J Herbmed Pharmacol. 2022; 11(3): 339-347.

http://www.herbmedpharmacol.com doi: 10.34172/jhp.2022.39

Journal of Herbmed Pharmacology

Screening of phytochemicals, toxicities, and activities of three *Dillenia* species

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 11 October 2021
Accepted: 2 April 2022

Keywords:
β-Sitosterol
Comet assay
Oleamide
Toxicity

ABSTRACT

Introduction: Plants containing β-sitosterol and oleamide are important for various diseases. So, *Dillenia indica*, *D. obovata*, and *D. pentagyna* were investigated for phytochemicals, cytotoxicity and genotoxicity levels on peripheral blood mononuclear cells (PBMCs) and Hela cells. The protective effect of *D. pentagyna* extract on a HepG2 cell line was also investigated.
Methods: Gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) were used for phytochemical analysis. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction (MTT) and comet assays were performed for toxicity testing and protective effects against DNA oxidative damage.
Results: The major components were oleamide and β-sitosterol at 38.464-58.247% and 5.585-6.887% with concentration and quantity of β-sitosterol at 0.2-0.37 mg/mL and 0.42-0.964 mg/g leaf. The *D. indica*, *D. obovata*, and *D. pentagyna* toxicities on PBMCs showed IC_{50} values at >430, >430, and 350 µg/mL respectively, with no significant DNA damage ($P > 0.05$) compared to the negative control group. All plant extracts showed toxic activity on Hela cell with IC_{50} values at <0.43 µg/mL and induced significant DNA damage ($P < 0.05$) compared to the negative control group. Conversely, the activity of the *D. pentagyna* extract indicated low cytotoxic activity against HepG2 (IC_{50}>430 µg/mL), no significant ($P > 0.05$) DNA damage induction, significantly ($P < 0.05$) decreased DNA damage level, and tremendous antioxidant effect. Additionally, a combined mixture of all plants in an equal proportion revealed no IC_{50} value and insignificant DNA damage.
Conclusion: All the studied species contained oleamide and β-sitosterol, with toxicity on Hela cells without toxicity on PBMC. The *D. pentagyna* species showed high antioxidant effects and no toxicity on HepG2.

Implication for health policy/practice/research/medical education:
This research emphasizes phytochemicals concentrated on oleamide and β-sitosterol, toxicities, and activities of *D. indica, D. obovata*, and *D. pentagyna* species. This information can support further uses in human health.
Please cite this paper as: Siripiyasing P, Silawong K, Thooptianrat T, Sudmoon R, Babayan N, Khondkaryan L, et al. Screening of phytochemicals, toxicities, and activities of three *Dillenia* species. J Herbmed Pharmacol. 2022;11(3):339-347. doi: 10.34172/jhp.2022.39.

Introduction

Plants, plant-derived extracts, and phytochemicals isolated from plants have long been used worldwide in the treatments of many diseases. For example, the alkaloid morphine, a strong painkiller, is used to treat severe pain after an operation, serious injury, and pain from cancer and heart attacks; the anti-tussive agent codeine; and the anti-spasmodic alkaloid papaverine isolated from poppy (*Papaver somniferum*) (1). Cannabidiol (CBD), tetrahydrocannabinol (THC), and

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other phytocannabinoids and non-phytocannabinoid chemicals, such as terpenes and flavonoids have myriad pharmacological properties (2-5). Hence, phytochemicals are natural resources, which lead to many beneficial uses with more sophisticated and modern preparation methods. Plants are important sources of novel pharmacologically active compounds, with 50% of the approved drugs being derived directly or indirectly from plants. Despite the current preoccupation with synthetic chemistry as a vehicle to discover and manufacture drugs, the contribution of plants to disease treatment and prevention is still enormous (6). Also, the two substances β-sitosterol and oleamide are important in human body function. Oleamide is a protective agent against scopolamine-induced memory loss and has been suggested to be useful as a preventive agent against Alzheimer's disease, insomnia, loss of appetite, inflammation, prevention and treatment of atherosclerosis, thrombosis, arthritis, and cancer (7-11). β-sitosterol is as important to health as oleamide; it possesses various biological actions such as anxiolytic and sedative effects, analgesic, immunomodulatory, antimicrobial, anticancer, anti-inflammatory, lipid-lowering effect, hepatoprotective, protective effect against non-alcoholic fatty liver disease and respiratory diseases, wound healing effect, antioxidant and anti-diabetic activities (12).

There are still many important plants and substances that have not yet been studied and mentioned. The more plant species we know and study, the more benefits we find for humans. Given this, the two substances β-sitosterol and oleamide in *D. indica*, *D. obovata* and *D. pentagyna* species were examined; the cytotoxicity and genotoxicity, as well as DNA protective activity, were also evaluated, aimed at plant compounds and usage.

Materials and Methods

Plant materials

The mature leaves of three *Dillenia* species, *D. indica*, *D. obovata* and *D. pentagyna* were collected. The plants were identified by a proficient botanist, prof. Dr. Aunrat Chaveerach, the specimens were kept at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand, collector no. A. Chaveerach 930, 931, and 934. Then the leaves were further processed following the below steps.

