

Impact of *Psidium Guajava* Leaf Extract on Phosphorylated Tau Protein in the Hippocampus of Scopolamine-Induced Alzheimer's Disease Wistar Rats

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ABSTRACT

The study investigated the effect of *Psidium guajava* leaf on tau protein accumulation in the CA3 region of the hippocampus of Alzheimer's disease scopolamine-induced rats. *P. guajava* leaves were obtained from a botanical garden in Agada 1 Abua, Rivers State, Nigeria. Leaves were washed in running tap water, air dried in shady place for one week then chopped into pieces with the aid of a knife. The chopped leaves were ground into fine particles by means of a mortar and pestle, then extracted using methanol. The study was done in two phases, phase one (acute) lasted for 14 days and phase two (chronic) 84 days. 80 male Wistar rats with weight 100 - 230 g were divided into 5 groups (n = 8). Group 1 served as normal control, group 2 received 1 mg/kg b.wt. i.p. of scopolamine plus distilled water, group 3 received 1 mg/kg scopolamine plus 100 mg/kg b.wt. of *P. guajava* leaf extract, group 4 received 1 mg/kg scopolamine plus 300 mg/kg b.wt. of *P. guajava* leaf extract and group 5 received 1 mg/kg scopolamine plus 5 mg/kg donepezil. The phytochemical screening and quantification of the bio-constituents of *P. guajava* leaf indicated the presence of flavonoids, alkaloid, tannins, saponin, glycoside, carbohydrate and anthraquinone; with flavonoid being the most abundant and anthraquinone the least. Also the methanol proved to be a better solvent for the extraction of the phytochemicals in *P. guajava* leaf. At the end of the experiment in the two phases, the rats were euthanised with 100 mg/kg b.wt. of ketamine (i.p.), decapitated to remove the brain. Brain tissues for brain-derived neurotrophic factor (BDNF) ELISA assay were rinsed in normal saline then homogenised in phosphate buffer using ceramic mortar and pestle placed on ice then transferred to the lab in ice pack. Brain tissues for histological study were fixed in 10 % neutral buffered formalin. The hippocampus was excised out, sliced into 10 µm thick slides using a microtome and stained in tau antibody immunohistochemistry. Both doses of *P. guajava* leaf extract significantly reduced tau accumulation in the CA3 region of the hippocampus. However, the 100 mg/kg b.wt. dose had better efficacy as it demonstrated high ameliorative ability comparable to the normal control rats in group 1 in both phases of the study, while the 300 mg/kg b.wt. performed poorly in the acute phase but improved its attenuative effect in the chronic study indicating a time dependent benefit. The extracts also significantly ($p < 0.05$) increased the BDNF level. Donepezil also improved the level of BDNF and reduced phosphorylated tau but not as compared with the extract.

KEYWORDS: Psidium guajava, tau protein, Alzheimer's disease, BDNF

ARTICLE DETAILS

Published On:
27 October 2025

Available on:
<https://ijpbms.com/>

INTRODUCTION

Neurodegenerative disease can be described as a heterogeneous group of disorders caused by progressive, long-lasting degradation and loss of neuronal cells in specific areas of the central nervous system (Pohl et al., 2018). Neurodegenerative diseases are known for their wide range of harmful conditions leading to progressive cell damage, nervous system connections and neuronal death. These pathologies culminate to loss of important motor and cognitive functions, such as mobility, learning and sensation, loss of memory, decision making, balance, talking, breathing and heart function (Silva et al., 2020). Neurodegeneration affects millions of people worldwide, yet no integral cure has been developed (Rojas-Garcia et al., 2023).

Alzheimer's disease (AD) is the most common cause of dementia among elderly people, constituting a major global health problem with huge implications for individuals and society (Frozza et al., 2018, Li et al., 2024; Rawat et al., 2022). Despite scientific breakthrough that have broaden our knowledge on the cellular and molecular bases of AD in the past decades, therapies that effectively halt the disease progression are still elusive (Frozza et al., 2018). AD is generally considered as a disorder related to intensified loss of neurons and synapses which proceeds in distinct anatomical loci, resulting in different phenotypes (Thakur et al., 2018). The pathophysiology of AD is caused by factors such as cholinergic dysfunction, amyloid/tau toxicity and oxidative stress/mitochondrial dysfunction (Thakur et al., 2018). Neurofibrillary tangles (NFTs) form paired helical filaments with hyperphosphorylated tau proteins. The accumulation of these paired helical filament (PHF) and neurofibrillary tangles (NFTs) are characterised by impaired physiological functions, apoptosis, neuronal and synaptic loss and some certain distinctive lesions which reflects as cognitive impairment (Sinsky, 2021; Thakur et al., 2018).

Dementia is the loss of mental abilities that affects daily life. It is usually promoted by other neurodegenerative diseases such as AD (60 – 80 % of all cases). AD is characterised by unavoidable loss of neurons, formation of neurofibrillary tangles, tau protein aggregation, amyloid β -protein (A β) deposition and low levels of acetylcholine (ACh) (Rawat et al., 2022; Sun et al., 2022). Common symptoms of AD are memory loss, inability to learn new things, loss of language function, impaired perception of space, inability to perform calculations, depression, delusions etc. (Pasinetti, 2010).

Tau, a microtubule-associated protein, under physiological conditions, regulates the assembly and maintenance of the structural stability of microtubules. In the diseased brain, however, phosphorylation of tau increases, reducing its affinity for microtubules and results in destabilisation of cytoskeleton in neurons (Guo et al., 2017; Rawat et al., 2022). This ultimately causes the microtubules to disassemble, and the free tau molecules aggregate into paired helical filaments

that accumulate as neurofibrillary tangles in AD and related tauopathies (Guo et al., 2017; Medeiro et al., 2011). A large body of evidence suggests that tau hyperphosphorylation results from perturbation of cellular signalling, mainly through imbalance in the activities of different protein kinases and phosphatases (Medeiro et al., 2010).

