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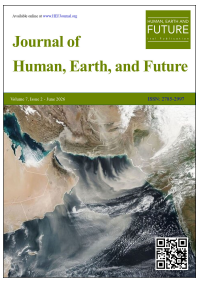

Gene Expression Analysis of Natural Oleamide Activities via Cannabinoid and Choline Acetyltransferase Detection

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Abstract

Oleamide, a naturally occurring compound that stimulates acetylcholine synthesis via the CB1 receptor, is vital for human physiology. This research aims to investigate the cannabinoid-like activity of oleamide-containing plants, focusing on their mechanism of action via the endocannabinoid-like receptor system. We quantified oleamide levels and evaluated the bioactivity, cytotoxicity, and genotoxicity of six plant extracts: the leaves of four *Dillenia* species (*D. obovata*, *D. ovata*, *D. indica*, and *D. pentagyna*), and the seeds of *Cucurbita moschata* and *Cannabis sativa* spp. *sativa*. Oleamide was detected in all extracts (0.06 to 0.11 mg/g). While no cytotoxicity was observed, the four *Dillenia* species extracts induced significant DNA damage in PBMCs and HepG2 cells ($p < 0.01$). The *D. indica* extract also damaged DNA in THLE-3 cells. Furthermore, all six extracts stimulated the expression of CB1 and ChAT genes in THLE-3 cells at various concentrations. Specifically, *D. obovata* increased CB1 gene expression by 318.2%–557.8% (at 1.7 and 0.017 mg/mL), while *Cucurbita moschata* increased ChAT gene expression by 236.3% at 0.263 mg/mL. These results indicate that the oleamide content in these extracts effectively promotes acetylcholine synthesis by inducing both the CB1 receptor and the ChAT enzyme in THLE-3 cells, suggesting potential applications in human health.

Keywords: Oleamide; *Dillenia species*; *Cucurbita moschata*; *Cannabis sativa* spp. *sativa*; Cannabinoid Gene; Choline Acetyltransferase Gene; Human Health.

1. Introduction

The high diversity of plant species and their complex chemical compositions offer a vast reservoir of bioactive metabolites. To date, these compounds have been utilized in their traditional forms, such as direct plant parts or as standardized extracts and powders. While advances in phytochemical analysis now allow for the synthesis of these structures, the human body's endogenous production of similar signaling molecules remains superior for maintaining physiological balance [1, 2].

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A critical concern in aging populations is the decline of these endogenous production mechanisms, which is a key factor in the onset of neurodegenerative diseases. For instance, oleamide (cis-9-octadecenamide), a primary fatty acid amide, serves as a vital modulator of the central nervous system. Functioning as an endocannabinoid-like molecule, oleamide modulates the endocannabinoid system (ECS) by inhibiting the degradation of anandamide and directly activating CB1 receptors, both of which are crucial for neuroprotective signaling pathways [3, 4]. This CB1 activation is intrinsically linked to the regulation of appetite, sleep-wake cycles, and cardiovascular stability [4]. Furthermore, research indicates that oleamide stimulates the choline acetyltransferase (ChAT) enzyme, which catalyzes the rate-limiting step in acetylcholine (ACh) synthesis [5, 6]. Decreased ACh levels are a hallmark of Alzheimer's disease (AD), often resulting from the depletion of ChAT and the overactivity of acetylcholinesterase (AChE) [7, 8]. Consequently, oleamide's ability to restore memory, relieve stress, and induce deep sleep, as demonstrated in foundational studies, positions it as a potent candidate for neuroprotective therapy [9, 10].

Given that the liver's capacity to synthesize choline, the ACh precursor, is often insufficient to meet metabolic demands, exogenous supplementation with oleamide presents a compelling therapeutic strategy [11, 12]. Our previous phytochemical screenings identified significant oleamide concentrations in several botanical genera, including *Ipomoea*, *Dillenia*, and *Tiliacora* [13, 14], highlighting their potential as natural sources.

However, a significant pharmacological gap exists between the study of pure compounds and complex plant extracts. While synthetic oleamide is well-characterized, its presence within a natural "phytochemical matrix" presents a unique profile. Unlike pure compounds, plant extracts contain multiple secondary metabolites that may act synergistically, a phenomenon often termed the "entourage effect", potentially enhancing the bioavailability, binding affinity to targets like CB1 receptors or PPARs, or overall bioactivity of oleamide [15, 16]. Conversely, these complex mixtures also carry the risk of unintended cytotoxicity or genotoxicity from co-occurring compounds. This critical safety consideration, often overlooked in traditional applications, justifies rigorous preclinical evaluation [17].

Therefore, the central question remains unaddressed: can plant-derived oleamide, within its native extract matrix, emulate the functional bioactivity and neuroprotective potential of its pure synthetic counterpart without inducing cellular damage? To address this, the present study employs a comprehensive translational research framework. We aim to quantify the oleamide content in selected promising sources: the leaves of four *Dillenia* species (*D. ovata*, *D. obovata*, *D. indica*, and *D. pentagyna*) and the seeds of *Cucurbita moschata* and *Cannabis sativa* spp. *sativa*. Subsequently, we will rigorously evaluate the safety profile of these extracts by assessing their cytotoxicity and genotoxicity in normal human cells and normal human cell lines (PBMCs, HepG2, and THLE-3). Finally, we will investigate their functional efficacy by measuring their capacity to upregulate key molecular targets, specifically CB1 and ChAT gene expression. This multi-faceted approach will provide the essential scientific evidence needed to validate these plants as effective, synergistic, and safe sources of oleamide for strategies aimed at mitigating neurodegenerative decline.

2. Materials and Methods

To provide a concise overview of the study's scope, a methodological flowchart is presented in Figure 1. Further comprehensive details for each step are elaborated in the subsequent sections.

