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In Vitro cytotoxic and antioxidant activities of Methanol, Chloroform and Aqueous Extracts of *Pithecellobium dulce* Seeds and their Comparative Analysis.

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Abstract
The imbalance between pro-oxidants and antioxidants in an organism leads to oxidative stress, which plays a significant role in the etiology of a number of degenerative illnesses, including cancer, Alzheimer's, arthritis, and cardiovascular diseases. Phytochemicals found in medicinal plants are used to treat a variety of ailments, including those associated with oxidative stress, because of their ease of use, low toxicity, and efficacy. In our present study, three extracts with different solvents like chloroform, methanol and aqueous extract of *Pithecellobium dulce* seeds have been evaluated for in vitro antioxidant activity using DPPH radical scavenging and superoxide anion radical scavenging assay, determination of superoxide dismutase (SOD), In-vitro cellular studies for neuroprotective abilities using Cell viability assessment, Cell-based in-vitro cellular protective effect and examination of cellular morphology of SHSY5Y and Aβ_SHSY5Y cells over the treatment of various solvent extracts. Among the extracts, Methanol showed the highest antioxidant capacity. Based on DPPH radical scavenging, superoxide anion radical scavenging, determination of superoxide dismutase, methanol extract has shown strong scavenging capacity in comparison to other extracts. The elevated cell viability observed in the methanol and Aqueous extracts indicates the existence of neuroprotective compounds that can counteract neurotoxins by boosting neuron survival processes. And in cell-based in-vitro test also *P. dulce* seed methanol extract, has shown highest neuroprotective capability of mitigating Aβ-induced neurotoxicity. The results showed that all the extracts of *P. dulce* seed possess significant free radical scavenging and reducing power properties at concentration-dependent manner, cell viability and restore neuronal morphology affected by amyloid-beta toxicity especially methanol extract. Hence, it can be concluded that the *P. dulce* seeds could be pharmaceutically exploited for antioxidant properties.

Keywords: Pithecellobium dulce, antioxidants, neurotoxicity, superoxide dismutase, neuroprotection.

Introduction:

All living cells produce free radicals as a typical byproduct of biological activity. Nonetheless, many diseases are brought on by the overproduction of free radicals, either from endogenous or external causes. Examples of free radical induced oxidative stress include ageing, immunosuppressant, and numerous chronic and degenerative illnesses, including as cancer, atherosclerosis, diabetes mellitus, and neurodegenerative disorders (Young and Woodside, 2001). Cells utilize various cellular antioxidant systems, including low molecular mass antioxidants (glutathione, tocopherols, ascorbic acid), enzymes that interact with reactive oxygen species (ROS), such as catalase, peroxidases, and superoxide dismutase, and other enzymes that produce reduced forms of antioxidants, to safeguard themselves against free radical-mediated oxidative stress (Blokhina et al., 2003). Since the pathophysiology of nearly all diseases is attributed to the imbalance of cellular redox homeostasis, which is the reactive oxygen species (ROS) and antioxidant system, an increasing body of antioxidant extracted from plants has been identified to date to support health and wellness (Manea et al., 2012, Gupta et al., 2012). According to earlier research, the free radicals produced have the potential to lower antioxidant substrates and enzymes and cause oxidative damage. In order to lower the risk of toxicity, it is critical to investigate antioxidant compounds in the food sector as well as in preventive medicine (Shen et al., 2009).

The food and pharmaceutical industries have demonstrated a rising interest in researching the metabolic characteristics of several medicinal plants in recent years. In addition to producing secondary metabolites to protect themselves from biotic and abiotic challenges, plants also create some of these substances to benefit the species that consume them. *Pithecellobium dulce* is a tree that belongs to the Fabaceae family of flowering plants. It can reach a maximum length of 15 m and has a spiky trunk. This plant is indigenous to regions of South and Central America as well as India, where it has long been utilized for culinary and occasionally medical uses (Pal et al., 2012). *P. dulce* has been used medicinally because it includes a number of compounds that have been shown to enhance human health. For instance, P. dulce seeds include calcium, magnesium, and phosphorus in addition to several minerals and antioxidants such flavonoids, quercetin, and vitamin C (Vargas et al., 2020; Pío et al., 2013). Therefore, this study aims to investigate the role of *P. dulce* of three solvent extracts of chloroform, methanol and aqueous have been evaluated

for in vitro antioxidant activity using DPPH radical scavenging and superoxide anion radical scavenging assay, determination of superoxide dismutase (SOD), In-vitro cellular studies for neuroprotective abilities using Cell viability assessment, Cell-based in-vitro cellular protective effect and examination of cellular morphology of SHSY5Y and Aβ_SHSY5Y cells over the treatment of various solvent extracts.

MATERIALS AND METHODS

In-vitro Anti-Oxidative Evaluation

DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging assay: The antioxidant activity of the various solvent extracts of *Pithecellobium dulce* seeds was determined by measuring their ability to decolorize the purple-coloredmethanolic solution of DPPH, as described by Wright & Shahidi,2017. In brief, 1 mL of a 0.2 mM DPPH methanol solution was added to 1 mL of various concentrations (50 to 750 µg/mL) of the extract and incubated at 25 ^{o}C for 30 min. The absorbance of the resulting mixture was measured against a blank at 517 nm using a microplate reader (BIO-RAD, Model 680, Japan). The percentage inhibition rate (I %) on the DPPH radical was calculated using the formula:

Percentage inhibition (I %) = $[(Abs_{control} - Abs_{extract})/Abs_{control}] \times 100$

Where $Abs_{control}$ is the absorbance of the control, $Abs_{extract}$ is the absorbance of the extract. The concentration of extracts causing 50% inhibition (IC_{50}) of DPPH radical was calculated using the non-linear regression coefficient method.

