RESEARCH PAPER

PHYTOCHEMICAL SCREENING, ANTIOXIDANT POTENTIAL, AND *IN-VITRO* CYTOTOXIC ACTIVITY OF *OCIMUM TENUIFLORUM* AND *PIPER BETEL* LEAVES AND THEIR COMBINED EXTRACTS

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Abstract

Objective: This study set out to characterize the phytochemical profile, DPPH antioxidant activity, and MTT cytotoxic selectivity of successive solvent extracts of *Ocimum tenuiflorum* and *Piper betel* leaves — evaluated individually and as 1:1 (v/v) polarity-matched combinations — against HeLa, MCF-7, and HEK293 cell lines, with the selectivity index (SI) serving as the quantitative measure of differential cancer-cell toxicity.

Methods: Leaves of both species underwent sequential Soxhlet extraction with solvents of ascending polarity (hexane, ethyl acetate, ethanol, aqueous). Each fraction was screened for major phytoconstituent classes, then evaluated for radical scavenging capacity by the DPPH assay and for cytotoxicity by the MTT assay against HeLa, MCF-7, and HEK293 cells, with cisplatin as the positive control. Polarity-matched extracts of the two species were combined 1:1 (v/v) and assayed under identical conditions.

Results: Phytochemical screening revealed a broad complement of alkaloids, flavonoids, tannins, phenolics, glycosides, saponins, and terpenoids, distributed across the four solvents in a pattern that tracked polarity closely, though not identically, between species. Ethanol produced the strongest antioxidant activity in both individual species (*O. tenuiflorum* IC_{50} = 154.42 µg/mL; *P. betel* IC_{50} = 341.28 µg/mL), and the combined ethanol extract surpassed both (IC_{50} = 95.71 µg/mL — a 1.6-fold improvement over *O. tenuiflorum* alone). Cytotoxicity followed the same arc: the combined ethanol fraction reached HeLa IC_{50} = 151.52 ± 2.28 µg/mL (SI = 2.66), again ahead of either parent extract, while the combined hexane fraction delivered the strongest MCF-7 result in the entire study (IC_{50} = 158.54 ± 1.62 µg/mL, SI = 2.33).

Conclusion: Combined preparations consistently outperformed their individual parent extracts across multiple polarity tiers, a pattern most plausibly explained by complementary phytochemical mechanisms — the rosmarinic acid–luteolin axis of *O. tenuiflorum* engaging apoptotic machinery alongside the hydroxychavicol–allylphenol complement of *P. betel* at distinct mechanistic nodes rather than the same one twice. On this evidence, the 1:1 ethanol combination stands as the priority candidate for bioassay-guided fractionation and mechanistic apoptosis profiling.

Keywords: *Ocimum tenuiflorum*; *Piper betel*; Phytochemical screening; DPPH assay; MTT assay; Polyherbal.

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Introduction

Cancer remains one of the foremost causes of mortality worldwide, with breast and cervical cancers together accounting for a substantial proportion of cancer-related deaths in women, particularly across low- and middle-income countries [1] (Sung et al., 2021). The clinical arsenal against these malignancies — dominated by platinum-based chemotherapeutics such as cisplatin, taxanes, and targeted biologics — is constrained by dose-limiting toxicity, acquired resistance, and restricted accessibility in resource-limited settings [2, 3]. Against this backdrop, medicinal plants have re-emerged as a tractable source of structurally diverse bioactive leads, several of which have already yielded clinically approved anticancer agents including paclitaxel, vinblastine, and camptothecin [4, 5].

Ocimum tenuiflorum L. (family Lamiaceae), commonly designated Tulsi or Holy Basil, is among the most medicinally revered plants of the Indian subcontinent. Its phytochemical repertoire — comprising eugenol, rosmarinic acid, ursolic acid,

oleanolic acid, luteolin, orientin, and vicenin — underpins a broad pharmacological profile encompassing antioxidant, anti-inflammatory, antimicrobial, antidiabetic, hepatoprotective, and anticancer activities [6,7]. Mechanistically, anticancer constituents of *O. tenuiflorum* have been linked to apoptosis induction, cell-cycle arrest, and reactive oxygen species (ROS)-mediated cytotoxicity across multiple tumour lineages; rosmarinic acid in particular suppresses NF-κB signalling and modulates Bcl-2 family proteins, while ursolic acid and luteolin independently induce caspase-dependent apoptosis in cancer cell lines [6].