Methods

Preparation of chemical extracts

To prepare the extracts, the leaf samples were rinsed with water and air-dried. A 20 g of sample was then ground into a powder, mixed with 125 mL of hexane (analytical grade) and filtered through a filter paper at room temperature. Next, 80 mL of the filtrate was obtained and stored at -20°C until use. The highest extract concentration was obtained at 4300 µg/mL. This concentration was used as a stock and 10-fold serial dilutions were performed to prepare a total of four testing concentrations, 430, 43, 4.3, and 0.43 µg/mL for cytotoxicity and genotoxicity assays.

The three study plants were combined in an equal proportion as a combined mixture, which was then extracted to obtain a concentration of 4,300 µg/mL. Then the four testing concentrations, 430, 43, 4.3, and 0.43 µg/mL were made and used for cytotoxicity and genotoxicity assays.

Gas chromatography-mass spectrometry (GC-MS) analysis and component identification

The GC-MS analysis of the crude extracts was performed using an Agilent Technologies GC 6890 N/5973 inert mass spectrometer fused with a capillary column (30.0 m × 250 µm × 0.25 µm). Helium gas was used as the carrier at a constant flow rate of 1 mL/min. The injection and mass-transferred line temperature was set at 280°C. The oven temperature was programmed for 70°C to 120°C at 3°C/min, then held isothermally for 2 minutes, and raised to 270°C at 5°C/min. A 1 µL aliquot of the crude extract was injected in split mode. The relative percentage of the crude constituents was expressed as a percentage using peak area normalization. Component identification was determined by comparing the obtained mass spectra with the reference compounds in the Wiley 7N.1 library.

Cell culture

In this study human peripheral blood mononuclear cells (PBMCs), human cervical carcinoma (HeLa) and human hepatoma (HepG2) cell lines were used. PBMCs were obtained from 24 mL blood sample collected from healthy human from the Central Blood Bank at Srinagarind hospital, (Khon Kaen University) into heparinized blood collection tubes using sterile techniques. PBMCs were isolated using Ficoll-Paque density gradient technique (Ficoll-Paque Plus, GE Healthcare). The cells were collected by centrifugation and resuspended in phosphate-buffered saline (PBS). The cells were cultured in growth medium RPMI (Sigma Aldrich, Germany) supplemented with 10% fetal bovine serum (HyClone, UK), 2 mM L-glutamine (Sigma Aldrich, Germany), 100 IU/mL penicillin (Sigma Aldrich, Germany), and 100 µg/mL streptomycin (Sigma Aldrich, Germany) at 37°C and 5% CO_2. The human HeLa and HepG2 cell lines were routinely maintained in the growth medium DMEM (Sigma Aldrich, Germany) supplemented with 10% fetal bovine serum (HyClone, UK), 2 mM L-glutamine (Sigma Aldrich, Germany), 100 IU/mL penicillin (Sigma Aldrich, Germany), and 100 µg/mL streptomycin (Sigma Aldrich, Germany) at 37°C and 5% CO_2.

Estimation of cytotoxicity

The cytotoxicity of plant extracts in PBMCs was assessed using trypan blue exclusion test (13). The cell suspension

was prepared at a concentration of viable cells at 4-6 × 10^5 cells/mL. The cell suspension was divided into 1.5 mL microtubes (500 µL/tube) and incubated with the plant extract (50 µL/tube) at 37°C for 24 hours. The cells were treated with different concentrations of the plant extract diluted with dimethyl sulfoxide (DMSO): 4,300, 430, 43, and 4.3 µg/mL. The untreated cells (negative control) were incubated in a culture medium only. The DMSO treated samples were used as vehicle control. Cells incubated with 100 µM of H_2O_2 for 15 minutes were used as a positive control. The cytotoxicity of plant extracts in HeLa and HepG2 cell lines was analyzed using MTT colorimetric cell viability assay (14). The cells were seeded at the density of 0.2-0.25 × 10^6 cell/mL into 96-well plates (Greiner, Germany) and incubated for 24 hours at 37°C and 5% CO_2. Afterwards, plant extracts were added to the cell cultures at concentrations ranging from 4.3 µg/mL to 430 µg/mL. Untreated cells were used as a negative control, while DMSO treated cells served as solvent control. After further incubation for 24 hours, the MTT assay was performed. The absorbance was measured using an ELISA plate reader (Human Reader HS, Germany) at a wavelength of 570 nm. Cell viability was expressed as a percentage of the negative control. Doses inducing 50% inhibition of cell viability (the IC_{50} value) were calculated using GraphPad Prism 5.01 (GraphPad Software, USA). Each experiment was performed in triplicate.

Genotoxicity assay

The alkaline single-cell gel electrophoresis (comet assay) was performed to evaluate DNA damages induced by plant extracts according to the method described by (15) with slight modifications. Briefly, after incubation with the extracts as described above, cell pellets were obtained by centrifugation and then resuspended in PBS. A 100 µL of 0.5% low melting point agarose (LMA) was mixed with40 µL of the cell suspension. The mixture was dropped onto slides that were precoated with 1% normal melting point agarose. Next, cover slips (22 mm × 50 mm) were placed on top of the slides and stored at 4°C. After 5-10 minutes, the cover slips were removed, and slides were submerged in a lysis solution (8 M NaCl, 0.6 M EDTA pH 8, 0.2 M Tris, 0.1% Triton X-100) for at least 1 hour. Slides were then soaked in electrophoresis buffer (6 mM EDTA pH 10, 0.75 M NaOH) for 40 minutes. Next, electrophoresis was performed for 25 minutes at 26 volts and 300 milliamps at 4°C. After electrophoresis, the slides were immediately neutralized with 0.4 M Tris buffer (pH 7.5) for 5 minutes. The neutralization process was repeated three times for 5 minutes each. The slides were then stained with 1 µg/mL ethidium bromide (60 µL per slide) overnight at 4°C in the dark. Comets were observed at 360x magnification with a fluorescence microscope Zeiss III RS (Germany) equipped with 560 nm excitation filter, 590 nm barrier filter and a CCD video camera PCO (Germany). At least 150 cells (50 cells for each of triplicate slides) were examined for each experimental point. Image analysis software Komet 4 (Perceptive instruments, UK) was used to analyze the content of DNA in the tail (by the relative tail fluorescence intensity in percent to the untreated control) and olive tail moment (OTM), representing the product of a tail length and the percentage of DNA content in the tail of comets.

