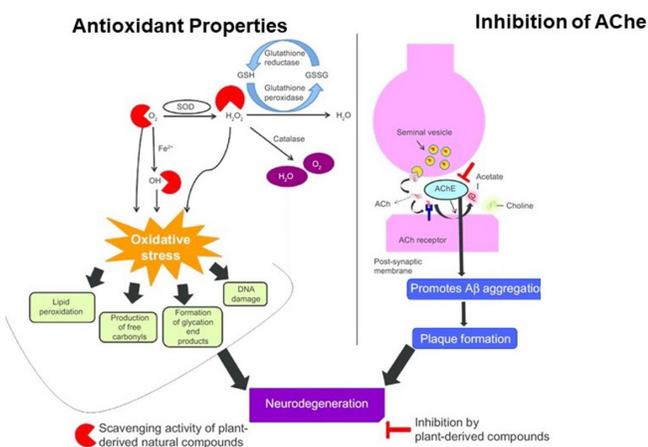
their potential as an alternative for the treatment of AD (Figure 11). Inhibition of  $\alpha$ -glucosidase enzymes, which in turn delay in the digestion of carbohydrates, is an effective approach for the management of carbohydrate metabolic disorders like DM. Due to the strong inhibition of  $\alpha$ -glucosidases, it is evident that the methanolic extracts and fractions BAC, HIC, PIC, and OOC have excellent antidiabetic potency.

Currently, the multifactorial biological pathways involved in AD and DM seem to share oxidative stress as a unifying factor. Oxidative stress may be either due to excessive production of ROS, loss of antioxidant defenses, or both. Consequently, scavenging of ROS has become highly beneficial and a promising strategy for the treatment of AD and DM. Presently, the increased activity against ABTS by the fractions BAC, HIC, PIC, and OOC show that the tested fractions have the capacity to prevent the potential damage by ROS (Figure 11).

The safety of the extract is absolutely crucial for a successful drug. Therefore, the possible toxicity effects of the active fractions of the four plants in the SK-N-SH cells were measured. Interestingly, in the cell viability assay, the escalating cell proliferation at even high concentrations suggested that fractions are nontoxic to SK-N-SH-cells and likely promote cell survival or delay the natural death of neurons in culture medium. (Figure 8) As fractions provided higher or almost similar neuroprotective profile to that of control at higher concentrations, BAC, HIC, PIC, and especially OOC, are considered to act as potential oxidative suppressors against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in SK-N-



**Figure 10. Spectrophotometer readings for Cell Neurodegeneracy.** (Data are mean +/- SD (n=3))



**Figure 11. How plant extracts can help with AD.** The plant extracts would help prevent degeneration by inhibiting AChE leading to increased levels of ACh while also scavenge for free radicals reducing oxidative stress (31)

### SH cells (Figure 10).

Currently there is no cure for Alzheimer's Disease. The few agents approved by the US Federal Drug Administration for the treatment of AD and DM have less potency and multiple side effects (23). Consequently, it has become a necessity to develop the new agents that are pharmacologically safe, cost-effective, and immediately available with minimal side effects. The World Health Organization has also recommended the development of improved and safer herbal medicines (24). The findings of this experiment serve as a promising starting point for studying the therapeutic potential of these natural agents to break the DM and Alzheimer's cycle. However, because responses observed *in vitro* can be magnified, diminished, or totally different in more complex integrated systems, *in vivo* work is vital for the analysis of drug action and development of new therapeutic agents. Future *in vivo* research, such as experiments using a mouse model, needs to be conducted to see how the plant extracts react in a living organism, in precise cellular conditions.

## METHODS

### Plant Extracts

Plant samples were taken, dried, and ground to a powder and stored in a cool, dark place.

Ground plant material (100 g) was extracted with 500 ml of 90% methanol by soaking for two days and then filtered through Whatman No.1 filter paper. For this experiment, the crude methanolic extracts (20 g) were also suspended in water (50 mL) and chloroform (100 mL) was added and shaken well, and the layers were allowed to separate for 6 hours in a separating funnel. The remaining methanolic extracts were dried for three days in an oven. Chloroform and water layers were then separated and evaporated to obtain the chloroform fraction and water fractions. All the extracts were stored in a cool, dark place until ready for use.

### Cholinesterase Enzyme Inhibition Assay (Methanolic and Chloroform Extracts)

Acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel) and Butyrylcholinesterase (BuChE) from equine serum were used in this assay. 10.85 mg of Acetylthiocholine iodide and Butyrylthiocholine iodide were mixed separately in 5 ml of Phosphate buffer (Substrate). 3.96 mg of Ellman's Reagent also known as DTNB (5,5'-dithio-bis-[2-nitrobenzoic acid]) and 1.5 mg of sodium bicarbonate were mixed to make DTNB solution. In a 96-well plate, 10  $\mu$ L of enzyme (AChE, 2 U/mL or BuChE, 2 U/mL), 10  $\mu$ L of plant extract/fraction (30, 90, 150  $\mu$ g/mL), 100  $\mu$ L of phosphate buffer, and 50  $\mu$ L of DTNB solution were added. As an additional control, 150  $\mu$ g/mL of galantamine was used. For a negative control, the test mixture without the plant samples was used. Three wells were used for each test sample (3 replicates for each methanolic, chloroform, and water fractions for each plant) and control. The plate was incubated for 5 minutes at 25°C. 15  $\mu$ L of the substrate was added, and the plate was incubated for another 5 minutes at 25°C. A spectrophotometer reading at 412 nm was taken.