Superoxide anion radical scavenging capacity: Determination of the superoxide anion scavenging effect of the various solvent extracts of *Pithecellobium dulce* seeds was conducted using the procedure of *Liu et al*., in 2013. Superoxide radicals were generated in 50 µL of Tris-HCl buffer (15 mM, pH 8.0) containing 50 µL of 50 mM nitrobluetetrazolium (NBT) solution, 50 µL of 75 mM nicotinamide adenine dinucleotide (NADH) and varying concentrations of the extracts (50 to 750 µg/mL). The reaction was initiated by adding 1 mL of 10 mM phenazinemethosulphate solution to the mixture. The reaction mixture was incubated at 25^{o}C for 5 min, and the absorbance was measured at 560 nm. Results were estimated from the calibration curve and expressed as IC_{50} value.

Determination of superoxide dismutase (SOD): The SOD activity was assessed with the SOD test kit-WST (Sigma-Aldrich®) as described by Sun *et al.*, 1988. This kit is commonly employed to quantify the extent of inhibition of the SOD enzyme. The reaction mixtures in the SOD kit were mixed with 100 µL of solvent extracts and subsequently, the mixtures were gently agitated and then incubated at a temperature of 37 °C for 20 min. The suppressive effect of SOD on the xanthine oxidase process, which produces superoxide, was assessed by utilizing a tetrazolium salt. The absorbance of the resulting mixtures was measured at a wavelength of 450 nm using a microplate reader. The positive control utilized in this study involved the substitution of the extracts with ascorbic acid at 10 mg/mL.

***In-vitro* Cellular studies for neuroprotective abilities**

Cell culture*:* Cell culture studies were conducted using SHSY5Y neuroblastoma cells provided by the ATCC, USA. SHSY5Y cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 1% NEAA (non-essential amino acids), 2 mM L-glutamine, and 1% PEST, 100 µg/mL streptomycin, and 100 IU/mL penicillin. Both chambers were maintained at 37 ^{o}C with a CO_2 humidity of 5%. SHSY5Y cells were differentiated using a method described by Gustafsson*et al.,* 2010. Overnight, the cells were cultured in 96-well plates at a density of 500 cells/mm^2. Distinguishing media (including 1 µM RA) was used to substitute for the original medium (a mixture of Ham's F12 and Dulbecco's modified Eagle's medium [1:1], 1% N_2 supplement, and 1% PEST). 3 to 6 days were used to distinguish the cells. Every 48 h, half of each well's media was replaced.

Cell viability assessment: The three solvent extracts of *P. dulce* seeds were tested for their cytotoxic effects using the MTT assay SHSY5Y cells at doses ranging from 10 to 500 µg/mL for 24 h as described by Zhang *et al.,* 2017. The SHSY5Y cells were seeded at a density of 3,500 and 5,000 and plated into each well of a 96-well culture plate. After incubation for 24 hours, the medium was changed out for new media, and the substances were administered to reach the necessary amounts. After incubation for 24 hours, the MTT reagent (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each group to assess cell survival. For 4 hours, both cell types were treated with 20 µL of MTT per well (at 5 mg/mL). After washing the culture plates to get rid of any leftover growth media, the formazan crystals were digested in DMSO. Quantitative analysis was performed using a Multiwell Microtitre Spectrophotometer

(Thermo Scientific, Multiskan^{TM} FC Microplate Photometer, Massachusetts, USA). Cell viability in the control group was quantified using absorbance intensity-based mortality analysis, and results are presented as the percentage of live cells. Experiments were performed in triplicate.

Cell-based in-vitro cellular protective effect : To find out how neuroprotective capabilities of three solvent extracts of *P. dulce* seeds against SHS5YS cells, the cells were exposed to 5-100 µg/mL for 24 h and then treated with 2 µM Amyloid β- (Aβ) for 48 h before the MTT assays as described by Adewusi*et al.,* 2013. After treatment, 5 mg/mL MTT was added to the cells for 4 hours at 37 °C. After carefully taking out the media, 100 µL of DMSO was added to solubilize the formazan crystals that had formed. A microplate reader (Thermo Scientific, Multiskan^{TM} FC Microplate reader) was used to measure the absorbance at 570 nm. Controls were cells exposed to 0.15% DMSO (vehicle control) and treated with 2 µM Aβ without being treated with test compounds (Aβ control).

Examination of cellular morphology of SHSY5Y and Aβ_SHSY5Y cells over the treatment of various solvent extracts of P.dulce seeds: To investigate morphological changes and potential neuroprotective effects of *P. dulce* seed extracts on SHSY5Y cells, with a specific focus on Aβ induced neurodegeneration, the following procedure was conducted (Chang et al. 2009). SHSY5Y neuroblastoma cells were cultured under standard conditions (37°C, 5% CO_2). A subset of cells was treated with Aβ at a concentration and duration known to induce significant, yet not total, neurotoxicity. Control groups included DMSO-treated cells (vehicle control) and untreated SHSY5Y cells (baseline morphology). After Aβ and DMSO treatments, cells were exposed to *P. dulce* seed extracts (in chloroform, methanol, and water) at various concentrations (5, 40, and 100 µg/mL). Post-treatment, cells were examined under phase-contrast microscopy to assess morphological changes in neurite outgrowth, cell body size, and confluency. Images documented these changes, and cell viability was measured using an MTT assay to correlate morphology with a quantitative viability measure. Morphological and viability data from extract-treated Aβ cells were compared to Aβ-only treated cells to assess neuroprotection. All experiments were performed in triplicate for reproducibility and statistical analysis.

STATISTICAL ANALYSIS

The data collected was analyzed using Sigma Plot 11.0 for Windows. Initially the data was organized into a line plot graph, accompanied with error bars. Subsequently, statistically test known as Analysis of variance (One way ANOVA) were performed with a confidence level of 95%. Any differences with a P-value less than 0.05 were deemed to be statistically significant.

RESULTS

In-vitro Anti-Oxidative Evaluation

DPPH radical scavenging assay: The data provided demonstrates the *in-vitro* antioxidant activity of *P. dulce* seed extracts, evaluated by DPPH radical scavenging assay at various concentrations (10, 25, 50, 100, 150, 200, and 250 µg/mL) as shown in Table.1 and Figure 1. The percentage inhibition of DPPH scavenging activity increased with the concentration for all three extracts: chloroform (Chf Ex.), methanol (MeOH Ex.), and aqueous (Aqueous Ex.). Methanol extract showed the highest scavenging activity across all concentrations, followed by the aqueous extract, and lastly the chloroform extract. At 250 µg/mL, the methanol extract exhibited close to complete DPPH radical scavenging activity (97.33 ± 9.23%), the aqueous extract showed significant activity (49.08 ± 5.09%), while the chloroform extract demonstrated the least activity (20.55 ± 1.99%).The results suggest a dose-dependent increase in antioxidant activity for all extracts, which is typical for such assays. As the concentration of the extract increases, more phytochemicals are available to react with and neutralize DPPH radicals.