Protective effect of plant extracts towards DNA oxidative damage in HepG2 cell line

Antioxidant activity of non-toxic plant extract, *D. pentagyna* against hydrogen peroxide-induced DNA damage was evaluated using comet assay in HepG2 cell line. The plant extract at the concentration of 430 µg/mL was added to the cultures of HepG2 cell line 24 hours after seeding. Experiments were performed in two experimental protocols: (I) co-treatment of cells with hydrogen peroxide and plant extract, which tested the ability of the plant extracts to directly scavenge ROS and (II) 24 hours pre-treatment with plant extract followed by co-treatment (pre+co-treatment) which, in addition to direct scavenging activity, allowed accumulation of plant extracts in the cell and induction of enzymatic and non-enzymatic cellular antioxidants and detoxifying enzymes. The untreated cells were used as a negative control (NC), cell cultures treated only with H_2O_2 (50 µM of H_2O_2 for 5 minutes) were used as a positive control (PC). The comet assay was performed as described above.

Statistical analysis

Data analysis was performed with GraphPad Prism 5.01 (GraphPad Software, USA). All experiments were repeated at least three times. At least triplicate cultures were scored for an experimental point. All values were expressed as means ± SE. Data were analyzed by non-parametric Mann-Whitney U test. $P < 0.05$ was considered as the statistically significant value.

Results

The phytochemical study on three *Dillenia* species, *D. indica*, *D. obovata,* and *D. pentagyna* by GC-MS indicated that oleamide was found as the most abundant constituent with 38-58% from the hexane leaf extracts. Additionally, β-sitosterol was also found at 6.887%, 5.679%, and 5.585%, respectively. The GC-MS chromatogram is shown in Figure 1, and the phytochemical contents are presented in Table 1. The HPLC analysis following chromatograms (Figure 2) reported that they contained β-sitosterol concentrations of 0.21 mg/mL, 0.25 mg/mL and 0.37 mg/mL, and quantities of 0.42 mg/g, 0.55 mg/g, and 0.964 mg/g dried leaf material, respectively (Table 2).

The results of dose dependent effect of plant extracts on the viability of PBMC, HeLa and HepG2 cells are shown in Figure 3, and the calculated IC_{50} values are presented in Table 3. The three *Dillenia* extracts showed slight or

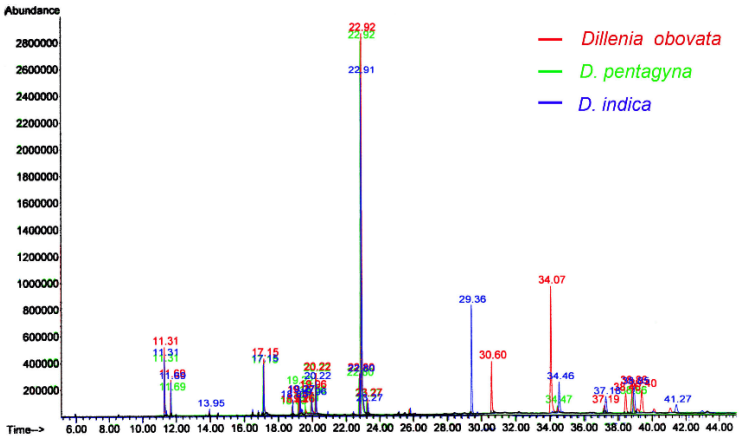

Figure 1. Chromatograms of hexane crude extracts from the leaves of *Dillenia indica*, *D. obovate*, and *D. pentagyna*.

no cytotoxic effect on PBMCs. In the case of *D. indica* and *D. obovata* extracts treatments, the cell viability at the highest tested concentration of 430 µg/mL was 77-79% demonstrating a non-cytotoxic profile for PBMC cells. For the *D. pentagyna* extract, the cell viability at the treatment concentration of 430 µg/mL was 48.10±7.02% with an estimated IC_{50} value of 350 µg/mL. The PBMCs viability incubated with 100 µM H_2O_2 (positive control) was 39.60 ± 7.09%.

For the genotoxicity assessment in terms of induction of DNA strand breaks in PBMCs, the extract concentrations of 430 µg/mL, 430 µg/mL, and 350 µg/mL were tested for *D. indica*, *D. obovata* and *D. pentagyna*, respectively, using comet assay (Figure 4). The three *Dillenia* extracts were shown to be non-genotoxic since the level of DNA damage was statistically insignificant compared to the negative control (Table 4). The dose-dependent cytotoxic effects of three species on HeLa and HepG2 cells are revealed in Figure 3a and 3b.