2.1. Plant Materials

Mature foliage from four wild *Dillenia* species (*D. indica*, *D. ovata*, *D. obovata*, and *D. pentagyna*) was obtained from various natural and ex-situ populations across Thailand. Additionally, seeds of *Cucurbita moschata* and *Cannabis sativa* spp. *sativa* were sourced from commercial cultivation sites. Botanical identification was performed by Professor Arunrat Chaveerach, Ph.D. For subsequent phytochemical analysis, the leaf samples were cleansed with tap water and dried either at ambient temperature (in the shade) or in a 60°C hot-air oven. Once dried, the specimens were sealed in plastic bags and maintained at room temperature.

2.2. Plant Extraction and Preparation

The dried plant materials were homogenized using an electric grinder. Samples (5 g each) were separately extracted with 50 mL of ethanol and hexane for 72 hours of maceration at room temperature in the absence of light. Following filtration through Whatman® No. 1 filter paper, a 2 mL aliquot was set aside for phytochemical analysis. The filtrate was concentrated using a rotary evaporator to obtain the crude extracts, which were then solubilized in 100% DMSO. All prepared samples were maintained at -20°C until further investigation [12].

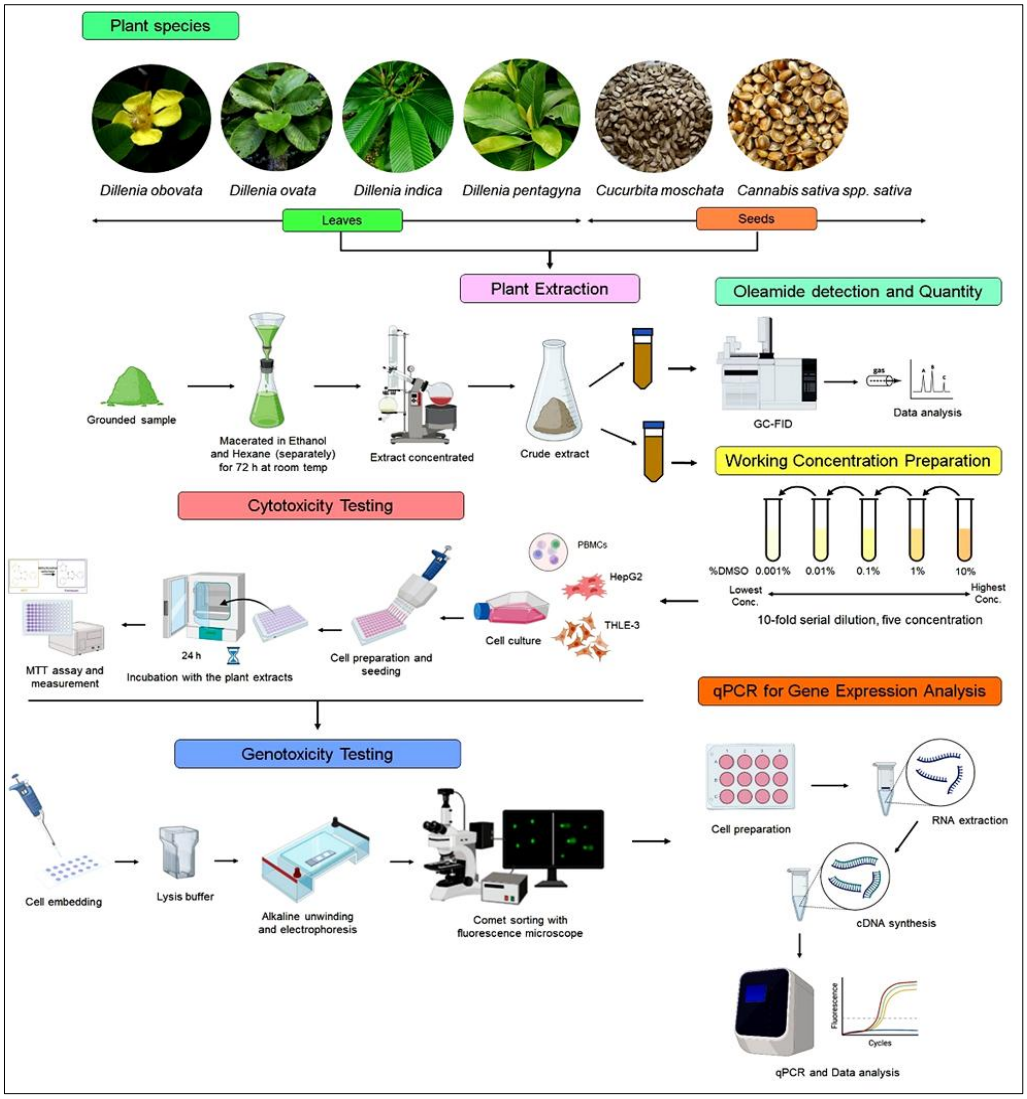

Figure 1. Methodology workflow

2.3. Oleamide Analysis Using GC–FID

Adapting the modified procedures of Farajzadeh et al. [18] and Kornpointner et al. [19], oleamide content was analyzed using an Agilent 7890B Gas Chromatograph coupled with a Flame Ionization Detector (FID). The separation was achieved on an HP–5 capillary column. Each 2 mL sample was clarified using a 0.45 µm syringe filter; subsequently, 1 µL was injected in split mode (1:10) at an inlet temperature of 290°C. The programmed thermal gradient started at 200°C for 3 min, followed by an increase to 300°C. The detector was maintained at a constant 300°C. To quantify the compound, oleamide concentrations in the samples were determined against a five-point calibration curve (0.0625–1 mg/mL) constructed via serial dilution of a 1 mg/mL stock solution.

2.4. Preparation of Phytochemicals for Biological Activity Testing

The extracts were first reconstituted in 100% DMSO and further diluted with sterile water to produce a range of five working concentrations (containing 0.001% to 10% final DMSO v/v). A 10-fold dilution series was employed for this purpose. These working solutions were stored under frozen conditions (-20 °C) and equilibrated to room temperature before use in biological studies.

