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IN VITRO SCREENING OF CYTOTOXIC ACTIVITY OF VIETNAMESE MEDICINAL PLANTS

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Summary

Cancer remains a leading cause of mortality worldwide, and the discovery of new anticancer agents is crucial for improving therapy. Natural products, including medicinal plants, are promising sources of bioactive compounds with potential anticancer effects. In this study, twenty-six 70% ethanol extracts from twenty-three species were initially screened for cytotoxicity against A549 and MCF-7 cells, leading to the selection of two promising extracts, *Piper longum* and *Cratoxylum cochinchinense*, for further evaluation against eight cell lines, including seven cancer cell lines and one non-cancerous cell line (JB6 CI41). Phytochemical investigation of the ethyl acetate fraction of *C. cochinchinense* yielded five xanthones with moderate cytotoxic activity, whereas the ethyl acetate fraction of *P. longum* afforded alkaloids with stronger cytotoxic activity. Among the compounds isolated from *P. longum*, guineensine was identified as the most potent compound (IC_{50}: 2.17–4.47 μM), followed by piperine (IC_{50}: 3.82–7.99 μM) and methyl piperate (IC_{50}: 4.37–9.45 μM). These compounds are proposed as bioactive markers for the development of standardized extraction procedures and quality control. Overall, this study expands the knowledge of bioactive compounds from medicinal plants and identifies potential anticancer agents.

Keywords: *Cytotoxicity; Vietnamese medicinal plants; Piper longum; Guineensine*.

1. Introduction

Currently, cancer remains one of the biggest challenges in modern medicine and is a leading cause of death worldwide. According to the International Agency for Research on Cancer (IARC, WHO), about 20 million new cancer cases were diagnosed in 2022, up from 18 million in 2020, with the most common types being lung (2.5 million), breast (2.3 million), colorectal (1.9 million), prostate (1.5 million), and stomach cancer (970,000). In Vietnam, cancer incidence is among the highest in the world, with 180,480 new cases and 409,144 existing cases in 2022. The most frequent cancers were breast, liver, lung, colorectal, and stomach cancer, with liver, lung, stomach, colorectal, and prostate cancers accounting for about 64.3% of all cases in men, and breast, lung, colorectal, stomach, and liver cancers accounting for about 60.1% in women [1]. Recent advances in science and technology have revolutionized cancer treatment, leading to more targeted and effective therapies. Concurrently, the development of novel drugs from medicinal plants has emerged as a promising frontier in the pharmaceutical industry. From 1981 to 2019, approximately 25% of all newly approved anticancer drugs were derived from natural products [2].

To date, few studies have investigated the anticancer activity of Vietnamese medicinal plants. In developed countries, research on screening and developing anticancer drugs from medicinal plants has been conducted since the 1950s. In Vietnam, such research has only attracted attention in the past decade. Despite the rich diversity of medicinal plants in Vietnam, studies on their anticancer potential are still limited. Therefore, twenty-three medicinal plant species were selected based on their traditional medicinal use, available evidence of anticancer activity, and their potential for the discovery of new anticancer agents. Species with reported anticancer activity were included for further evaluation, whereas those with limited or no previous anticancer studies were selected to explore their cytotoxic potential. The investigated

plant parts were chosen according to their traditional medicinal use and available phytochemical or pharmacological evidence. In this study, we evaluated the effects of 70% ethanol extracts, fractionated extracts, and isolated compounds from the selected medicinal plants against eight cancer cell lines (A549, MCF-7, HT-29, MB-49, HepG2, MDA-MB-231, K-562, and HL-60) and one non-cancerous cell line (JB6 Cl41) using the MTT assay, aiming to identify potential candidates for the development of anticancer agents.

2. Materials and Methods

2.1. Plant material

The medicinal plants (Table 1) were collected in Vietnam between 2018 and 2021. The plants were taxonomically identified by the Center of Medicinal Material Resources, National Institute of Medicinal Materials (NIMM), where the voucher specimens were deposited. The dried samples were ground into fine powder and stored at the Department of Analytical Chemistry and Standardization, NIMM.