Piper betel L. (family Piperaceae), the betel vine, is equally entrenched in South and Southeast Asian ethnomedicine. Its leaves are biochemically complex — rich in hydroxychavicol, eugenol, chavibetol, allylpyrocatechol, and diverse flavonoid glycosides — and this chemical diversity underpins well-documented antioxidant, antimicrobial, anti-inflammatory, and antimutagenic activities [8,9]. Ethanolic leaf extracts induce dose-dependent apoptosis in colorectal carcinoma cells through upregulation of p53 and downregulation of Bcl-XL, while hydroxychavicol and related phenylpropanoids show selective cytotoxicity across multiple cancer lineages [10]. Both species have been studied together previously — Sivareddy et al. (2019) [11] reporting broad-spectrum anticandidal activity for their solvent extracts — though combined-fraction anticancer evaluation remains largely unexplored.

Extraction solvent polarity is a determinant — frequently underappreciated — of phytochemical yield and, consequently, of biological outcome. Sequential extraction with solvents of increasing polarity partitions secondary metabolites according to their hydrophilicity, concentrating lipophilic terpenoids and steroids in non-polar fractions while directing phenolics, flavonoids, alkaloids, and saponins into polar fractions [12, 13]. Ethanol fractions consistently recover the broadest polar metabolite complement and outperform other solvents in phenolic yield and DPPH radical scavenging potency, a pattern documented across angiosperm families including *Polygonum glabrum* and *Cistus creticus* [14]. Whether this hierarchy holds for polarity-matched combinations of two complementary species — and whether mixing amplifies or attenuates individual-plant activity — is a question the present work addresses directly.

Polyherbal formulations exploit the structural and mechanistic complementarity of constituent phytochemical classes. Evidence is accumulating that combinations of botanicals with non-overlapping antioxidant mechanisms produce augmented, and occasionally synergistic, radical scavenging activity relative to individual preparations [15, 16]. In the anticancer domain, polyherbal ethanolic combinations have consistently matched or exceeded single-plant performance against HeLa and MCF-7 cell lines, with enhancement attributed to multi-target engagement of apoptotic pathways [17, 18]. Against this background, the present study aimed to characterize the phytochemical profile, DPPH antioxidant activity, and MTT cytotoxic selectivity of successive solvent extracts of *O. tenuiflorum* and *P. betel*, individually and in 1:1 (v/v) combination, against HeLa, MCF-7, and HEK293 cell lines, with the selectivity index (SI) computed as a quantitative indicator of differential cancer-cell toxicity.

Materials and methods

Plant Material Collection, Authentication, and Processing

Fresh leaves of *Ocimum tenuiflorum* L. and *Piper betel* L. were collected from a local cultivation site, washed with distilled water to remove surface contaminants, and authenticated by a qualified botanist. Voucher specimens were retained for future reference. Leaves were shade-dried at ambient temperature (25–30 °C) for 7–10 days to minimize degradation of thermolabile secondary metabolites, then coarsely powdered using a mechanical grinder and stored in airtight containers at room temperature prior to extraction.

Successive Solvent Extraction

Approximately 100–500 g of powdered plant material per species was subjected to sequential Soxhlet extraction using solvents in ascending order of polarity: hexane (non-polar), ethyl acetate (semi-polar), ethanol (polar), and water (highly polar). Each cycle was conducted for 6–8 hours, or until the siphon-tube solvent ran colourless. After each cycle, the marc was air-dried to remove residual solvent before transfer to the next. Aqueous extraction was accomplished by prolonged reflux or maceration (6–8 h). Each extract was filtered and concentrated under reduced pressure using a rotary evaporator at ≤ 50 °C; the aqueous extract was concentrated using a water bath or lyophilization. Concentrated extracts were desiccated and stored at 4 °C until use.

Preparation of Combined Extracts

Polarity-matched extracts of *O. tenuiflorum* and *P. betel* were combined in a 1:1 (v/v) ratio — hexane with hexane, ethyl acetate with ethyl acetate, ethanol with ethanol, and aqueous with aqueous — yielding four combined preparations. All combinations were prepared fresh and used immediately for biological assays to prevent constituent redistribution.