2.5. Cell Culture and Preparation

Human PBMCs were isolated from whole blood units provided by the Central Blood Bank at the Faculty of Medicine, Khon Kaen University. The density gradient centrifugation was conducted using Ficoll–Paque Plus®, adhering to the protocol established by Freshney [20]. Following isolation, the PBMCs were maintained in RPMI medium supplemented with 10% FBS and 1% penicillin–streptomycin. The HepG2 (HB–8065) and THLE–3 (CRL–

3583) cell lines were acquired from the ATCC. After thawing, these lines were propagated in DMEM containing 10% FBS and 1% antibiotics. All cultures were incubated in a humidified 5% CO_2 environment at 37 °C, with the medium refreshed every 3–4 days. Upon reaching approximately 70% confluence, cells were detached using 1 mL of TrypLE™ Express Enzyme.

2.6. Cytotoxicity Testing Through MTT Assay

For the cytotoxicity assay, PBMCs, HepG2, and THLE–3 cells were seeded at densities of 1×10^6, 5×10^5, and 2×10^5 cells/well, respectively, in a 100 µL volume. Following a 24-hour incubation at 37 °C with 5% CO_2, the cells were treated with 12.5 µL of varying extract concentrations for an additional 24 hours. After treatment, the supernatant was removed and replaced with 10 µL of MTT reagent (5 mg/mL). After a 4-hour incubation, the resulting formazan crystals were solubilized in 100 µL of DMSO. Absorbance was recorded at 570 nm using an Agilent BioTek Epoch 2NS microplate reader. Percentage cell viability was determined relative to the DMSO control, followed by IC_{50} and LD_{50} estimations to classify toxicity according to WHO standards [21–23].

2.7. Comet Assay for Genotoxicity Detection

The DNA fragmentation was assessed via the alkaline comet assay, adhering to the experimental design of Singh et al. [24]. The cells were treated at their respective IC_{50} concentrations; in cases where no IC_{50} was reached, the highest working concentration was utilized. To quantify the extent of DNA damage, 150 cells per treatment were analyzed using CASP software (version 1.2.3, CASPlab, Poland). The primary parameter measured was the Olive Tail Moment (OTM), calculated as the product of the percentage of tail DNA and the distance between the intensity centers of the head and tails [22].

2.8. Gene Expression Analysis via qRT–PCR

THLE–3 cells were seeded into 6-well plates at a density of 2×10^5 cells/mL. Following a 24-hour incubation, the cells were treated at specific concentrations for an additional 24 hours prior to RNA harvesting. Total RNA was isolated using the GF–1 RNA extraction kit (Vivantis, Malaysia), adhering to the manufacturer's instructions. RNA integrity and quantity were assessed using a Nanodrop spectrophotometer (DeNovix, USA).

For first-strand cDNA synthesis, the Viva 2-steps RT–PCR kit was employed, utilizing M-MuLV reverse transcriptase and Oligo(dT)18 primers. The resulting cDNA templates were stored at -20 °C.

Quantitative analysis of gene expression was performed on a CFX Duet Real–Time PCR system (BIO-RAD, USA). The qPCR program commenced with initial denaturation at 95 °C for 10 minutes, followed by 45 cycles of 95 °C (30 s), 60 °C (30 s), and 70 °C (60 s). A Melting curve analysis was conducted to ensured the specificity of the amplification products. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Cq}$ method, with *GAPDH* as the internal control for normalization. Table 1 lists the specific primer sequences used.

Table 1. List of genes employed in the study and their primer sequences

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Ref.
GAPDH	TGCCACCCAGAAGACTGTGG	TTCAGCTCTGGGATGACCTT	Aizawa et al. [25]
CB1	CATGCGCGCTTGGTCTGG	CGTGGGCAGCCTGTTCCTCA	Moaddel et al. [26]
ChAT	GCCAGTGGAAGAATCGTCAT	TTGTGCATGTGAGTGTGTGG	Aizawa et al. [25]

2.9. Statistical Analysis

Experimental results are presented as mean ± standard deviation (SD). To assess statistical differences, non-parametric tests were employed, the Kruskal–Walli's test was used for comparisons across multiple groups, followed by the Mann–Whitney U test for pairwise comparisons between groups. Data processing was conducted using GraphPad Prism (version 8.0.2), with a p-value < 0.05 considered statistically significant.

3. Results and Discussion

Phytochemicals are numerous and display a variety of pharmacological effects when present in sufficient concentration or quantity. These include antioxidant, anti-inflammatory, and immune-boosting properties as well as stress-reducing effects. A mong these, oleamide is a particularly significant phytochemical for humans. It has been reported in several plant species in Thailand. Interestingly, oleamide is an amide of oleic acid that is also naturally produced in the animal body; it accumulates in the cerebrospinal fluid and regulates functions such as stress relief,

memory restoration, and the induction of deep sleep and appetite. Furthermore, its potential anti-Alzheimer's properties are of clinical interest, as natural production often declines with age [1, 27–29]. Additionally, previous research has demonstrated that oleamide possesses potent anti-inflammatory effects [6–8].

The mechanism of action for oleamide involves stimulating the production of choline acetyltransferase (ChAT), the enzyme responsible for synthesizing the neurotransmitter acetylcholine. Yang et al. [29] demonstrated that oleamide induces sleep in murine models, while Mishra et al. [10] reported that Alzheimer's disease patients exhibit reduced acetylcholine levels due to a deficit in ChAT activity. Consequently, oleamide shows therapeutic potential for the prevention or management of Alzheimer's disease. While these effects have been validated through *in vitro* and *in vivo* studies using either pure oleamide or extracts from jujube seeds [28], the bioactive potential of oleamide derived from the six plant species investigated here has not yet been explored.