Table 1: *Percentage of scavenging activity of DPPH radical over the treatment of various solvent extracts of P. dulce seeds.*

Conc in µg/mL	Chf Ex.	MeOH Ex.	Aqueous Ex.
10	3.96 ± 0.28	9.85 ± 0.85	6.23 ± 0.85
25	8.25 ± 0.96	19.66 ± 1.47	12.35 ± 1.04
50	13.24 ± 1.08	36.25 ± 2.96	18.22 ± 1.77
100	15.06 ± 1.33	59.21 ± 4.22	31.87 ± 2.58
150	17.45 ± 1.25	81.34 ± 6.39	39.54 ± 3.32
200	18.96 ± 1.69	94.86 ± 8.47	45.88 ± 4.18
250	20.55 ± 1.99	97.33 ± 9.23	49.08 ± 5.09

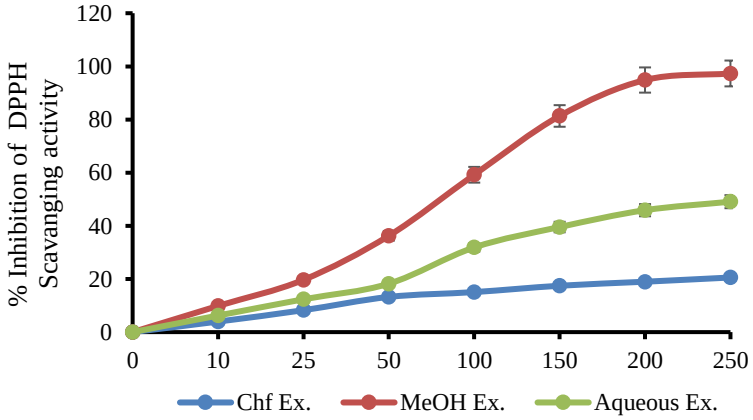


Figure 1: Percentage of scavenging activity of DPPH radical over the treatment of various solvents extracts of *P. dulce* seeds; The results are represented as mean ± standard deviation of triplicate readings (n=3). All the data indicated having p value less than 0.05 ($p<0.05$) represent statistically significant.

2. Superoxide anion radical scavenging capacity: The superoxide anion radical scavenging assay is critical in determining the antioxidant capacity of plant extracts, as superoxide anion radicals are among the primary reactive oxygen species formed in living systems, potentially leading to oxidative stress and tissue damage (Babior, 1978). The results (Table 2 and Figure 2) demonstrate the superoxide anion radical scavenging capacity of *P. dulce* seed extracts using three different solvents: chloroform (Chf Ex.), methanol (MeOH Ex.), and water (Aqueous Ex.). The activity increased with the concentration of the extracts, which is presented at various

concentrations ranging from 10 to 250 µg/mL. The methanol extract showed the highest scavenging activity at all concentrations, with a near complete scavenging activity at the highest concentration (95.99 ± 9.41% at 250 µg/mL). The aqueous extract exhibited moderate scavenging activity (45.96 ± 4.28% at 250 µg/mL), while the chloroform extract demonstrated the least activity (19.06 ± 1.45% at 250 µg/mL). The increase in scavenging activity with concentration for all extracts indicates a dose-response relationship, which is typical of antioxidant assays. It suggests that a higher concentration of active components in the extracts leads to greater scavenging abilities.

Table 2: *Percentage of superoxide radical scavenging activity over the treatment of various solvent extracts of P. dulce seeds.*

Conc in µg/mL	Chf Ex.	MeOH Ex.	Aqueous Ex.
10	2.63 ± 0.33	10.87 ± 0.95	6.89 ± 0.54
25	5.86 ± 0.69	22.69 ± 1.66	13.47 ± 0.99
50	8.57 ± 0.91	37.42 ± 2.35	23.99 ± 1.86
100	12.05 ± 1.07	59.18 ± 5.28	31.56 ± 2.54
150	15.39 ± 1.22	78.12 ± 6.96	39.65 ± 2.95
200	17.88 ± 1.69	90.66 ± 8.12	43.85 ± 5.12
250	19.06 ± 1.45	95.99 ± 9.41	45.96 ± 4.28

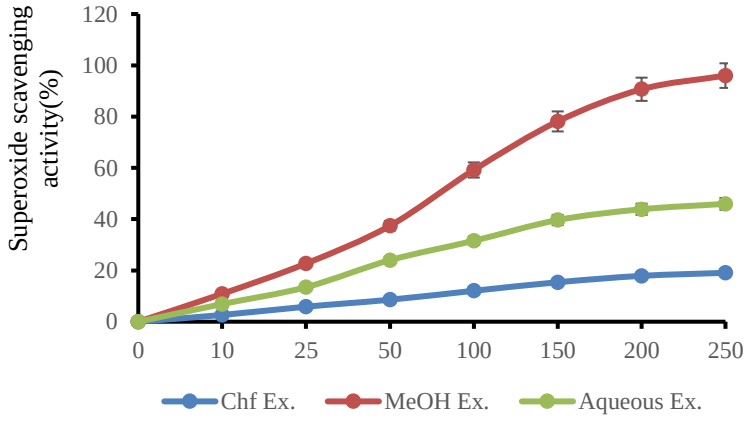


Figure 2: Percentage of superoxide radical scavenging activity over the treatment of various solvents extracts of *P. dulce* seeds; the results are represented as mean ± standard deviation of triplicate readings (n=3). All the data indicated having p value less than 0.05 ($p<0.05$) represent statistically significant.