Preliminary Phytochemical Screening

All four solvent fractions of both individual species were screened for alkaloids (Dragendorff's/Mayer's test), flavonoids (Shinoda/alkaline reagent test), tannins and phenolics (ferric chloride test), glycosides

(Keller–Killiani test), saponins (froth test), terpenoids (Salkowski test), steroids (Liebermann–Burchard test), fixed oils and fats (spot test/Sudan III), proteins (Biuret test), and carbohydrates (Molisch's test).

DPPH Free Radical Scavenging Assay

Antioxidant activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay following the method of Blois (1958) [19], standardised by Brand-Williams et al. (1995) [20] and Molyneux (2004) [21]. A 0.1 mM DPPH solution was freshly prepared in methanol and protected from light. Each extract (25–800 µg/mL) was mixed with an equal volume of DPPH solution, vortexed, and incubated for 30 min at 25 ± 2 °C in darkness. Absorbance was recorded at 517 nm against extract-specific blanks. Ascorbic acid served as the reference antioxidant standard. Percentage radical scavenging activity (% RSA) was calculated as:

$$\% \text{ RSA} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 is the control absorbance and A_1 is the sample absorbance. IC_{50} values were derived by linear interpolation of dose–response curves and are reported as mean ± standard deviation (SD) of triplicates.

MTT Cytotoxicity Assay

Cytotoxic activity against HeLa (human cervical carcinoma), MCF-7 (human breast adenocarcinoma), and HEK293 (human embryonic kidney) cell lines was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay [22, 23]. Cells were seeded at 5×10^4 cells/well in 96-well plates in DMEM supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin, and allowed to adhere for 24 h at 37 °C in 5% CO_2. Extracts were dissolved in DMSO (final concentration ≤ 0.5%) and diluted to 25–800 µg/mL in culture medium. After 48 h of treatment, 10 µL of MTT solution (5 mg/mL in PBS) was added per well and incubated for 4 h at 37 °C. Formazan crystals were dissolved in 100 µL DMSO and absorbance read at 570 nm (reference: 630 nm). Cisplatin served as the positive control. Cell viability (%) was calculated as:

$$\% \text{ Viability} = [(\text{Abs\_sample} - \text{Abs\_blank}) / (\text{Abs\_control} - \text{Abs\_blank})] \times 100$$

IC_{50} values were determined by regression analysis and expressed as mean ± SD (n = 3). The selectivity index (SI) was calculated as IC_{50} (HEK293) / IC_{50} (cancer cell line); SI > 2.0 is the widely adopted threshold for meaningful differential toxicity in plant-extract screening (Aslan et al., 2016). It should be noted that HEK293 is an adenovirus-transformed, immortalized cell line rather than a primary normal kidney cell line; SI values reported here should therefore be regarded as indicative of relative selectivity, not as definitive safety margins.

Results

Preliminary Phytochemical Screening

The polarity-dependent distribution of secondary metabolites in *O. tenuiflorum* and *P. betel* was characterized across four solvent fractions; results appear in Tables 1 and 2, respectively.

Table 1. Preliminary phytochemical screening of *Ocimum tenuiflorum* leaf extracts.

Phytoconstituent	Test	Hexane	Ethyl Acetate	Ethanol	Aqueous
Alkaloids	Dragendorff's/Mayer's	−	+	+	+
Flavonoids	Shinoda/Alkaline Reagent	−	+	+	+
Tannins	Ferric Chloride	−	+	+	+
Phenolics	Ferric Chloride	−	+	+	+
Glycosides	Keller–Killiani	−	+	+	+
Saponins	Froth Test	−	+	+	+
Terpenoids	Salkowski	+	+	+	−
Steroids	Liebermann–Burchard	+	+	−	−
Fixed Oils/Fats	Spot/Sudan III	+	+	−	−
Proteins	Biuret	−	−	−	+
Carbohydrates	Molisch's	−	−	−	+

Table 2. Preliminary phytochemical screening of *Piper betel* leaf extracts.