However, HeLa cells demonstrated higher sensitivity

Table 1. Percentage of chemical constituents in relative content by GC-MS of the three *Dillenia* species

Common name	Chemical formula	Relative content (%)		
		D. indica	*D. obovata*	*D. pentagyna*
Oleamide	$C_{18}H_{35}NO$	42.21	38.46	58.25
Squalene	$C_{30}H_{50}$	11.29	-	-
β-Sitosterol	$C_{29}H_{50}O$	6.89	5.68	5.59
BHT quinone methide	$C_{15}H_{22}O$	5.43	4.50	-
Vitamin E	$C_{29}H_{50}O2$	5.30	-	-
Palmitic acid or myristic acid or tridecanoic acid	$C_{16}H_{32}O_2$	5.00	4.11	5.55
Stigmasterol	$C_{29}H_{48}O$	3.90	1.38	-
Palmitamide	$C_{16}H_{33}NO$	3.21	3.27	5.00
Cycloartenol	$C_{30}H_{50}O$	2.34	-	-
n-Hentriacontane	$C_{19}H_{40}$	0.49	17.13	-
1,5-Dimethyl-6-(1,5-dimethylhexyl)	$C_{28}H_{46}O_2$	-	5.68	-
Taraxerol	$C_{30}H_{50}O$	-	5.03	-
D-Friedoolean-14-en-3-one	$C_{30}H_{48}O$	-	3.65	-
n-Heptadecane	$C_{17}H_{36}$	-	3.39	-
Stearic acid	$C_{18}H_{36}O_2$	-	2.31	2.80
Butylated hydroxytoluene (BHT)	$C_{15}H_{24}O$	-	1.81	-
Phytol	$C_{20}H_{40}O$	-	1.24	3.37
2,6-Di-tert-butylbenzoquinone	$C_{15}H_{22}O$	-	-	5.73
Linolenic alcohol or α-linolenic acid	$C_{18}H_{32}O$	-	-	2.02
2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	-	-	1.87
Unknown		13.95	2.35	9.82

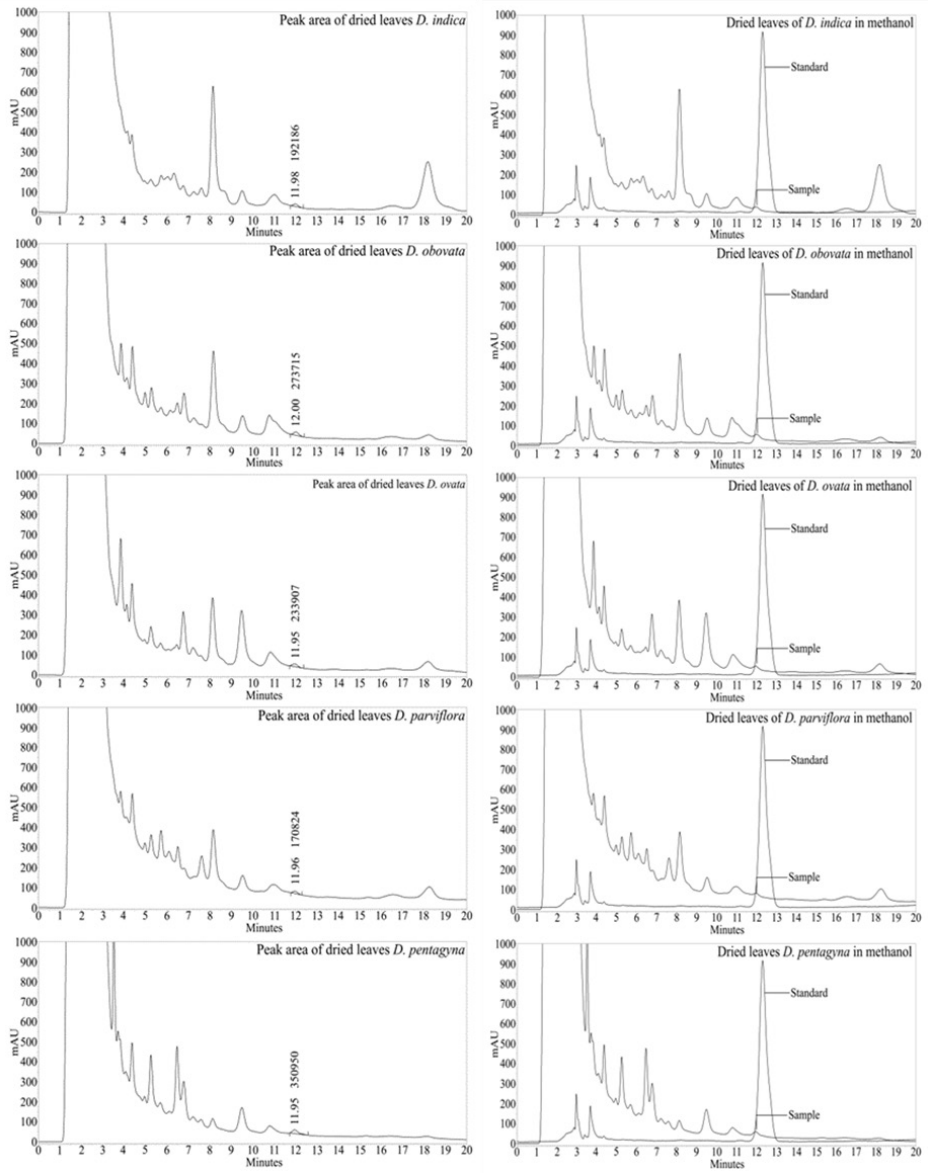

Figure 2. Chromatograms showing peaks and peak areas of β-sitosterol in *Dillenia indica, D. obovata,* and *D. pentagyna.*