Oxidative stress is one of the major reasons for tauopathies. Oxidative stress plays an important role in tau hyperphosphorylation, polymerisation, and tau toxicity which lead to cell death in neurodegeneration and cause tau to aggregate (Alavi & Soussi-Yanicostas, 2015; Bhatia & Sharma, 2021). Phosphorylation and dephosphorylation of tau are the major alterations of physiological tau that changes its relation with microtubules and likely other mechanisms (Chang et al., 2018). Oxidative stress, mainly produced by mitochondria, peroxisomes and endoplasmic reticulum (Alavi & Soussi-Yanicostas, 2015), produces oxidized fatty acids that stimulate in vitro tau polymerisation (Gamblin et al., 2000). Long-term mitochondrial stress induces a cytosolic adaptive response, which is responsible for increased tau aggregation (Samluk et al., 2022). Oxidative stress activates multiple kinases, such as glycogen synthase kinase-3 (GSK3), and mitogen-activated protein kinases, including extracellular receptor kinase, and P38 mitogen-activated protein kinases (P38MAPK) and have the potential to phosphorylate tau and formation of neurofilaments (Zhu et al., 2001). The major cause of tau deposition is the impairment of its degradation pathway (Khanna et al., 2016). In humans there are six isoforms of tau generated by alternative splicing of exon 2, 3, and 10. The incorporation of exon 10 leads to four microtubule binding repeats (4R tau) instead of three (3R tau), altering how tightly the protein binds to microtubule and its propensity to aggregate (Gao et al., 2018). Healthy humans express similar amount of 3R and 4R tau. The ratio between the two isoforms may impact disease, with higher 4R isoforms leading to greater degeneration.

In AD, there is a higher ratio of 4R to 3R, and reported downstream consequences including transcriptional alteration in the Wnt signalling pathway (Chen et al., 2011), and altered axonal transport (Combs et al., 2019). Prior to NFT formation, tau become hyperphosphorylated, and tau phosphorylation not only plays a large role in regulating tau function, but could be the key change resulting in the accumulation, and potential toxicity of this protein. In fact, multiple tauopathy mutations cause tau to be more readily phosphorylated (Iqbal et al., 2010). Tau has been hypothesized to induce neurotoxicity via loss of function, gain of function and / or mis-localisation. Loss of function of tau occurs when tau is no longer able to stabilise microtubules have an impact on neuronal cytoskeleton, and similarly could lead to deficiencies in axonal transport (Noble et al., 2011). Higher levels of tau have also been implicated in inhibiting

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vesicle and organelle trafficking, including those carrying APP, and increase levels of oxidative stress as well as have effect on axonal transport. The mis-localisation of tau to dendritic spines has been shown to affect cognition and synapses in vivo (Miller *et al.*, 2014).

Tau neurotoxicity provokes alterations in brain-derived neurotrophic factors (BDNF)/ tropomyosin receptor kinase B (TrkB/cAMP – response-element binding protein (CREB) signalling to contribute to neurodegeneration. Compounds activating TrkB may therefore provide beneficial effects in tauopathies (Te-Hsien *et al.*, 2022).

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor that promotes neuronal survival and growth (Pisani *et al.*, 2023). In the brain, BDNF is mainly synthesized in cell bodies of neurons and glial cells and then transported to presynaptic terminals and postsynaptic dendrites. The localisation of BDNF and its receptors, tropomyosin receptor kinase B (TrkB), to glutamate synapses regulate neurotransmitter release, ion channel activity, axonal pathfinding and neuronal excitability (Schinder & Poo, 2000). BDNF binds to tropomyosin-receptor kinase B (TrkB) to induce dimerisation and phosphorylation of TrkB, and subsequent activation of downstream extracellular-signal regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signalling pathway (Huang *et al.*, 2003). ERK phosphorylates cAMP responsive element binding protein 1 (CREB 1) to promote transcription of genes, such as BDNF and B-cell lymphoma 2 (BCL 2) anti-apoptosis regulator for neuronal survival, neurite outgrowth and neuroplasticity (Walton & Dragunow, 2000). In addition, PI3K – AKT pathway stimulates target gene expression via CREB phosphorylation to promote cell survival. Enhancement of BDNF expression rescues neuronal death and improves learning and memory in rodent and primate models of AD (Hsiao *et al.*, 2014).

The diagnosis of AD is by evaluating the location, distribution and abundance of characteristic brain lesions. These neuroimaging techniques are evolving and capable of both quantifying AD-associated cerebral atrophy and detecting amyloid beta (A β) peptide, phosphorylated tau or other β -pleated sheet proteins in the brain while patients are alive, these techniques also help when disease is completely asymptomatic or at early stages of neurodegeneration (Kumar *et al.*, 2015).

AD affects many regions of the brain such as hippocampus, cerebellum, thalamus, amygdala, frontal lobe etc. (Martin, 2015). The hippocampus is the first area of the brain to be affected by AD resulting in loss of short-term memory. A study showed that the volume and ratio of this region was reduced by 25 % in AD, 21 % in mixed dementia, 11 % in vascular dementia, and 5 % in normal pressure hydrocephalus (Martin, 2015). The hippocampus is a complex brain structure embedded deep into the temporal lobe. It can be distinguished externally as a layer of densely packed neurons, which curls

into S-shaped structure on the edge of temporal lobe. In adult humans, the hippocampus on each side of the brain is about 3 – 3.5 cm³ as compared to 320 – 420 cm³ for the volume of the neocortex. The hippocampus plays a major role in learning, memory, and spatial navigation and emerging studies have also indicated it to be part of ‘moral brain’ (Fumagalli & Priori, 2012).

The hippocampal formation appears after removal of the entorhinal cortex as C-shaped hippocampus proper, dentate gyrus, and subiculum (Khaled & Randa, 2020). When viewed in transverse (coronal) section, the hippocampus is subdivided into four zones designated CA1 – CA4 (CA – Cornu Ammonis). The hippocampus is composed primarily of pyramidal cells with dendrite and axon. The CA1 field contains the pyramidal cells located closest to the subiculum, whereas the two other fields CA2 and CA3 are closer to the surface. With histochemical preparations, the fields of the hippocampus consist of four main layers and a fifth one mainly in the CA3. These layers are molecular layer, radiate layer, pyramidal layer, orient layer and alveus (Khaled & Randa, 2020; Roberto, 2022).