Phytoconstituent	Test	Hexane	Ethyl Acetate	Ethanol	Aqueous
Alkaloids	Dragendorff's/Mayer's	−	−	+	+
Flavonoids	Shinoda/Alkaline Reagent	−	+	+	+
Tannins	Ferric Chloride	−	+	+	+
Phenolics	Ferric Chloride	+	+	+	−
Glycosides	Keller–Killiani	−	+	+	+
Saponins	Froth Test	−	+	+	+
Terpenoids	Salkowski	+	+	+	−

Steroids	Liebermann–Burchard	+	+	−	−
Fixed Oils/Fats	Spot/Sudan III	+	−	−	−
Proteins	Biuret	−	−	−	+
Carbohydrates	Molisch's	−	−	−	+

(+) = present; (−) = absent

Solvent polarity governed which metabolite classes turned up where, in both species, more or less as expected. Not entirely, though. In *O. tenuiflorum* (Table 1), the hexane fraction tested positive only for terpenoids, steroids, and fixed oils/fats — nothing polar came through at all. Ethyl acetate and ethanol recovered the widest complement of the four solvents, both testing positive for alkaloids, flavonoids, tannins, phenolics, glycosides, and saponins alike. Then the aqueous fraction did something the other three didn't: it picked up proteins and carbohydrates, detected nowhere else in the panel, while losing terpenoid positivity entirely.

P. betel (Table 2) broke from the *O. tenuiflorum* pattern at exactly two points. Phenolics tested positive in the hexane fraction here — a result the equivalent *O. tenuiflorum* fraction did not produce. And alkaloids tested negative at ethyl acetate but positive in both ethanol and aqueous fractions, a gap that has no counterpart in *O. tenuiflorum*, where alkaloid detection began already at the ethyl acetate tier.

DPPH Free Radical Scavenging Activity

Dose–response data — absorbance (mean ± SD) and % RSA at six concentrations — for individual and combined extracts of both species appear in Table 3 (hexane and ethyl acetate fractions) and Table 4 (ethanol and aqueous fractions); Figure 1 consolidates IC_{50} values across all fractions and preparations for direct comparison.

Hexane went nowhere. Neither individual hexane preparation, nor their combination, crossed the 50% RSA threshold at any tested concentration up to 800 µg/mL; extrapolated IC_{50} values exceeded 1800 µg/mL for all three. Ethyl acetate did better, though not by much — *P. betel* still fell short of 50% RSA at the top concentration (38.70% at 800 µg/mL), and *O. tenuiflorum* (50.04%) and the combination (51.05%) only just cleared it, landing all three IC_{50} values in the 660–740 µg/mL range.

Ethanol is where the fractions separate. At 100 µg/mL — the midpoint of the tested range — *O. tenuiflorum* reached 42.28% RSA, *P. betel* only 28.23%, and the combined preparation had already crossed 50% (51.81%). Its IC_{50}, 95.71 µg/mL, undercut both individual fractions by a wide margin: *O. tenuiflorum* ethanol sat at 154.42 µg/mL, *P. betel* ethanol at 341.28 µg/mL. At the highest tested concentration (800 µg/mL), the combination reached 82.41% RSA against 72.29% and 63.52% for the individual ethanol fractions — a gap that widened, not narrowed, as concentration climbed.

Aqueous fractions were more modest across the board. The combined aqueous preparation (IC_{50} = 497.62 µg/mL) crossed 50% RSA only at 800 µg/mL (55.08%); *O. tenuiflorum* aqueous just managed the same (52.52%), while *P. betel* aqueous never got there (42.92% at 800 µg/mL, IC_{50} = 879.26 µg/mL). Pooling helped a lot against *P. betel* aqueous and only a little against *O. tenuiflorum* aqueous.

Table 3. DPPH radical scavenging activity — absorbance (mean ± SD, n = 3) and % RSA for hexane and ethyl acetate fractions of *O. tenuiflorum*, *P. betel*, and their 1:1 combination.

Hexane Extracts						
Conc. (µg/mL)	***O. tenuiflorum* Mean ± SD**	**% RSA**	***P. betel* Mean ± SD**	**% RSA**	**Combined Mean ± SD**	**% RSA**
25	0.758 ± 0.0036	4.53	0.714 ± 0.0026	4.93	0.756 ± 0.0031	4.83
50	0.727 ± 0.0060	8.48	0.698 ± 0.0030	7.06	0.726 ± 0.0031	8.52
100	0.690 ± 0.0040	13.06	0.654 ± 0.0038	12.96	0.687 ± 0.0038	13.43
200	0.658 ± 0.0056	17.13	0.612 ± 0.0042	18.46	0.654 ± 0.0056	17.63
400	0.627 ± 0.0040	20.99	0.596 ± 0.0045	20.59	0.622 ± 0.0066	21.66
800	0.600 ± 0.0031	24.48	0.535 ± 0.0040	28.76	0.594 ± 0.0067	25.15