compared to HepG2 cells, since at the lowest tested concentration (0.43 µg/mL) the viability of HeLa cells was estimated to be 23.7 ± 2.3%, 34.2 ± 1.8%, and 42.5 ± 3.4% for *D. pentagyna, D. indica,* and *D. obovata,* respectively. For the positive control, 100 µM H_2O_2, the HeLa cells' viability was 2.68 ± 1.15%. The final concentration used to analyze the induction of DNA damages in HeLa cells after treatment with *D. indica*, *D. obovata,* and *D. pentagyna* extracts, was 0.43 µg/mL. The statistically significant increase in the level of DNA damage was observed with *D. indica* and *D. obovata* extracts treatment; however, it was notably lower compared to the positive control (Table 4). The extract from *D. pentagyna* was shown to be non-genotoxic against HeLa cells. As mentioned, the dose-dependent cytotoxic effect was observed in case of HepG2 cells after treatment with plant extracts (Figure 3b). The IC_{50} values were possible to estimate for *D. indica* and *D. obovata* species, whereas *D. pentagyna* was shown to be non-cytotoxic at the highest tested concentration (430 µg/mL) (Table 3). The slight genotoxic potential in HepG2 cells was revealed for the *D. obovata* extracts, whereas *D. indica* and *D. pentagyna* demonstrated non-genotoxic potential (Table 4, Figure 4).

Table 2. Concentration and amount of β-sitosterol measured by HPLC in the three methanol *Dillenia* species extracts

Plant	Concentration (mg/mL)	Amount (mg/g sample)
Dillenia indica	0.21	0.42
D. obvata	0.29	0.59
D. pentagyna	0.37	0.96

The PBMC cells treated with a combined mixture

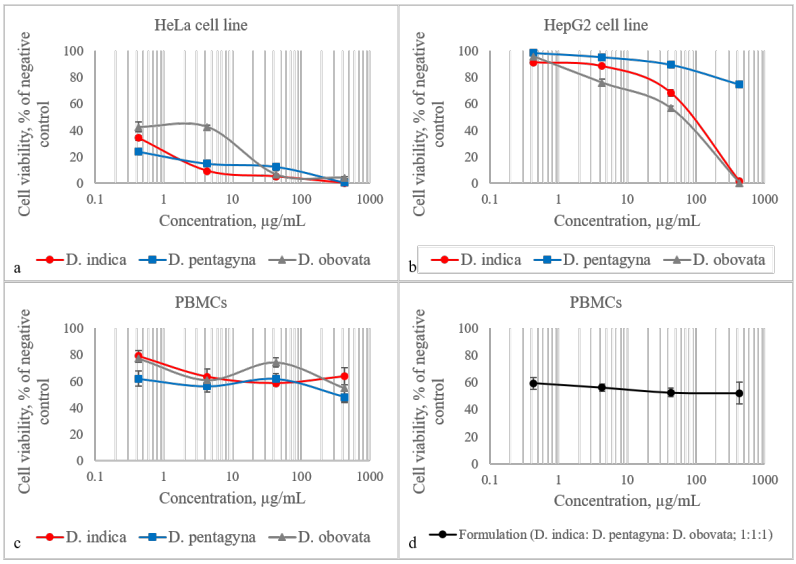

Figure 3. Cytotoxic potential of hexane leaf extracts of *Dillenia indica, D. pentagyna,* and *D. obovata* species in HeLa (a), HepG2 (b), and PBMC (c). The cytotoxicity of formulation in PBMCs (d).

showed no sign of dose-dependent decrease of cell viability (Figure 3d). The cell viability percentages were 52.19 ± 14.01% (430 µg/mL), 52.59 ± 5.57% (43 µg/mL), 56.18 ± 4.36% (4.3 µg/mL), and 59.36 ± 7.37% (0.43 µg/mL). The cell viability did not drastically differ between low and high concentrations. Because of no IC_{50} value, the highest concentration (430 µg/mL) of the combined mixture was selected for the comet assay. PBMCs did not show significant sign of DNA damage, the Olive tail moment value was 0.01 ± 0.002, while in the case of negative control it was 0.15 ± 0.01. The results of protective and antioxidant activity against hydrogen peroxide-induced DNA damage of non-toxic plant extract of *D. pentagyna* in HepG2 cells are presented in Table 5. It was shown that the extent of DNA damage significantly increased in hydrogen peroxide treated cultures (OTM: 13.2 ± 0.96) as compared to the negative control (OTM: 5.9 ± 0.65; $P < 0.05$). Both pre-treatment and co-treatment of cell

Table 3. The IC_50 values of hexane leaf extracts of *Dillenia indica, D. pentagyna,* and *D. obovata* species estimated for HeLa, HepG2, and PBMC cells

Plant species	IC50 values, µg/mL		
	HeLa cells	HepG2 cells	PBMCs
D. indica	<0.43	139±2.6	>430
D. pentagyna	<0.43	>430	350
D. obovata	<0.43	81±2.1	>430
Combined mixture (*D. indica: D. pentagyna: D. obovata;* 1:1:1)	-	-	>430