The present study adopts a targeted, hypothesis-driven framework focused on the bioactivity of oleamide, rather than a general phytochemical inventory. This approach is based on two core pharmacological premises: first, that exogenous oleamide from plant sources acts as a functional agonist within the endogenous cannabinoid system by binding to CB1 receptors, thereby modulating neurological functions such as sleep and stress responses; and second, that it concurrently strengthens the cholinergic system by upregulating choline acetyltransferase (ChAT) activity, which is crucial for acetylcholine synthesis and cognitive health. This dual-pathway theory provides the mechanistic rationale for investigating whether oleamide-rich extracts from novel botanical sources (including the four *Dillenia* species, pumpkin seeds, and hemp seeds) can elicit these receptor- and enzyme-mediated effects, linking their traditional uses to a plausible molecular mode of action.

3.1. Oleamide Authentication and Quantification in the Studied Extracts

GC–FID analysis of the ethanol extracts from *Dillenia obovata*, *D. ovata*, *D. pentagyna*, *D. indica*, *Cucurbita moschata*, and *Cannabis sativa* subsp. *sativa* seeds identified oleamide at a retention time (R*t*) of approximately 7.5 min (Table 2). A standard curve for oleamide was generated using five concentration levels, yielding a correlation coefficient (R^2) of 0.9999 (Figure 2). The oleamide content in each extract was quantified as 0.06, 0.07, 0.10, 0.11, 0.11, and 0.09 mg/g of dry weight, with corresponding concentrations of 0.03, 0.03, 0.03, 0.04, 0.03, and 0.03 mg/mL, respectively (Table 2).

Table 2. Details of oleamide detection in the plant extracts studied, showing the retention time, peak area, concentration and amount of the compound

Plant Samples	Filtrate volume (mL)	Retention time (min)	Peak area (pA x s)	Concentrations (mg/mL)	Amounts	
					mg/ g sample	mg/ 100 g sample
Dillenia obovata	1.9	7.62	2.04	0.03	0.06	0.551
D. ovata	2.4	7.53	2.10	0.03	0.07	0.697
D. pentagyna	3.4	7.69	4.35	0.03	0.10	1.039
D. indica	2.9	7.51	14.50	0.04	0.11	1.084
Cucurbita moschata	3.7	7.56	3.66	0.03	0.11	1.114
Cannabis sativa spp. *sativa*	3.2	7.69	1.18	0.03	0.09	0.910

In this study, six plant species were evaluated for their oleamide content using GC-FID. Among the extracts, D. indica exhibited the highest level of oleamide at 0.11 and 0.04 mg/mL. These findings align with earlier research by Siripiyasing et al. [5], who reported high relative percentages of oleamide (38.46–58.25%) in three *Dillenia* species: *D. indica*, *D. obovata*, and *D. pentagyna*. The methodological focus of this study was strictly limited to the quantification of oleamide, a known cannabinoid–like compound, to evaluate the potential cannabinoid-like effects of these species. A comprehensive phytochemical inventory was deemed unnecessary for this specific objective. Furthermore, two of the materials, pumpkin and hemp seeds, are established food sources that have been extensively characterized in previous literature [30–41]. Similarly, the four *Dillenia* species investigated here were part of a detailed GC-MS phytochemical analysis by Thooptianrat et al. [15], which identified oleamide as a major constituent across nine *Dillenia* species. Consequently, further broad-spectrum screening was unwarranted. This research thus serves as a targeted extension of existing knowledge, specifically quantifying oleamide concentrations to establish a clear link between these botanical sources and their corresponding cannabinoid-like bioactivity.