IC_{50} (µg/mL)	**1813.88**		**1846.65**		**1802.49**	
Ethyl Acetate Extracts						
Conc. (µg/mL)	***O. tenuiflorum* Mean ± SD**	**% RSA**	***P. betel* Mean ± SD**	**% RSA**	**Combined Mean ± SD**	**% RSA**
25	0.756 ± 0.004	4.83	0.720 ± 0.003	4.13	0.741 ± 0.004	6.72
50	0.718 ± 0.002	9.61	0.694 ± 0.002	7.55	0.704 ± 0.002	11.38
100	0.641 ± 0.003	19.23	0.638 ± 0.006	15.05	0.628 ± 0.003	20.86
200	0.562 ± 0.004	29.22	0.585 ± 0.004	22.10	0.551 ± 0.004	30.60
400	0.455 ± 0.003	42.65	0.522 ± 0.004	30.54	0.446 ± 0.003	43.79
800	0.397 ± 0.001	50.04	0.460 ± 0.003	38.70	0.389 ± 0.001	51.05
IC_{50} (µg/mL)	**692.30**		**739.68**		**660.96**	
				± SD		
25	0.682 ± 0.003	14.06	0.697 ± 0.004	7.15	0.655 ± 0.003	17.46
50	0.573 ± 0.005	27.83	0.625 ± 0.004	16.78	0.550 ± 0.005	30.73
100	0.458 ± 0.004	42.28	0.539 ± 0.007	28.23	0.383 ± 0.003	51.81
200	0.345 ± 0.003	56.55	0.442 ± 0.004	41.10	0.275 ± 0.002	65.41
400	0.259 ± 0.002	67.42	0.348 ± 0.003	53.71	0.194 ± 0.001	75.52
800	0.220 ± 0.003	72.29	0.274 ± 0.004	63.52	0.140 ± 0.002	82.41
IC_{50} (µg/mL)	**154.42**		**341.28**		**95.71**	
Aqueous Extracts						
Conc. (µg/mL)	***O. tenuiflorum* Mean ± SD**	**% RSA**	***P. betel* Mean ± SD**	**% RSA**	**Combined Mean ± SD**	**% RSA**
25	0.729 ± 0.004	8.23	0.723 ± 0.004	3.73	0.680 ± 0.004	14.36
50	0.667 ± 0.005	15.99	0.685 ± 0.003	8.83	0.646 ± 0.002	18.64

Table 4. DPPH radical scavenging activity — absorbance (mean ± SD, n = 3) and % RSA for ethanol and aqueous fractions of *O. tenuiflorum*, *P. betel*, and their 1:1 combination.

100	0.598 ± 0.003	24.73	0.644 ± 0.005	14.25	0.577 ± 0.003	27.29
200	0.530 ± 0.002	33.29	0.578 ± 0.003	22.99	0.506 ± 0.004	36.27
400	0.460 ± 0.002	42.11	0.514 ± 0.005	31.51	0.410 ± 0.002	48.36
800	0.377 ± 0.003	52.52	0.429 ± 0.004	42.92	0.357 ± 0.001	55.08
IC_{50} (µg/mL)	**662.67**		**879.26**		**497.62**	

Figure 1 puts the whole picture side by side. Ethanol was the most productive solvent for radical-scavenging extraction across all three preparations, full stop, and the combined ethanol fraction (IC_{50} = 95.71 µg/mL) beat both individual species — roughly a 1.6-fold improvement over *O. tenuiflorum* alone, 3.6-fold over *P. betel* alone. At 100 µg/mL, the combined ethanol preparation quenched 51.81% of DPPH radicals against 42.28% for *O. tenuiflorum* and 28.23% for *P. betel* at that same concentration; the combination crossed the 50% mark at a dose neither individual fraction reached until 200 µg/mL or beyond. Ethyl acetate and aqueous combinations (IC_{50} = 660.96 and 497.62 µg/mL) each modestly outperformed their respective individual fractions. Hexane combinations did not: IC_{50} = 1802.49 µg/mL, and % RSA stayed below 30 across the entire concentration range tested (Table 3).