Psidium guajava commonly known as guava in English is called goba in Hausa, guaba or gilofa in Yoruba, ugwoba in Igbo (Okoye, 2021) and ugwomba in Abua language. *P. guajava* is an evergreen shrub native to the Caribbean, Central America and South America and Mexico. It is widely cultivated in tropical and subtropical regions around the world. *P. guajava* belongs to the phylum magnoliophyta, class magnoliopsida and myrtaceae family (Dakappa *et al.*, 2013; Okoye, 2021). The plant has a wide spreading network of branches. Its leaves have opposite arrangement with small petioles of about 3 to 16 cm. The leaves have clear green colour with prominent veins (Rouseff *et al.*, 2008). The plant produces white flowers with incurved petals having a nice fragrant. The flowers have four to six petals and yellow coloured anthers and pollination is by means of insects especially bees. *P. guajava* have been used in traditional medicine in many cultures throughout central America, the Caribbean, Africa and Asia for the treatment of inflammation, diabetes, hypertension, caries, wounds, pain relief, fever, diarrhoea, rheumatism, lung diseases and ulcers (Gutierrez *et al.*, 2008). Several studies have demonstrated the potential of *P. guajava* in the management of AD in area of inhibiting acetylcholinesterase (Bhattacharya *et al.*, 2024; Lorena *et al.*, 2022; Silva *et al.*, 2022) and inhibiting neurotoxicity and cognitive dysfunction (Usen & Enogieru, 2023).

Studies on the correlation of the cognitive impairment to the histopathological changes have consistently demonstrated that the number of neurofibrillary tangles, and not the plaques, correlates best with the presence and or the degree of dementia in AD. Whereas, neurofibrillary degeneration appears to be required for the clinical expression of the disease, dementia, β -amyloidosis alone in the absence of neurofibrillary degeneration does not produce the disease

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clinically (Quiroz et al., 2018). Also, available drugs used for the management of AD were found to cause some side effects such as nausea, diarrhoea and insomnia (Breijyeh & Karaman, 2020). Hence, the development of novel efficacious medicines with least or no side effects for AD is imperative.

MATERIALS AND METHODS

Plant Preparation

The fresh leaves of *P. guajava* were obtained from Agada 1 in Abua/Odual Local Government Area of River State, Nigeria. The fresh leaves were washed in running tap water and air dried in a shady place for two weeks. Thereafter, the leaves were chopped into pieces with the aid of a knife then pulverised using laboratory mortar and pestle into powder form. The pulverised leaves were extracted using methanol as a solvent. The extract was dried using water bath at 60 °C then stored in a refrigerator at 4 °C until used.

Gas Chromatography Mass Spectrometer (GC-MS) Analysis

GC-MS analysis was carried out using a gas chromatography clarus 500 Perkin Elmer system comprising an AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column Elite-1 fused silica capillary column (30 × 0.25 mm ID (Inner Diameter) × 1 µ Mdf (mass defect filtering), composed of 100 % Dimethyl polysiloxane), operating in electron impact mode at 70 eV, Helium gas (99.999 %) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 µl was employed (split ratio of 10:1) injector temperature 250 °C; ion-source temperature 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min.) with an increase of 10 °C/min, to 200 °C, then 5 °C/min to 280 °C, ending with nine minutes isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 450 Da (Dalton). Total gas chromatography running time was 36 minutes. And the relative percentage amount of each compound was calculated by comparing its average peak area to the total areas. Turbomas version 5.2.0 software was adopted to handle mass spectra and chromatograms (Srinivasan et al., 2013; Thenmozhi & Rajan, 2015). The interpretation of mass spectrometer GC-MS was done using

a database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components were compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test material were ascertained.

Phytochemical Screening

The phytochemical screening of the *P. guajava* leaf extract were done using methods of Sofowore (1993) and Trease and Evans (1989). The various phytoconstituents of the extract were presented in a table.

Animals

Eighty (80) male Wistar rats of weight 100 – 230 g were used for the study. Forty (40) rats each were used for phase one and phase two of the study. After two weeks of acclimatisation, the animals were divided into five groups in plastic wire meshed cages. Group 1 served as normal control and received distilled water only, group 2 received 1 mg/kg b.wt.i.p. of scopolamine and distilled water, group 3 received 1 mg/kg b.wt. i.p. of scopolamine and 100 mg/kg b.wt. of *P. guajava* leaf extract, group 4 received 1 mg/kg b.wt. i.p. of scopolamine and 300 mg/kg b.wt. of *P. guajava* leaf extract, and group 5 received 1 mg/kg b.wt. i.p. of scopolamine and 5 mg/kg b.wt. of donepezil. Throughout the experiment, the animals were allowed free access to water and feed (vital grower®) *ad libitum*. Animals were maintained in twelve hours of light and dark cycles at room temperature.

Induction of Alzheimer's disease

Apart from the animals in the control group (group one), all other animals from group 2 to group 5 were induced Alzheimer's disease with 1 mg/kg b.wt. of scopolamine (i.p.) injection for 7 days. Scopolamine hydrobromide purchased from Sigma – Aldrich (USA), was dissolved in normal saline (0.9 % NaCl) (El-Marasy et al., 2018).

Experimental Design

The animals were divided into five groups (n = 8) and the treatment regimen was as indicated in Table 1. The experiment was done in two phases. Phase one (acute study) lasted for 14 days (2 weeks) following a 7 days of Alzheimer's disease induction while phase two (chronic study) took 84 days (12 weeks).

Table 1: Treatment Regimen of the Drug and Extract.

