



Phytochemical profiling, antioxidant capacity, and preliminary cytotoxicity of *Diospyros dasyphylla* Kurz leaf extract

[Perfil fitoquímico, capacidad antioxidante y citotoxicidad preliminar del extracto de hojas de *Diospyros dasyphylla* Kurz]

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Abstract

Context: Natural products remain valuable sources of bioactive compounds. *Diospyros dasyphylla* Kurz is poorly characterized with respect to its phytochemical composition and biological activities.

Aims: To evaluate the antioxidant activity, preliminary MTT-based cytotoxicity, phytochemical profile, and predicted molecular interactions of *D. dasyphylla* leaf extract.

Methods: Leaves were extracted with 95% ethanol. Phenolic, flavonoid, and tannin contents were determined colorimetrically. Antioxidant activity was assessed using DPPH, ABTS, and FRAP assays. Preliminary cytotoxicity was assessed in MCF-7, HCT-116, and CCD-841 cells using the MTT assay. Major constituents were tentatively identified by GC-MS. Drug-likeness, pharmacokinetic properties, and docking interactions with CASP3, AKT-1, and BCL-2 were predicted *in silico*.

Results: The extract exhibited DPPH and ABTS IC₅₀ values of 171.36 ± 1.53 and 140.52 ± 0.32 µg/mL, respectively, and a FRAP value of 49.51 ± 1.71 mmol Fe²⁺ equivalents/100 g extract. MTT-based cytotoxic activity was observed against MCF-7 and HCT-116 cells, with IC₅₀ values of 39.70 ± 1.50 and 25.70 ± 1.50 µg/mL, respectively. Limited selectivity was observed relative to CCD-841 cells. GC-MS tentatively identified ten major constituents, with uvaol tentatively identified as the predominant compound. Docking predicted favorable interactions of triterpenoids with AKT-1 and BCL-2.

Conclusions: *D. dasyphylla* leaf extract contained triterpenoid-rich constituents and showed measurable antioxidant activity and preliminary MTT-based cytotoxic activity. This appears to be the first comprehensive report describing the phytochemical composition, antioxidant activity, preliminary MTT-based cytotoxicity, and molecular docking profile of this species. Complementary cell-death and pathway-validation assays are required.

Keywords: antioxidants; *Ebenaceae*; molecular docking; phytochemicals; triterpenes.

Resumen

Contexto: Los productos naturales son fuentes valiosas de compuestos bioactivos. *Diospyros dasyphylla* Kurz ha sido poco estudiada en cuanto a su composición fitoquímica y a sus actividades biológicas.

Objetivos: Evaluar la actividad antioxidante, la citotoxicidad preliminar mediante el ensayo de MTT, el perfil fitoquímico y las interacciones moleculares predichas del extracto de hojas de *D. dasyphylla*.

Métodos: Las hojas se extrajeron con etanol al 95%. Los compuestos fenólicos, flavonoides y taninos se determinaron colorimétricamente. La actividad antioxidante se evaluó mediante DPPH, ABTS y FRAP, y la citotoxicidad preliminar mediante MTT en células MCF-7, HCT-116 y CCD-841. Los principales constituyentes se identificaron de forma tentativa mediante GC-MS. Las propiedades farmacocinéticas y las interacciones con CASP3, AKT-1 y BCL-2 se predijeron *in silico*.

Resultados: El extracto presentó valores de IC₅₀ de 171.36 ± 1.53 y 140.52 ± 0.32 µg/mL para DPPH y ABTS, respectivamente, y un valor de FRAP de 49.51 ± 1.71 mmol Fe²⁺/100 g. La actividad citotóxica basada en MTT mostró valores de IC₅₀ de 39.70 ± 1.50 y 25.70 ± 1.50 µg/mL en MCF-7 y HCT-116, respectivamente, con selectividad limitada frente a CCD-841. GC-MS identificó tentativamente diez constituyentes principales, siendo el uvaol el predominante. El acoplamiento molecular predijo interacciones favorables entre los triterpenos y AKT-1 y BCL-2.

Conclusiones: El extracto de hojas de *D. dasyphylla* presentó constituyentes ricos en triterpenos, actividad antioxidante y citotoxicidad preliminar determinada mediante MTT. Este parece ser el primer informe integral sobre el perfil fitoquímico, antioxidante, citotóxico y molecular de esta especie. Se requieren ensayos complementarios para validar estos hallazgos.

Palabras Clave: acoplamiento molecular; antioxidantes; *Ebenaceae*; fitoquímicos; triterpenos.

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ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **ADME:** absorption, distribution, metabolism and excretion; **AKT-1:** AKT serine/threonine kinase 1; **BAX:** Bcl-2-associated X protein; **BCL-2:** B-cell lymphoma 2; **CASP3:** caspase-3; **CCD-841:** human normal colonic epithelial cell line; **CYP:** Cytochrome P450; **DMSO:** dimethyl sulfoxide; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **EI:** electron impact; **FRAP:** ferric reducing antioxidant power; **GAE:** gallic acid equivalent; **GC-MS:** gas chromatography-mass spectrometry; **HBA:** hydrogen bond acceptor; **HBD:** hydrogen bond donor; **KI:** Kovats indices; **Ki:** inhibition constants; **MTT:** 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; **P-gp:** P-glycoprotein; **PDB:** protein data bank; **RMSD:** root mean square deviation; **ROS:** reactive oxygen species; **TAE:** tannic acid equivalent; **TFC:** total flavonoid content; **TPC:** total phenolic content; **TPSA:** Topological polar surface area.

INTRODUCTION

Cancer remains a major global health problem and continues to impose a substantial burden on healthcare systems worldwide. Although advances in diagnosis and treatment have improved patient outcomes, the clinical application of conventional chemotherapeutic agents is often limited by adverse effects and non-selective toxicity toward normal cells (Yousef et al., 2021). In addition, therapeutic resistance and limited target specificity further constrain long-term clinical efficacy, highlighting the need for safer and multi-targeted therapeutic strategies. These limitations have prompted increasing interest in natural products as alternative or complementary sources of anticancer agents.

