



OPEN Pharmacological profiling of *Gnetum gnemon* var. *tenerum* extracts exhibits antibacterial, antioxidant, cytotoxic and anti-inflammatory activities

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This study conducted a comprehensive in vitro pharmacological evaluation of the crude extract and solvent-partitioned fractions of *Gnetum gnemon* var. *tenerum*. LC–MS metabolite profiling identified 77 compounds, including flavonoids and phenylethanamine derivatives, that exhibit the antioxidant, cytotoxic, and anti-inflammatory activities described in this paper. The total phenolic and flavonoid contents (TPC/TFC) were quantified, showed that the ethyl acetate and n-butanol fractions contained the highest levels of these phytochemicals. These fractions exhibited strong antioxidant activity in both ABTS and DPPH assays. The antibacterial activity of the extracts was assessed using disk diffusion, MIC, and MBC assays against five pathogenic bacteria, where the chloroform and ethyl acetate fractions demonstrated the highest potency, with MIC values as low as 0.049 mg/mL against *Acinetobacter baumannii*. Cytotoxicity testing on human gastrointestinal cancer cell lines (AGS, HT-29) and a normal intestinal epithelial cell line (HIEC-6) showed dose-dependent cytotoxicity toward cancer cells while exerting markedly lower effects on normal cells, suggesting selective action. Anti-inflammatory activity, evaluated by nitric oxide (NO) inhibition in LPS-stimulated RAW264.7 macrophages, revealed that the chloroform and ethyl acetate fractions most effectively suppressed NO production. These findings provide scientific support for the traditional uses of *G. gnemon* and highlight its extracts as a promising source of bioactive compounds with multi-target pharmacological potential against oxidative stress, bacterial infections, inflammation, and cancer.

Keywords *Gnetum Gnemon* var. *Tenerum*, Cytotoxicity, Antioxidant, Anti-inflammatory, Antibacterial activity

Natural products, particularly those of plant medicinal origin, have been and continue to be a nonreplaceable and inexhaustible source for the discovery, development, and innovation of new therapeutic agents¹. Their chemical heterogeneity upon isolation from the natural source and the large number of pharmacologically active entities associated with them provide them as optimal lead structures for the meeting of unmet medical needs. Throughout human history, different systems of traditional medicine all over the world have relied on the intricate properties of plants for their medicinal and health-protecting effects and hence accumulated a wealth of ethnobotanical heritage that continues to guide and instruct scientific innovation today. Modern pharmacological research, as a matter of course, attempts to scientifically validate these centuries-old traditional uses and meticulously enumerate the intricate molecular mechanisms behind their attributed health benefits². This rigorous and systematic process is critical to the process of converting empirical traditional knowledge to

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evidence-based clinical practice. Furthermore, the process facilitates the creation of the identification of potential lead compounds that could be advanced to pharmaceutical drugs further. Natural product libraries remain a target of research for pharmaceuticals, whose activities continue to give rise to new therapeutic remedies for diseases having wide spectra.

Gnetum gnemon (Linn.) var. *tenerum*, otherwise referred to by names such as phak liang (Thailand), melinjo (Indonesian), is a cosmopolitan and widely recognized species of plant, widely spread predominantly in Southeast Asia, belonging to the ancient and interesting Gnetaceae family. This extraordinary plant is of significant cultural and economic value in its original environments, both because of its unique culinary application and because of its profound role as a traditional medicine³. Various plant anatomical structures, including the seeds, leaves, and stem, have been utilized profoundly in traditional medicines for their perceived nutritional value and therapeutic qualities. In traditional medical practice, *G. gnemon* has been traditionally utilized for the cure of a great number of human ailments, such as the treatment of inflammatory diseases, the cure of gastrointestinal disturbances, and its utilization as a general health tonic or restorative remedy, therefore solidifying its perceived therapeutic diversity and broad application scope⁴. Considering the well-documented traditional importance and overall availability of the general *G. gnemon* varieties, extensive scientific study particularly targeted to the elucidation of its individual biological activities and distinctive phytochemical profiles of its varieties, such as *G. gnemon* var. *tenerum*, regrettably still remain less studied compared to the overall species. Such information deficiency points to the dire need for specialized research into unearthing the special therapeutic potentiality inherent within this individual variety.

The general destruction of chronic diseases, including the relentless procession of cancer, the treacherous onset of oxidative stress disorders, and the debilitating expressions of chronic inflammatory disorders, all represent superior world health concerns that impose a significant burden on the health care systems of the world. Cancer, by its very nature, characterized by its hallmark feature of out-of-control cell proliferation and metastasis, still remains the leading cause of death globally and consequently needs a continued and desperate quest for very powerful, highly selective, and well-tolerated anticancer drugs that ideally are less harmful in terms of side effects than existing agents⁵. Oxidative stress, the consequence of an intricate imbalance between the generation of extremely reactive oxygen species (ROS) and the capacity of endogenous or exogenous antioxidant protective systems to counteract them, plays a causal role in the multifaceted pathogenesis and relentless course of a vast array of chronic diseases, to cause extensive cellular damage, molecular dysfunction, and, finally, organ failure⁶. In addition, longstanding chronic inflammation, a widespread and often dysregulated immune response, is an underlying pathological mechanism in a wide range of crippling human disease, from autoimmune disease and metabolic syndrome to various cancers and neurodegenerative disorders⁷. The persistent and uncontrolled activation of inflammatory mechanisms, usually at the expense of excessive production of primary inflammatory mediators like NO and prostaglandins, ineluctably leads to irreparable tissue damage, fibrosis, and severe disease exacerbation. With the inherent interconnectedness and shared molecular processes of these multifaceted disease processes, natural product-derived compounds with intrinsic multi-faceted bioactivities, such as synergistic cytotoxic, potent antioxidant, and superior anti-inflammatory activity, are of profound and increasing interest for targeted therapeutic use. Such compounds can potentially address multiple disease aspects simultaneously, with the potential for offering more effective and holistic treatments.

Building upon the recognized traditional uses and broader pharmacological potential of *G. gnemon*, this study conducted extensive in vitro pharmacological screen of the crude extract and solvent-partitioned fractions of *G. gnemon* var. *tenerum*. Given the growing global challenge of antimicrobial resistance and the limited scientific evidence describing the antibacterial properties of this species, we also aimed to evaluate its antibacterial activity to determine whether it may serve as a source of natural antimicrobial agents. Our objectives were to assess their cytotoxic activity against gastrointestinal cancer (AGS, HT-29) and normal (HIEC-6) cell lines using the MTT assay in order to determine selective toxicity. We also determined their TPC and TFC, evaluated their antioxidant activity based on ABTS and DPPH radical scavenging assay, and measured their anti-inflammatory activity through NO inhibition in Lipopolysaccharide (LPS)-induced RAW264.7 macrophages. The integrated results are anticipated to scientifically validate the traditional applications and identify promising fractions to isolate lead compounds for potential therapeutic application against cancer, oxidative stress, and inflammatory diseases.