Group	Treatment	Dosage	Duration	
			Phase 1	phase 2
1	distilled water	2 mL/kg	14 days	84 days
2	scopolamine + distilled water	1 mg/kg + 2 mL/kg	14 days	84 days
3	scopolamine + <i>P. guajava</i>	1 mg/kg + 100 mg/kg	14 days	84 days
4	scopolamine + <i>P. guajava</i>	1 mg/kg + 300 mg/kg	14 days	84 days
5	scopolamine + donepezil	1 mg/kg + 5 mg/kg	14 days	84 days

n = 16

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Sample Collection

At the end of the experiment at the 14th day (week 2) and 84th day (week 12), the rats were euthanised using 100 mg/kg of ketamine administered intraperitoneally by means of a 2 mm needle syringe. The rats were then decapitated and the brains were removed for immunohistochemistry and biochemical analysis. The brains for the biochemical assay were rinsed in normal saline then homogenised in phosphate buffer under ice blocks using ceramic mortar and pestle. The homogenates were transferred into the test bottles and stored in ice pack then transferred to the laboratory for the ELISA. The brains for the immunohistochemistry were rinsed in normal saline to remove blood then transferred into plane bottles containing 10 % neutral buffered formalin for preservation.

Biochemical Assay of BDNF

A 100 µL of dilution of standard solution was added to blank and sample wells each. The plates were covered with the sealer, incubated for 90 min at 37°C. The liquid from each well were decanted without washing. 100 µL of biotinylated detection Ab working solution was immediately added to each well and covered with a new sealer then incubated for 1 hour at 37°C. the solution from each well was decanted and 350 µL of was buffer was added to each well , soaked for 1min and the solution aspirated from each well the patted dry against clean absorbent paper. This step was repeated 3 times. 100 µL of HRP conjugate working solution was added to each well, covered with a new sealer and incubated for 30 min at 37°C. The solution was decanted from each well and the process repeated for 5 times. 90 µL of substrate reagent was added to 37°C. The plate was protected from light. The microplate reader was preheated for about 15 min before optical density measurement. 50 µL of stop solution was added to each well in the same order as the substrate solution. The optical density value of each well was determined at once with a micro-plate reader set at 450 nM.

RESULTS

Table 2: Result of the quantitative analysis of *P. guajava* leaf extract

Phytochemicals	N-Hexane (Pg/mL)	Methanol (Pg/mL)	Hydro-methanol (Pg/mL)
Alkaloids	0.0012	0.0014	0.0012
Flavornoids	0.0019	0.0020	0.0015
Carbohydrate	0.0017	0.0019	0.0022
Saponin	0.0012	0.0014	0.0012
Glycoside	0.0019	0.0020	0.0018
Tannin	0.0012	0.0012	0.0014
Anthraquinnone	0.0009	0.0010	0.0009

Table 2 provides a quantitative screening of various phytochemicals present in *Psidium guajava* leaf in the three solvents extracts. The results indicate that methanol is the most effective solvent for extracting flavonoids, alkaloids, saponin, glycosides and anthraquinone yielding the highest

Tau immunohistochemistry

The fixed harvested brains were excised to remove the hippocampus then sliced into 10 µm thick paraffin embedded sections using rotary microtome. The sections were deparaffinised and hydrated in descending grades of alcohol (100 % - 70 % alcohol) then stained for hyperphosphorylated tau protein using tau antibody. Sections were blocked with 10 % goat serum in Tris buffered saline (TBS, 150 mM NaCl, 50 mM Tris, and pH 7.6). Sections were then incubated with primary antibodies (tau antibody) diluted in 1 % BSA in TBS with 0.1 % Tween 20 (TBS – T) overnight at room temperature. Bound antibodies were labelled by avidin and biotinylated HRP and developed with 3, 3 diaminobenzidine (DAB) in the presence of hydrogen peroxide. Stained and mounted slides were visualised using a microscope and Leica ICC50 E camera to develop the photomicrographs.

Ethical Permit

Ethical permit was sought for and obtained from the Ethical Permit Committee for Animal Use of the Faculty of Basic Medical Sciences, Rivers State University with approval number RSU/FBMS/REC/25/267, and the experiment was done in accordance with the guidelines of the permit.

Statistical Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) software package (version 28). A one way ANOVA was employed to determine the significance of the mean of the data obtained from the biochemical analysis and a multiple comparisons (LSD) *post-hoc* test was used to compare individual groups. Results were presented as mean ± SEM (standard error of the mean) and difference were considered significant at the level of $p < 0.05$.

concentration at 0.0020 Pg/mL, 0.0014 Pg/mL, 0.0014 Pg/mL, 0.0020 Pg/mL and 0.0010 Pg/mL respectively. While hydro-methanol is the most effective solvent for extracting carbohydrate yielding the highest concentration of 0.0022 Pg/mL and tannin at 0.0014 Pg/mL. N-Hexane did better than

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hydro-methanol in extracting flavonoids at 0.0019 Pg/mL compared to 0.0012 Pg/mL; and glycoside at a concentration of 0.0019 Pg/mL compared to 0.0018 Pg/mL. Overall, these findings highlight the importance of solvent selection in maximizing the extraction of specific phytochemicals from *Psidium guajava* leaves, with methanol being particularly

effective for flavonoids, alkaloids, saponins, glycoside and anthraquinone. Hydro-methanol being optimal for carbohydrates and tannins. The table shows that *P. guajava* leaf extract contained higher quantities of carbohydrate, glycoside and flavonoids in that order while anthraquinone was the least.

Table 3: GC-MS Identified Chemical Compounds in *Psidium guajava* Methanol Leaf Extract