Table 4. The level of DNA damage evaluated by comet assay based on Olive tail moment after treatment with hexane leaf extracts of *D. indica, D. pentagyna,* and *D. obovata* species

	NC	PC	*D. indica*	*D. obovata*	*D. pentagyna*	Combined mixture
HeLa cells						
Concentration	-	100 µM	0.43 µg/mL	0.43 µg/mL	0.43 µg/mL	-
OTM	0.02±0.006	26.07±2.00*	0.08±0.005*	0.81±0.05*	0.06±0.01ns	-
HepG2 cells						
Concentration	-	50 µM	139 µg/mL	81 µg/mL	430 µg/mL	-
OTM	5.2±0.89	15.6±1.4*	7.4±1.82 ns	8.2±2.12*	4.2±0.07 ns	-
PBMCs						
Concentration	-	100 µM	430 µg/mL	430 µg/mL	350 µg/mL	430 µg/mL
OTM	0.15±0.01	9.69±1.26*	0.22±0.04ns	0.09±0.01 ns	0.14±0.01 ns	0.01±0.002 ns

OTM, olive tail moment; NC, negative control; PC, positive control (H2O2); PBMCs, peripheral blood mononuclear cells.
* $P < 0.05$ compared to negative control; ns – $P > 0.05$ compared to negative control

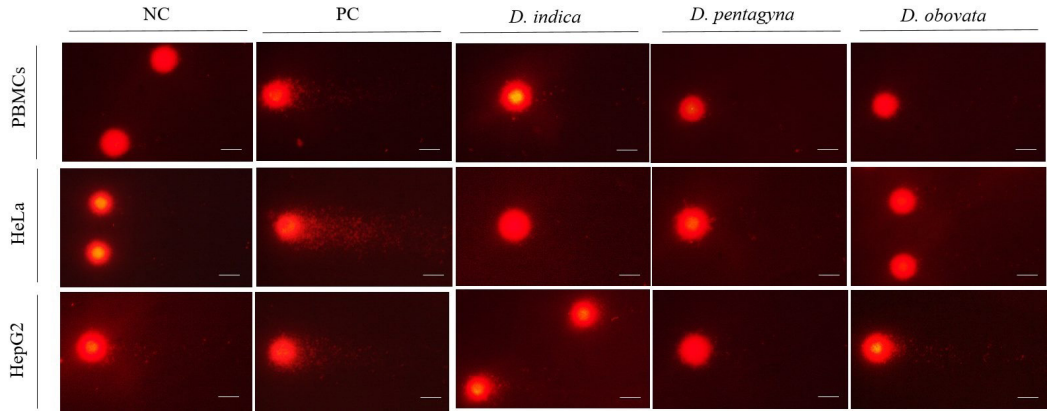

Figure 4. The comet assay performed in PBMCs, HeLa, and HepG2 cells treated with hexane leaf extracts of *D. indica, D. pentagyna*, and *D. obovata* species at IC50, 430 µg/mL or 0.43 µg/mL (if IC_{50} value was higher or lower of the tested concentrations) concentrations. NC: negative control; PC: positive control; The scale bar is 10 µM.

cultures by the extract resulted in a significant ($P < 0.05$) DNA repair (OTM: 3.6 ± 0.27 and 7.3 ± 0.34 compared to the positive control hydrogen peroxide treated cells). However, pre-treatment of cultures with the plant extracts was more efficient in reducing DNA damage levels than co-treatment.

Discussion

The research results showed that the three *Dillenia* species, including *D. indica*, *D. obovata,* and *D. pentagyna* are new potential resources for improving human health to use given the high levels of oleamide and β-sitosterol they contain. Aside from the three studied *Dillenia* species, *D. indica*, *D. obovata,* and *D. pentagyna*, nine *Dillenia* species found in Thailand have been studied for their oleamide contents (16), but β-sitosterol was not studied in this plant group. This study is the first to show the two important substances found together in these three *Dillenia* species, which can be used in various diseases given the substances' physiological properties. Beta-sitosterol in the form of plant, substance, or plant extract can be used in supplements, cosmetics, medical settings, modern herbal products, etc. The physiological actions in the human body include anti-inflammatory, chemopreventive, hypocholesterolemic, wound healing, antioxidant, and anti-diabetic effects. It is also used for benign prostatic hyperplasia and prostatic cancer treatment (17-21). The plants or extracts can be used in natural product creation such as hair supplements for hair growth improvement and hair loss reduction, given the better absorption and improved activity for the treatment of alopecia in shampoos and hair serums (22-24). In this regard *Polygonum multiform* and saw palmetto (*Serenoa repens*) have been used in hair care products (23). In addition to the β-sitosterol substance contained in the *Dillenia* species, there were high quantities of oleamide percentage-wise as elucidated by the GC-MS method. However, HPLC was used for the amount and concentration measurements and showed oleamide at 1.01 mg/g plant sample and 0.326 mg/mL, 1.12 mg/g plant sample and 0.374 mg/mL, and 1.17 mg/g plant sample and 0.262 mg/mL in *D. indica*, *D. obovata,* and *D. pentagyna*, respectively (7). The role of oleamide in human body is very important, such as stress-reducing, memory improvement used for Alzheimer's, inducing deep sleep, improving appetite, and as an anti-inflammatory without toxicity (7,8,9,16).