Materials and methods

Plant collection and identification

Fresh leaves of *Gnetum gnemon* var. *tenerum* were collected in Nakhon Si Thammarat, Thailand, in January 2024 with permission from the local administrative authority under the Plant Genetic Conservation Project (RSPG), Walailak University. The species is not listed as a protected or endangered plant under Thai national regulations. The plant species were taxonomically identified by a specialized botanist, Mrs. Ruamporn Kongjan, at Walailak University, Thailand. Voucher specimens consisting of leaves, stems, and roots were deposited in the Walailak University Herbarium (Nakhon Si Thammarat, Thailand), where they are publicly accessible and serve as references for future botanical verification. Experimental research and field studies complied with institutional and national guidelines and legislation governing plant research and biodiversity conservation in Thailand. All procedures were conducted under local and national regulations, and formal ethical approval was not required for this study.

Extraction of bioactive compounds from *G. gnemon* leaves

The extraction and solvent-partitioning procedures were performed according to a previously reported with minor modifications, as described by Kooltheat et al.⁸ The leaves were washed thoroughly with clean water to remove debris, air-dried, and homogenized with 2 L of deionized water (DI) using an electric blender (Philips

HR3652, Netherlands). A total of 500 g of air-dried leaf material was used for extraction, corresponding to a solvent-to-solid ratio of 2 L : 500 g (equivalent to 4 mL of solvent per gram of dry material). The homogenate was macerated in 2 L of methanol (RCI Labscan Ltd., Bangkok, Thailand) at room temperature for 7 days with occasional stirring. After maceration, the extract was separated from the plant residue by filtration through muslin cloth, ensuring maximum recovery of the filtrate. The methanol extract was concentrated using a hot-air oven (Memmert UN55, Germany) at 60 °C until a viscous residue was obtained. The crude methanol extract was reconstituted in 600 mL of DI water and 100 mL of this solution was reserved as the crude methanol extract. The remaining 500 mL was subjected to sequential liquid–liquid partitioning with organic solvents of increasing polarity.

First, the aqueous extract was extracted three times with an equal volume (500 mL) of diethyl ether using a magnetic stirrer (IKA C-MAG HS7, Germany) for 30 min, followed by centrifugation at 2500 rpm for 15 min (Eppendorf 5810R, Germany) to obtain the diethyl ether fraction and the remaining aqueous phase. Sodium bicarbonate (RCI Labscan Ltd., Bangkok, Thailand) was added to the aqueous phase to adjust the pH to 8–9, converting phenolic acids to their sodium salt forms. The alkaline aqueous phase was then extracted with 500 mL of chloroform under the same stirring and centrifugation conditions to yield the chloroform fraction and aqueous phase. The pH of the aqueous phase was subsequently adjusted to 3–4 with acetic acid (RCI Labscan Ltd., Bangkok, Thailand) to reconvert phenolic salts to their acid form, followed by extraction with 500 mL of ethyl acetate to obtain the ethyl acetate fraction and aqueous phase. The remaining aqueous phase was then extracted with 500 mL of *n*-butanol to yield the *n*-butanol fraction and the final aqueous residual fraction. All solvent fractions were evaporated to dryness at 60 °C, weighed, and their extraction yield (%) calculated as:

$$\text{Yield (\%)} = [\text{Weight of dried extract (g)} / \text{Initial fresh weight of leaves (g)}] \times 100$$

Total phenolic content

The total phenolic content of *G. gnemon* extracts were determined using the Folin–Ciocalteu method as described by Sornsene P et al.⁹ with slight modifications. Briefly, 50 µL of each extract (400 µg/mL in methanol) or gallic acid standard was mixed with 50 µL of Folin–Ciocalteu reagent in a 96-well microplate. After 5 min, 50 µL of 7.5% Na₂CO₃ solution was added, and the mixture was incubated at room temperature for 1 h in the dark. Absorbance was then measured at 750 nm using a microplate reader. All measurements were performed in triplicate. The TPC was expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g).

Total flavonoid content (TFC)

The total flavonoid content of *G. gnemon* extracts were quantified using the aluminium chloride (AlCl₃) colorimetric method as described by Sornsene P et al.⁹ with slight modifications. In a 96-well microplate, 50 µL of each extract (400 µg/mL in methanol) or quercetin standard was mixed with 50 µL of 2% AlCl₃ solution. The mixture was gently shaken and incubated at room temperature for 30 min. Absorbance was measured at 415 nm against a blank using a microplate reader. All measurements were performed in triplicate. The TFC was expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g).

LC-MS analysis

LC–MS/MS analysis was carried out to characterize the metabolite composition of the *G. gnemon* var. *tenerum* crude extract and solvent-partitioned fractions. The profiling was performed using a high-resolution LC–MS system equipped with an electrospray ionization (ESI) source operating in both positive and negative modes. Chromatographic separation was achieved on a Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 µm; Agilent Technologies), maintained at 50 °C. The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), delivered at a flow rate of 0.4 mL/min under a linear gradient from 5% to 95% B over 20 min before re-equilibration. An injection volume of 10 µL was used for all samples. Mass spectrometric detection was performed using MS1 scans in the *m/z* range 40–1700, followed by data-dependent MS/MS scans between *m/z* 25–1000. Ionization parameters included a drying gas temperature of 325 °C, sheath gas temperature of 275 °C, gas flow of 13 L/h, nebulizer pressure of 45 psi, and a capillary voltage of 4000 V. Collision energies of 20 eV (positive mode) and 10 eV (negative mode) were applied for fragmentation. Reference ions were continuously infused to ensure mass accuracy throughout the run. For each analysis cycle, up to ten precursors were selected for MS/MS acquisition based on intensity and charge state. Raw spectral data were processed using MS-Dial version 5.3, and peak alignment was performed against pooled quality-control samples. An internal standard (sulfadimethoxine) was used for normalization, and metabolite annotation was based on MS-Dial's ESI (+/–) MS/MS library and precursor-fragment matching. To ensure data reliability, only features meeting the following criteria were retained: an identification score greater than 1.0, Pearson correlation with QC dilution ≥ 0.8, and coefficient of variation (CV) < 30% among QC replicates. The analysis detected a total of 3456 features in positive mode and 1039 features in negative mode across all samples.