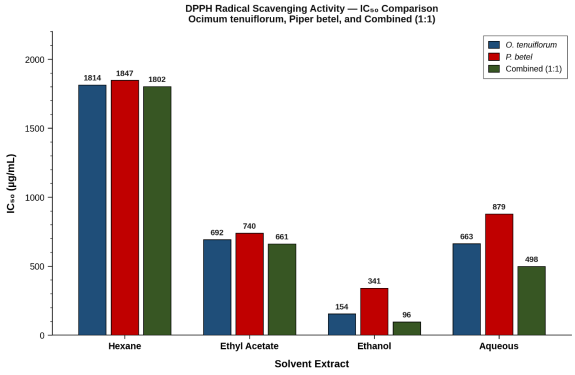

Figure 1: Summary of DPPH IC_{50} values (µg/mL) for all fractions and preparations of *O. tenuiflorum*, *P. betel*, and their 1:1 combination.

***In Vitro* Cytotoxicity and Selectivity Against HeLa, MCF-7, and HEK293 Cell Lines**

MTT cytotoxicity data for individual *O. tenuiflorum* and *P. betel* fractions appear in Tables 5 and 6, respectively; Table 7 reports IC_{50} values and SI for the 1:1 combined preparations alongside the cisplatin reference.

Table 5. IC_{50} values (µg/mL, mean ± SD, n = 3) and selectivity indices for individual *Ocimum tenuiflorum* extracts against HeLa, MCF-7, and HEK293 cell lines.

Extract	**HeLa IC_{50}**	**MCF-7 IC_{50}**	**HEK293 IC_{50}**	**SI (HeLa)**	**SI (MCF-7)**
Hexane	381.28 ± 2.75	409.47 ± 3.94	469.15 ± 3.94	1.23	1.15
Ethyl Acetate	487.22 ± 3.55	422.15 ± 2.97	705.36 ± 2.24	1.45	1.67
Ethanol	207.36 ± 3.64	195.27 ± 2.18	568.91 ± 4.73	2.74	2.91
Aqueous	537.81 ± 4.18	498.54 ± 1.95	704.89 ± 3.88	1.31	1.41

Table 6. IC_{50} values (µg/mL, mean ± SD, n = 3) and selectivity indices for individual *Piper betel* extracts against HeLa, MCF-7, and HEK293 cell lines.

Extract	**HeLa IC_{50}**	**MCF-7 IC_{50}**	**HEK293 IC_{50}**	**SI (HeLa)**	**SI (MCF-7)**
Hexane	288.19 ± 3.77	194.35 ± 2.05	442.17 ± 2.93	1.53	2.27
Ethyl Acetate	408.51 ± 2.27	378.82 ± 1.95	608.61 ± 1.77	1.49	1.61
Ethanol	186.74 ± 2.88	233.58 ± 2.49	509.45 ± 2.14	2.73	2.18
Aqueous	485.62 ± 3.27	508.63 ± 5.04	707.13 ± 3.78	1.45	1.39

Table 7. IC_{50} values (µg/mL, mean ± SD, n = 3) and selectivity indices for the combined (1:1 v/v) extracts of *O. tenuiflorum* and *P. betel*, with cisplatin as positive control.

Extract	**HeLa IC_{50}**	**MCF-7 IC_{50}**	**HEK293 IC_{50}**	**SI (HeLa)**	**SI (MCF-7)**
Hexane	227.67 ± 2.98	**158.54 ± 1.62**	369.31 ± 2.31	1.62	2.33

Ethyl Acetate	322.72 ± 1.79	302.27 ± 1.54	470.80 ± 1.40	1.46	1.55
Ethanol	151.52 ± 2.28	198.53 ± 1.97	402.47 ± 1.69	2.66	2.03
Aqueous	383.64 ± 2.58	401.82 ± 3.98	545.63 ± 2.99	1.42	1.35
Cisplatin	25.83 ± 1.27	39.75 ± 1.06	103.87 ± 1.14	4.02	2.61

Individual extract cytotoxicity. One pattern holds across Tables 5 and 6 without exception: the ethanol fraction of each species produced the lowest cancer-cell IC_{50} and the highest HEK293 IC_{50}, and these were the only individual preparations to clear an SI of 2.0. For *O. tenuiflorum* (Table 5), ethanol reached HeLa IC_{50} = 207.36 ± 3.64 µg/mL and MCF-7 IC_{50} = 195.27 ± 2.18 µg/mL against a HEK293 IC_{50} of 568.91 ± 4.73 µg/mL — SI values of 2.74 and 2.91, the highest recorded for either individual species. *P. betel* ethanol (Table 6) came close: HeLa IC_{50} = 186.74 ± 2.88 µg/mL, MCF-7 IC_{50} = 233.58 ± 2.49 µg/mL, SI values of 2.73 and 2.18.