2.2. Preparation of extracts, fractionated extracts, and compounds

Plant materials were cut or ground into coarse powder (<0.5 cm) and extracted twice with 70% ethanol under heating at 70°C for 2 h each (solvent-to-material ratio 8:1, v/w). A 70% ethanol solution was selected because it efficiently extracts a broad range of phytochemicals with different polarities. The combined filtrates were concentrated under reduced pressure to yield the crude extract, from which the extraction yield and moisture content were determined. The extract was sealed in polyethylene bags, labeled, and stored at 4–8°C for further assays.

The crude extract was suspended in water (1 g/2 mL) and successively partitioned with *n*-hexane and ethyl acetate (three times each, solvent-to-aqueous ratio 1:1, v/v). Successive partitioning with *n*-hexane and ethyl acetate was performed to separate compounds according to their polarity, facilitating bioactivity-guided fractionation and the isolation of active constituents. The organic layers were concentrated under reduced pressure to obtain the *n*-hexane and ethyl acetate fractions, while the residual aqueous layer was concentrated at 75°C to yield the water fraction. All fractions were obtained as solid extracts with moisture content below 20%.

Based on the cytotoxic screening results, the active fractions were selected for further phytochemical investigation. The compounds were isolated from the potential fractions using chromatographic separation methods, including column chromatography, and their structures were identified based on spectroscopic analyses and comparison with reported data. The isolated compounds were subsequently evaluated for their cytotoxic activities against cancer cell lines.

2.3. Instruments, chemicals, and cancer cell lines

An ELISA plate reader (Molecular Devices, Menlo Park, CA, USA), 96-well plates (Corning Inc., USA; Tecan Group Ltd., Switzerland), and pipettes (Eppendorf, Germany) were used. Dimethyl sulfoxide (DMSO), doxorubicin, and MTT (M2003-1G) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and Fetal calf serum (FCS) (#10309433) were obtained from Fisher Scientific (USA). Cancer cell lines including human cancer cell lines A549 (lung adenocarcinoma), MCF-7 (breast adenocarcinoma), HT-29 (colorectal adenocarcinoma), HepG2 (hepatocellular carcinoma), MDA-MB-231 (triple-negative breast cancer), K562 (chronic myelogenous leukemia), and HL-60 (promyelocytic leukemia), as well as murine MB-49 (bladder carcinoma) and JB6 CI41 (mouse epidermal cells), were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

2.4. Cytotoxic assay

The 70% ethanol extracts, fractionated extracts (*n*-hexane, ethyl acetate, and water), and isolated compounds were dissolved in DMSO to prepare stock solutions and evaluated for cytotoxicity using the MTT assay as previously described [3]. Briefly, the cell lines (A549, MCF-7, HT-29, MB-49, HepG2, MDA-MB-231, JB6 CI41, K562, and HL-60) were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated at 37°C with 5% CO_2 for 24 h. Cells were then treated for 48 h with the 70% ethanol extract (20 μg/mL), fractionated extracts (1–50 μg/mL), or isolated compounds (1–50 μM). After treatment, MTT solution (0.5 mg/mL in phosphate-buffered saline) was added, and the plates were further

incubated for 4 h. Formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using an ELISA plate reader. The percentage of growth inhibition was calculated as:

$$(\%)\ \text{inhibition} = 100\% - \frac{OD_{sample} - OD_{blank}}{OD_{DMSO} - OD_{blank}} \times 100$$

Doxorubicin was used as a positive control and was tested at various concentrations. Cytotoxic effects were assessed in five independent experiments, and IC_{50} values were calculated from the percent inhibition of cell growth using nonlinear regression analysis by GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

2.5. Statistical analysis

Quantitative data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test, using GraphPad Prism. A p-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Extract preparation results

Twenty-six 70% ethanol extracts from 23 Vietnamese medicinal plants were prepared and evaluated for their cytotoxic activity. The results are presented in **Table 1**.