### $\alpha$ -Glucosidase Inhibitory Assay (Methanolic and Chloroform Fractions)

In a 96-well plate, 50  $\mu$ L of enzyme  $\alpha$ -glucosidase (0.15 unit/mL), 10  $\mu$ L of plant extract/fraction (30, 90, 150  $\mu$ g/mL), and 100  $\mu$ L of phosphate buffer were added. For positive control, 150  $\mu$ g/mL of Acarbose was used and for negative control. The test mixture without the plant samples was used. Three wells were made for each test sample (3 replicates for each methanolic, chloroform, and water fractions for each plant) and the control. The well was incubated for 15 minutes at 37°C. 50  $\mu$ L of the substrate 4-Nitrophenyl- $\alpha$ -D-glucopyranoside was added, and the plate was incubated further at 37°C for 15 minutes. 50  $\mu$ L of sodium bicarbonate was added to terminate the reaction. A spectrophotometer reading at 415 nm was taken to measure enzyme activity.

### Antioxidant Activity Assay (Chloroform Fractions)

In a 96-well plate, 10  $\mu$ L of Metmyoglobin (1 mg/mL), 10  $\mu$ L of plant fraction (30, 90, 150  $\mu$ g/mL), and 150  $\mu$ L of Chromogen were added. For the positive control, 150  $\mu$ g/mL of Trolox (a water-soluble analog of vitamin E) was used and for the negative control, the test mixture without the plant samples was used. Only chloroform fractions were used for this assay as the methanol could have an adverse reaction with the chemicals used for the assay. Three wells were used for each test sample (3 replicates for chloroform fraction for each plant) and the control. Different concentrations of Trolox (45, 90, 135, 180, 225  $\mu$ g/mL) for the Trolox standard were prepared. The reaction was initiated by adding 40  $\mu$ L of hydrogen peroxide (final concentration in the assay is 250  $\mu$ M) as quickly as possible, and the plate was incubated on shaker for 5 minutes at room temperature. Absorbance at 405 nm was read using a plate reader.

### Cell Culture

SK-N-SH cells were cultured in MEM supplemented with 10% FBS and maintained at 37°C in a humidified 5% carbon dioxide incubator. Cells were passaged every 4 days to get 6 flasks. When the cells reached 80% confluence, they were counted. Only plant extracts in chloroform fractions were used in the cell assays as methanol would react adversely with the cells.

### Cell Viability Assay

Cytotoxic effects of selected plant fractions on the cell viability were measured using MTT assay. SK-N-SH cells were counted using a hemocytometer and cells ( $2 \times 10^5$  cells per well) in 200 mL of corresponding medium with 10% FBS were seeded into 96-wellplate. 1  $\mu$ L of cells was added per well. Medium was removed and replaced with 100  $\mu$ L of fresh medium along with plant fractions in chloroform at various concentrations (50, 100, 200, 400  $\mu$ g/mL). 10  $\mu$ L of the MTT labeling reagent was added to each well, including the ones with medium alone for a negative control. The microplate was incubated at 37°C for 4 hours in a humidified incubator. 100  $\mu$ L of Formazan solubilization solution was added to each well and mixed thoroughly, and the plate was incubated for 24 hours at 37°C in a humidified incubator. The spectrophotometrical absorbance of the samples were measured at 570 nm.

### Protection against Hydrogen Peroxide induced Cell Death Assay

1  $\mu$ L of cells was added per well. Medium was removed and replaced with 100  $\mu$ L of fresh medium along with plant fractions in chloroform at various concentrations (50, 100, 200, 400 $\mu$ g/mL). 10  $\mu$ L of the MTT labeling reagent was added to each well, including the ones with medium alone for negative control. The microplate was incubated at 37°C for 4 hours in a humidified incubator.

To induce oxidative stress, 1 mM of hydrogen peroxide was added to each well. (Figure 3). 100  $\mu$ L of Formazan solubilization solution was added to each well and mixed thoroughly. The plate was incubated for 36 hours at 37°C in a humidified incubator. The spectrophotometer absorbance of the samples was measured at 570 nm.

### Equipment used

A spectrophotometer is able to determine what substances are present in a target and exactly how much through calculations of observed wavelengths. Colorimetry was used as a principle means of measuring the effectiveness of the extracts. Most spectrophotometers apply a logarithmic function to the linear transmittance ratio to calculate the absorbency of the sample, a value which is proportional to the concentration of the chemical being measured. The higher optical density readings indicated higher enzyme activity. In the cholinesterase and glucosidase assays, the effectiveness of the plant extracts in inhibiting enzyme activity was indicated

by lower spectrophotometer readings. For the cell assays, the lighter the color, the less proliferation/viability of the cells (Figure 11).

### Statistical Analysis

A student's t-test was used to determine if two sets of data are significantly different from each other. The null hypothesis tested here is that the experimental mean and the control mean are identical. Statistical hypothesis testing was performed by student's t-test and the values were considered as statistically significant when *p*-values were less than 0.05.

**Received:** March 18, 2019

**Accepted:** May 27, 2019

**Published:** November 13, 2019

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