One further result in Table 6 stands apart. The *P. betel* hexane fraction reached MCF-7 IC_{50} = 194.35 ± 2.05 µg/mL with SI = 2.27, clearing the selectivity threshold and substantially outperforming *O. tenuiflorum* hexane against the same cell line (MCF-7 IC_{50} = 409.47 µg/mL, SI = 1.15; Table 5).

Cisplatin (Table 7) produced far lower IC_{50} values than any plant preparation — HeLa 25.83 ± 1.27 µg/mL, MCF-7 39.75 ± 1.06 µg/mL — with a HEK293 IC_{50} of 103.87 ± 1.14 µg/mL and SI values of 4.02 (HeLa) and 2.61 (MCF-7). Aqueous and ethyl acetate fractions of both species (Tables 5 and 6) consistently failed to reach SI > 2.0 against either cancer line.

Combined extract cytotoxicity and selectivity. The 1:1 combined extracts beat at least one individual preparation — and, in three of the four solvent tiers, both — against the cancer cell lines (Table 7). Combined ethanol reached HeLa IC_{50} = 151.52 ± 2.28 µg/mL, below both the *O. tenuiflorum* ethanol figure (207.36 µg/mL) and the *P. betel* ethanol figure (186.74 µg/mL), for SI (HeLa) = 2.66. Against MCF-7, combined ethanol (IC_{50} = 198.53 ± 1.97 µg/mL, SI = 2.03) beat *O. tenuiflorum* ethanol (IC_{50} = 195.27 µg/mL, SI = 2.91) on raw potency but not on SI, because the combined preparation's own HEK293 IC_{50} (402.47 µg/mL) came in lower than either individual ethanol fraction's. SI held above 2.0 against both cell lines for combined ethanol, though it was not maximised against MCF-7.

Combined hexane produced an MCF-7 IC_{50} of 158.54 ± 1.62 µg/mL — the lowest MCF-7 value recorded anywhere in the MTT dataset — with SI = 2.33. Combined aqueous (HeLa IC_{50} = 383.64 ± 2.58 µg/mL, SI = 1.42) and combined ethyl acetate (HeLa IC_{50} = 322.72 ± 1.79 µg/mL, SI = 1.46) beat some individual fractions but never reached SI > 2.0 against either cancer line.

Discussion

The polarity-tracking pattern in Tables 1 and 2 is expected in outline, but two divergences are chemically informative. The *O. tenuiflorum* hexane fraction's restriction to terpenoids, steroids, and fixed oils matches the known dominance of eugenol, β-caryophyllene, and linalool in its non-polar partition (Bhattarai et al., 2024) [6], while terpenoid loss in the aqueous fraction reflects the thermal sensitivity and poor water-solubility of this constituent class (Usman et al., 2022) [13]. In *P. betel*, phenolics in the hexane fraction are consistent with the partial lipophilicity of hydroxychavicol and eugenol aglycones (Gupta et al., 2023) [8], and delayed alkaloid recovery until the ethanol tier suggests piperine-class alkaloids require higher polarity for efficient extraction than *O. tenuiflorum*'s alkaloid complement does — a pattern with precedent elsewhere in the genus [24] and in other angiosperm families (Rahman et al., 2022) [14].

The combined ethanol extract's DPPH enhancement is best explained by complementary, non-overlapping antioxidant chemistry: rosmarinic acid and flavonoids in *O. tenuiflorum* act as hydrogen-donating and metal-chelating antioxidants [6], while *P. betel*'s hydroxychavicol and eugenol-class allylphenols quench DPPH via a distinct one-electron transfer route [8]. That the combined-versus-individual gap widens rather than narrows with concentration (Table 4) argues against the two extracts competing for a shared radical pool, and Gyawali et al. (2022) [15] reported comparable synergism for mechanistically complementary polyherbal antioxidant pairs. Weaker enhancement at the ethyl acetate and aqueous tiers, and its near-absence at the hexane tier, tracks the shrinking phenolic content of these fractions and the generally poor radical-quenching capacity of terpenoids and steroids (Prior et al., 2005) [25].