Table 1. Results of preparation of 70% ethanol extract and fractions

No.	Code	Scientific name	Extract	$m_{Plant\ material}$ (g)	$m_{Extract}$ (g)	Extraction yield (%)	Moisture content (%)
1	**N1**	Twigs and leaves of *Psychotria asiatica*	70% Ethanol	500	149.55	29.91	12.5
2	**N2**	Fruits of *Canarium album*	70% Ethanol	2065	292.10	14.15	4.5
3	**N3**	Bulbs of *Allium ascalonicum*	70% Ethanol	1157	221.10	19.11	15.6
4	**N6**	Stems of *Gomphandra tetrandra*	70% Ethanol	500	46.95	9.39	14.1
5	**N7**	Roots of *Gomphandra tetrandra*	70% Ethanol	500	120.15	24.03	12.6
6	**N8**	Stems and leaves of *Wikstroemia indica*	70% Ethanol	500	28.45	5.69	11.5
7	**N9**	Roots of *Wikstroemia indica*	70% Ethanol	5000	74.95	1.50	15.7
8	**N10**	Fruiting body of *Ganoderma cochlear*	70% Ethanol	500	30.95	6.19	7.2
9	**N11**	Fruiting body of *Ganoderma applanatum*	70% Ethanol	500	22.00	4.40	8.1
10	**N12**	Fruits of *Piper longum*	70% Ethanol	520	52.40	10.08	23.6
	N12.1		*n*-Hexane		0.50	2.50	
	N12.2		Ethyl acetate		5.65	28.25	
	N12.3		Aqueous		5.85	29.25	
11	**N13**	Rhizomes of *Abelmoschus rhodopetalus*	70% Ethanol	250	100.90	40.36	17.4
12	**N14**	Twigs and leaves of *Cratoxylum cochinchinense*	70% Ethanol	500	149.70	29.94	9.2
	N14.1		*n*-Hexane		0.10	0.50	
	N14.2		Ethyl acetate		5.10	25.50	
	N14.3		Aqueous		5.20	26.00	
13	**N15**	Fruits of *Amomum aromaticum*	70% Ethanol	500	41.20	8.24	10.6
14	**N16**	Fruits of *Zanthoxylum rhetsa*	70% Ethanol	750	118.10	15.75	18.9
15	**N17**	Rhizomes of *Panax vietnamensis* var. *fuscidiscus*	70% Ethanol	100	29.80	29.80	9.2
16	**N18**	Aerial parts of *Isodon lophanthoides*	70% Ethanol	500	50.80	10.16	13.6

17	**N19**	Twigs and leaves of *Dregea volubilis*	70% Ethanol	400	42.00	10.50	12.6
18	**N20**	Fruits of *Dregea volubilis*	70% Ethanol	250	22.40	8.96	10.4
19	**N21**	Fruiting body of *Coriolus versicolor*	70% Ethanol	1000	55.15	5.52	8.9
20	**N22**	Fruiting body of *Ganoderma tropicum*	70% Ethanol	500	30.15	6.03	10.2
21	**N24**	Aerial parts of *Launaea sarmentosa*	70% Ethanol	500	126.40	25.28	8.0
22	**N29**	Leaves of *Clausena indica*	70% Ethanol	1000	134.80	13.48	14.5
23	**N30**	Stem and leaves of *Perilla frutescens*	70% Ethanol	2500	88.30	3.53	25.1
24	**N33**	Rhizomes of *Paris vietnamensis*	70% Ethanol	176	31.40	17.84	6.3
25	**N37**	Rhizomes of *Curcuma zedoaroides*	70% Ethanol	367	22.40	6.10	7.5
26	**N38**	Leaves of *Boehmeria nivea*	70% Ethanol	2500	53.50	2.14	8.1

3.2. Bioassay results

The cytotoxic activity of twenty-six 70% ethanol extracts was evaluated against two human cancer cell lines, A549 and MCF-7, with the results summarized in **Figs. 1** and **2**.