Determination of SOD: The SOD inhibitory assay is a measure of a substance's ability to mimic the activity of superoxide dismutase, an enzyme that catalytically quenches superoxide radicals (O2•−), thus protecting the cellular components from oxidative damage (McCord &Fridovich, 1969). The superoxide anion radical scavenging assay offers valuable insights into a plant extract's antioxidant potential, given that superoxide anion radicals are highly reactive and can contribute to oxidative stress and cellular damage in biological systems (Babior, 1978). The *in-vitro* antioxidant activity data presents the SOD inhibitory activity of *P. dulce* seed extracts across three different solvents: chloroform (Chf Ex.), methanol (MeOH Ex.), and aqueous (Aqueous Ex.), as depicted in Table 3 and Figure 3. At increasing concentrations of 10 to 250 µg/mL, the MeOH Ex. exhibited the highest inhibition, peaking at 85.42 ± 8.09% at 250 µg/mL. The Aqueous Ex. showed moderate inhibition, with a maximum of 49.23 ± 4.12% at 250 µg/mL. The Chf Ex. demonstrated the lowest inhibitory activity, reaching 21.56 ± 1.99% at 250 µg/mL. The data indicates a positive correlation between concentration and SOD inhibitory activity for all extracts, consistent with the dose-dependent response often observed in antioxidant assays (Kedziora-Kornatowska et al., 1995). The superior performance of the MeOH Ex. in the SOD assay, when compared to the Aqueous and Chf Ex., underscores the presence of potent antioxidants within the methanol-soluble fraction of *P. dulce* seeds (Sun et al., 2002). Even though the Chf Ex. showed the lowest activity, it still exhibits some level of SOD inhibition, indicating that even non-polar components of *P. dulce* seeds might possess antioxidant capabilities, albeit less pronounced (Das & Das, 2002) .

Table 3: *Percentage of inhibition of SOD activity over the treatment of various solvent extracts of P. dulce seeds.*

Conc in µg/mL	Chf Ex.	MeOH Ex.	Aqueous Ex.
10	3.99 ± 0.28	9.54 ± 0.88	4.87 ± 0.65
25	6.88 ± 0.92	14.69 ± 1.13	11.05 ± 0.89
50	9.69 ± 1.03	34.88 ± 2.56	25.74 ± 2.14
100	15.09 ± 1.24	55.12 ± 4.88	32.86 ± 2.76
150	19.47 ± 1.87	73.22 ± 6.23	38.24 ± 3.55
200	18.74 ± 1.72	81.34 ± 7.85	46.02 ± 3.87
250	21.56 ± 1.99	85.42 ± 8.09	49.23 ± 4.12

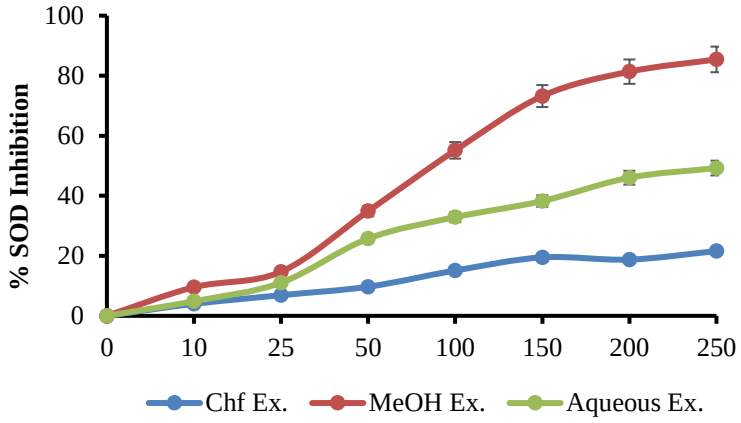


Figure 3: Percentage of Superoxide dismutase (SOD) Inhibition over the treatment of various solvents extracts of *P. dulce* seeds; the results are represented as mean ± standard deviation of triplicate readings (n=3). All the data indicated having p value less than 0.05 ($p<0.05$) represent statistically significant.

In-vitro Cellular Studies Evaluating Neuroprotective Abilities

Cell viability assessment: To know the neuroprotective capabilities of solvent extracts of *P. dulce* seeds, initially, the extracts were examined for cellular cytotoxic. The high cell viability percentages observed across all concentrations and solvent extracts suggest that *P. dulce* seed extracts possess neuroprotective effects, maintaining the survival of SH-SY5Y neuroblastoma cells under experimental conditions. The vitality of SH-SY5Y cells treated with *P. dulce* seed extracts (Chloroform, Methanol, and Aqueous) was assessed, and it was found that the cells remained highly viable at all tested concentrations, which ranged from 10 to 500 µg/mL as shown in Table 4 and Figure 4.

The chloroform extract (Chf Ex.) exhibited a decrease from 99.91 ± 6.25% at a concentration of 10 µg/mL to 96.57 ± 7.99% at a concentration of 500 µg/mL. The methanol extract (MeOH Ex.) exhibited a cell viability of over 98% even at the highest concentration tested (98.21 ± 5.24% at 500 µg/mL). The aqueous extract showed the smallest range of variation in cell viability, with values ranging from 99.91 ± 8.66% to 98.34 ± 9.05%.The study assesses the antioxidant activity of seed extracts from *P. dulce* by employing the DPPH radical scavenging assay at various doses. The methanol extract had the most potent scavenging activity, with the aqueous and chloroform extracts showing lower activity. At a concentration of 250 µg/mL, the MeOH example exhibited almost whole DPPH radical scavenging action, but the aqueous extract displayed noteworthy activity. The chf ex. exhibited the lowest level of activity.

This indicates that these extracts possess promising antioxidant capabilities. The elevated cell viability observed in the MeOH and Aqueous ex. at all concentrations indicates the existence of neuroprotective compounds in the extracts, that can counteract neurotoxins by boosting neuron survival processes (Lipton, 1998).