Medicinal plants represent a rich source of bioactive secondary metabolites, including phenolics, flavonoids and terpenoids, many of which have been reported to exhibit antioxidant and cytotoxic activities relevant to cancer prevention and treatment (Kim et al., 2021). Antioxidant activity is of particular interest, as oxidative stress plays a critical role in cancer initiation and progression through excessive production of reactive oxygen species (ROS), leading to oxidative damage and genomic instability (Alkadi, 2020). Beyond redox modulation, ROS imbalance is closely associated with dysregulation of key signaling pathways involved in apoptosis and cell survival, including caspase activation and the PI3K/AKT axis, which are central to cancer cell proliferation and resistance mechanisms (Liu et al., 2021).

The genus *Diospyros* (Ebenaceae) comprises numerous species traditionally used in herbal medicine and is known to contain diverse bioactive constituents, particularly triterpenoids and phenolic compounds. Several *Diospyros* species have been reported to possess antioxidant, anti-inflammatory and cytotoxic properties (Chen et al., 2024; Nguyen et al., 2024). Notably, pentacyclic triterpenoids such as amyrins and related derivatives have been associated with apoptosis induction, mitochondrial dysfunction and modulation of survival-related proteins. However, *Diospyros dasyphylla* Kurz, locally known as Chan Dong in Thailand, remains poorly investigated, with limited scientific evidence regarding its phytochemical composition and biological activities. Moreover, no comprehensive

study has yet integrated phytochemical profiling, cytotoxic evaluation against both cancer and normal cell lines, and mechanistic *in silico* analysis for this species.

In recent years, the integration of experimental bioassays with computational approaches has become increasingly valuable in natural product research. *In silico* techniques, such as ADME prediction and molecular docking, are widely used to support biological findings and to provide mechanistic insight into potential ligand-protein interactions, particularly for apoptosis- and survival-related targets including caspase-3, AKT-1 and BCL-2 (Ramachandran et al., 2013). Such integrated approaches are particularly useful for identifying multi-target interactions of complex phytochemical mixtures and for prioritizing bioactive compounds with potential pharmacological relevance.

To date, no study has simultaneously investigated the phytochemical composition, antioxidant potential, preliminary MTT-based cytotoxic activity in cancer and normal cell lines, and molecular target interactions of *D. dasyphylla* leaves. Therefore, the present study aimed to evaluate the antioxidant activity, preliminary MTT-based cytotoxicity, and phytochemical composition of *D. dasyphylla* leaf extract using an integrated *in vitro* and *in silico* approach. Special emphasis was placed on comparing MTT-based cytotoxic activity between cancer and normal cells and exploring potential molecular interactions with targets associated with apoptosis and cell survival pathways. Antioxidant capacity and preliminary cytotoxicity in selected human cancer and normal cell lines were assessed using established *in vitro* assays; phytochemical constituents were characterized by quantitative analysis and GC-MS profiling; and molecular docking was performed to explore potential interactions with key molecular targets. The findings provide baseline data on the phytochemical composition and biological activities of *D. dasyphylla* and may support further investigation of this underexplored medicinal plant.

MATERIAL AND METHODS

Chemicals

All chemicals and reagents used in this study were of analytical grade. Ethanol was obtained from ACI Labscan (Bangkok, Thailand). Aluminum chloride (AlCl₃) was purchased from QReC™ (New Zealand).

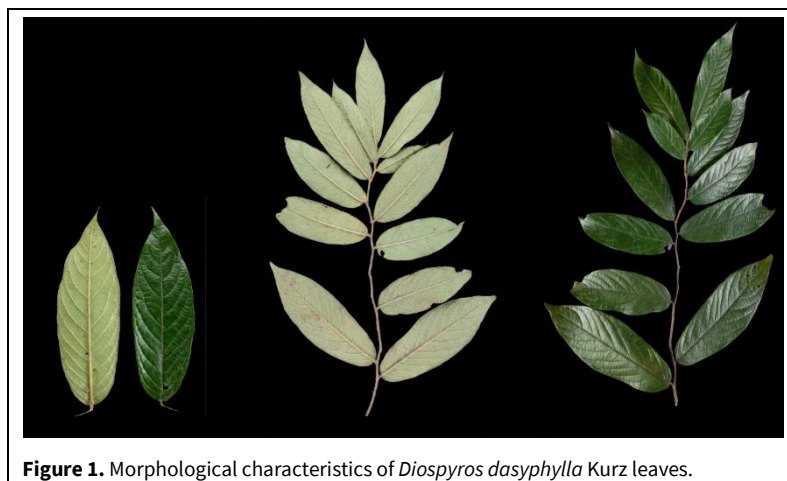


Figure 1. Morphological characteristics of *Diospyros dasyphylla* Kurz leaves.

Iron (II) sulfate (FeSO_4) was obtained from Thermo Scientific (Mumbai, India), while hydrochloric acid (HCl) was supplied by Carlo Erba (Milan, Italy). Sodium acetate (NaOAc), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), sodium carbonate (Na_2CO_3), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma-Aldrich (Bangalore and Hamburg, Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (Buchs, Switzerland). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco (Invitrogen, Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany).

Plant material and crude extract preparation

Mature leaves of *D. dasyphylla* Kurz (Ebenaceae) were collected from Ban Ron, Khao Kaeo Subdistrict, Lan Saka District, Nakhon Si Thammarat Province, Thailand, in June 2024 (8°21'24.1"N, 99°44'57.7"E). The plant material was taxonomically authenticated by Dr. Rumrada Meeboonya, Suan Sunandha Rajabhat University, Thailand (Fig. 1). Botanical nomenclature and scientific names were verified using the Plants of the World Online database (<https://powo.science.kew.org/>). Voucher specimens of *D. dasyphylla* (SSRU 005) were deposited at the Department of Cannabis Health Sciences, College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram, Thailand. The identification was conducted in accordance with standard taxonomic procedures, and the voucher specimen was preserved to ensure reproducibility and traceability of the plant material.