When plants contain vital phytochemicals, the combined mixture needs to be examined in terms of toxicity, both cytotoxicity and genotoxicity. The mixture showed non-toxicity on PBMCs and on the cell level because the mixture had greater proportions of the non-toxic *D. indica* and *D. obovata* than the proportion of the more toxic *D. pentagyna*. However, even though there was an IC_{50} value of 350 µg/mL for *D. pentagyna*, this predicted an LD_{50} of 934.100 mg/kg, which categorized it as a WHO Class II (50-2,000 mg/kg body weight, oral), and Class

Table 5. The level of induced oxidative DNA damage in human hepatoma HepG2 cell line after treatment with *D. pentagyna* hexane leaf extract

Plant extract	Pre-treatment		Co-treatment	
	%DNA in tail	OTM	%DNA in tail	OTM
Negative control (un-treated cells)	13.3 ± 1.3	5.9 ± 0.65	13.3 ± 1.3	5.9 ± 0.65
Positive control (PC)	29.8 ± 1.84	13.2 ± 0.96	29.8 ± 1.84	13.2 ± 0.96
Dillenia pentagyna	6.74 ± 0.81#*	3.6 ± 0.27#*	16.5 ± 0.67*	7.3 ± 0.34*

#$P < 0.05$ in comparison with negative control; * $P < 0.05$ in comparison with the positive control; PC: cells treated with 50 µM H_2O_2 for 5 min. OTM: Olive tail moment.

Ib (50-200 mg/kg body weight, dermal) moderately to highly hazardous toxic chemical. In order to meet this classification, a 50 kg body weight person would have to consume a 2500-100 000 mg dose or apply on the skin a 2500 -10 000 mg dose. In terms of humans, who are quite different from rats both in body size and genetics, it is very difficult for people to consume or apply to the skin such a high dose corresponding to going over the threshold for no toxicity in our DNA results.

The study of protective effects of *D. pentagyna* extract showed that pre-treatment of the cultures with the extract was more efficient in reducing DNA damage levels than co-treatment. An especially remarkable protective effect was found in the case of cells pre-treated with *D. pentagyna*, as DNA damage level significantly decreased even in comparison with the level of background DNA damage in the negative control ($P<0.05$). Therefore, stronger protection was obtained via pre-treatment than in co-treated cells for the tested extract, indicating that in addition to direct scavenging of ROS, the accumulation of plant extract in the cell increased the induction of enzymatic and non-enzymatic cellular antioxidants and detoxifying enzymes, thus leading to the elevation of cellular antioxidative defenses. Here the tremendous antioxidant effect on the cellular level, based on biological response (DNA-damage) was shown. It is assumed that phenolics and flavonoids appear to be major contributors to the antioxidant potential of those extracts. However, the major constituents of *D. pentagyna* extract, oleamide and β-sitosterol, and their role in cell antioxidant defenses cannot be ignored. Indeed, oleamide is a ligand of thioredoxin reductase enzyme known to catalyze the reduction of thioredoxin, a component of the cellular defense system against oxidative damage (25).

The experiment of the combined mixture further assures that the utilization of these three plants for health promotion and disease treatment is good, non-toxic, and is likely to be highly effective based on the properties of the two active ingredients.

Conclusion

All of the experimental aspects of this study after phytochemical measurements, including toxicity testing in PBMCs, HeLa, and HepG2 cell lines plus antioxidant activity, were done to ensure that they will be able to be fully utilized for human health. Given this, *D. indica*, *D. obovata,* and *D. pentagyna* may be used in traditional and modified forms according to modern scientific measurements that are presently available. They can be potentially used as natural resources with several advantages stemming from the β-sitosterol and oleamide compounds that the plants contained.

Authors' contributions

Conceptualization: AC and NB. Data curation: PS, KS, and TT. Formal analysis: PS, TiT. Formal analysis: AC and NB. Investigation: RS and TaT. Methodology: PS, TiT, LK, LA. Project administration: RS. Supervision: AC. Manuscript writing, review & editing: AC, RS, and NB.

Conflict of interests

The funders had no role in the design of the study, collection, analyses, interpretation of the data, writing of the manuscript, or in the decision to publish the results.

Ethical considerations

Ethical issues including text plagiarism, data fabrication, double publication, falsification and etc. have been carefully observed by the authors. All the experiments were performed in accordance with relevant guidelines and regulations.

Funding/Support

RS and AC were financially supported for the research by Research and Graduate Studies, Khon Kaen University (Grant number is RS and AC, 2021).