Antioxidant activity

2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS⁺) radical cation scavenging activity
The ABTS⁺ radical cation scavenging activity of *G. gnemon* extracts were determined using the ABTS decolorization assay described by Sornsene P et al.⁹ with slight modifications. The ABTS⁺ radical cation was generated by mixing 7 mM ABTS with 2.45 mM potassium persulfate at a ratio of 2:3 (v/v) and allowing the mixture to stand in the dark at 25 °C for 18 h. The ABTS⁺ solution was diluted with absolute methanol to an absorbance of 0.70 ± 0.02 at 734 nm. In a 96-well microplate, 20 µL of each extract or fraction (100 µg/mL in absolute ethanol) or ascorbic acid standard (1.56–100 µg/mL) was mixed with 180 µL of ABTS⁺ working solution and incubated at room temperature for 45 min. Absorbance was then measured at 734 nm using a

microplate reader. All experiments were carried out in triplicate. The ABTS⁺ radical cation scavenging activity was expressed as milligram ascorbic acid equivalents per gram of dry extract (mg AAE/g). IC₅₀ of the extract and fractions against ABTS cation radical was calculated by curve fitting analysis.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant activity of *G. gnemon* extracts and solvent fractions was evaluated using the DPPH radical scavenging assay, with Trolox (Sigma-Aldrich, St. Louis, MO, USA) as the standard, following the method of Dunkhunthod B et al.¹⁰ with minor modifications. Briefly, 20 µL of each extract (100 µg/mL in absolute methanol) or ascorbic acid standard (1.56–100 µg/mL) was mixed with 180 µL of DPPH solution (0.2 mM in methanol) in a 96-well microplate. The reaction mixtures were incubated in the dark at room temperature for 30 min, and absorbance was measured at 517 nm using a microplate reader. All determinations were performed in triplicate. The DPPH radical scavenging activity was expressed as milligram ascorbic acid equivalents per gram of dry extract (mg AAE/g). IC₅₀ of the extract and fractions against DPPH radical was calculated by curve fitting analysis.

Antibacterial activity

Bacterial strains

The antibacterial activity of the extracts was evaluated using bacterial strains representing both Gram-positive and Gram-negative groups. *Enterococcus faecalis* DMST 4331 was included as the Gram-positive strain. The Gram-negative group consisted of *Acinetobacter baumannii* DMST 49,606, *Salmonella enterica* serovar Typhi DMST 22,842, *Shigella flexneri* ATCC 44,237, and *Klebsiella pneumoniae* ATCC 27,731. All strains were cultured on tryptic soy agar (TSA; HiMedia, Mumbai, India) and incubated at 37 °C for 24 h. For broth preparation, single colonies were inoculated into tryptic soy broth (TSB; HiMedia, Mumbai, India) and incubated at 37 °C for 18 h. Stock cultures were maintained by mixing bacterial suspensions with sterile 30% glycerol and stored at –80 °C until further use.

Disk diffusion assay

The antibacterial activity of *G. gnemon* extracts and solvent fractions was evaluated using the disk diffusion method as described by Arun LB et al.¹¹, with modifications. Briefly, overnight cultures of each bacterial strain were adjusted to a turbidity equivalent to the 0.5 McFarland standard in Mueller–Hinton broth (MHB; HiMedia, Mumbai, India). Sterile paper disks (6 mm in diameter) were impregnated with each fraction to obtain a final loading of 2 mg/disk and placed on Mueller–Hinton agar (MHA; HiMedia, Mumbai, India) plates previously spread with the test bacterial suspension. Amikacin disks were used as the positive control, while disks containing 0.01% dimethyl sulfoxide (DMSO) served as the negative control. Plates were incubated at 37 °C for 24 h, and antibacterial activity was assessed by measuring the inhibition zone diameter (mm). All experiments were performed in triplicate.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of the *G. gnemon* extracts against each bacterial strain were determined following the Clinical and Laboratory Standards Institute (CLSI) guidelines¹². In a sterile 96-well microtiter plate, extracts were serially diluted twofold in MHB to final concentrations ranging from 250 to 0.98 mg/mL. Bacterial suspensions were adjusted to 5×10^5 CFU/mL and inoculated into each well. Amikacin and 0.01% DMSO served as positive and negative controls, respectively. Plates were incubated at 37 °C for 18 h, after which 0.05% resazurin (Thermo Fisher Scientific, Lancashire, UK) was added to each well and incubated for 2 h. MIC was recorded as the lowest concentration showing no visible growth and a color change from pink to blue. MBC values were determined by subculturing 10 µL from wells showing no growth onto TSA plates and incubating at 37 °C for 24 h.

Cytotoxicity assay

Cell lines and culture conditions

Human gastric adenocarcinoma cells (AGS), human colorectal adenocarcinoma cells (HT-29), and normal human intestinal epithelial cells (HIEC-6) were obtained from ATCC. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin, maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Murine macrophage-like cells (RAW 264.7) were maintained in DMEM with the same supplements and conditions.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cytotoxic activity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay¹³. Cells were seeded in 96-well plates and incubated for 24 h. Various concentrations of crude extract and fractions (6.25–200 µg/mL) were added and incubated for an additional 48 h. MTT solution (5 mg/mL, 20 µL) was added to each well and incubated for 4 h at 37 °C. The formazan crystals were dissolved in 100 µL DMSO, and absorbance was measured at 570 nm using a microplate reader (Bio-Rad, USA). The MTT assay measures mitochondrial metabolic activity, which is widely used as an indirect indicator of cell viability in cytotoxicity studies. Cell viability (%) was calculated relative to untreated control cells. LC₅ and LC₁₀ represent the concentrations at which 5% and 10% of cells were non-viable, respectively, and were used to assess anti-inflammatory effects under sub-cytotoxic conditions.

Anti-inflammatory activities

Nitric oxide (NO) production Inhibition assay

The anti-inflammatory potential of *G. gnemon* extracts was determined by measuring NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells using the Griess reaction. RAW 264.7 cells were seeded into 96-well plates and incubated for 24 h at 37 °C in a humidified 5% CO₂ atmosphere. Cells were pretreated for 2 h with each extract at their respective LC₅ and LC₁₀ concentrations, as determined from the cytotoxicity assay, and then stimulated with LPS (1 µg/mL) for 22 h. Following incubation, 100 µL of culture supernatant from each well was combined with 100 µL of Griess reagent in a new 96-well plate. The absorbance was measured at 540 nm using a microplate reader. Sodium nitrite was used to prepare a standard curve, and NO production was expressed as a percentage relative to the untreated LPS-stimulated control. All assays were conducted in triplicate.

Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test in GraphPad Prism (version 9). A *p*-value < 0.05 was considered statistically significant.

Results

Total phenolic and flavonoid content of *G. gnemon* var. *Tenerum* extracts

The total phenolic content (TPC) and total flavonoid content (TFC) of *G. gnemon* var. *tenerum* crude extract and solvent-partitioned fractions were quantified using the Folin–Ciocalteu and aluminium chloride colorimetric assays, respectively, and expressed as mg gallic acid equivalents per g of dry extract (mg GAE/g DE) and mg quercetin equivalents per g of dry extract (mg QE/g extract). As shown in Table 1, the ethyl acetate fraction exhibited the highest TPC and TFC values (878.78 ± 50.68 mg GAE/g DE and 878.78 ± 50.68 mg QE/g extract), indicating a strong enrichment of polyphenolic compounds. The crude extract also contained substantial phenolic and flavonoid contents (427.89 ± 19.10 mg GAE/g DE and 427.89 ± 19.10 mg QE/g extract), followed by the chloroform fraction (333.93 ± 21.82 mg GAE/g DE and 333.93 ± 21.82 mg QE/g extract). Moderate levels were observed in the *n*-butanol fraction (217.17 ± 32.43 mg GAE/g DE and 217.17 ± 32.43 mg QE/g extract), whereas the diethyl ether and aqueous residual fractions exhibited comparatively lower phenolic and flavonoid contents. These results demonstrate that moderately polar solvents, particularly ethyl acetate, were most effective in concentrating phenolic and flavonoid constituents, which may contribute to the biological activities observed in subsequent assays.

Metabolites in the extract and fractions as detecting by LC-MS analysis

The metabolites/compounds presented in the extract and different fractions of *G. gnemon* leaves were investigated by LC-MS analysis. A total of 77 metabolites were detected in both the extract and different fractions according to the relative standard deviation of the denoising method. The results revealed phenylethanolamine was detected in all the extract/and fractions (Table 2). In addition, many amino acid molecules were also detected in both the extract/and fractions. In addition, choline, a monomer that used in the synthesize acetylcholine, was also presented in the crude extract and aqueous residual fraction. In addition, flavonoid derivatives were also detected in the fractions.

Antioxidant potential of *G. gnemon* var. *Tenerum* extracts

The antioxidant activity of the crude extract and solvent-partitioned fractions was evaluated using ABTS and DPPH radical scavenging assays, with results expressed as mg ascorbic acid equivalents per gram of dry extract (mg AAE/g) and IC₅₀ values (Table 3). The ethyl acetate fraction demonstrated the strongest antioxidant activity in both assays, exhibiting the highest ABTS (809.43 ± 3.51 mg AAE/g extract) and DPPH (807.11 ± 2.60 mg AAE/g extract) scavenging capacities, along with the lowest IC₅₀ values against ABTS (125.30 ± 0.67 µg/mL) and DPPH radicals (77.11 ± 0.31 µg/mL). The *n*-butanol fraction also showed pronounced antioxidant activity, whereas the crude extract and aqueous residual fraction displayed comparatively weak effects. So, the antioxidant capacity of the fractions correlated well with their phenolic and flavonoid contents, suggesting that these compounds play a major role in the radical scavenging activity of *G. gnemon* var. *tenerum* extracts.

Fraction	Extraction yield (% w/w of dry weight)	Total Phenolic Content (mg GAE/g of dry extract)	Total Flavonoid Content (mg QE/g of dry extract)
Crude extract	0.09	427.89 ± 19.10	427.89 ± 19.10
Diethyl ether	0.01	264.38 ± 23.80	264.38 ± 23.80
Chloroform	0.02	333.93 ± 21.82	333.93 ± 21.82
Ethyl acetate	0.01	878.78 ± 50.68	878.78 ± 50.68
<i>n</i> -Butanol	0.06	217.17 ± 32.43	217.17 ± 32.43
Aqueous residual	1.28	108.69 ± 17.42	108.69 ± 17.42

Table 1. Extraction yield, total phenolic content (TPC), and total flavonoid content (TFC) of *G. gnemon* var. *Tenerum* crude extract and solvent-partitioned fractions.