The leaves were air-dried at room temperature and ground into a fine powder. Approximately 20 g of powdered leaves were extracted with 95% ethanol using an ultrasonic sonication bath (GT SONIC-D3, 100 W, 40 kHz; Guangdong GT Sonic Co., China) at room temperature (25–28°C) for 30 min. This sonication-assisted extraction facilitates solvent penetration into plant tissues and enhances the release of phytochemicals from plant matrices. The extract was filtered through Whatman No. 1 filter paper (GE Healthcare, UK), and the solvent was removed under reduced pressure using a rotary evaporator (Rotavapor® R-210, Büchi, Switzerland). The crude ethanolic extract was obtained with a yield of 1.50 g (7.40%, w/w) and stored at 4°C until further analysis. All extracts were protected from light and moisture to minimize degradation prior to subsequent analyses.

Chemical composition

Determination of total phenolic content (TPC)

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method as previously described (Mungkunkoth et al., 2026). Briefly, 20 μL of extract solution was mixed with 100 μL of 10% (v/v) Folin-Ciocalteu reagent and 80 μL of 7% (w/v) sodium carbonate solution into a 96-well plate. The reaction mixture was incubated at room temperature for 30 min, and absorbance was measured at 760 nm using a microplate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). A calibration curve was constructed using gallic acid at different concentrations, and TPC was expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract). All measurements were performed in triplicate.

Determination of total flavonoid content (TFC)

Total flavonoid content (TFC) was determined according to the method described by Choirunnisa et al. (2025), with minor modifications. The extract solution (100 μ L) was mixed with 2% (w/v) aluminum chloride solution (100 μ L) into a 96-well plate, followed by incubation at room temperature for 10 min. Absorbance was measured at 415 nm using a microplate reader. Quercetin was used to generate a standard calibration curve, and TFC was expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g extract). All measurements were performed in triplicate.

Determination of total tannin content (TTC)

Total tannin content (TTC) was determined using the Folin–Ciocalteu method following Baali et al. (2024). The extract solution (20 μ L) was mixed with 10% Folin–Ciocalteu reagent (55 μ L), distilled water (70 μ L) and 7% (w/v) sodium carbonate solution (55 μ L) into a 96-well plate, and the reaction mixture was incubated in the dark at room temperature for 30 min. Absorbance was measured at 750 nm using a microplate reader. A calibration curve was prepared using tannic acid as the reference standard, and TTC was expressed as milligrams of tannic acid equivalents per gram of extract (mg TAE/g extract). All measurements were performed in triplicate.

GC–MS analysis

The chemical composition of the ethanolic leaf extract of *D. dasphylla* was analyzed using a gas chromatography–mass spectrometry (GC–MS) system (Shimadzu QP-2010, Kyoto, Japan). Separation was carried out on a fused-silica capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μ m film thickness; 5% phenylmethylpolysiloxane stationary phase) using helium as the carrier gas at a constant flow rate of 1.0 mL/min. The injection volume was 1 μ L in split mode (split ratio 1:20). The oven temperature was programmed from 70°C (2 min hold) to 200°C at 5°C/min (10 min hold), then increased to 230°C (10 min hold), 250°C (5 min hold), and finally to 320°C with a final hold of 20 min. The ion source temperature was set at 250°C, and mass spectra were acquired in electron impact (EI) ionization mode (70 eV) over a scan range of 35–500 m/z. Compounds were tentatively identified by comparing their mass spectra and retention times with those in the NIST 17 mass spectral library, following the procedure described by Yongram et al. (2025). Only compounds with acceptable spectral matching scores were included. The relative abundance of each compound was expressed as the percentage of the total peak area.

Antioxidant activity assays

DPPH radical scavenging assay

The free radical scavenging activity of the extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay as previously described (Siriparu et al., 2026). Briefly, 100 μ L of 200 μ M DPPH solution was mixed with 100 μ L of extract at concentrations ranging from 5–1000 μ g/mL in a 96-well plate. The reaction mixtures were incubated in the dark at room temperature for 30 min. Absorbance was measured at 490 nm using a microplate reader, with Trolox used as the reference standard. A calibration curve of Trolox was constructed for comparison of antioxidant capacity. The radical scavenging activity was calculated as the percentage inhibition relative to the control, and the IC₅₀ value (μ g/mL) was determined from the dose–response curve. All measurements were performed in triplicate.

ABTS radical scavenging assay

The ABTS radical scavenging assay was conducted according to the method described by Siriparu et al. (2026). The ABTS^{•+} radical cation was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate and incubating the mixture in the dark at room temperature (12–16 h). Subsequently, 100 μ L of ABTS^{•+} solution was mixed with 100 μ L of extract at concentrations ranging from 5–1000 μ g/mL in a 96-well plate and incubated for 10 min in the dark. Absorbance was measured at 415 nm using a microplate reader. Trolox was used as a reference standard, and a calibration curve was prepared accordingly. The percentage of ABTS radical scavenging activity and IC₅₀ values (μ g/mL) were calculated using the same equation as described for the DPPH assay. All measurements were performed in triplicate.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was performed according to Yongram et al. (2025). The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM FeCl₃ solution in a ratio of 10:1:1 (v/v/v). An aliquot of the extract solution (20 μ L) was mixed with FRAP reagent (80 μ L) into a 96-well plate. After incubation at room temperature for 5 min, absorbance was measured at 595 nm using a microplate reader. The antioxidant reducing capacity was expressed as millimoles of Fe²⁺ equivalents per 100 g extract (mmol Fe²⁺/100 g extract), calculated from a ferrous sulfate (FeSO₄) standard calibration curve. All measurements were performed in triplicate.

MTT-based cytotoxicity screening

Cell culture

Normal human colonic epithelial cells (CCD-841), human breast carcinoma cells (MCF-7), and human colorectal carcinoma cells (HCT-116) were used in this study. These cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO₂, as previously described (Jia et al., 2021). Cells were routinely monitored for morphology and confluency prior to experimental use. The cell lines were not independently authenticated after receipt, and mycoplasma testing was not performed.