S/N	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Retention Time (min)	Area %
1	Phytol	C ₂₀ H ₄₀ O	296.53	14.674	15.08
2	Undecanoic acid, ethyl ester	C ₁₃ H ₂₆ O ₂	214.34	13.707	12.38
3	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	C ₁₈ H ₃₂ O	264.45	15.075	11.29
4	3-Chloropropionic acid, heptadecyl ester	C ₂₀ H ₃₉ ClO ₂	346.98	12.008	8.24
5	5-Octadecene, (E)-	C ₁₈ H ₃₆	252.48	10.143	8.21
6	5-Tetradecene, (E)-	C ₁₄ H ₂₈	196.38	8.065	8.06
7	10-Octadecenoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296.49	14.56	3.98
8	Palmitoleic acid	C ₁₆ H ₃₀ O ₂	254.41	15.109	2.9
9	Octadecanoic acid, ethyl ester	C ₂₀ H ₄₀ O ₂	312.53	15.252	2.68
10	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-	C ₁₀ H ₁₆	136.23	12.42	2.68
11	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.45	13.152	2.41
12	9,15-Octadecadienoic acid, methyl ester, (Z,Z)-	C ₁₉ H ₃₄ O ₂	294.47	14.52	2.32
13	Carbonic acid, dodecyl prop-1-en-2-yl ester	C ₁₆ H ₃₀ O ₃	270.41	12.483	2.28
14	1,4-Benzenediol, 2-methyl-	C ₇ H ₈ O ₂	124.14	12.191	2.26
15	1-Decene	C ₁₀ H ₂₀	140.27	5.731	2.2
16	2,4-Di-tert-butylphenol	C ₁₄ H ₂₂ O	206.33	9.456	2.07
17	2-Dodecene, (Z)-	C ₁₂ H ₂₄	168.32	5.754	2.02
18	9,12-Octadecadienoic acid (Z,Z)-	C ₁₈ H ₃₂ O ₂	280.45	15.023	1.88
19	Dodeca-1,6-dien-12-ol, 6,10-dimethyl-	C ₁₄ H ₂₆ O	210.36	12.792	1.64
20	7H-Purin-6-amine, 7-methyl-	C ₆ H ₇ N ₅	149.16	13.519	1.23

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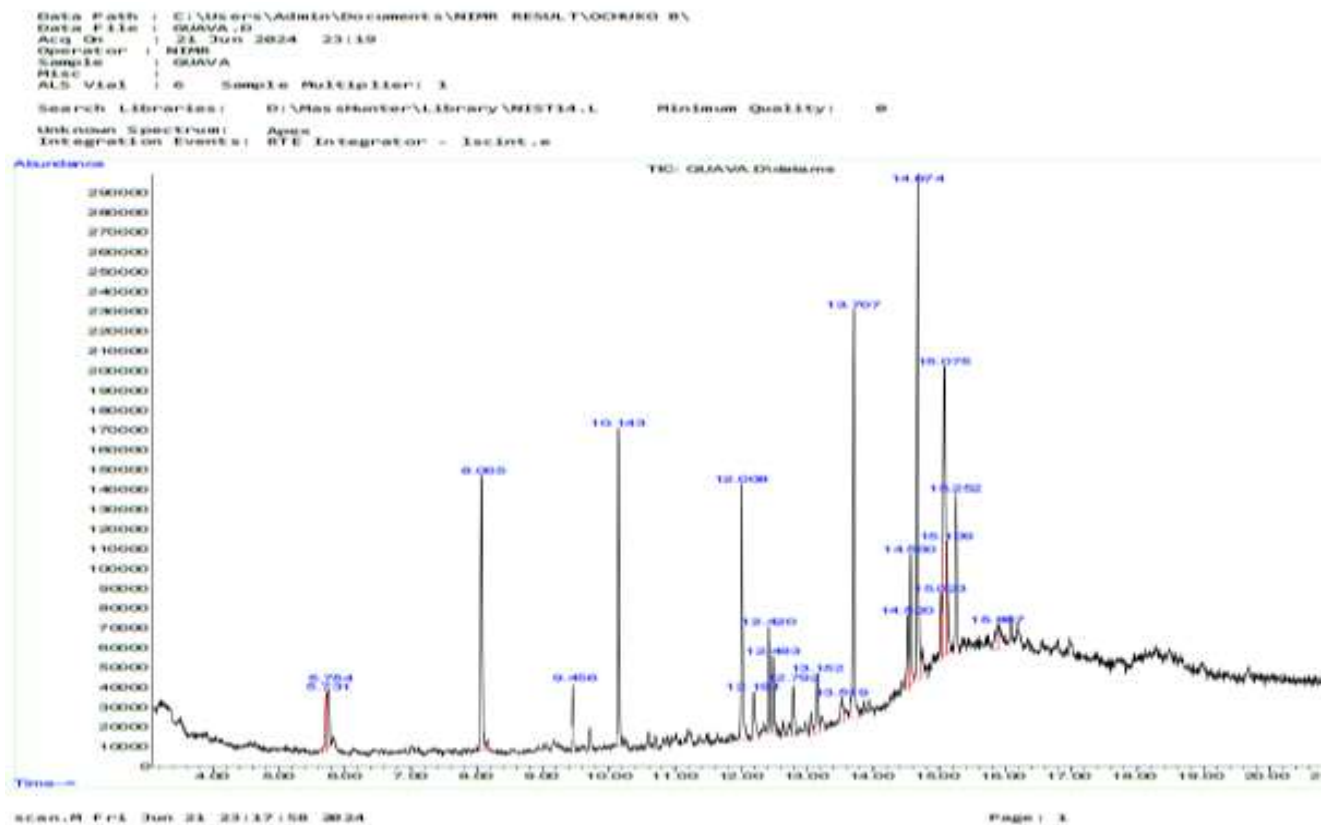


Figure 1: Chromatogram from GC-MS screening of the methanol extract of *Psidium guajava* leaf
Source: (Researcher)

Table 3 lists the chemical compounds identified in the methanolic leaf extract of *Psidium guajava* through gas chromatography-mass spectrometry (GC-MS) analysis. A total of 20 compounds were detected, each characterised by its molecular formula, molecular weight, retention time, and area percentage, which indicates the relative abundance of each compound in the extract. The most abundant compound identified was phytol (C₂₀H₄₀O), which constituted 15.08% of the total area with a retention time of 14.674 minutes and a molecular weight of 296.53 g/mol. Following closely was undecanoic acid, ethyl ester (C₁₃H₂₆O₂) at 12.38% with a retention time of 13.707 minutes. Other significant compounds included 9, 12, 15-octadecatrien-1-ol, (Z, Z, Z) - (C₁₈H₃₂O) at 11.29%, 3-chloropropionic acid, heptadecyl ester (C₂₀H₃₉ClO₂) at 8.24%, and 5-octadecene, (E) - (C₁₈H₃₆) at 8.21%.