Fractions	Types of detection	Average Rt (min)	Average Mz	Metabolite name	Total score	Ontology
Crude extract	Positive	1.758	120.08589	Phenylethanolamine	1.11	Aralkylamines
		2.332	177.15318	geraniol	1.06	Acyclic monoterpenoids
		1.788	103.05708	(R)-4-aminoisoxazolidin-3-one	1.10	Alpha amino acids and derivatives
		0.617	104.10857	Choline	1.57	Cholines
		3.048	144.09688	2-Naphthylamine	1.85	Naphthalenes
		3.521	739.20685	Kaempferol-3-O-galactoside-6"-rhamnoside-3"-rha	1.48	Flavonoid-3-O-glycosides
	Negative	0.934	290.08719	N-Fructosyl pyroglutamate	1.59	Proline and derivatives
		0.945	128.03532	L-Pyroglutamic acid	1.72	Alpha amino acids and derivatives
		5.527	619.16943	6-[(benzoyloxy)methyl]-3,4,5-trihydroxyoxan-2-yl]oxy-6-hydroxy-8-methyl-9,10-dioxatetracyclo[4.3.1.0]decan-2-yl]methyl benzoate	1.55	Terpene glycosides
		1.258	130.08727	Leucine	1.74	Leucine and derivatives
		6.321	307.07175	3-Hydroxy-N-(3-nitrophenyl)naphthalene-2-carboxamid	1.40	Naphthalene-2-carboxanilides
		1.816	164.07208	L-(-)-Phenylalanine	1.90	Phenylalanine and derivatives
		7.903	327.21576	9,12,13-trihydroxyoctadeca-10,15-dienoic acid	1.48	Lineolic acids and derivatives
Diethyl ether	Positive	1.758	120.08589	Phenylethanolamine	1.11	Aralkylamines
		7.385	275.20737	8-[(1 S,5R)-4-Oxo-5-[(2Z)-2-penten-1-yl]-2-cyclopenten-1-yl] octanoic acid	1.54	Prostaglandins and related compounds
		7.386	351.22089	Andrographolide	1.10	Gamma butyrolactones
	Negative	11.541	293.21225	9-HOTrE	1.80	Lineolic acids and derivatives
		9.958	311.22156	FA 18:2 + 2O	1.66	Lineolic acids and derivatives
		8.009	327.21954	9,12,13-trihydroxyoctadeca-10,15-dienoic acid	1.58	Lineolic acids and derivatives
		8.699	329.23135	FA 18:1 + 3O	1.46	Long-chain fatty acids
		13.949	555.28217	Dronedarone	1.68	Aryl-phenylketones
		8.554	329.242	FA 18:1 + 3O	1.68	Long-chain fatty acids
		1.772	100.08282	2-Piperidone	1.53	Piperidinones
Chloroform	Positive	1.758	120.08589	Phenylethanolamine	1.11	Aralkylamines
		2.332	177.15318	Geraniol	1.06	Acyclic monoterpenoids
		2.051	171.06076	Propylthiouracil	1.17	Pyrimidones
		0.656	177.14709	1-Benzylpiperazine	1.65	Phenylmethylanines
	Negative	13.949	555.28217	Dronedarone	1.68	Aryl-phenylketones
		0.945	128.03532	L-Pyroglutamic acid	1.72	Alpha amino acids and derivatives
		3.521	739.20685	Kaempferol-3-O-galactoside-6	1.48	Flavonoid-3-O-glycosides
		1.258	130.08727	Leucine	1.74	Leucine and derivatives
	Positive	1.772	100.08282	2-Piperidone	1.53	Piperidinones
		1.287	136.06839	Adenine	1.89	6-aminopurines
		1.758	120.08589	Phenylethanolamine	1.11	Aralkylamines
		0.667	136.07545	Adenine	1.82	6-aminopurines
		1.149	136.06178	Adenine	1.77	6-aminopurines
		2.051	171.06076	Propylthiouracil	1.17	Pyrimidones
		0.888	136.06174	Adenine	1.74	6-aminopurines
Ethyl acetate	Negative	1.825	241.08203	Thymidine	1.53	Pyrimidine 2'-deoxyribonucleosides
		0.737	96.96143	Phosphoric acid	1.83	Non-metal phosphates
		0.637	134.04745	Adenine	1.82	6-aminopurines
		13.949	555.28217	Dronedarone	1.68	Aryl-phenylketones
	Positive	1.788	103.05708	4-aminoisoxazolidin-3-one	1.10	Alpha amino acids and derivatives
		2.442	188.07964	4-hydroxy-4-(pyridin-2-yl)butan-2-one	1.07	Pyridines and derivatives
		1.287	136.06839	Adenine	1.89	6-aminopurines
		1.758	120.08589	Phenylethanolamine	1.11	Aralkylamines
		0.656	177.14709	low score: 1-Benzylpiperazine	1.65	Phenylmethylanines
		1.816	164.07208	L-Phenylalanine	1.90	Phenylalanine and derivatives
n-Butanol	Negative	3.521	739.20685	Kaempferol-3-O-galactoside	1.48	Flavonoid-3-O-glycosides
		1.258	130.08727	Leucine	1.74	Leucine and derivatives
		3.915	433.20511	Hexose + C ₁₃ H ₂₁ O ₂	1.55	Fatty acyl glycosides of mono- and disaccharides
		1.825	241.08203	Thymidine	1.53	Pyrimidine 2'-deoxyribonucleosides
		0.945	128.03532	L-Pyroglutamic acid	1.72	Alpha amino acids and derivatives
	Positive	1.788	103.05708	4-aminoisoxazolidin-3-one	1.10	Alpha amino acids and derivatives
		2.442	188.07964	4-hydroxy-4-(pyridin-2-yl)butan-2-one	1.07	Pyridines and derivatives
		1.287	136.06839	Adenine	1.89	6-aminopurines
		1.758	120.08589	Phenylethanolamine	1.11	Aralkylamines
		0.656	177.14709	low score: 1-Benzylpiperazine	1.65	Phenylmethylanines
		1.816	164.07208	L-Phenylalanine	1.90	Phenylalanine and derivatives

Continued

Fractions	Types of detection	Average Rt (min)	Average Mz	Metabolite name	Total score	Ontology
Aqueous residual	Positive	1.758	120.08589	Phenylethanolamine	1.11	Aralkylamines
		2.332	177.15318	Geraniol	1.06	Acyclic monoterpenoids
		1.788	103.05708	4-aminoisoxazolidin-3-one	1.10	Alpha amino acids and derivatives
		0.617	104.10857	Choline	1.57	Cholines
		3.527	741.2276	Kaempferol-3-O-robinoside-7-O-rhamnoside	1.63	Flavonoid-7-O-glycosides
	Negative	3.521	739.20685	Kaempferol-3-O-galactoside-6"-rhamnoside-3	1.48	Flavonoid-3-O-glycosides
		0.934	290.08719	N-Fructosyl pyroglutamate	1.59	Proline and derivatives
		0.945	128.03532	L-Pyroglutamic acid	1.72	Alpha amino acids and derivatives
		6.321	307.07175	3-Hydroxy-N-(3-nitrophenyl) naphthalene-2-carboxamide	1.40	Naphthalene-2-carboxanilides
		1.258	130.08727	Leucine	1.74	Leucine and derivatives
		1.816	164.07208	L-Phenylalanine	1.90	Phenylalanine and derivatives
		5.527	619.16943	6-[(benzoyloxy)methyl]-3,4,5-trihydroxyoxan-2-yl] oxy}-6-hydroxy-8-methyl-9,10-dioxatetracyclo [4.3.1.0] decan-2-yl] methyl benzoate	1.55	Terpene glycosides
		0.649	116.07169	L-Valine	1.40	Valine and derivatives

Table 2. Metabolites in *Gnetum Gnemon* var. *Tenerum* crude extract and fraction as detected by LC-MS analysis.

Fraction	ABTS Radical Cation Scavenging Activity (mg AAE/g of dry extract)	IC50 Against ABTS (µg/ml)	DPPH Free Radical Scavenging Activity (mg AAE/g of dry extract)	IC50 Against DPPH (µg/ml)
Crude extract	439.77 ± 23.09	2245.00 ± 0.77	234.43 ± 6.55	4,043.00 ± 0.66
Diethyl ether	200.34 ± 15.11	3180.00 ± 0.43	690.47 ± 16.00	114.30 ± 0.21
Chloroform	422.06 ± 23.22	651.90 ± 0.59	632.11 ± 7.94	3,230.00 ± 1.09
Ethyl acetate	809.43 ± 3.51	125.30 ± 0.67	807.11 ± 2.60	77.11 ± 0.31
n-Butanol	690.10 ± 16.16	337.50 ± 0.58	794.23 ± 2.94	274.70 ± 0.35
Aqueous residual	149.66 ± 11.61	1697.00 ± 0.19	430.48 ± 15.93	905.30 ± 0.29

Table 3. The antioxidant activity of the crude extract and solvent-partitioned fractions of *G. gnemon* var. *Tenerum* at a concentration of 100–800 mg/mL was determined by DPPH and ABTS radical scavenging assays.