Cytotoxicity assay

The cytotoxic activity of *D. dasyphylla* leaf extract was evaluated using the MTT assay. The crude extract was dissolved in 100% DMSO and the concentration of DMSO in the final culture medium was <2%. Because exact final DMSO concentrations were not recorded retrospectively, the cytotoxicity results should be interpreted as preliminary MTT-based screening data. CCD-841, MCF-7 and HCT-116 cells were seeded into a 96-well plate at a density of 2×10^4 cells/well (100 µL per well) and allowed to attach for 24 h. Cells were then treated with the ethanolic leaf extract of *D. dasyphylla* at concentrations ranging from 5–50 µg/mL and incubated for 48 h. Following treatment, MTT solution (5 mg/mL stock solution, diluted to working concentration) was added to each well, and the plates were incubated for an additional 4 h at 37°C to allow for formazan crystal formation. The culture medium was carefully removed, and the resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 490 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells, and the half-maximal inhibitory concentration (IC₅₀, µg/mL) values were determined according to the method described by Yang et al. (2021) and Yahuafai et al. (2023). All cytotoxicity measurements were performed in technical triplicate. Cytotoxic effects were evaluated in both cancer and normal cell lines to assess selective cytotoxicity. The selectivity index (SI) was calculated as the IC₅₀ value in normal cells divided by the IC₅₀ value in cancer cells.

In silico prediction

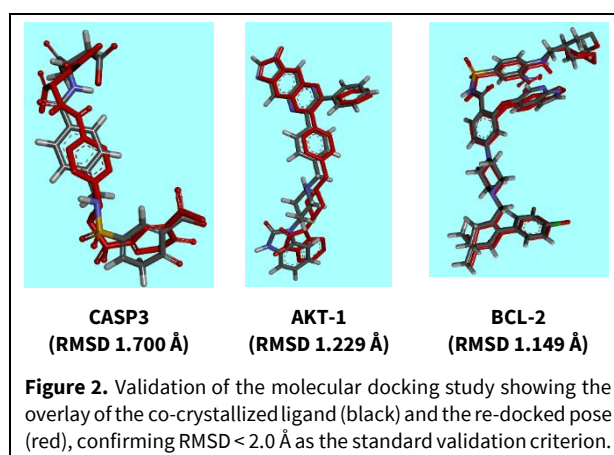
ADME prediction

Drug-likeness and pharmacokinetic properties of the major phytochemicals identified by GC-MS were

evaluated using the SwissADME web-based tool. Simplified molecular-input line-entry system (SMILES) codes of the selected compounds were retrieved and submitted to SwissADME to calculate key physico-chemical descriptors, including molecular weight, hydrogen bond donors and acceptors, lipophilicity, solubility and gastrointestinal absorption. Additional parameters, including topological polar surface area (TPSA) and number of rotatable bonds, were also considered to assess drug-likeness (Alifiyah et al., 2026). Lipinski's rule of five and additional drug-likeness filters were applied to evaluate the pharmacokinetic feasibility of the identified compounds, as previously reported.

In silico molecular docking prediction and visualization

Molecular docking studies were conducted to predict potential interactions between selected phytochemicals and cancer-related molecular targets. The three-dimensional crystal structures of caspase-3 (CASP3; PDB ID: 1NMS), AKT-1 (PDB ID: 3O96) and BCL-2 (PDB ID: 6O0K) were retrieved from the Protein Data Bank (Fig. 2).



These targets were selected based on their established roles in apoptosis and cell survival pathways. Docking validation was performed by re-docking the respective co-crystallized ligands into their original binding pockets. RMSD values below 2.0 Å were considered indicative of a reliable docking protocol (Bissantz et al., 2000). Protein structures were prepared by removing water molecules, adding polar hydrogen atoms, and assigning Kollman charges at physiological pH (7.4). Bioactive phytochemicals identified from *D. dasyphylla* were obtained in three-dimensional format from the PubChem database. Ligands were protonated at pH 7.4 using MarvinSketch and converted to PDBQT format using AutoDock Tools. The predicted binding poses were superimposed onto the corresponding crystallographic conformations, and the resulting RMSD values of 1.700 Å (CASP3), 1.229 Å

(AKT-1), and 1.149 Å (BCL-2) confirmed the reliability of the docking protocol. Docking simulations were performed using AutoDock 4.2 with the Lamarckian Genetic Algorithm (LGA). Each ligand was subjected to 100 independent docking runs using a population size of 100, a maximum of 1,000,000 energy evaluations, 27,000 maximum generations, a mutation rate of 0.02, a crossover rate of 0.80, and an elitism value of 1. Grid box parameters were defined according to the coordinates of the respective co-crystallized ligands (Table 1). Doxorubicin was included only as a reference compound for comparative docking analysis and was not used as the native ligand for docking validation. Binding affinities were expressed as binding energy (ΔG , kcal/mol) and inhibition constant (K_i). Ligand-protein interactions were analyzed and visualized in two- and three-dimensional formats using BIOVIA Discovery Studio Visualizer (Morris et al., 2009).

Statistical analysis

All experiments were performed in technical replicates, and data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA). Differences between control and treated groups were evaluated using Student's *t*-test, and $p < 0.05$ was considered statistically significant. Cytotoxicity data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test ($p < 0.05$).

RESULTS

Phytochemical composition

Quantitative phytochemical analysis showed that *D. dasyphylla* leaves contained measurable levels of phenolics, flavonoids and tannins (Table 2). The total

phenolic content was 23.13 ± 0.95 mg GAE/g extract, while total flavonoid and tannin contents were 111.27 ± 1.47 mg QE/g extract and 36.60 ± 1.56 mg TAE/g extract, respectively. Among the analyzed classes of secondary metabolites, flavonoids were present at the highest concentration.