Table 4:SHSY5Y cells viability studies

Conc. in µg/mL	Chf Ex.	MeOH Ex.	Aqueous Ex.
10	99.91± 6.25	99.88 ± 7.48	99.91 ± 8.66
50	99.53 ± 8.69	99.66 ± 8.54	99.72 ± 6.35
100	99.24 ± 8.96	99.31 ± 9.03	99.55 ± 7.47
200	98.75 ± 9.23	99.05 ± 9.15	99.34 ± 8.57
300	98.31 ± 9.07	98.68 ± 8.47	99.24 ± 7.26
400	97.13 ± 8.75	98.42 ± 9.23	98.76 ± 7.69
500	96.57 ± 7.99	98.21 ± 5.24	98.34 ± 9.05

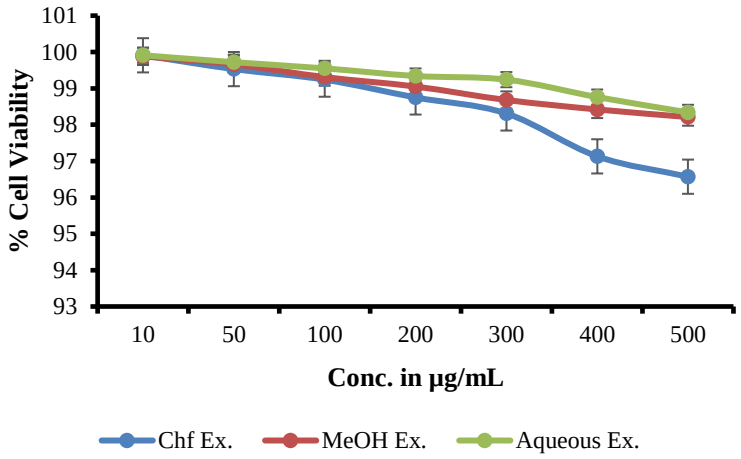
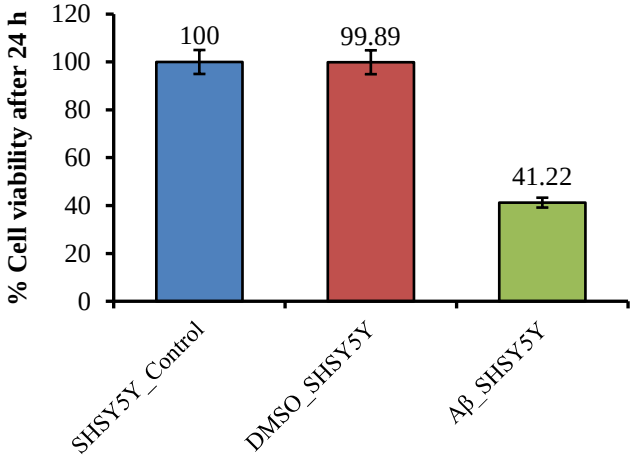


Figure 4: *Cell viability studies against SHSY5Y neuroblastomacells over the treatment of various solvent extracts of P. dulce seeds using MTT assay; The results are represented as mean ± standard deviation of triplicate readings (n=3). All the data indicated having p value less than 0.05 ($p<0.05$) represent statistically significant.*

Cell-based in-vitro cellular protective effect of P. dulce seed extract: The study sought to assess the neuroprotective properties of *P. dulce* seed extracts on SHSY5Y cells displaying neurodegenerative features caused by amyloid-beta (Aβ) protein. The restoration abilities of Chf Ex., MeOH Ex., and Aqueous extracts were evaluated by measuring cell viability and the results were shown in Table 5 and Figure 5. The extracts were tested at doses ranging from 5 to 100 µg/mL. The SHSY5Y control cells that were not treated exhibited 100% vitality, whereas the DMSO vehicle control exhibited a viability of 99.89%. The viability of SHSY5Y cells treated with Aβ was drastically reduced to 41.22%. After being treated with *P. dulce* seed extracts, the MeOH Ex. showed the highest level of cell viability restoration. It increased from 40.85% ± 3.56 at a concentration of 5 µg/mL to 83.96% ± 7.04 at a concentration of 100 µg/mL. The MeOH Ex. proved most potent, showing the strongest cell restoration across most concentrations, with a notable increase at 40 µg/mL and above. This dose-dependent effect suggests a direct relationship between extract concentration and improved cell health. The aqueous extract also promoted cell restoration, although less pronouncedly than the methanol extract. Interestingly, this restoration seems to plateau around 40-60 µg/mL. The Chf Ex. displayed the weakest restorative effect, with only a gradual increase in cell viability at higher concentrations.



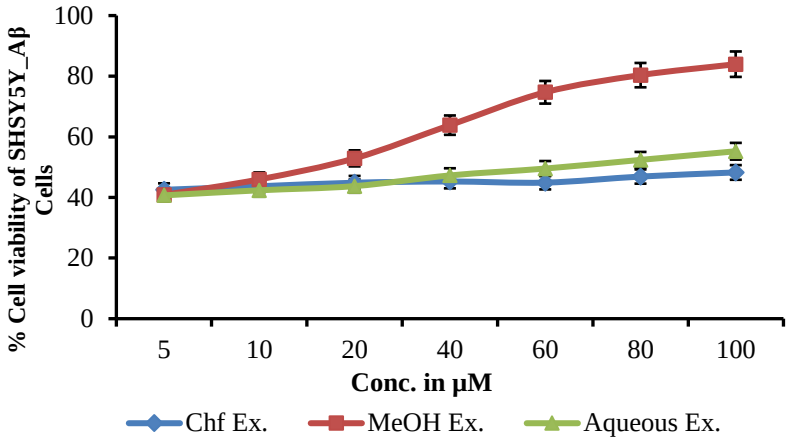


Figure 5: Cell viability of various test controls of the study; (b) Restoring SHSY5Y_Aβ cell viability over treating Chloroform, Methanol, and Aqueous extracts of *P. dulce* seeds. The results are represented as mean ± standard deviation of triplicate readings (n=3). All the data indicated having p value less than 0.05 ($p<0.05$) represent statistically significant.