Extraction solvent (2 mg/disk)	Inhibition zone Mean ± SD (mm)				
	<i>E. faecalis</i> DMST 4331	<i>A. baumannii</i> DMST 49,606	<i>S. Typhi</i> DMST 22,842	<i>S. flexneri</i> ATCC 44,237	<i>K. pneumoniae</i> ATCC 27,731
Crude extract	6.5 ± 0.71	6.5 ± 0.71	6 ± 0.00	6 ± 0.00	6.5 ± 0.71
Diethyl ether	6 ± 0.00	20.5 ± 0.71	9 ± 1.41	8.5 ± 2.12	6 ± 0.00
Chloroform	19 ± 0.00	28 ± 0.00	16 ± 0.00	16 ± 0.00	14 ± 0.00
Ethyl acetate	14 ± 0.00	23 ± 1.41	11.5 ± 0.71	11.5 ± 0.71	7 ± 0.00
n-Butanol	6 ± 0.00	6 ± 0.00	8.5 ± 0.71	8.5 ± 0.71	6 ± 0.00
Aqueous residual	6 ± 0.00	6 ± 0.00	7 ± 1.41	6.5 ± 0.71	6 ± 0.00
Amikacin	9.5 ± 0.71	21 ± 0.00	23.5 ± 3.54	21 ± 0.00	20 ± 0.00
10% DMSO	6 ± 0.00	6 ± 0.00	6 ± 0.00	6 ± 0.00	6 ± 0.00

Table 4. The antibacterial activity of *G. gnemon* var. *Tenerum* extracts, determined by the disk diffusion method, varied depending on the bacterial strain and extraction solvent.

Antibacterial activity of *G. gnemon* against pathogens

The antibacterial activities of *G. gnemon* extracts obtained using different solvents were evaluated against five pathogenic bacterial strains using the disk diffusion method, and the inhibition zones are summarized in Table 4. Among the tested extracts, the chloroform fraction exhibited the strongest overall activity, producing the largest inhibition zone against *A. baumannii* DMST 49,606 (28.0 ± 0.00 mm), followed by *E. faecalis* DMST 4331 (19.0 ± 0.00 mm) and both *S. Typhi* DMST 22,842 and *S. flexneri* ATCC 44,237 (16.0 ± 0.00 mm each). The ethyl acetate fraction also demonstrated considerable antibacterial potential, with notable inhibition against *A. baumannii* DMST 49,606 (23.0 ± 1.41 mm), *E. faecalis* DMST 4331 (14.0 ± 0.00 mm), and *S. Typhi* DMST 22,842/*S. flexneri* ATCC 44,237 (11.5 ± 0.71 mm each).

	E. faecalis DMST 4331		A. baumannii DMST 49,606		S. Typhi DMST 22,842		S. flexneri ATCC 44,237		K. pneumoniae ATCC 27,731	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
Crude extract	12.5	>25	6.25	>25	12.5	>25	6.25	>25	12.5	>25
Diethyl ether	6.25	>25	0.39	25	3.125	12.5	1.563	12.5	1.536	25
Chloroform	0.195	>25	0.049	>25	0.195	>25	0.39	3.125	0.39	>25
Ethyl acetate	0.195	>25	0.098	6.25	0.39	>25	0.195	6.25	0.781	12.5
n-Butanol	0.781	>25	3.125	25	3.125	>25	6.25	25	12.5	>25
Aqueous residual	25	>25	6.25	>25	12.5	>25	12.5	>25	12.5	>25
Tetracycline	12.5	>25	0.098	25	25	>25	12.5	>25	1.563	25

Table 5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *G. gnemon* var. *Tenerum* extracts.

Concentration (mg/mL)	AGS cell						HIEC-6 cell						HT-29 cell					
	6.25	12.5	25	50	100	200	6.25	12.5	25	50	100	200	6.25	12.5	25	50	100	200
Crude extract	105.73	107.28	59.54	23.37	23.35	10.94	104.11	98.7	74.94	61.76	55.34	9.82	117.07	101.88	81.49	57.17	42.21	101
Diethyl ether	102.24	90.49	55.22	22	17.81	10.76	94.16	77.65	62.74	44.29	37.11	8.9	102.36	89.51	70.68	48.36	37.23	12.21
Chloroform	112.33	99.3	68.3	23.39	21.71	10.67	83.48	79.33	68.48	56.16	38.95	9.57	106.22	98.62	75.78	53.44	33.02	11.18
Ethyl acetate	116.63	124.99	69.24	31.9	21.15	10.24	107.69	98.26	79.59	57.21	42.89	9.77	114.35	101.26	82.23	55.64	38.37	12.19
n-Butanol	108.98	108.96	73.44	21.85	14.69	10.83	79.2	74.13	66.33	52.05	47.95	9.44	100.18	98.41	78.31	54.85	43.34	11.83
Aqueous residual	93.99	95.92	60.96	23.52	24.6	11.84	85.64	87.75	74.26	61.53	60.16	9.42	118.32	103.79	80.44	54.67	45.54	11.91

Table 6. Effects of crude extract and solvent-partitioned fractions of *G. gnemon* var. *Tenerum* on MTT-based metabolic activity of AGS, HT-29, and HIEC-6 cells following treatment with extract concentrations of 6.25–200 mg/mL.