The chemical composition of the ethanolic leaf extract was further characterized by GC-MS analysis, which tentatively identified ten major constituents accounting for 88.79% of the total peak area (Table 3). Corresponding chromatograms and mass spectra are provided in the Supplementary Data (Figs. S1-S11). The chromatographic profile was dominated by pentacyclic triterpenoids, with uvaol (42.82%) as the most abundant compound, followed by α -amyrin (21.42%) and β -amyrin (8.73%). Additional constituents included vitamin E (1.94%), γ -sitosterol (1.82%) and betulin (2.89%). Minor components such as neophytadiene, phytol and cis-3,14-clerodadien-13-ol were also detected in lower proportions. Collectively, the GC-MS data indicate that triterpenoid-type compounds represent the predominant chemical class in *D. dasyphylla* leaves, complementing the quantitative phytochemical results.

Antioxidant activity

The antioxidant activity of *D. dasyphylla* leaf extract was evaluated using DPPH, ABTS and FRAP assays (Table 2). The extract exhibited IC_{50} values of 171.36 ± 1.53 μ g/mL in the DPPH assay and 140.52 ± 0.32 μ g/mL in the ABTS assay, indicating relatively higher scavenging efficiency against ABTS radicals. The ferric reducing capacity determined by the FRAP assay was 49.51 ± 1.71 mmol Fe^{2+} /100 g extract. In all assays, the antioxidant activity of the extract was significantly lower than that of the reference antioxidant Trolox ($p < 0.05$). Low standard deviation values were observed among replicate measurements.

Table 1. Parameters used for molecular docking study.

Target protein	PDB ID	Co-crystallized ligand	Grid box center (x, y, z)	Grid box size	Spacing (Å)	RMSD (< 2Å)
CASP3	1NMS	Ligand ID: 161	-9.09, -4.02, 24.24	30x50x60	0.231	1.700
AKT-1	3O96	IQO (allosteric inhibitor)	8.37, -6.82, 12.62	44x48x48	0.342	1.229
BCL-2	6O0K	Venetoclax	-15.35, 2.24, -9.56	32x38x60	0.375	1.149

CASP3: caspase-3; AKT-1: AKT serine/threonine kinase 1; BCL-2: B-cell lymphoma 2. The co-crystallized ligands were re-docked into their respective binding sites for docking validation. The resulting RMSD values (< 2.0 Å) confirmed the reliability of the docking protocol.

Table 2. Antioxidant activities and chemical contents of *D. dasyphylla* leaves.

Parameter	Leaves	Trolox
DPPH; IC ₅₀ (μg/mL)	171.36 ± 1.53*	6.19 ± 0.04
ABTS; IC ₅₀ (μg/mL)	140.52 ± 0.32*	7.35 ± 0.06
FRAP (mmol Fe ²⁺ /100 g extract)	49.51 ± 1.71*	711.73 ± 8.03
TPC (mg GAE/g extract)	23.13 ± 0.95	–
TFC (mg QE/g extract)	111.27 ± 1.47	–
TTC (mg TAE/g extract)	36.60 ± 1.56	–

Data are presented as mean ± SD (n = 3). *p<0.05 is significantly different between the sample and Trolox by Student's t-test.

Table 3. Chemical composition of *D. dasyphylla* leaves by GC-MS.

No.	Retention time (min)	Formula	MW	KI ^a	KI ^b	Similarity score (%)	%Area	Compound name	Fragmentation Pattern m/z (decreasing order of abundance)
1	26.55	C ₂₀ H ₃₈	278	1774	1842	94	0.94	Neophytadiene	68(100), 95, 82, 43, 57, 81, 67, 69, 55, 41, 83, 123, 97, 71, 96, 79, 53, 111, 65, 77, 137
2	33.31	C ₂₀ H ₄₀ O	296	2045	2111	97	2.84	Phytol	71(100), 43, 81, 57, 41, 55, 69, 123, 95, 70, 68, 84, 56, 66, 82, 97, 85, 111, 107, 137
3	69.57	C ₂₉ H ₅₀ O ₂	430	3149	3149.4	86	1.94	Vitamin E	164(100), 165, 430, 205, 43, 166, 121, 57, 137, 39, 136, 55, 135, 71, 91, 149, 109, 191, 281, 341
4	72.85	C ₂₉ H ₅₀ O	414	2731	3351.3	82	1.82	γ-Sitosterol	43(100), 213, 95, 105, 81, 55, 133, 329, 160, 199, 41, 119, 231, 255, 149, 273, 303, 414, 396
5	73.37	C ₃₀ H ₅₀ O	426	2886	-	91	8.73	β-Amyrin	218(100), 203, 189, 95, 69, 119, 55, 81, 107, 175, 136, 147, 41, 161, 257, 426
6	74.17	C ₃₀ H ₅₀ O	426	2873	-	93	21.42	α-Amyrin	218(100), 203, 189, 122, 95, 135, 147, 107, 55, 161, 81, 69, 41, 175, 257, 426, 272, 411
7	75.52	C ₂₀ H ₃₄ O	290	2021	2051.4	76	0.55	cis-3,14-Clerodadien-13-ol	135(100), 95, 109, 119, 208, 189, 175, 145, 81, 67, 161, 55, 43, 267, 251, 218, 281, 229
8	78.19	C ₃₀ H ₅₀ O ₂	442	3128	-	90	4.84	(3β)-Olean-12-ene-3,28-diol	203(100), 204, 95, 105, 175, 69, 119, 81, 131, 55, 189, 147, 43, 216, 234, 159, 411, 282, 442
9	79.06	C ₃₀ H ₅₀ O ₂	442	3116	3692.4	91	42.82	Uvaol	203(100), 133, 234, 119, 95, 81, 189, 105, 69, 43, 147, 175, 159, 216, 411, 255, 442
10	79.35	C ₃₀ H ₅₀ O ₂	442	3090	3760.5	73	2.89	Betulin	203(100), 95, 109, 81, 177, 69, 43, 133, 191, 121, 147, 161, 237, 219, 286, 355, 327, 250, 381, 401, 341, 297, 268

MW: molecular weight; KI^a: Kovats indices from this research. KI^b: Kovats indices from the reference, previously reported at <https://webbook.nist.gov/chemistry/>

MTT-based cytotoxicity screening

The cytotoxicity of the *D. dasyphylla* leaf extract was evaluated in MCF-7, HCT-116, and CCD-841 cells using the MTT assay. IC₅₀ values of 39.70 ± 1.50 and 25.70 ± 1.50 μg/mL were observed in MCF-7 and HCT-116 cells, respectively (Table 4). The calculated selectivity indices were 0.96 for MCF-7 and 1.48 for HCT-116, indicating limited selectivity under the tested conditions. Doxorubicin exhibited greater cytotoxic potency, with selectivity indices of 0.63 for MCF-7 and 4.00 for HCT-116. Under the tested conditions, the extract exhibited moderate cytotoxic activity, with the lowest IC₅₀ observed against HCT-116 cells. However, because the MTT assay reflects cellular metabolic activity rather than direct cell death, complementary assays are required to confirm the underlying cytotoxic mechanism and cancer cell selectivity.