Table 5: *Restoring SHSY5Y_Aβ cell viability over the treatment of various solvent extracts of P. dulce seeds*

Conc. in µg/mL	Chf Ex.	MeOH Ex.	Aqueous Ex.
5	42.53 ± 3.85	40.85 ± 3.56	40.69 ± 3.87
10	43.69 ± 4.66	45.96 ± 3.47	42.36 ± 4.66
20	44.86 ± 5.23	52.89 ± 4.12	43.74 ± 5.28
40	45.26 ± 4.81	63.85 ± 5.66	47.26 ± 4.78
60	44.89 ± 3.96	74.69 ± 7.87	49.53 ± 6.23
80	46.89 ± 6.05	80.35 ± 6.23	52.39 ± 5.82
100	48.25 ± 5.55	83.96 ± 7.04	55.24 ± 6.55

Examination of cellular morphology of SHSY5Y and Aβ_SHSY5Y cells over the treatment of various solvent extracts of *P. dulce* seeds: SHSY5Y cells are a well-established model for studying neurodegenerative diseases like Alzheimer's due to their neuronal-like properties and ability to express disease-relevant proteins such as amyloid precursor protein (APP) (Kovalevich & Langford, 2013). These alterations can also indicate the onset of cellular demise, which a

potent neuroprotective drug aims to mitigate. The morphology of SHSY5Y cells can reflect cellular health, with intact neuritic networks indicating a healthy neuronal phenotype, while neurodegenerative features include neurite retraction and cell body shrinkage (Cheung et al., 2009). The microscopic images depict the morphology of SHSY5Y cells under various conditions: untreated control cells, amyloid-beta (Aβ) treated cells to model neurodegeneration, DMSO vehicle control cells, and cells treated with various concentrations (5, 40, and 100 µg/mL) of solvent extracts from *P. dulce* seeds as shown in Figure 6. The control SHSY5Y cells show normal neuronal-like morphology with extensive neuritic processes. The Aβ treated cells exhibit clear signs of neurodegenerative morphology, characterized by cell shrinkage, loss of neuritic processes, and reduced confluence. The DMSO-treated cells resemble the control cells, suggesting that DMSO does not adversely affect cell morphology. Cells treated with *P. dulce* extracts show varying degrees of morphological restoration, depending on the extract concentration and solvent type (Figure 6). The study indicates that *P. dulce* seed extracts, particularly those extracted with methanol may contain neuroprotective agents capable of mitigating Aβ-induced neurotoxicity.

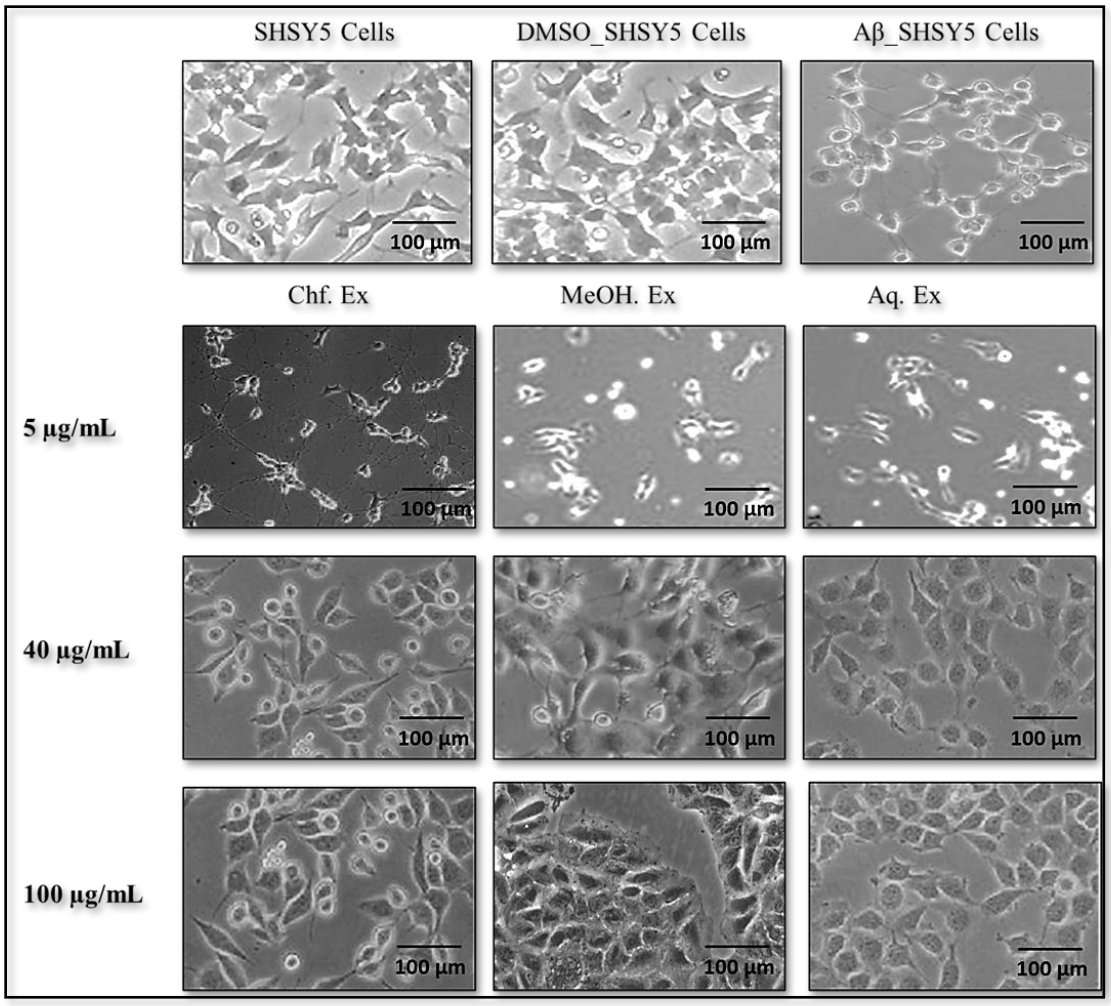


Figure 6: Cellular Morphology of SHSY5Y Cells and Aβ treated SHSY5Y cells over the treatment of various solvent extracts of PDME.