The diethyl ether fraction showed the highest activity against *A. baumannii* DMST 49,606 (20.5 ± 0.71 mm), followed by *S. Typhi* DMST 22,842 (9.0 ± 1.41 mm) and *S. flexneri* ATCC 44,237 (8.5 ± 2.12 mm). In contrast, the methanol, n-butanol, and aqueous residual extracts exhibited weak antibacterial activity, with inhibition zones ranging from 6.0 to 8.5 mm, and no remarkable effect against most tested bacteria. Overall, chloroform extract displayed the most potent and broad-spectrum antibacterial activity, followed by ethyl acetate and diethyl ether fractions, suggesting that moderately non-polar solvent extracts contain the most active antibacterial constituents in *G. gnemon* var. *tenerum* leaves.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *G. gnemon* var. *Tenerum* extracts

The MIC and MBC values of *G. gnemon* var. *tenerum* extracts against *E. faecalis* DMST 4331, *A. baumannii* DMST 49,606, *S. enterica* serovar Typhi DMST 22,842, *S. flexneri* ATCC 44,237, and *K. pneumoniae* ATCC 27,731 are summarized in Table 5. For *E. faecalis*, chloroform and ethyl acetate extracts exhibited the lowest MIC values (0.195 mg/mL), followed by n-butanol extract (0.781 mg/mL). In *A. baumannii*, chloroform extract showed the highest activity with a MIC of 0.049 mg/mL, followed by ethyl acetate (0.098 mg/mL) and diethyl ether (0.39 mg/mL). For *S. Typhi*, the lowest MIC values were observed for chloroform and ethyl acetate extracts (0.195 mg/mL), followed by diethyl ether (3.125 mg/mL). In *S. flexneri*, ethyl acetate extract demonstrated the strongest inhibition (0.195 mg/mL), followed by chloroform (0.39 mg/mL) and diethyl ether (1.563 mg/mL). *K. pneumoniae*, chloroform extract exhibited the lowest MIC value (0.39 mg/mL), followed by ethyl acetate (0.781 mg/mL) and tetracycline (1.563 mg/mL). These results suggest that the chloroform and ethyl acetate extracts exhibited the most potent antibacterial activity against the tested pathogens, with MIC values ranging from 0.049 to 0.781 mg/mL. In contrast, MBC values were generally higher, often exceeding 25 mg/mL, indicating predominantly bacteriostatic rather than bactericidal effects.

Cytotoxic effects of *G. gnemon* var. *Tenerum* extracts on Gastrointestinal cell lines

The cytotoxic potential of the crude extract and solvent-partitioned fractions of *G. gnemon* var. *tenerum* was assessed against three gastrointestinal cell lines: AGS (gastric adenocarcinoma), HIEC-6 (normal intestinal epithelial), and HT-29 (colorectal adenocarcinoma) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were treated with extract concentrations ranging from 6.25 µg/mL to 25 µg/mL (Table 6). In AGS gastric adenocarcinoma cells, all extracts demonstrated minimal cytotoxicity at the lower concentration of 6.25 µg/mL, with cell viability observed to exceed 100%. This suggests a lack of toxicity or potentially a slight proliferative effect at low doses. However, at the highest concentration of 25 µg/mL, a notable dose-dependent reduction in cell viability was observed. Specifically, the crude extract, diethyl ether fraction, and chloroform fraction reduced AGS cell viability to 59.5%, 55.2%, and 68.3%, respectively. The ethyl

Sample	Nitric oxide concentration (mM) (Mean $\pm$ SEM)	
	LC ₅	LC ₁₀
Crude extract	26.19 $\pm$ 2.62	28.23 $\pm$ 0.72
Diethyl ether	24.25 $\pm$ 1.48	21.41 $\pm$ 0.92
Chloroform	5.36 $\pm$ 4.08	0.75 $\pm$ 1.01
Ethyl acetate	28.63 $\pm$ 1.43	15.48 $\pm$ 2.88
n-Butanol	29.83 $\pm$ 1.93	25.68 $\pm$ 1.95
Aqueous residual	30.89 $\pm$ 2.33	28.23 $\pm$ 0.72
Aspirin (5.42 mg/mL) + LPS	4.45 $\pm$ 1.00	
Negative control + LPS	29.12 $\pm$ 0.56	
Negative control wo LPS	-0.37 $\pm$ 1.20	

Table 7. Anti-inflammatory activity of crude extract and solvent-partitioned fractions of *G. gnemon* var. *tenerum*, measured as nitric oxide (NO) inhibition in LPS-stimulated RAW264.7 macrophages at LC₅ and LC₁₀ extract concentrations.

acetate and n-butanol fractions also exhibited moderate cytotoxicity at this concentration, leading to viabilities of 69.2% and 73.4%, respectively. HIEC-6 normal intestinal epithelial cells demonstrated a comparatively higher tolerance to the extracts. At 25 μ g/mL, the ethyl acetate and n-butanol fractions-maintained cell viability above 74%, indicating a more attenuated cytotoxic impact on normal cellular structures compared to cancerous cell lines. In contrast, HT-29 colorectal adenocarcinoma cells displayed significant sensitivity, particularly to certain fractions. At 25 μ g/mL, the crude extract and diethyl ether fractions decreased HT-29 cell viability to 81.5% and 70.7%, respectively. These findings collectively indicate a dose-dependent cytotoxic effect of *G. gnemon* var. *tenerum* extracts, with a promising degree of selectivity towards cancerous cell lines (AGS and HT-29).

Anti-inflammatory potential of *G. gnemon* var. *Tenerum* extracts

To assess the anti-inflammatory potential, the ability of the extracts to suppress NO production was evaluated in LPS-stimulated RAW264.7 macrophage-like cells. This evaluation was performed at concentrations corresponding to LC₅ and LC₁₀ of the extracts. Among all fractions, the ethyl acetate fraction showed the most pronounced inhibition of NO production, reducing NO levels to 15.48 $\pm$ 2.88 mM at LC₁₀. This reduction was statistically significant compared to control (18.01 $\pm$ 0.77 mM). The chloroform fraction was also highly effective, particularly at LC₁₀ (0.75 $\pm$ 1.01 mM), suggesting a strong suppressive effect on NO synthesis. The crude extract and diethyl ether fraction demonstrated moderate inhibition, while the aqueous residual fraction exhibited the least anti-inflammatory effect. These findings indicate that specific fractions of *G. gnemon* var. *tenerum*, particularly the ethyl acetate and chloroform fractions, possess promising anti-inflammatory properties, likely mediated via the suppression of inducible nitric oxide synthase (iNOS) activity (Table 7).

Discussion

The present study evaluated the pharmacological properties of the crude extract and solvent-partitioned fractions of *G. gnemon* var. *tenerum*. To establish the chemical basis underlying these activities, the investigation began with quantifying the TPC and TFC across all fractions, together with LC-MS metabolite profiling to identify major constituents within each fraction. The ethyl acetate and n-butanol fractions exhibited the highest TPC and TFC values, indicating that moderately polar solvents were most effective in concentrating polyphenolic constituents. This pattern is consistent with extensive evidence demonstrating that phenolics and flavonoids contribute significantly to antioxidant, anti-inflammatory, and cytotoxic responses^{14,15}. These compounds exert their biological effects through multiple mechanisms, including radical scavenging, modulation of cell-signaling pathways, and regulation of gene expression¹⁶. The comparatively lower TPC and TFC values observed in the crude extract and in the non-polar (diethyl ether) or highly polar (aqueous residual) fractions further validate the effectiveness of the fractionation strategy employed in this study.