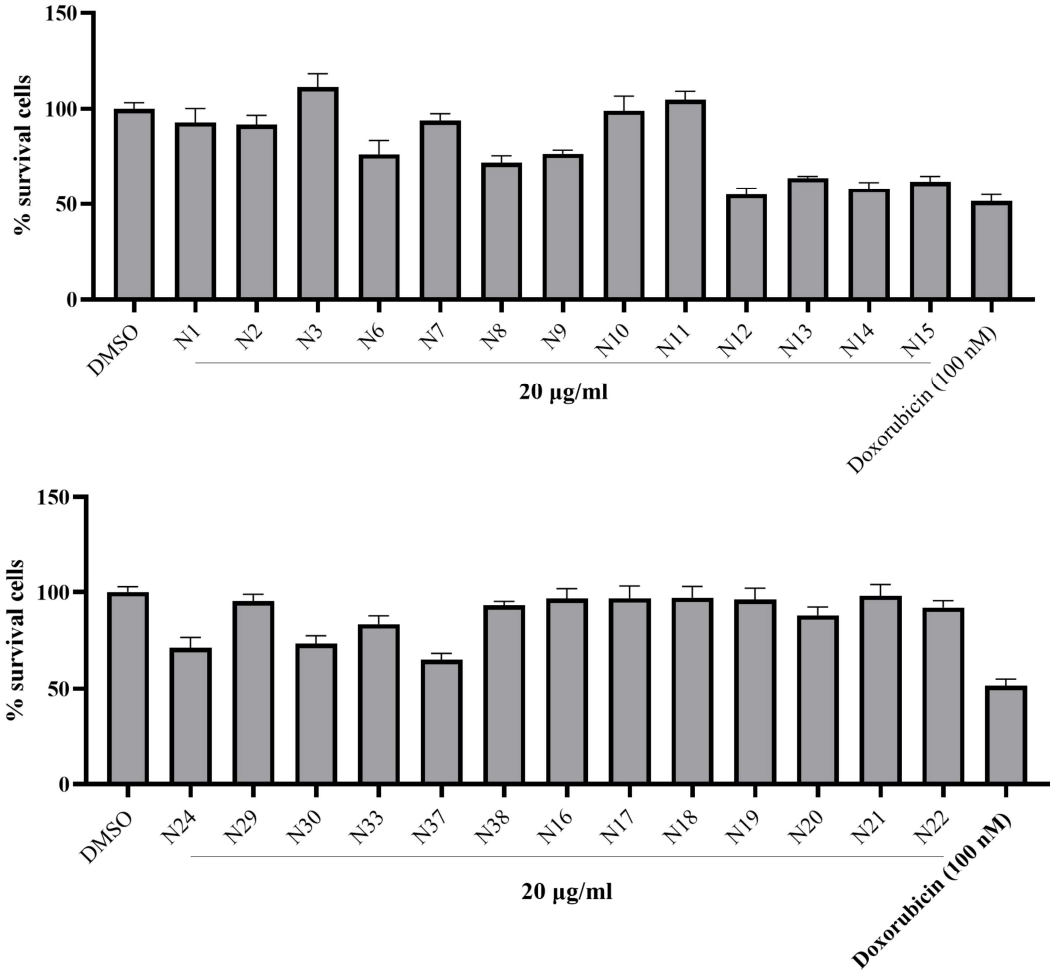


Fig. 1. *In vitro* cytotoxic activity of 70% ethanol extracts against A549 cell lines

Fig. 1 shows that the 70% ethanol extracts (20 µg/mL) exhibited varying cytotoxicity against A549 cells. Among them, N12 (fruits of *P. longum*), N13 (rhizomes of *A. rhodopetalus*), N14 (twigs and leaves of *C. cochinchinense*), N15 (fruits of *A. aromaticum*), and N37 (rhizomes of *C. zedoaroides*) showed similar effects, with surviving cell percentages of 55.00%, 63.82%, 58.26%, 61.80%, and 64.96% respectively, comparable to Doxorubicin (100 nM, 51.52%). Some extracts exhibited moderate cytotoxicity, including N24 (aerial parts of *L. sarmentosa*, 71.06%), N8 (twigs and leaves of *W. indica*, 71.84%), N30 (stem and leaves of *P. frutescens*, 73.20%), N6 (twigs of *G. tetrandra*, 76.30%), and N9 (roots of *W. indica*, 76.48%). Others displayed weaker or no effects.