References

1. Calixto JB. The role of natural products in modern drug discovery. An Acad Bras Cienc. 2019;91 Suppl 3:e20190105. doi: 10.1590/0001-3765201920190105.
2. Pellati F, Borgonetti V, Brighenti V, Biagi M, Benvenuti S, Corsi L. *Cannabis sativa* L. and nonpsychoactive cannabinoids: their chemistry and role against oxidative stress, inflammation, and cancer. Biomed Res Int. 2018;2018:1691428. doi: 10.1155/2018/1691428.
3. Atakan Z. Cannabis, a complex plant: different compounds and different effects on individuals. Ther Adv Psychopharmacol. 2012;2(6):241-54. doi: 10.1177/2045125312457586.
4. Bautista JL, Yu S, Tian L. Flavonoids in *Cannabis sativa*: biosynthesis, bioactivities, and biotechnology. ACS Omega. 2021;6(8):5119-23. doi: 10.1021/acsomega.1c00318.
5. Lim XY, Tan TYC, Muhd Rosli SH, Sa'at MNF, Sirdar Ali S, Syed Mohamed AF. *Cannabis sativa* subsp. sativa's pharmacological properties and health effects: a scoping review of current evidence. PLoS One. 2021;16(1):e0245471. doi: 10.1371/journal.pone.0245471.
6. Veeresham C. Natural products derived from plants as a source of drugs. J Adv Pharm Technol Res. 2012;3(4):200-1. doi: 10.4103/2231-4040.104709.
7. Ameamsri U, Tanee T, Chaveerach A, Peigneur S, Tytgat J, Sudmoon R. Anti-inflammatory and detoxification activities of some *Ipomoea* species determined by ion channel inhibition and their phytochemical constituents. J Sci Soc Thailand. 2021;47(3):321-9. doi: 10.2306/scienceasia1513-1874.2021.042.
8. Hachisu M, Konishi K, Hosoi M, Tani M, Tomioka H, Inamoto A, et al. Beyond the hypothesis of serum anticholinergic activity in Alzheimer's disease: acetylcholine neuronal activity modulates brain-derived neurotrophic factor production and inflammation in the brain. Neurodegener Dis. 2015;15(3):182-7. doi:

10.1159/000381531.
9. Martínez-González D, Bonilla-Jaime H, Morales-Otal A, Henriksen SJ, Velázquez-Moctezuma J, Prospéro-García O. Oleamide and anandamide effects on food intake and sexual behavior of rats. Neurosci Lett. 2004;364(1):1-6. doi: 10.1016/j.neulet.2004.03.080.
10. Moon SM, Lee SA, Hong JH, Kim JS, Kim DK, Kim CS. Oleamide suppresses inflammatory responses in LPS-induced RAW264.7 murine macrophages and alleviates paw edema in a carrageenan-induced inflammatory rat model. Int Immunopharmacol. 2018;56:179-85. doi: 10.1016/j.intimp.2018.01.032.
11. Oh YT, Lee JY, Lee J, Lee JH, Kim JE, Ha J, et al. Oleamide suppresses lipopolysaccharide-induced expression of iNOS and COX-2 through inhibition of NF-kappaB activation in BV2 murine microglial cells. Neurosci Lett. 2010;474(3):148-53. doi: 10.1016/j.neulet.2010.03.026.
12. Babu S, Jayaraman S. An update on β-sitosterol: a potential herbal nutraceutical for diabetic management. Biomed Pharmacother. 2020;131:110702. doi: 10.1016/j.biopha.2020.110702.
13. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2015;111: A3.B.1-A3.B.3. doi: 10.1002/0471142735.ima03bs111.
14. van de Loosdrecht AA, Beelen RH, Ossenkoppele GJ, Broekhoven MG, Langenhuijsen MM. A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. J Immunol Methods. 1994;174(1-2):311-20. doi: 10.1016/0022-1759(94)90034-5.
15. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175(1):184-91. doi: 10.1016/0014-4827(88)90265-0.
16. Thooptianrat T, Chaveerach A, Sudmoon R, Tanee T, Liehr T, Babayan N. Screening of phytochemicals and toxicity of medicinal plants, *Dillenia* species, reveals potential natural product resources. J Food Biochem. 2017;41(3):e12363. doi: 10.1111/jfbc.12363.
17. Saeidnia S, Manayi A, Gohari AR, Abdollahi M. The story of beta-sitosterol-a review. European J Med Plants. 2014;4(5):590-609.
18. Ras RT, Geleijnse JM, Trautwein EA. LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: a meta-analysis of randomised controlled studies. Br J Nutr. 2014;112(2):214-9. doi: 10.1017/s0007114514000750.
19. Novotny L, Abdel-Hamid ME, Hunakova L. Anticancer potential of β-sitosterol. Int J Clin Pharmacol Pharmacother. 2017;2:129. doi: 10.15344/2017/2456-3501/129.
20. Rakel D. Benign prostatic hyperplasia. In: Integrative Medicine. 4th ed. Philadelphia, PA: Elsevier; 2018.
21. Goel A. Lower urinary tract symptoms and benign prostatic hyperplasia: from research to bedside. Indian J Med Res. 2019;149(5):684-5. doi: 10.4103/ijmr.IJMR_323_19.
22. Upadhyay K, Gupta NK, Dixit VK. Development and characterization of phyto-vesicles of β-sitosterol for the treatment of androgenetic alopecia. Arch Dermatol Res. 2012;304(7):511-9. doi: 10.1007/s00403-011-1199-8.
23. Kalwat JI. The use of *Serenoa repens* (*Saw palmetto*) in hair care products. Biomed J Sci Tech Res. 2019;13(1):9725-8. doi: 10.26717/bjstr.2019.13.002348.
24. Nguyen J, Korta DZ, Mesinkovska NA. Hair supplements. In: Miteva M, ed. Alopecia. Elsevier; 2019. p. 295-304. doi: 10.1016/b978-0-323-54825-0.00028-4.
25. Munigunti R, Nelson N, Mulabagal V, Gupta MP, Brun R, Calderón AI. Identification of oleamide in *Guatteria recurvisepala* by LC/MS-based *Plasmodium falciparum* thioredoxin reductase ligand binding method. Planta Med. 2011;77(15):1749-53. doi: 10.1055/s-0030-1271080.