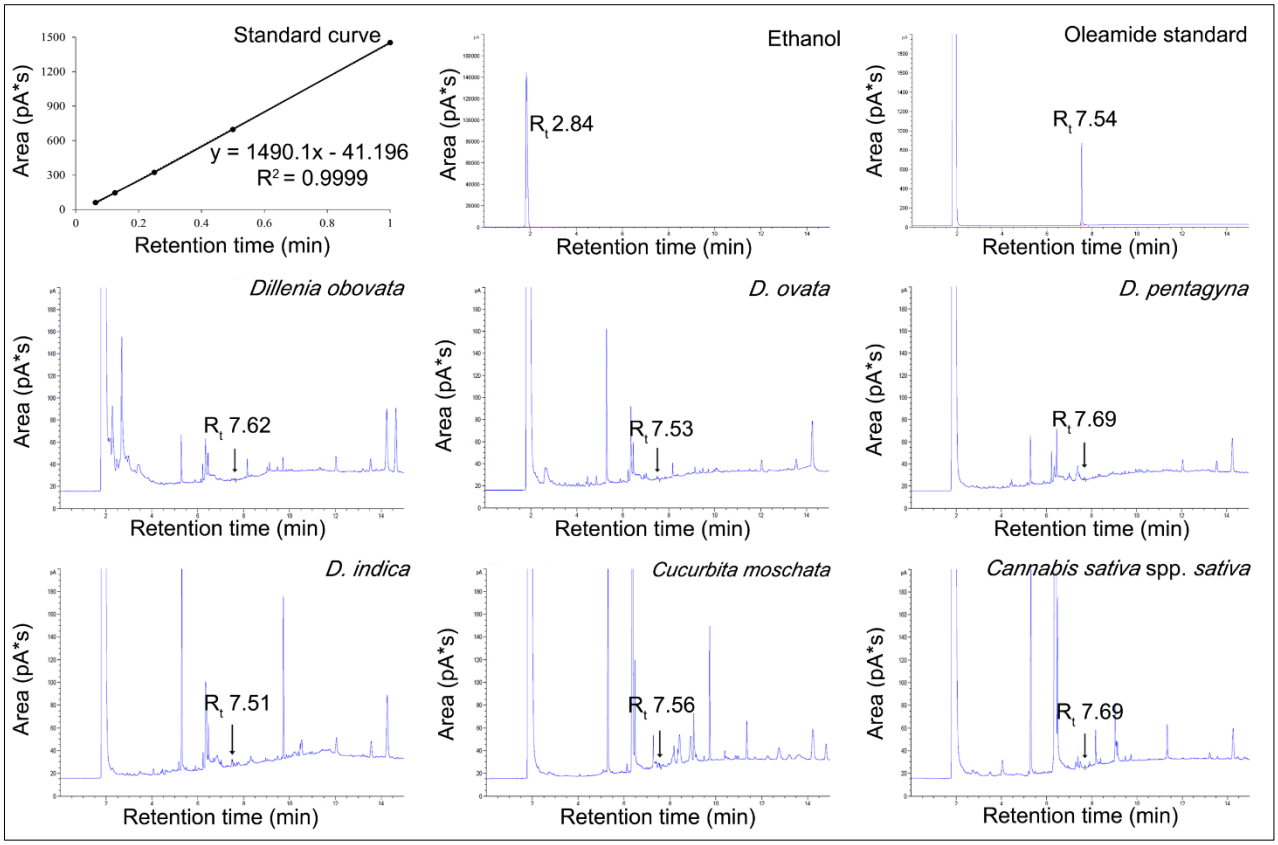

Figure 2. GC–FID Chromatograms showing standard calibration curve of oleamide and its retention time, and oleamide detection in *Dillenia obovata*, *D. ovata*, *D. indica* and *D. pentagyna* leaf extract, and *Cucurbita moschata* and *Cannabis sativa* spp. *sativa* seed extracts.

3.2. Cytotoxicity and Genotoxicity Evaluation of the Extracts Containing the Oleamide Compound

Following the preparation of working concentrations (Table 3), cytotoxicity and genotoxicity were evaluated in peripheral blood mononuclear cells (PBMCs), hepatocellular carcinoma cells (HepG2), and human normal liver cells (THLE-3). The MTT assay results indicated no cytotoxicity across all three cell lines; no IC_{50} values were reached, and the cells maintained high viability percentages (Figure 3 and Table 4).

Table 3. The initial and working concentration of the sample extracts studied

Plant Samples	The initial concentrations (mg/mL)	Working concentrations (mg/mL)				
		10x	1x	0.1x	0.01x	0.001x
Dillenia obovata	17.0	1.70	0.17	0.017	0.0017	0.00017
D. ovata	14.0	1.40	0.14	0.014	0.0014	0.00014
D. pentagyna	15.0	1.50	0.15	0.015	0.0015	0.00015
D. indica	21.0	2.10	0.21	0.021	0.0021	0.00021
Cucurbita moschata	26.3	2.63	0.263	0.0263	0.00263	0.000263
Cannabis sativa spp. *sativa*	35.0	3.50	0.35	0.035	0.0035	0.00035

Genotoxicity was further evaluated via the comet assay. Since IC_{50} values were not reached, the highest working concentrations were utilized for testing, yielding results that varied by cell type. In PBMCs and HepG2 cells, extracts from *D. obovata*, *D. ovata*, *D. pentagyna*, and *D. indica* significantly induced DNA damage compared to the negative control ($p < 0.01$), as measured by the Olive Tail Moment (OTM). In contrast, extracts from *Cucurbita moschata* and *Cannabis sativa* subsp. *sativa* did not elicit genotoxic effects in these lines. Regarding the normal liver cells (THLE-3), no DNA toxicity was observed for *D. obovata*, *D. ovata*, *D. indica*, *Cucurbita moschata* and *Cannabis sativa* spp. *sativa* seeds. However, the *D. pentagyna* extract did significantly increase OTM levels compared to the negative control (Figure 4 and Table 5), indicating DNA fragmentation.

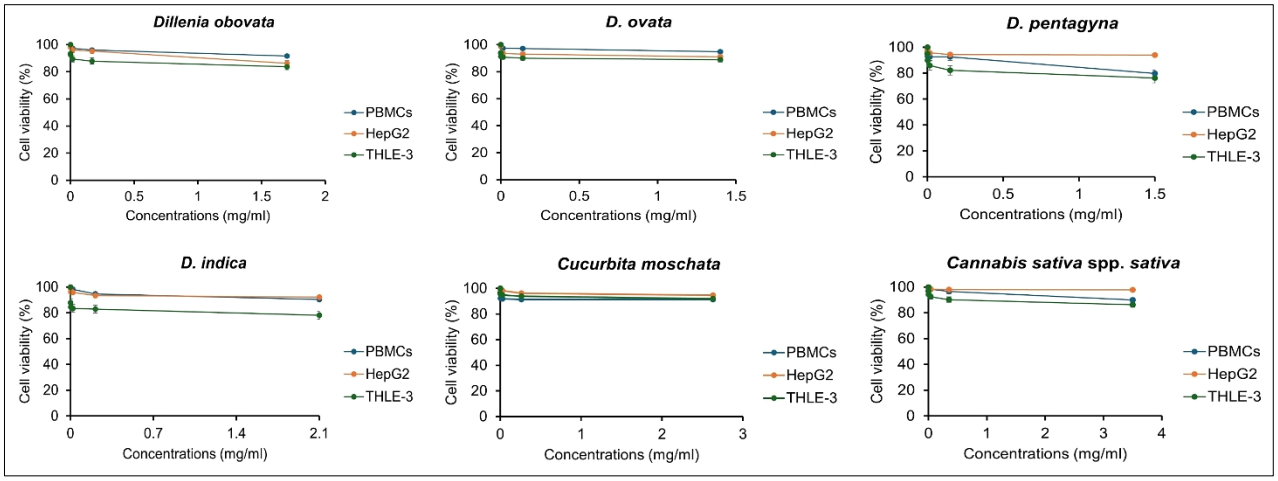

Figure 3. Cytotoxicity evaluation of the extracts on PBMCs, HepG2 and THLE–3 cells with no IC_{50} value using the MTT assay

Table 4. The sample plants showed the results from cytotoxicity testing as MTT assay in PBMCs, HepG2 and THLE–3 cells which did not get toxicity, indicating no IC_{50} value