In silico ADME and pharmacokinetic properties

The physicochemical and pharmacokinetic properties of the identified compounds were predicted using SwissADME (Tables 5 and 6). Most compounds exhibited molecular weights below 500 Da, meeting a key criterion for drug-likeness. However, high lipophilicity values (cLogP > 5) were observed for the majority of triterpenoids and phytosterols, resulting in one Lipinski rule violation per compound. Cis-3,14-clerodadien-13-ol exhibited comparatively lower lipophilicity (cLogP 4.95), placing it close to the Lipinski threshold.

Predicted intestinal absorption values ranged from 95.04% to 109.00%, indicating predicted intestinal absorption was high for most compounds. Only cis-3,14-clerodadien-13-ol was predicted to be blood-brain barrier permeant, whereas the remaining compounds were not expected to penetrate the central nervous system. Several compounds were predicted to interact

with cytochrome P450 enzymes, particularly CYP2C19 and CYP2C9, while others showed no inhibitory effects on major CYP isoforms. Doxorubicin, used as a reference compound, exhibited lower predicted absorption and multiple Lipinski rule violations.

Molecular docking analysis

Molecular docking analysis was conducted to evaluate the binding affinities of major phytochemicals identified from *D. dasyphylla* leaves toward three cancer-related target proteins, namely caspase-3 (CASP3), AKT serine/threonine kinase 1 (AKT-1) and B-cell lymphoma-2 (BCL-2). Docking protocol validation indicated acceptable accuracy, with RMSD values below 2.0 Å for all protein–ligand complexes, confirming the reliability of the docking parameters (Table 1).

The binding affinities (ΔG) of the tested compounds ranged from -1.56 to -11.43 kcal/mol (Table 7). For CASP3, γ -sitosterol (-8.17 kcal/mol) and vitamin E (-8.09 kcal/mol) exhibited the strongest binding affinities among the tested phytochemicals, whereas neophytadiene and phytol showed weaker interactions (Fig. 3). Docking predicted favorable binding energies for several triterpenoid compounds against AKT-1. Among these compounds, α -amyrin (-11.43 kcal/mol), γ -sitosterol (-11.27 kcal/mol), and uvaol (-11.11 kcal/mol) showed the lowest predicted binding energies. The corresponding K_i values represent theoretical estimates generated by the docking software and should not be interpreted as experimentally determined inhibition constants (Fig. 4).

Table 4. Cytotoxic activity of *D. dasyphylla* leaves.

Sample	IC ₅₀ (μg/mL)		
	CCD-841	MCF-7 (SI)	HCT-116 (SI)
Leaves	38.10 ± 1.50 ^{a,b}	39.70 ± 1.50 ^{a,b} (0.96)	25.70 ± 1.50 ^{a,a} (1.48)
Doxorubicin	1.20 ± 0.10 ^a	1.90 ± 0.20 ^a (0.63)	0.30 ± 0.05 ^a (4.00)

Data are presented as mean ± SD (n = 3). *p<0.05 indicates a significant difference between the leaf extract and doxorubicin within the same cell line (Student's t-test). Different lowercase letters (^{a,b}) indicate significant differences among cell lines within the same treatment, as determined by one-way ANOVA followed by Tukey's HSD test (p<0.05). CCD-841: Normal human colonic epithelial cells; MCF-7: human breast carcinoma cells; HCT-116: human colorectal carcinoma cells. The SI = IC₅₀ normal cell/IC₅₀ cancer cell.

Table 5. Physicochemical properties of compounds identified in *D. dasyphylla* leaves.

Compound	Physicochemical properties							
	MW	cLogP	cLogS	TPSA	NORTB	HBA	HBD	Lipinski violations
Criteria	<500	-	-	≤140	≤10	≤5	≤10	≤0
Neophytadiene	278.52	7.07	-9.53	0	13	0	0	1
Phytol	296.53	6.25	-8.47	20.23	13	1	1	1
Vitamin E	430.71	8.29	-11.27	29.46	12	2	1	1
γ -Sitosterol	414.71	7.24	-9.67	20.23	6	1	1	1
β -Amyrin	426.72	7.18	-9.47	20.23	0	1	1	1
α -Amyrin	426.72	7.05	-9.33	20.23	0	1	1	1
cis-3,14-Clerodadien-13-ol	290.48	4.95	-5.82	20.23	4	1	1	1
(3 β)-Olean-12-ene-3,28-diol	442.72	6.3	-8.25	40.46	1	2	2	1
Uvaol	442.72	6.15	-8.1	40.46	1	2	2	1
Betulin	442.72	6.39	-8.99	40.46	2	2	2	1
Doxorubicin (Positive control)	543.52	0.52	-5.2	206.07	5	12	6	3

MW: molecular weight; cLogP: calculated octanol/water partition coefficient; cLogS: solubility parameter; TPSA: topological polar surface area; NORTB: number of freely rotatable bonds; HBA: hydrogen bond acceptors; HBD: hydrogen bond donors.

Table 6. Predicted pharmacokinetic properties of compounds in *D. dasyphylla* leaves.