The analysis also revealed various fatty acids and their esters, such as 10-octadecenoic acid, methyl ester (C₁₉H₃₆O₂) at 3.98% and palmitoleic acid (C₁₆H₃₀O₂) at 2.9%. Other compounds, including bicyclo [3.1.1] heptane, 2, 6, 6-trimethyl- (C₁₀H₁₆) and hexadecanoic acid, methyl ester (C₁₇H₃₄O₂), contributed smaller percentages to the overall composition, indicating a diverse range of phytochemicals present in the methanol extract of *P. guajava* leaf. Overall, the GC-MS analysis highlights the presence of various bioactive compounds, including fatty acids, esters, and phenolic compounds, which may contribute to the medicinal properties associated with *P. guajava* leaf. These findings suggest the potential of this plant extract for further exploration in pharmacological applications.

Table 3: Effect of *P. guajava* leaf extract on brain-derived neurotropic factor (BDNF)

Groups/Treatment	BDNF (Pg/mL) 14 th day (2 weeks)	BDNF (Pg/mL) 90 th day (12 weeks)
Group1 (control group)	202.96 ± #0.17	206.00 ± #44.79
Group 2 (1 mg/kg Scopolamine)	177.73 ± *0.72	149.67 ± 5.44*
Group 3 (100 mg/kg <i>P. guajava</i>)	195.49 ± 0.56	490.33 ± 17.02#

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Group 4 (300 mg/kg <i>P. guajava</i>)	295 ± *1.64	480.67 ± 18.96#
Group 5 (5 mg/kg Donepezil)	194.57 ± #0.27	394.32 ± 16.29#

Values are presented in mean ± SEM, n= 5. * Means values are statistically significant ($P \leq 0.05$) when compared to the control group 1, # means values are statistically significant ($P \leq 0.05$) when compared to scopolamine only group.

Table 3 presents the levels of brain-derived neurotrophic factor (BDNF) measured in various treatment groups at both 14th day (2nd week) and 84th day (12th week) of the study, providing insight into neurotrophic support in response to different interventions. The control group (group 1) exhibited a stable BDNF response, starting at 202.96 ± 0.17 Pg/mL at the 2nd week and slightly increasing to 206.00 ± 44.79 Pg/mL by the 12th week, indicating a consistent neurotrophic environment. In contrast, the Scopolamine group (group 2) showed significantly reduced BDNF levels, decreasing from 177.73 ± 0.73 Pg/mL at week two to 149.67 ± 5.44 Pg/mL at the 12th week (both $P \leq 0.05$), suggesting that scopolamine impairs neurotrophic support and potentially contributes to neurodegeneration. The 100 mg/kg *P. guajava* group (group 3) exhibited a favourable response, with initial BDNF levels at 195.49 ± 0.56 Pg/mL, increasing dramatically to 490.33 ± 17.02 Pg/mL by the 12th week ($P \leq 0.05$ compared to the control group), indicating a significant enhancement in neurotrophic factors through *P. guajava* treatment. Similarly, the 300 mg/kg treated group (group 4) showed a higher starting point of 295 ± 1.64 Pg/mL and an impressive increase to 480.67 ± 18.96 Pg/mL by the end of the study, reflecting the potent neuroprotective effects associated with this treatment. The donepezil group (group 5) also demonstrated

a notable improvement in BDNF, with levels starting at 194.57 ± 0.27 Pg/mL at week 2 and rising to 394.32 ± 16.29 Pg/mL by week 12 of treatment, showing a significant increase compared to the scopolamine group.

Tau Immunohistochemistry

Sections from group 1, in phase one and two (G1A: tau and G1B: Tau) indicated negative tau protein expression in the CA3 region of the hippocampus. Sections from group 2 (scopolamine-induced Alzheimer's disease, 1 mg/kg b.wt.) showed intense tau protein expressions in phase one (G2A: Tau) which festers with progression of time in phase two (G2B: Tau) in the pyramidal cells and other neurocytes (+3 degree). Sections from groups 3, Alzheimer's rats treated with 100 mg/kg b.wt. of *P. guajava* leaf extract indicated very low tau expression in phase one (G3A: Tau) and negative tau reaction in phase tow (G3B: Tau). Sections from group 4, Alzheimer's rats treated with 300 mg/kg b.wt. of *P. guajava* leaf extract showed moderate tau expression in phase one (G4A: Tau) and low tau expression in phase two (G4B: Tau) while sections from group 5, Alzheimer's rats treated with 5 mg/kg b.wt. of donepezil indicated low tau expression in phase one (G5A: Tau) and moderate expression in phase two (G5B: Tau) of the experiment as shown in figures 2 and 3.