Discussion

The antioxidant study assesses the activity of seed extracts from *P. duce* by employing the DPPH radical scavenging assay at various doses. The methanol extract exhibited the most potent scavenging activity, with the aqueous and chloroform extracts showing lower levels of activity. As a concentration of 250 µg/ml, the methanol extract exhibited almost whole DPPH radical scavenging action, but the aqueous extract showed noteworthy activity. The chloroform extract exhibited the lowest level of activity. The research indicates that these extracts possess promising antioxidant capabilities. The DPPH radical scavenging assay offers a reliable method for evaluating the antioxidant potential of plant extracts and other substances (Brand et al., 1995).

This assay measures an antioxidant's ability to donate a hydrogen atom, neutralizing the stable DPPH radical and leading to a decrease in absorbance. The methanol (MeOH) extract showed the strongest DPPH scavenging activity, likely due to its high content of phenolic compounds and flavonoids, known for their antioxidant properties (Prior et al., 2005). Methanol's polarity makes it adept at extracting these potent antioxidants from *P.dule* seeds. The aqueous extract also exhibited notable activity, attributable to the partial solubility o certain phenols and flavonoids in water (Rice et al., 1996). The non-polar chloroform extract, less efficient in extracting these antioxidants, expectedly displayed the weakest activity (Molyneux, 2004). The observed dose-dependent increase in activity across all extracts aligns with the principle that higher extract concentrations provide more phytochemicals to neutralize DPPH radical (Ou et al., 2001). Methanol's superior scavenging power, compared to chloroform and aqueous extracts, probably stems from its compositional advantage in extracting compounds like phenols and flavonoids (Cai et al., 2004). Despite their lesser potency, chloroform and aqueous extracts still retain some free-radical scavenging capabilities (Slinkard, 1997). In conclusion, this study highlights the potent antioxidant activity of *P.dule's* methanol seed extract, warranting further exploration for therapeutic uses (Huang et al., 2005). Solvent choice significantly influences the extraction of these antioxidants. Future research should prioritize methanol and aqueous extracts while fully characterizing their active constituents, as these could become valuable natural antioxidants for the pharmaceutical or food industries (Heim et al., 2002). These findings support P. dulce seeds as a potential source of health-promoting compounds (Nijveldt et al., 2001).

The SOD scavenging study demonstrates the scavenging capacity of P. dulce seed extracts in three solvents: chloroform, methanol, and water. The extracts' activity increased with concentration, with methanol showing the highest scavenging activity at all concentrations. The aqueous extract showed moderate scavenging activity, while the chloroform extract showed the least activity. The study suggests that the extracts' scavenging capacity can be improved by increasing the concentration of the extracts. The superoxide anion radical scavenging assay offers valuable insights into a plant extract's antioxidant potential, given that superoxide anion radicals are highly reactive and can contribute to oxidative stress and cellular damage in biological systems (Babior, 1978). The methanol extract's superior scavenging activity suggests it possesses a higher concentration of compounds effective against superoxide anions. This

aligns with methanol's polarity, enabling it to extract a wide range of antioxidants (Halliwell and Gutteridge, 1992). The aqueous extract exhibited moderate activity, likely due to the partial water solubility of certain polar antioxidants (Hodgson, 1997). Chloroform, being non-polar, expectedly showed the lowest activity, potentially extracting fewer antioxidant compounds or those with weaker superoxide scavenging capabilities (Halliwell and Gutteridge, 1984). The observed dose-dependent increase across all extracts highlights the typical relationship between increased extract concentration and heightened antioxidant activity (Beckman and Ames, 1998). The differences in scavenging abilities support the idea that each solvent yields a distinct phytochemical profile, with methanol seemingly the most effective for extracting superoxide anion scavengers from P. dulce seeds (Prior et al., 1996). While less potent, even the chloroform and aqueous extracts exhibit some antioxidant activity, hinting at a diverse range of phytochemicals within P. dulce seeds (Marklund, and Marklund, 1974).

The superoxide dismutase (SOD) inhibitory assay measures a substance's ability to mimic the activity of superoxide dismutase, an enzyme that quenches superoxide radicals, protecting cellular components from oxidative damage. In-vitro antioxidant activity data shows that P. dulce seed extracts exhibit SOD inhibitory activity across three solvents: chloroform (Chf Ex.), methanol (MeOH Ex.), and aqueous (Aqueous Ex.). MeOH Ex. showed the highest inhibition, while Aqueous Ex. showed moderate inhibition. Chf Ex. showed the lowest inhibitory activity. The SOD inhibitory assay evaluates a substance's capacity to mimic the action of superoxide dismutase, a crucial enzyme that safeguards cells by neutralizing superoxide radicals (02-) (McCord and Fridovich, 1969). The methanol (MeOH) extract's potent SOD inhibitory activity indicates it contains compounds with strong antioxidant properties, capable of mimicking SOD's function (Fukai and Ushio, 2011; Kedziora et al., 1992). Methanol's polar nature allows it to extract a diverse range of potential antioxidants. The aqueous extract's moderate activity suggests the presence of water-soluble antioxidants with SOD-mimetic capabilities, though likely less potent than those extracted by methanol (McCord and Fridovich, 1969, Halliwell, 1992). Chloroform, being non-polar, expectedly exhibited the weakest activity, as it's less efficient at extracting the polar antioxidants likely responsible for SOD-like activity (Bors et al., 1990). The positive correlation between concentration and SOD inhibition in all extracts aligns with the dose-dependent nature of antioxidant assays (Kedziora et al., 1992). Methanol's superior

performance in this assay highlights the presence of powerful antioxidants in its extract of P. dulce seeds (Sun et al., 2002). While displaying lesser activity, even the chloroform extract's slight SOD inhibition hints at some antioxidant potential within the less-polar compounds of P. dulce seeds (Das and Das,2002).

The viability study examined SH-SY5Y cells treated with *P.dulce* seed extracts (Chloroform, Methanol, and Aqueous) at various doses. The chloroform extract showed a decrease in cell viability, while the methanol extract showed over 98% cell viability even at the highest concentration. The aqueous extract showed the smallest variation in cell viability. The study also assessed the antioxidant activity of *P.dulce* seed extracts using the DPPH radical scavenging assay. The methanol extract showed the most potent scavenging activity, while the aqueous and chloroform extracts showed lower activity. At a concentration of 250 μg/mL, the methanol extract showed almost whole DPPH radical scavenging action, while the aqueous extract showed noteworthy activity.