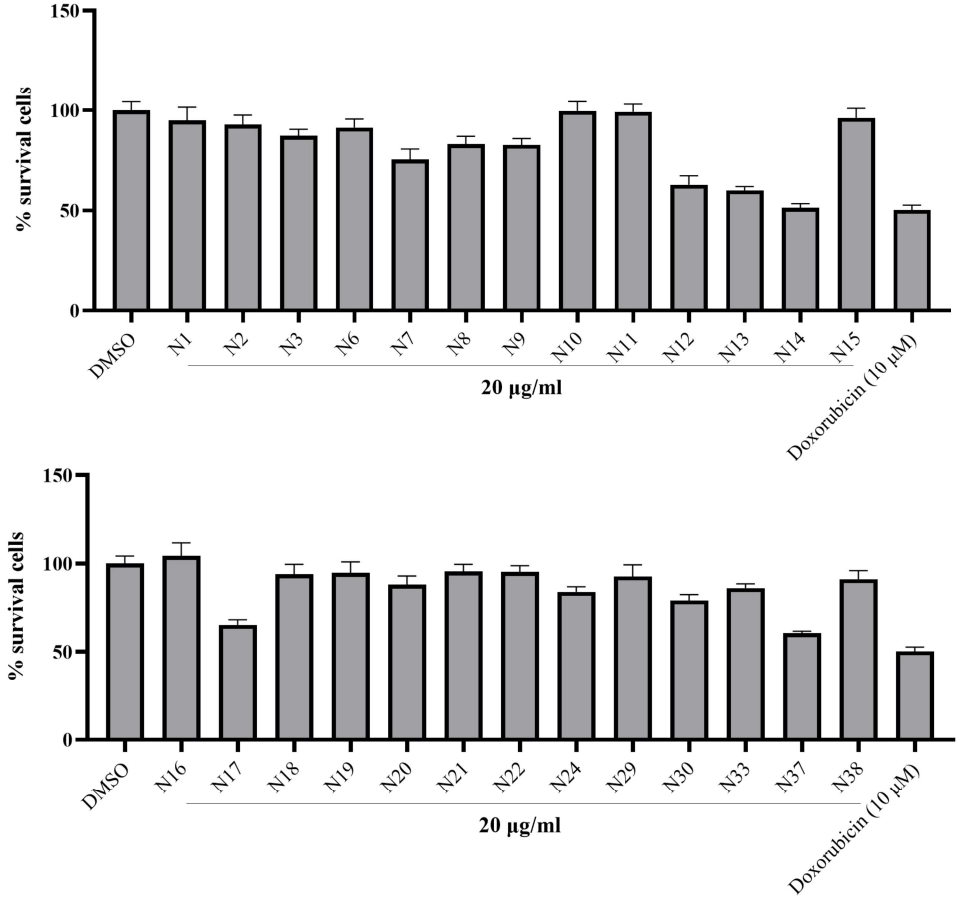


Fig. 2. *In vitro* cytotoxic activity of 70% ethanol extracts against MCF-7 cell lines

Fig. 2 illustrates the cytotoxic effects of the 70% ethanol extracts (20 µg/mL) on MCF-7 cells, showing variation in their activity. In particular, N12, N13, N14, N17 (rhizomes of *P. vietnamensis* var. *fuscidiscus*), and N37 exhibited activity, with cell viability of 62.81%, 59.90%, 51.25%, 65.25%, and 60.58%, respectively, comparable to doxorubicin (10 µM, 50.21%). Several extracts showed moderate activity, including N7 (roots of *G. tetrandra*, 75.37%) and N30 (79.07%). Other extracts possessed weaker or negligible effects.