Plant Samples	% Cell viability (Mean ± S.D.)			IC_{50} (mg/mL)
	PBMCs	HepG2	THLE–3 cell	
Dillenia obovata	99.91±0.05–91.53±0.04	98.54±0.34–86.16±0.17	93.28±0.13–83.68±0.16	–
D. ovata	99.55±0.04–94.85±0.04	99.08±0.24–91.03±0.23	93.81±0.16–88.85±0.14	–
D. pentagyna	94.78±0.04–79.69±0.02	97.43±0.17–93.95±0.18	94.98±0.10–76.12±0.12	–
D. indica	99.49±0.05–90.26±0.05	98.88±0.17–92.13±0.15	87.85±0.11–78.05±0.13	–
Cucurbita moschata	96.77±0.04–91.29±0.05	99.34±0.24–94.51±0.21	96.09±0.13–91.84±0.12	–
Cannabis sativa spp. *sativa*	99.65±0.03–89.97±0.02	99.62±0.14–97.87±0.15	96.80±0.14–86.27±0.15	–

Although no IC_{50} values were reached at the cellular level, DNA-level analysis revealed that extracts from *D. obovata*, *D. ovata*, *D. pentagyna*, and *D. indica* induced significant DNA damage ($p < 0.01$) in PBMCs and HepG2 cells; furthermore, the *D. pentagyna* extract also exhibited genotoxicity in THLE-3 cells.

The observed absence of cytotoxicity concurrent with the induction of DNA damage in certain extracts is a plausible toxicological outcome. In the absence of determinable IC_{50} values, the highest concentrations utilized in the MTT assay were employed for the comet assay following the standard protocol to ensure a robust genotoxicity assessment. This approach is consistent with the subsequent quantitative toxicological evaluations used to predict the LD_{50}. This parameter, determined in alignment with World Health Organization (WHO) guidelines using rat models [23], serves to classify substances according to their hazard potential and determines whether a compound poses a realistic risk at human exposure levels. Therefore, the LD_{50} evaluation provides the necessary toxicological context to interpret *in vitro* genotoxicity data regarding safe human consumption. Therefore, the highest working concentrations of these four plant extracts (1.70, 1.40, 1.50, and 2.10 mg/mL) were used to calculate the predicted LD_{50} values, which are the amounts of these plants that would cause 50% mortality in a rat population after oral consumption, according to the WHO, 2009 class of hazard. The results predicted an LD_{50} of 1681.62, 1564.44, 1605.11, and 1819.14 mg/kg of rat body weight, respectively, as shown in Table 5.

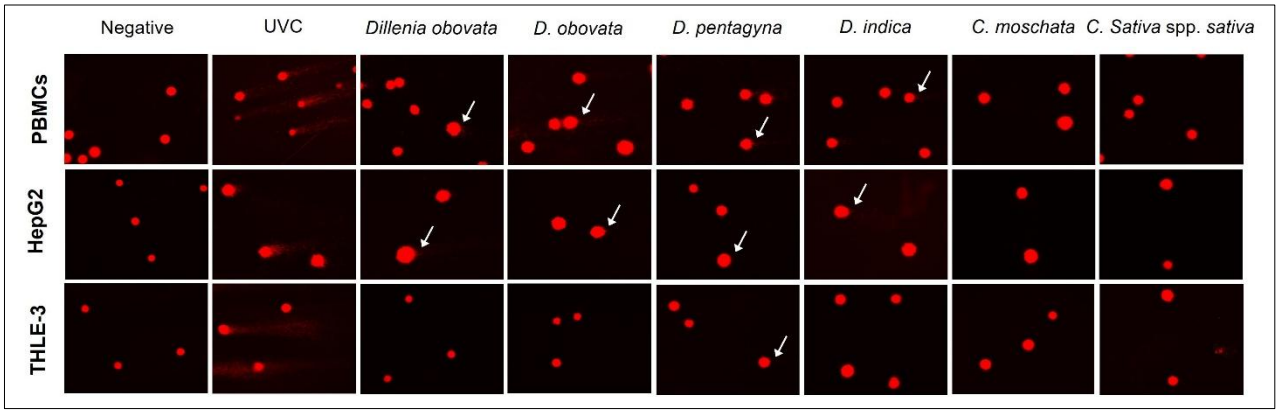

Figure 4. Genotoxicity testing via comet assay at 200x magnification revealed significant DNA damage (indicated by white arrows) compared to the negative control, as indicated by an olive tail moment (OTM) (p<0.05), after treatment with some extracts.

Table 5. The sample plants showed the results from genotoxicity testing as the comet assay, which induced significant (p<0.01) DNA damage in PBMCs, HepG2, and THLE–3 cells by four *Dillenia* species, but did not induce DNA damage by *Cucurbita moschata* and *Cannabis sativa* spp. *Sativa.*

Plant Samples	Working Conc. (mg/mL)	PBMCs		HepG2		THLE–3 cell		LD_{50} (mg/kg rat)
		OTM (mean ± S.D.)	p–values	OTM (Mean ± S.D.)	p–values	OTM (Mean ± S.D.)	p–values	
Negative control	–	0.15±0.03	–	0.04±0.09	–	0.05±0.15	–	–
D. obovata	1.70	3.13±0.70	<0.01	0.15±0.20	<0.01	0.03±0.28	0.1112	1,681.62[a,b]
D. ovata	1.40	4.88±0.71	<0.01	0.18±0.31	<0.01	0.04±0.20	0.9655	1,564.44[a,b]
D. pentagyna	1.50	3.21±0.74	<0.01	0.12±0.56	<0.01	0.12±0.21	< 0.01	1,605.11[a,b,c]
D. indica	2.10	1.17±0.31	<0.01	0.14±0.13	<0.01	0.11±0.14	0.0112	1,819.14[a,b]
C. moschata	2.63	0.15±0.15	0.4892	0.05±0.14	0.1509	0.05±0.20	0.7691	–
C. sativa spp. *sativa*	3.50	0.15±0.06	0.8590	0.05±0.12	0.4028	0.08±0.13	0.1257	–

Note; a = PBMCs cell, b = HepG2 cell, c = THLE-3 cell, OTM = Olive Tail Moment.