Compound	Pharmacokinetic properties							
	%ABS	BBB permeant	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
Neophytadiene	109.00	No	Yes	No	No	Yes	No	No
Phytol	102.02	No	Yes	No	No	Yes	No	No
Vitamin E	98.84	No	Yes	No	No	No	No	No
γ-Sitosterol	102.02	No	No	No	No	No	No	No
β-Amyrin	102.02	No	No	No	No	No	No	No
α-Amyrin	102.02	No	No	No	No	No	No	No
cis-3,14-Clerodadien-13-ol	102.02	Yes	No	No	Yes	Yes	Yes	No
(3β)-Olean-12-ene-3,28-diol	95.04	No	No	No	No	No	No	No
Uvaol	95.04	No	No	No	No	No	No	No
Betulin	95.04	No	No	No	No	No	No	No
Doxorubicin (Positive control)	37.91	No	Yes	No	No	No	No	No

%ABS: %absorption; BBB: blood-brain barrier; Pgp: p-glycoprotein; CYP: cytochrome P450.

Table 7. Molecular docking binding affinities (ΔG , kcal/mol) and inhibition constants (K_i) of *D. dasyphylla* phytochemicals against CASP3, AKT-1, and BCL-2 proteins.

No.	Compound	Protein targets					
		CASP3		AKT-1		BCL-2	
		Score binding (ΔG)	K_i	Score binding (ΔG)	K_i	Score binding (ΔG)	K_i
1	Neophytadiene	-5.87	49.92 ^a	-6.56	15.66 ^c	-5.31	127.50 ^a
2	Phytol	-6.17	29.82 ^a	-7.02	7.10 ^c	-5.63	74.17 ^a
3	Vitamin E	-8.09	1.17 ^a	-9.93	52.47 ^c	-6.93	8.28 ^a
4	γ-Sitosterol	-8.17	1.02 ^a	-11.27	5.49 ^c	-8.90	301.02 ^c
5	β-Amyrin	-7.67	2.38 ^a	-11.03	8.32 ^c	-8.51	580.93 ^c
6	α-Amyrin	-7.72	2.19 ^a	-11.43	4.15 ^c	-9.17	189.55 ^c
7	cis-3,14-Clerodadien-13-ol	-7.50	3.21 ^a	-8.49	598.21 ^c	-7.34	4.19 ^a
8	(3β)-Olean-12-ene-3,28-diol	-7.64	2.55 ^a	-10.55	18.45 ^c	-8.44	713.62 ^c
9	Uvaol	-7.67	2.41 ^a	-11.11	7.33 ^c	-9.31	149.22 ^c
10	Betulin	-1.56	71.75 ^b	-11.20	6.21 ^c	-7.56	2.86 ^a
11	Co-crystallized ligand (Ligand ID: 161)	-9.62	89.51 ^c	-	-	-	-
12	Doxorubicin (Reference compound)	-	-	-13.40	150.00 ^d	-7.79	1.95 ^a

Binding affinity values are expressed in kcal/mol. Inhibition constants (K_i) are reported in different units: a = micromolar (μM), b = millimolar (mM), c = nanomolar (nM), and d = picomolar (pM). The co-crystallized ligand (Ligand ID: 161) was used for docking validation of CASP3. Docking validation of CASP3, AKT-1, and BCL-2 was performed using their respective co-crystallized ligands (RMSD = 1.700, 1.229, and 1.149 Å, respectively). Doxorubicin was included only as a reference compound for comparative docking analysis and was not used as the native ligand for docking validation.

generally showed more favorable predicted binding energies than the non-triterpenoid constituents.

DISCUSSION

Phytochemical analysis revealed a relatively high flavonoid content, accompanied by appreciable levels of tannins and phenolics, which likely contribute to the observed biological activities. GC-MS profiling further revealed that pentacyclic triterpenoids and phytosterols constituted the major chemical classes, with uvaol tentatively identified as the predominant compound (42.82%), followed by α -amyrin and β -amyrin, together with γ -sitosterol, betulin and vitamin E. Comparable chemical patterns have been reported in other *Diospyros* species and are frequently associated with apoptosis-related bioactivities (Rauf et al., 2024). In addition, previous studies have highlighted that species within the genus *Diospyros*, including *Diospyros mollis*, contain diverse classes of secondary metabolites such as triterpenoids, sterols, and quinone derivatives, supporting the chemical diversity observed in the present study (Chen et al., 2024); this is further supported by broader reviews demonstrating the wide ethnomedicinal applications and pharmacological potential of *Diospyros* species across different regions (Ribeiro et al., 2023).

The predominance of triterpenoid constituents, particularly uvaol, suggests that these compounds may contribute to the biological activities observed in this study. Consistently, triterpenoid-rich fractions isolated from *Diospyros kaki* leaves have demonstrated significant bioactivities, including cytotoxic, anti-inflammatory, antioxidant, and enzyme inhibitory effects, supporting the pharmacological relevance of this compound class (Nguyen et al., 2024). Taken together, these findings suggest that pentacyclic triterpenoids may contribute to the observed bioactivities, although further mechanistic studies are required to confirm their specific contributions.

The antioxidant activity observed across DPPH, ABTS and FRAP assays indicates the presence of diverse redox-active constituents with complementary mechanisms, a pattern comparable to that reported for other *Diospyros* species (Ojha et al., 2023). According to the classification proposed by Sukweenadhi et al. (2020), antioxidant activity can be categorized as very strong ($IC_{50} < 50 \mu\text{g/mL}$), strong ($50\text{--}100 \mu\text{g/mL}$), moderate ($101\text{--}150 \mu\text{g/mL}$), and weak ($IC_{50} > 150 \mu\text{g/mL}$). Based on this criterion, the extract can be classified as weak in the DPPH assay and moderate in the ABTS assay, indicating assay-dependent variation in radical scavenging activity. The comparatively stronger activity observed in the ABTS assay suggests differential reactivity of the extract toward distinct radical systems, potentially reflecting contributions from

both hydrophilic and moderately lipophilic constituents.