Screening of cytotoxic activity against A549 and MCF-7 cell lines identified four samples (N12, N13, N14, and N37) as the most active, consistent with previous reports on these species. N12 (*Piper longum*) contains alkaloids such as piplartin, piperine, and piperlonguminin, which have been reported to induce apoptosis, inhibit metastasis, suppress tumor growth *in vivo*, and exhibit chemopreventive effects, including protection of cell membranes and inhibition of inflammatory responses [4],[5],[6],[7]. N13 (*A. rhodopetalus*) remains less studied; However, related species (e.g., *H. sabdariffa*, *H. taiwanensis*) have demonstrated antiproliferative activity, induction of apoptosis *via* mitochondrial ROS, and inhibition of Bcl-2 and phosphorylated

RB in various cancer cell lines [8],[9],[10]. N14 (*Cratoxylum cochinchinense*) displayed pronounced cytotoxicity across multiple cancer cell lines, attributed mainly to its xanthone content, with some compounds demonstrating greater potency than cisplatin and selective effects against A549 and leukemia cell lines [11],[12],[13],[14],[15]. N37 (*Curcuma zedoaroides*) has been less studied, but within the *Curcuma* genus, sesquiterpenes such as furanodiene and curcuminoids have shown strong cytotoxicity, induced cell cycle arrest and apoptosis, enhanced the effect of chemotherapeutics, and inhibited tumor growth in xenograft models [16]. Nevertheless, N13 is challenging to standardize due to difficulties in isolating and identifying chemical markers, and N37's constituents are unstable [17], complicating reproducible extraction. To focus on both identifying potential anticancer agents and establishing standardized extraction procedures and quality control for raw materials and final products, we selected N12 and N14 for further evaluated on six additional cell lines (MDA-MB-231, HepG2, MB-49, JB6 CI41, K562, and HT-29). The results are presented in **Table 2**.

Table 2. *In vitro* cytotoxic activity of the fractionated extracts against eight cancer cell lines

Samples	IC_{50} (µg/mL)							
	A549	**MCF-7**	**MDA-MB-231**	**HepG2**	**MB-49**	**JB6 CI41**	**K562**	**HT-29**
N12.1	10.11 ± 0.13	7.80 ± 0.74	10.37 ± 1.17	12.86 ± 1.12	8.81 ± 1.15	10.62 ± 0.85	8.44 ± 0.67	12.09 ± 0.85
N12.2	4.78 ± 0.39	7.97 ± 0.65	7.19 ± 1.24	6.74 ± 1.35	8.96 ± 1.34	6.52 ± 0.71	6.03 ± 0.85	9.50 ± 0.76
N12.3	9.20 ± 1.06	9.44 ± 1.17	9.07 ± 1.02	9.10 ± 1.67	10.60 ± 1.62	12.72 ± 1.22	7.47 ± 1.28	14.25 ± 1.71
N14.1	10.09 ± 1.41	13.26 ± 1.21	16.35 ± 1.28	14.45 ± 1.57	10.74 ± 1.52	10.07 ± 2.57	13.96 ± 1.71	10.47 ± 1.34
N14.2	5.16 ± 0.67	5.91 ± 0.57	8.16 ± 0.98	6.86 ± 0.89	4.30 ± 0.82	8.33 ± 1.01	6.51 ± 0.73	7.65 ± 0.96
N14.3	12.94 ± 1.63	8.99 ± 1.52	11.79 ± 1.02	14.59 ± 0.31	6.96 ± 0.35	12.24 ± 0.93	9.67 ± 1.03	13.79 ± 1.31
Doxorubicin	0.043 ± 0.003	4.28 ± 1.03	3.56 ± 0.96	0.13 ± 0.02	0.57 ± 0.13	0.94 ± 0.19	2.65 ± 0.51	7.51 ± 0.45

Note: *N12.1, N12.2, and N12.3 are the n-hexane, ethyl acetate, and aqueous fractions, respectively, of the 70% ethanol extract of P. longum fruits. N14.1, N14.2, and N14.3 are the n-hexane, ethyl acetate, and aqueous fractions, respectively, of the 70% ethanol extract of C. cochinchinense twigs and leaves.*