For the cellular toxicity study using the MTT assay, no IC_{50} values were found for any of the six plant extracts on any of the tested cells. However, extracts from four species, *D. obovata*, *D. ovata*, *D. pentagyna*, and *D. indica*, were found to cause DNA damage to PBMCs and HepG2 cells. The *D. pentagyna* extract also caused statistically significant DNA damage to THLE–3 cells ($p < 0.01$) compared to the negative control. An important finding was that extracts from pumpkin seeds, *Cucurbita moschata* and hemp seeds (*Cannabis sativa* spp. *sativa*) showed no toxicity to either the cells or DNA in any of the tested cell lines. Since the amount and concentration of oleamide did not vary significantly among the six plant extracts, all of them can likely be beneficial for human health. The highest concentrations used in the genotoxicity tests, 1.70, 1.40, 1.50, and 2.10 mg/mL, were used to calculate the LD_{50} (the concentration that would be lethal to 50% of test mice) according to the WHO's 2009 evaluation criteria [23]. The calculated LD_{50} values were 1682.62, 1564.44, 1605.11, and 1819.14 mg/kg of the rat's body weight, respectively. This classifies the compound as WHO Class II, indicating they are moderately hazardous toxic chemicals when orally consumed at doses of 50–2,000 mg/kg of body weight. As an example of toxicity, a person weighing 50 kg would likely have to consume a dose of 2,500–100,000 mg (2.5–100 g dry weight) to reach a toxic level. This is a very high amount that is unlikely to be consumed. Therefore, it can be concluded that there are no adverse effects on humans with normal consumption. This suggests that all these plants can be used beneficially in humans, especially the pumpkin seed and hemp seed extracts. However, the question of whether these six plants with oleamide can be effective in the human body needs to be addressed. To answer this, we must go back to the meaning and processing of oleamide formation.

The observed absence of cytotoxicity concurrent with the induction of DNA damage in certain extracts is a plausible toxicological outcome. This is because, in the absence of a determinable IC_{50} value, the highest available test concentration was employed in the comet assay for genotoxicity assessment. The relevance of this condition should not be a concern, as these same high concentrations were subsequently used in the quantitative toxicological evaluation to predict the LD_{50}. This parameter, determined in alignment with World Health Organization (WHO) guidelines, serves to classify substances according to their potential hazard and informs the assessment of whether a substance poses a realistic risk at plausible human exposure levels. The LD_{50} evaluation thus provides the critical toxicological context for interpreting the in vitro genotoxicity data regarding safe human consumption.

3.3. The Bioactivity of Plant Extracts Containing Oleamide Compound on *CB1* and *ChAT* Gene Expressions

Based on the study of the effects of plant extracts on stimulating the expression of the *CB1* and *ChAT* enzyme genes, it was found that *D. obovata*, *D. ovata*, *D. pentagyna*, *D. indica*, *Cucurbita moschata*, and *Cannabis sativa* spp. *sativa* were able to significantly stimulate the expression of both the *CB1* and *ChAT* genes in normal liver cells of the THLE–3 type (Figure 5A and 5B). The percentage of stimulation was much higher than that of the negative control for all plant extracts, as shown in Table 6.

Oleamide is an endogenous fatty acid amide in the body that accumulates in the cerebrospinal fluid during sleep deprivation and induces sleep in animals. It acts as a full agonist of the CB1 cannabinoid receptor. While CB1 receptors are most abundant in the brain, they are also present in other tissues, including the liver [17, 42, 43]. Activation of these receptors can lead to changes in gene expression. Therefore, THLE–3 cell line was selected to prove that CB1 acts as a receptor for natural oleamide-containing plant extracts, along with ChAT, ChAT is an enzyme that catalyzes the conversion of choline to the neurotransmitter acetylcholine, which is the primary mediator through the non-neuronal cholinergic system to relieve stress, restore memory, induce deep sleep, and increase appetite in the elderly (Figure 5C).