Cytotoxic selectivity followed the same polarity logic. Ethanol fractions of both species were the only individual preparations to clear SI > 2.0 (Tables 5, 6), consistent with the caspase-dependent, cell-cycle-arresting activity reported for ursolic acid and luteolin in *O. tenuiflorum* [6] and the p53/Bcl-XL-mediated apoptosis reported for ethanolic *P. betel* (Radhalakshmi et al., 2024) [9]. The *P. betel* hexane fraction's unexpected MCF-7 selectivity (SI = 2.27) is plausibly attributable to β-sitosterol, a known MCF-7 inhibitor concentrated in this lipophilic partition (Tutu et al., 2022) [10], against a monoterpene-dominated and less active *O. tenuiflorum* hexane fraction —

though constituent-level confirmation would need bioassay-guided isolation. Against the cisplatin benchmark (SI = 4.02 HeLa, 2.61 MCF-7, reflecting its narrow, nephrotoxicity-limited therapeutic window), the plant ethanol fractions' SI values in the 2.5–3.0 range are consistent with typical crude-extract performance ahead of fractionation (Aslan et al., 2016) [24].

Combination enhanced selectivity most clearly for HeLa: the lower combined-ethanol IC_{50} (Table 7) is consistent with multi-node apoptotic engagement — rosmarinic acid/luteolin via NF-κB suppression and Bcl-2 modulation [6] alongside hydroxychavicol/eugenol-driven p53 upregulation and Bcl-XL downregulation [9] — a rationale consistent with reports of enhanced polyherbal activity elsewhere [17, 18]. The MCF-7 result was more equivocal, since combined ethanol's SI gain was offset by a parallel drop in HEK293 IC_{50}. The combined hexane fraction's MCF-7 result (SI = 2.33, the lowest MCF-7 IC_{50} recorded here) is consistent with additive or synergistic action between *P. betel* β-sitosterol/allylphenols and *O. tenuiflorum* β-caryophyllene, a CB2-receptor-mediated antiproliferative agent in breast cancer lines [6,10,27]; distinguishing synergism from additivity would require Chou–Talalay combination index analysis [26]. Combined aqueous and ethyl acetate preparations did not clear SI > 2.0, indicating that complementarity between the two species is expressed mainly at the ethanol and hexane polarity tiers.

The convergence of the strongest DPPH and cytotoxicity results in the same combined ethanol fraction (Tables 4, 7) is consistent with a contribution from redox-balance perturbation to cancer-cell-selective toxicity, since tumour cells operating near their oxidative-stress ceiling are typically more vulnerable to pro-oxidant loading than non-transformed cells [21, 25]. This remains an inference from parallel patterns rather than a mechanistically confirmed link; Annexin V/PI apoptosis profiling, caspase-3/7 activation assays, and intracellular ROS quantification in HeLa and MCF-7 cells are needed to establish it directly.

Conclusion

Sequential Soxhlet extraction partitioned secondary metabolites by polarity across both *O. tenuiflorum* and *P. betel*, with the ethanol fraction recovering the broadest phytochemical complement and delivering the strongest antioxidant and cytotoxic performance in individual and combined preparations alike. Among all preparations evaluated, the 1:1 combined ethanol extract achieved the lowest DPPH IC_{50} recorded (95.71 µg/mL) and the lowest HeLa IC_{50} in its solvent tier (151.52 µg/mL, SI = 2.66), outperforming either individual ethanol fraction. The combined hexane preparation produced the most potent MCF-7 inhibition across the entire dataset (IC_{50} = 158.54 µg/mL, SI = 2.33), implicating complementary lipophilic cytotoxic constituents — β-sitosterol and related triterpenes from *P. betel* acting in concert with β-caryophyllene from *O. tenuiflorum* — as candidates for isolation. Individual ethanol fractions of both species retained SI values > 2.0, and the *P. betel* hexane fraction alone reached SI = 2.27 against MCF-7, establishing it as a secondary fractionation priority. The enhancement observed in combined relative to individual preparations — consistently and across multiple polarity tiers — constitutes the most compelling evidence that the 1:1 combination strategy warrant escalation to mechanism-focused investigation. Formal combination index analysis, LC-MS/MS-guided constituent dereplication, and mechanistic apoptosis profiling in treated cancer cell lines are the logical continuation of this work.

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Conflict of Interest

Authors disclose no conflict of interest with this research work.

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