Table 2 shows that the extracts exhibited varying cytotoxic activities against the eight cell lines. For the fruits of *P. longum*, N12.2 showed the highest cytotoxic activity against A549, MDA-MB-231, HepG2, JB6 CI41, K562, and HT-29 cells, whereas N12.1 was the most active against MCF-7 and MB-49 cells. For the twigs and leaves of *C. cochinchinense*, N14.2 showed the highest cytotoxic activity against all eight cell lines. Overall, the ethyl acetate fractions N12.2 and N14.2 exhibited generally strong and comparable cytotoxic activities, with IC_{50} values ranging from 4.78 to 9.50 µg/mL and 4.30 to 8.33 µg/mL, respectively. These fractions were therefore selected for further compound isolation and cytotoxic evaluation.

The isolation of compounds from N14.2 and their cytotoxicity were reported in a previous study [18]. Five xanthones were obtained, and their structures were determined as 1,8-dihydroxy-3-methoxy-6-methylxanthone (**CC1**), cochinchinone A (**CC2**), 1,3,7-trihydroxy-2,4-diisoprenylxanthone (**CC3**), *α*-mangostin (**CC4**), and 1,3,5,6-trihydroxy-7-isoprenylxanthone (**CC5**). Among these, **CC2**–**CC4** demonstrated cytotoxic effects against three cancer cell lines (A549, MCF-7, and HepG2), with IC_{50} values ranging from 10.24 to 16.86 µM. In contrast, **CC5** exhibited selective cytotoxicity against MCF-7 and HepG2 cancer cell lines, with IC_{50} values of 13.55 ± 0.74 and 16.69 ± 0.81 µM, respectively. These results indicated that the compounds isolated from *C. cochinchinense* were obtained in low amounts and showed moderate cytotoxic activity.

In addition, seven compounds were isolated from the ethyl acetate extract of the fruits of *P. longum* (N12.2) (**PL1**–**PL7**). These compounds

were identified as avenalumic acid methyl ester (**PL1**), methyl piperate (**PL2**), piperine (**PL3**), pipernonaline (**PL4**), (2*E*,4Z,8*E*)-*N*-[9-(3,4-methylenedioxyphenyl)-2,4,8-nonatrienoyl] piperidine (**PL5**), pipercide (**PL6**), and guineensine (**PL7**) [19]. Five compounds (**PL2**–**PL4**, **PL6**, and **PL7**, **Fig. 3**) were tested *in vitro* for their cytotoxic activity against eight cancer cell lines: A549, MCF-7, MDA-MB-231, HepG2, MB-49, JB6 CI41, K562, and HL-60. The results are shown in **Table 3**.

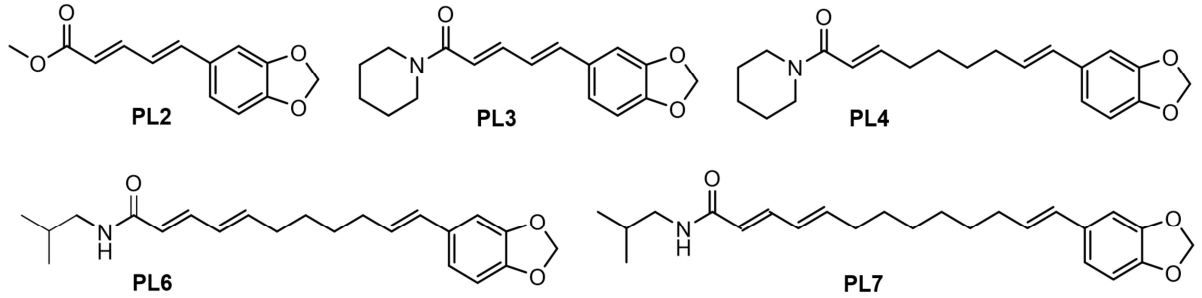


Fig. 3. Chemical structure of compounds (**PL2**–**PL4**, **PL6**, and **PL7**) isolated from the fruits of *P. longum*

Table 3. *In vitro* cytotoxic activity of compounds isolated from *P. longum* fruits against cancer cell lines