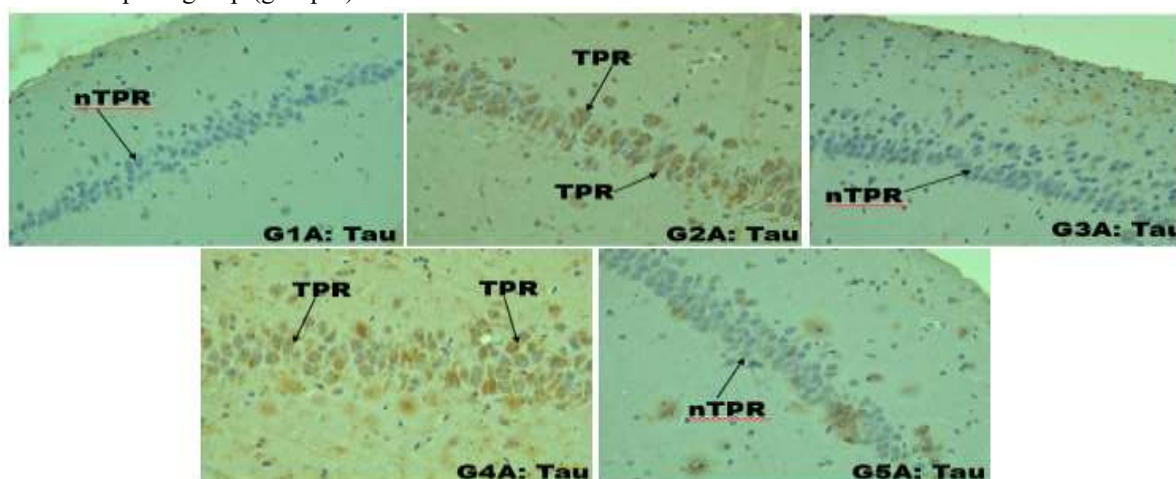


Figure 2: Immunohistochemical expression of hyperphosphorylated Tau accumulation in the pyramidal cells of CA3 region of the hippocampus at the 14th day of the study (phase one). Group 1 (G1A: Tau) control, indicating negative tau protein reaction (nTPR) in the pyramidal cells, group 2 (G2A: Tau) Alzheimer's disease rats induced with 1 mg/kg b.wt. of scopolamine indicating high tau protein reaction (TPR) (brown) +2 degree in the pyramidal cells, group 3 (G3A: Tau) Alzheimer's disease rats treated with 100 mg/kg b.wt. of *P. guajava* leaf extract indicating negative tau protein reaction (nTPR) with very low reaction in the pyramidal cells, group 4 (G4A: Tau) Alzheimer's rats treated with 300 mg/kg b.wt. of *P. guajava* leaf extract indicating moderate tau protein reaction (+1 degree) in the pyramidal cells and group 5 (G5A: Tau) Alzheimer's rats treated with 5 mg/kg b.wt. of donepezil indicating negative tau protein reaction (nTPR) with low reaction in the pyramidal cells. IHC (Tau antibody), X: 400.

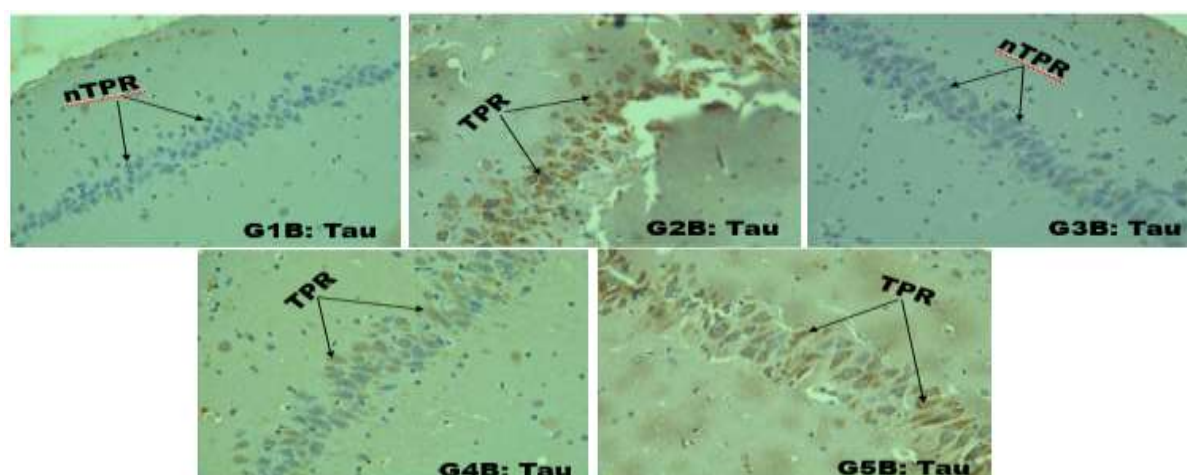


Figure 3: Immunohistochemical expression of hyperphosphorylated Tau accumulation in the pyramidal cells of CA3 region of the hippocampus at the 84th day of the study (phase two). Group1 (G1B: Tau) control, indicating negative tau protein reaction in the pyramidal cells, group 2 (G2B: Tau) Alzheimer's disease rats induced with 1 mg/kg b.wt. of scopolamine indicating very high Tau protein reaction (brown) (TPR) (+3 degree), group 3 (G3B: Tau) Alzheimer's disease rats treated with 100 mg/kg b.wt. of *P. guajava* leaf extract indicating negative tau protein reaction, group 4 (G4B: Tau) Alzheimer's disease rats treated with 300 mg/kg b.wt. of *P. guajava* leaf extract indicating low tau protein reaction (+ 1 degree) and group 5 (G5B: Tau) Alzheimer's disease rats treated with 5 mg/kg b.wt. of donepezil indicating moderate tau protein reaction (+ 2 degree). IHC (Tau antibody), X: 400.

DISCUSSION

Impairment of learning and memory and lack of coordination are some of the clinical implications of Alzheimer's disease (Silva et al., 2020). Pathologically, tau accumulation recognized as neurofibrillary tangles resulting from hyperphosphorylated tau proteins and neuroinflammation are some of the hallmarks of Alzheimer's disease (Reddy et al., 2017; Tackenberg et al., 2020). In this study, many spatial memory and learning; and muscle coordination tests were employed to investigate the effect of *P. guajava* leaf extract on spatial learning and memory as well as coordination in AD rats. Also, tau immunohistochemistry was used to evaluate the effect of *P. guajava* on tau accumulation (neurofibrillary tangles) in the CA3 region of the hippocampus of scopolamine-induced Alzheimer's disease rats.