The MTT assay, a reliable indicator of cell viability, provides valuable insights into the potential neuroprotective properties of *P.dulce* seed extracts. The minimal cytotoxicity exhibited by SHSY5Y neuroblastoma cells, a widely used model for studying neurotoxicity (Kaufman et al., 1995), across all extracts is a promising finding. While the chloroform extract (Chf Ex.) displayed a dose-dependent reduction in cell viability, the overall viability remained within a range considered non-toxic (Repetto, 2008). The slightly higher viability observed with the methanol extract (MeOH Ex.) could imply the presence of neuroprotective compounds or a reduced concentration of cytotoxic compounds within that extract (Liu et al., 1997). Notably, the aqueous extract (Aqueous Ex.) consistently showed the highest cell viability, suggesting water-soluble compounds might be either beneficial for cellular health or less toxic than those extracted by other solvents (Burlando and Cornara, 2004). The high overall cell viability, especially in the methanol and aqueous extracts, hints at potential neuroprotective compounds within P. dulce seeds, which might combat neurotoxins or enhance cellular processes that promote neuronal survival (Lipton, 1998). The differences in cell viability across extracts likely reflect the distinct chemical compositions selectively extracted by each solvent, showcasing their varying extraction capabilities (Houghton et al., 2007).

All three *P.dulce* seed extracts demonstrated cell-restoring abilities, enhancing the viability of SHSY5Y Aß cells compared to the untreated control (which likely had lower viability). The methanol extract (MeOH Ex.) proved most potent, showing the strongest cell restoration across most concentrations, with a notable increase at 40 µg/mL and above. This dose-dependent effect suggests a direct relationship between extract concentration and improved cell health. The aqueous extract (Aqueous Ex.) also promoted cell restoration, although less pronouncedly than the methanol extract. Interestingly, this restoration seems to plateau around 40-60 µg/mL. The chloroform extract (Chf Ex.) displayed the weakest restorative effect, with only a gradual increase in cell viability at higher concentrations. Amyloid-beta-induced cytotoxicity in SHSY5Y cells provides a well-established in vitro model for investigating mechanisms of neurodegeneration and screening potential neuroprotective compounds (Cheignon et a., 2018). The observed restoration of cell viability after treatment with *P. dulce* extracts suggests these extracts contain neuroprotective compounds that mitigate Aß-induced damage. The methanol extract (MeOH Ex.) exhibited the most pronounced dose-dependent increase in neuroprotection. This could stem from methanol's ability to extract bioactive constituents with antioxidant properties or the ability to directly target Aß pathways (Butterfield and Boyd-Kimball, 2005; Behl and Moosmann, 2002). While less potent, the chloroform (Chf Ex.) and aqueous (Aqueous Ex.) extracts also displayed neuroprotective effects, hinting at a diverse array of protective compounds with varying extraction efficiencies across solvents (Sultana et al 2009). The high viability observed in the DMSO control confirms the vehicle's non-toxicity (Chen et al., 2011), while the drastic viability reduction in Aß-treated cells emphasizes the cytotoxic effects of amyloid-beta and validates the model for studying neurodegeneration (Hardy and Selkoe, 2002). These findings suggest that *P. dulce* seed extracts, particularly the methanol extract, harbor compounds with potential therapeutic applications for neurodegenerative diseases linked to amyloid-beta, such as Alzheimer's disease (Hardy and Higgins, 1992). Future investigations should focus on isolating and identifying the active neuroprotective components within the methanol extract and elucidating their mechanisms of action to advance the development of targeted therapies.

Microscopic images show the morphology of SHSY5Y cells under different conditions, including untreated control cells, amyloid-beta (Aß) treated cells, DMSO vehicle control cells,

and cells treated with solvent extracts from P. dulce seeds. Control cells show normal neuronal-like morphology, while Aß treated cells show neurodegenerative signs. DMSO-treated cells resemble control cells, suggesting no adverse effects. Cells treated with P. dulce extracts show varying degrees of morphological restoration. SHSY5Y cells offer a valuable model for investigating neurodegenerative conditions like Alzheimer's disease due to their neuronal characteristics and ability to express relevant proteins like amyloid precursor protein (APP) (Kovalevich and Langford, 2013). Changes in SHSY5Y morphology reflect their health, with healthy neurons exhibiting extensive neurite networks, while signs of degeneration include neurite retraction and cell shrinkage (Cheung et al., 2009). The observed neurodegenerative changes in AB- treated SHSY5Y cells align with Aß's known mechanisms of toxicity, including oxidative stress, mitochondrial dysfunction, and apoptosis (Mattson,19970). The DMSO control's healthy morphology confirms its suitability as a non-toxic vehicle at low concentrations (Yu et al., 2002). Treatment with *P. dulce* seed extracts induced morphological changes suggestive of neuroprotection. The methanol extract (MeOH Ex.) at its highest concentration (100 µg/mL) resulted in cells most closely resembling the untreated control, implying significant restoration of neuronal health. The less pronounced improvement at lower extract concentrations (5 and 40 µg/mL) suggests a dose-dependent effect (Singh et al., 2009). Differences in efficacy between the solvent extracts likely stem from the distinct chemical profiles and biological activities of the compounds each solvent selectively extracts (Gupta, 2003). Methanol's ability to solubilize a wider array of potentially neuroprotective compounds might explain its superior restorative effects at higher concentrations. These findings support the hypothesis that *P. dulce* seeds, especially components within the methanol extract, possess neuroprotective compounds capable of countering AB-induced toxicity. This warrants further investigation to identify specific active compounds, elucidate their mechanisms of action and explore their potential as therapeutic interventions for neurodegenerative diseases.

Conclusion

The study highlights the significant antioxidant and the potential neuroprotective effects of *P. dulce* seed extracts on SHSY5Y cells, particularly the methanol extract, which demonstrated the highest scavenging activity, cell viability and restore neuronal morphology affected by amyloid-beta toxicity. These findings suggest that *P. dulce* seeds could be a valuable source of natural

compounds for therapeutic applications in combating oxidative stress and neurodegenerative diseases.

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