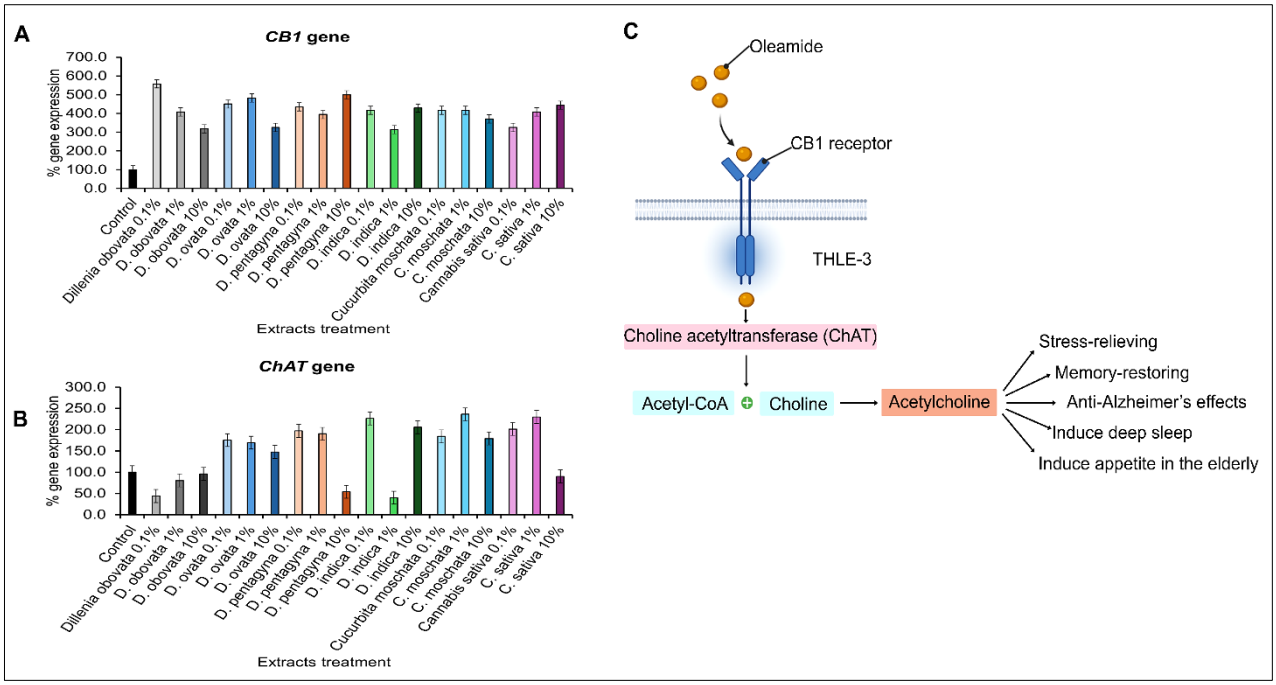

Figure 5. The effect of plant extracts containing oleamide on the expression of (A) *CB1* and (B) *ChAT* genes, measured by the level of mRNA using qRT–PCR analysis, (C) the cascade mechanism of oleamide on acetylcholine production, regulated by the CB1 receptor and ChAT

Table 6. The relative expression of the target genes, *CB1* and *ChAT,* was stimulated by six plant extracts containing the oleamide compound. Expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method, normalized with GAPDH as the reference gene, and compared to a control group (untreated cells).

Plant sample extract	Concentration (mg/mL)	THLE–3		
		Cq value of *GAPDH*	Gene expression value (%)	
			CB1	*ChAT*
Negative control	-	20.89	100	100
Dillenia obovata	0.017	21.51	557.8	43.8
	0.17	20.98	408.4	80.7
	1.70	20.62	318.2	96.0
D. ovata	0.014	21.01	450.0	175.4
	0.14	20.73	482.3	169.4
	1.40	21.28	324.9	147.5
D. pentagyna	0.015	20.77	434.7	197.3
	0.15	21.67	394.5	190.6
	1.50	21.04	499.3	54.0
D. indica	0.021	21.04	416.9	226.6
	0.21	21.27	313.8	40.1
	2.10	21.25	428.7	205.7
Cucurbita moschata	0.0263	20.97	416.9	184.1
	0.263	20.82	416.9	236.3
	2.63	20.92	370.6	179.1
Cannabis sativa spp. *sativa*	0.035	22.69	324.9	201.4
	0.35	20.81	408.4	229.8
	3.50	21.07	443.8	90.1

The results confirm that all the plant extracts induced a very high percentage of *CB1* gene expression, ranging from 313.8% to 557.8%. They also increased the expression of the ChAT enzyme in healthy liver cells (THLE–3) for synthesis of the neurotransmitter acetylcholine, with gene expression ranging from 43.8% to 236.3% compared to the control. Therefore, it can be concluded that the extracts from all six plants, the leaves of *D. obovata*, *D. ovata*, *D. pentagyna* and *D. indica*, as well as the seeds of *Cucurbita moschata* (pumpkin) and *Cannabis sativa* spp. *sativa* (hemp) are capable of exhibiting the effects of oleamide. This indicates that these plant extracts can be developed into medicines or various health products beneficial to humans.

4. Conclusion

This study successfully identified and quantified the endogenous fatty acid amide oleamide in six plant species using GC-FID analysis, with *Dillenia indica* leaf extract containing the highest concentration (0.11 mg/g). A comprehensive toxicological profile revealed a critical distinction: while none of the extracts showed cytotoxicity, the four *Dillenia* species induced significant genotoxic DNA damage in peripheral blood mononuclear cells and hepatoma cells, with *D. pentagyna* also affecting normal liver cells. In contrast, pumpkin and hemp seed extracts demonstrated no genotoxicity, establishing a superior safety profile. Predicted oral LD_{50} values classified the *Dillenia* extracts as moderately hazardous (WHO Class II), though the doses required for human toxicity remain far above plausible dietary intake.

Most significantly, the research provides novel functional validation by demonstrating that all six plant extracts potently stimulate the gene expression of two key neuromodulatory targets in normal human liver cells. The extracts upregulated the cannabinoid receptor CB1, with expression increases ranging from 314% to 558%, confirming activation of the endocannabinoid pathway linked to sleep and neuroprotection. Concurrently, they enhanced the expression of the enzyme choline acetyltransferase (ChAT) by 44% to 236%, indicating a capacity to boost acetylcholine synthesis relevant to memory and cognitive function. This dual-pathway activation offers a clear mechanistic rationale for the purported benefits of these plants. Consequently, this work transforms these botanicals from ordinary species into validated sources of a specific bioactive compound. The non-genotoxic pumpkin and hemp seed extracts, in particular, emerge as highly promising candidates for development into safe nutraceuticals or functional foods aimed at supporting neurological health, sleep quality, and cognitive resilience, representing a successful translation of phytochemical discovery into potential therapeutic applications.

5. Declarations

5.1. Author Contributions

Conceptualization, A.C., R.S., and S.D.; methodology, S.K.; software, S.Y.L.; validation, A.C., R.S., and S.D.; formal analysis, N.S.; investigation, N.S.; resources, T.T.; data curation, A.C.; writing—original draft preparation, N.S. and A.C.; writing—review and editing, S.K.; visualization, N.S., S.K., and A.C.; supervision, A.C.; project administration, R.S. and S.D.; funding acquisition, A.C. All authors have read and agreed to the published version of the manuscript.

5.2. Data Availability Statement

The data presented in this study are available on request from the corresponding author.

5.3. Funding

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5.5. Institutional Review Board Statement

Not applicable.

5.6. Informed Consent Statement

Not applicable.

5.7. Declaration of Competing Interest

The authors declare that there are no conflicts of interest concerning the publication of this manuscript. Furthermore, all ethical considerations, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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