Phytochemical screening for possible bioactive and biochemical compounds of medicinal values in plants has been a traditional practice in trying to evaluate the efficacy of plant parts on certain diseases. The phytochemical screening of *P. guajava* leaf extract using N-hexane, methanol and hydro-methanol solvents indicated the presence of alkaloids, flavonoids, carbohydrate, anthraquinones, glycoside, saponin, tannin and phlobatannin. It was found that methanol had a better extraction potential yielding a moderate (+ +) level of alkaloids, flavonoids, carbohydrate, glycoside, saponin, and phlobatannin, while the others yielded only slightly (+). Quantification of these phytochemicals indicates that methanol extracted the highest quantity with flavonoids and glycoside being the highest (0.0020 Pg/mL apiece) and anthraquinone the least (0.0010 Pg/mL). Although, hydro-methanol had carbohydrate at (0.0022 Pg/mL), methanol extracted *P. guajava* leaf extract was used for the study, since carbohydrate has not been shown to have antioxidant or

medicinal properties. This result agrees with Thenmozhi & Rajan (2015) and Usen & Enogieru (2023). Phytochemicals such as those present in *P. guajava* leaf have therapeutic effects on AD and dementia through different mechanisms (Brodowska, 2017; Liu et al., 2020; Shishta et al., 2020; Ullah et al., 2020). Hence, *P. guajava* is an important plant with the potentials to treat certain diseases. The GC-MS analysis also confirmed the presence of various bioactive compounds such as fatty acids and phenolic compounds further corroborating the possible medicinal properties of *P. guajava* leaf extract. The GC-MS indicated that phytol (C₂₀H₄₀O) was the most abundant with 15.08 % of the total area with a retention time of 14.674 minutes and a molecular weight of 296.53 g/mol. The brain-derived neurotrophic factor analysis at the 14th and 84th days of the study showed a drastic significant (P < 0.05) reduction of BDNF in the homogenised brain tissues of scopolamine-induced rats compared to the normal control rats. Deficiency of BDNF in the brain is a well-known biochemical hallmark of AD (Shimada et al., 2014). Reduced levels of BDNF have been implicated as a key factor leading to the dementia due to AD (Hock et al., 2000; Laske et al., 2006; Peng et al., 2005; Xue et al., 2022). Treatment with the *P. guajava* leaf extract demonstrated a good and consistent elevation of the level of BDNF in the brain. The 100 mg/kg b.wt. dose significantly improved the BDNF level compared to the scopolamine group. The 300 mg/kg b.wt. dose showed an earlier efficacious potential by elevating the BDNF level compared to the scopolamine group. The standard drug, donepezil was also effective in raising the BDNF levels in the brain. These results have shown the benefit of *P. guajava* leaf extract in mitigating memory impairment in scopolamine-induced rat model of AD.

Impact of Psidium Guajava Leaf Extract on Phosphorylated Tau Protein in the Hippocampus of Scopolamine-Induced Alzheimer's Disease Wistar Rats

Tau pathology, hyperphosphorylated tau (tau accumulation) was studied using Bielschowsky silver stain and tau antibody immunohistochemistry. Both stains were analysed qualitatively to see their reactions with neurofibrillary tangles (phosphorylated tau protein accumulation level). The photomicrograph of the CA3 region of the hippocampus of scopolamine (group 2) rats stained with tau antibody immunohistochemistry indicated a significant high expression of tau protein accumulation (+ 3 degree) in the pyramidal cells compared with the normal control (group 1) which showed negative reaction to tau antibody. At the end of the 14th day of the study, treatment with the extract showed that the 100 mg/kg b.wt. dose reduced the tau expression comparable with the normal control, the 300 mg/kg b.wt. dose had a low level of reduction with a +2 degree of expression while donepezil had moderate reduction of tau with a +1 degree of expression at the end of the second week of treatment (Figure 2). With the elongation of treatment, the 100 mg/kg b.wt. dose maintained the high level of reduction of tau protein expression. The 300 mg/kg b.wt. dose impressively improved the ameliorative prowess by reducing the phosphorylated tau protein expression to a moderate level (+1 degree) while the donepezil dropped in performance with a low level of reduction (+2 degree). However, rats in the scopolamine-induced group placed on placebo had worsened phosphorylated tau protein expression in the soma and fibres of the pyramidal cells (Figure 3).

This indicated that the low dose of *P. guajava* leaf extract is a sure bet for the management of tau protein accumulation in the hippocampus of scopolamine-induced AD rats as a way of treating AD. It had also agreed with others results of this study that the 300 mg/kg b.wt. dose was better with prolongation of period of treatment. This dose-duration dependent performance of the extract may be attributed to lots of factors including; enzyme induction, where the 300 mg/kg may induce enzymes that metabolise the drug but at a slower rate than the 100 mg/kg leading to increased efficacy of the 300mg/kg dose over time and vice versa; the body's feedback mechanisms may respond differently to the 100 mg/kg versus 300 mg/kg b.wt. doses leading to different efficacy over time, the two doses may have different receptor binding affinity leading to variation in effects.

The ability of the *P. guajava* leaf extract to mitigate the pathological alterations, spatial learning and memory deficits, and neurofibrillary tangles (phosphorylated tau protein) in the hippocampus may be attributed to the different phytochemicals with antioxidant properties contained in the extract. Flavonoids are antioxidants with anti-inflammatory properties (Brodowska, 2017) known to possess neuroprotective ability owing to their ability to cross the blood-brain barrier, scavenge reactive oxygen species and control signalling pathways involved in cognition, neuroinflammation and neuronal survival (Tachakittirungrod et al., 2007; Ullah et al., 2020). Flavonoids also enhance the

production of short-chain fatty acids hence, offering neuroprotection. Alkaloids have also demonstrated neuroprotective and anti-AD properties and butyrylcholinesterase anti-agonists. They also inhibit α -synuclein aggregation and function as agonists of dopaminergic and nicotinic cholinergic receptors, conferring their anti-neurodegenerative properties (Kim et al., 2024). The abundance of phytol (C₂₀H₄₀O) and considerable quantities of 2, 4-di-tert-butylphenol and 1, 4 -benzendiol, which are phenolic compounds in *P. guajava* leaf is an indication of its medicinal value as these compounds are antioxidants that have been found to decrease the development of interleukin 1 β , myeloperoxidase (MPO), tumours necrosis factor alpha (TNF- α), malondialdehyde (MDA) levels and increased levels of glutathione (GSH), thereby mitigating inflammation (Carvalho et al., 2020).

CONCLUSION

The *P. guajava* leaf extract was able to reduce the level of phosphorylated tau protein accumulation (neurofibrillary tangle) in the CA3 region of the hippocampus and elevate the level of BDNF in the brain. Hyperphosphorylated tau accumulation low levels of BDNF are significant hallmarks in the development of AD. Hence, the extract may be beneficial in the management of AD and other tauopathies.

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