The ethanolic extract of *D. dasyphylla* exhibited moderate cytotoxic effects against MCF-7 and HCT-116 cancer cell lines, with comparable effects observed in normal CCD-841 cells, indicating limited selectivity under the tested conditions. Although the extract was less potent than the reference drug doxorubicin, its activity is within the range typically reported for crude plant extracts and underscores its value as a source of bioactive constituents rather than a single therapeutic agent. Similar antiproliferative trends have been described for *D. lotus* and *D. kaki*, where growth inhibition was linked to oxidative imbalance and mitochondrial dysfunction (Bianchini et al., 2024; Loizzo et al., 2009). The observed cytotoxic effects may therefore be associated with modulation of oxidative stress and apoptosis-related pathways, although further mechanistic validation is required to confirm these effects. However, this research should be regarded as a preliminary MTT-based evaluation of the cytotoxic activity of *D. dasyphylla* leaf extract.

In silico ADME evaluation indicated that most identified compounds exhibit favorable predicted intestinal absorption, although high lipophilicity resulted in a single Lipinski rule violation for the majority of triterpenoids and phytosterols. Such physicochemical constraints are commonly associated with terpenoid-based natural products and may limit oral bioavailability; however, they do not preclude pharmacological relevance and can often be addressed through formulation or prodrug strategies (Daina et al., 2017). This observation highlights the importance of formulation strategies to improve the pharmacokinetic properties of bioactive triterpenoids.

Among the identified constituents, uvaol is a candidate for further investigation because it was tentatively identified as the most abundant GC-MS constituent (42.82%) and showed favorable predicted interactions in the docking analysis. In HepG2 cells, purified uvaol has been reported to increase apoptosis, increase BAX expression, decrease BCL-2 expression, downregulate PI3K/AKT signaling, and reduce intracellular ROS levels (Bonel-Pérez et al., 2020). ROS-dependent apoptosis has also been reported for synthetic pentacyclic triterpenoid derivatives (Cheng et al., 2023), although those findings cannot be attributed directly to uvaol or to the present crude extract. The docking results therefore provide a rationale for future pathway studies but do not establish the mechanism responsible for the MTT-based effects observed here.

Molecular docking analysis further suggested that triterpenoid compounds preferentially interacted with key cancer-related targets, including AKT-1 and BCL-

2, whereas linear terpenoids showed comparatively weaker binding to these proteins. From a structure–activity perspective, the rigid polycyclic framework of pentacyclic triterpenoids appears to favor hydrophobic interactions within survival-related protein pockets (Ahmad et al., 2022). In contrast, some linear terpenoids were predicted by molecular docking to interact with CASP3; however, this finding should be regarded solely as an *in silico* observation and should not be directly linked to the antiproliferative activity observed in MCF-7 cells, which are widely reported to be caspase-3 deficient because of a deletion in the CASP3 gene. Consequently, the predicted CASP3 interactions may be biologically relevant only to cell lines expressing functional caspase-3, such as HCT-116, and require experimental validation before any mechanistic conclusions can be drawn. Overall, the present findings suggest that the MTT-based cytotoxicity and docking results are consistent with possible interactions involving AKT-1 and BCL-2, but these predicted interactions require biochemical and cellular validation. Such complementary targeting of cancer-related pathways is increasingly recognized as a favorable characteristic of natural anticancer agents (Newman & Cragg, 2020).

The present findings highlight *D. dasyphylla* leaves as a chemically rich source of triterpenoids and phenolic compounds associated with moderate antioxidant activity and moderate cytotoxic effects with limited selectivity. The cytotoxicity assessment was based solely on the MTT assay, which reflects metabolic activity and may not directly confirm cell death. In addition, the cell lines were not independently authenticated after receipt, and mycoplasma testing was not performed. Accordingly, the cytotoxicity findings should be interpreted with appropriate caution, as undetected cell-line misidentification or mycoplasma contamination may influence metabolic activity and cytotoxicity measurements. In addition, the extract showed limited selectivity toward cancer cells compared with CCD-841 cells. The proposed involvement of apoptosis- and survival-related pathways was inferred from molecular docking predictions and has not yet been experimentally validated. Therefore, complementary assays, including LDH release, Annexin V/PI staining, mitochondrial membrane potential analysis, intracellular ROS measurement, and Western blot analysis of AKT, BCL-2, BAX, and caspase-related proteins, are required before mechanistic conclusions regarding the cytotoxic effects of the extract can be drawn.

CONCLUSION

The ethanolic leaf extract of *D. dasyphylla* contained triterpenoid-rich constituents, with uvaol tentatively identified as the predominant compound by GC–MS.

The extract exhibited measurable antioxidant activity and cytotoxic potential, with the greatest activity observed against HCT-116 cells, although selectivity toward cancer cells was limited. Molecular docking analysis suggested potential interactions between triterpenoids and cancer-related targets, particularly AKT-1 and BCL-2. This appears to be the first study to comprehensively characterize the phytochemical composition, antioxidant activity, cytotoxic potential, and molecular docking profile of *D. dasyphylla* leaf extract, providing baseline scientific evidence for this underexplored medicinal species. Collectively, these findings suggest that *D. dasyphylla* leaf extract contains bioactive phytochemicals warranting further investigation. Future studies should focus on compound isolation, mechanistic validation, and complementary cell-based assays to confirm the biological activities observed in the present study.

CONFLICT OF INTEREST

The research was conducted entirely free from any involvement in commercial, financial, or personal matters that might be perceived as a possible conflict of interest or that could have influenced the outcomes or interpretation of the study.

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The authors used ChatGPT (OpenAI, San Francisco, CA, USA) to improve the language and readability of the manuscript. The authors reviewed and edited the content and take full responsibility for its accuracy. No images were generated or manipulated using AI tools.

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AUTHOR CONTRIBUTION:						
Contribution	Yongram C	Krutchangthong S	Meeboonya R	Kamoltham T	Puthongking P	Sripan P
Concepts or ideas	x	x	x	x	x	x
Design	x	x	x	x	x	x
Definition of intellectual content						x
Literature search	x	x				x
Experimental studies	x	x			x	x
Data acquisition	x					x
Data analysis	x	x				x
Statistical analysis	x	x				x
Manuscript preparation	x	x				x
Manuscript editing	x	x			x	x
Manuscript review	x	x	x	x	x	x

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Supplementary data