The potent antioxidant potential observed in the ABTS and DPPH assays, particularly in the ethyl acetate and n-butanol fractions, correlates strongly with their phenolic and flavonoid content. Phenolic compounds exert antioxidant effects primarily through hydrogen atom or electron donation, thereby neutralizing reactive oxygen species and interrupting oxidative chain reactions¹⁷. Oxidative stress, characterized by an imbalance between reactive species and antioxidant defense mechanisms, is implicated in the pathogenesis of numerous diseases including cancer, cardiovascular disorders, neurodegeneration, and chronic inflammation¹⁸. Given the pronounced radical-scavenging capacity of the studied fractions, particularly the ethyl acetate fraction, these extracts hold promise as prophylactic agents against oxidative stress-related conditions.

LC-MS analysis provided further insights into the metabolite composition of the extracts and fractions. The detection of flavonoid glycosides, particularly kaempferol derivatives, aligns with the strong antioxidant and anti-inflammatory activities observed, as these compounds are well-established radical scavengers and modulators of NF- κ B/iNOS signaling pathways^{19,20}. The presence of phenylethanamine, detected across several fractions, may contribute to both antimicrobial and cytotoxic effects through its roles in membrane interaction and cellular stress signaling¹⁹. Additionally, monoterpenoids such as geraniol and alkaloid-like

metabolites including 2-piperidone, found predominantly in the chloroform and ethyl acetate fractions, are known to exert antibacterial activity by disrupting microbial membranes and impairing energy metabolism^{21,22}. Collectively, these metabolites provide a plausible mechanistic basis for the antioxidant, antibacterial, and anti-inflammatory properties demonstrated in this study.

Moreover, the crude extract and solvent fractions exhibited promising antibacterial effects, consistent with the presence of bioactive phytochemicals capable of disrupting microbial growth and survival. Such activity highlights the potential application of these fractions as natural antimicrobial agents, which may serve as alternatives or adjuncts to conventional antibiotics. The antibacterial potency was further supported by the MIC and MBC assays, which confirmed that certain fractions were able to inhibit bacterial proliferation and, at higher concentrations, eradicate bacterial cells. These effects may be attributed to phenolic²³ and flavonoid-rich constituents that are known to disrupt bacterial cell membranes and interfere with essential metabolic enzymes, leading to impaired cellular integrity and reduced energy production²⁴. Nevertheless, the generally high MBC values observed for most fractions indicate a predominantly bacteriostatic rather than bactericidal effect, which is an important consideration for future therapeutic applications.

Our investigation demonstrated that both the crude extract and solvent-derived fractions exert dose-dependent cytotoxic effects on gastrointestinal cancer cell lines, including AGS (gastric adenocarcinoma) and HT-29 (colorectal adenocarcinoma). The diethyl ether and chloroform fractions were notably effective in reducing AGS cell viability, while the diethyl ether fraction also displayed pronounced activity against HT-29 cells. These findings align with previous reports highlighting the anticancer potential of phytochemicals from natural sources, which often function via multiple mechanisms including induction of apoptosis, inhibition of proliferation, and modulation of redox status²⁵. Of particular interest was the observed selectivity of these extracts, as significantly lower cytotoxic effects were recorded in normal intestinal epithelial cells (HIEC-6) compared to cancerous cell lines. This differential activity is consistent with earlier studies suggesting that certain phytochemicals preferentially target malignant cells due to their altered metabolism and signaling pathways²⁶. Such selectivity is a desirable trait in novel anticancer agents, potentially reducing systemic toxicity and improving therapeutic index.

An intriguing observation in AGS cells was the enhancement of cell viability beyond 100% at the lowest tested dose (6.25 µg/mL), suggesting a potential hormetic effect. This biphasic response, whereby low doses stimulate beneficial effects while higher doses are inhibitory, is well documented in toxicological and pharmacological studies of natural products^{27,28}. This underscores the complex dose–response relationships characteristic of plant-derived compounds and warrants further mechanistic investigation.

Significant anti-inflammatory activity was also demonstrated by the extracts, as evidenced by their suppression of NO production in LPS-stimulated RAW264.7 macrophage-like cells. Elevated NO levels, driven by inducible nitric oxide synthase (iNOS), are central mediators of inflammatory processes and tissue injury in chronic inflammatory diseases²⁹. The ethyl acetate and chloroform fractions significantly reduced NO production, suggesting the presence of bioactive compounds capable of interfering with inflammatory signaling pathways. Prior research has shown that plant-derived flavonoids and phenolics can downregulate iNOS expression or directly inhibit its catalytic activity^{30,31}, mechanisms that may underlie the observed effects.

Thus, the selective cytotoxicity toward cancer cells, strong antioxidant capacity, and robust anti-inflammatory activity suggest a multi-target therapeutic potential for *G. gnemon* var. *tenerum* extracts. However, certain limitations of this study must be acknowledged. All bioactivity assays were conducted in vitro, which, while informative, do not fully replicate in vivo physiological complexity. In addition, although the TPC and TFC were quantified, the specific phytochemicals responsible for the observed bioactivities remain unidentified. Further studies employing the detailed mechanistic studies, including gene expression profiling, enzyme activity assays, and pathway-specific investigations, are warranted to delineate the precise biological targets and modes of action.

Conclusion

In conclusion, this study highlights the pharmacological potential of *G. gnemon* var. *tenerum*. The ethyl acetate and n-butanol fractions exhibited the highest phenolic and flavonoid contents, corresponding with strong antioxidant activity. LC–MS profiling revealed flavonoid glycosides, phenylethanamine derivatives, and other bioactive metabolites that support these effects. The extracts demonstrated antibacterial activity, particularly in the chloroform and ethyl acetate fractions. Cytotoxicity assays showed dose-dependent inhibition of AGS and HT-29 cancer cells with lower effects on normal cells, indicating selectivity. Anti-inflammatory assays further confirmed nitric oxide suppression in LPS-stimulated macrophages. Future work should focus on isolating active compounds, clarifying molecular mechanisms, and validating efficacy and safety in in vivo models.

Data availability

The data supporting the findings of this study are available within the article.

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Author contributions

Conceptualization CR and MS methodology: NK, and CR formal analysis: TT, NK, WM and CR investigation: TT, WM and CR resource: CR data curation: TT, NK, WM and CR writing—original draft preparation: TT, NK, WM and CR writing—review and editing: TT, NK, WM, MS and CR supervision: CR project administration: CR funding acquisition: CR. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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