Samples	IC_{50} (μM)							
	A549	**MCF-7**	**MDA-MB-231**	**HepG2**	**MB-49**	**JB6 CI41**	**K562**	**HL-60**
PL2	**4.81 ± 0.92**	7.42 ± 0.96	7.94 ± 0.68	9.45 ± 1.08	5.16 ± 1.97	**4.37 ± 2.10**	9.14 ± 1.68	7.49 ± 1.24
PL3	5.21 ± 1.03	7.52 ± 0.84	7.99 ± 0.85	7.70 ± 1.19	**4.54 ± 1.32**	**3.82 ± 2.49**	7.77 ± 1.94	7.56 ± 0.98
PL4	**4.73 ± 0.84**	10.43 ± 1.34	7.35 ± 1.45	6.90 ± 1.93	9.55 ± 1.84	8.44 ± 1.68	9.10 ± 1.60	6.50 ± 2.32
PL6	8.13 ± 1.34	10.86 ± 1.48	11.51 ± 1.94	11.09 ± 0.68	7.07 ± 1.17	6.12 ± 1.48	11.31 ± 1.01	11.25 ± 1.84
PL7	**2.85 ± 0.84**	**4.25 ± 0.54**	**4.47 ± 0.63**	**4.32 ± 0.91**	**2.57 ± 0.70**	**2.17 ± 0.96**	**3.51 ± 0.79**	**4.15 ± 0.74**
Doxorubicin	0.079 ± 0.005	7.87 ± 1.89	6.56 ± 1.76	0.24 ± 0.28	1.04 ± 0.24	1.72 ± 0.35	4.87 ± 0.93	0.065 ± 0.002

Table 3 shows that the five compounds exhibited different levels of cytotoxicity against the eight tested cell lines. Compound **PL7** (guineensine) was the most potent, with IC_{50} values of 2.17–4.47 μM, followed by compounds **PL3** (3.82–7.99 μM) and **PL2** (4.37–9.45 μM). Compounds **PL4** (4.73–10.43 μM) and **PL6** (6.12–11.51 μM) displayed weaker activity compared to the other compounds. Most compounds showed the strongest effects on the JB6 CI41 cell line, with activities differing among the other cell lines.

These findings are consistent with previous evidence suggesting that *P. longum* constituents, particularly guineensine and piperine, play an important role in its bioactivities, although their anticancer potential has not been fully characterized [20],[21],[22]. In our previous study [23], we evaluated the standardized extract of *P. longum* fruit (PLF-SE) and its components (piperine (**PL3**), pipernonaline (**PL4**), and guineensine (**PL7**)) against human breast cancer stem cells (CSCs). HPLC-DAD analysis showed that **PL3**, **PL4**, and **PL7** accounted for 25.04%, 2.91%, and 0.61% of PLF-SE, respectively. Treatment with PLF-SE (20 μg/mL), piperine (2.5 μM), or guineensine (5 μM) for 48 h significantly reduced the CSC population, inhibited spheroid formation, and downregulated key CSC-related proteins (Oct4, Nanog, Sox2, EpCAM, PARP). Molecular docking of two active compounds supported these *in vitro* findings [23]. Together, these results suggest that *P. longum* fruits and their major constituents possess promising anticancer potential, warranting further *in vivo* evaluation. In addition, **PL3**, **PL4**, and **PL7** have been further investigated for their anticancer mechanisms and proposed as chemical markers for standardized extraction and quality control.

Therefore, this study expands current knowledge on the biological activities of *P. longum* and highlights specific compounds with potential as anticancer agents.

4. Conclusion

In this study, twenty-six 70% ethanol extracts from twenty-three species and six fractionated extracts were screened for cytotoxic activity against various cancer cell lines. Among the tested samples, the ethyl acetate fractions of *P. longum* and *C. cochinchinense* exhibited the strongest cytotoxic activities. The phytochemical investigation of these active fractions revealed xanthones from *C. cochinchinense* with moderate cytotoxic activity and alkaloids from *P. longum* with stronger cytotoxic activity. Among the isolated alkaloids from *P. longum*, guineensine was the most potent, followed by piperine and methyl piperate.

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