งานวิจัยเรื่อง Anticancer effects of piperine-free Piper nigrum extract on cholangiocarcinoma cell lines

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Anticancer effects of piperine-free Piper nigrum extract on cholangiocarcinoma cell lines

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Description: Background

Black pepper (Piper nigrum L.) is widely used as a traditional medicine, including usage for pain relief, fevers, as well as an anticancer agent. Previously, we reported that piperine-free P. nigrum extract (PFPE) inhibited breast cancer in vitro and in vivo.

Objective

In this present study, we explored the anticancer effects of PFPE on cholangiocarcinoma (CCA).

Materials and Methods

3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay was performed to analyze cytotoxic potential of PFPE whereas deoxyribonucleic acid (DNA) fragmentation followed by Western blot analysis were used.

Results

PFPE composed of alkaloid, flavonoid, amide, lignans, opioid, and steroid. This crude extract represented cytotoxic effect against CCA cells which stronger than dichloromethane P. nigrum crude extract and piperine, especially on KKU-M213 (median

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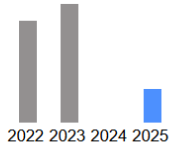

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Background: Pra-Sa-Ka-Phrao (PSKP) is a traditional Thai remedy recognized in the National List of Essential Herbal Medicines of Thailand. It is traditionally used for treating …

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Review

Harnessing the anticancer potential of *Piper nigrum*: a synergistic approach to chemotherapy enhancement and reduced side effects

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Abstract
Cancer therapy continues to face critical challenges, including drug resistance, recurrence, and severe side effects, which often compromise patient outcomes and quality of life. Exploring novel, cost-effective approaches, this review highlights the potential of *Piper nigrum* (black pepper) extract (PNE) as a complementary anticancer agent. *Piper nigrum*, a widely available spice with a rich history in traditional medicine, contains bioactive compounds such as piperine, which have demonstrated significant anticancer activities including cell cycle arrest, apoptosis induction, and inhibition of tumor growth and metastasis. The review evaluates the recent findings from in vitro, in vivo, and clinical studies, emphasizing PNE's capacity to enhance the efficacy of conventional chemotherapeutic agents while mitigating their side effects. Key mechanisms underlying these effects include oxidative stress modulation, suppression of pro-metastatic factors, and synergistic interactions with established drugs like doxorubicin and paclitaxel. These interactions suggest that PNE could play a pivotal role in overcoming chemoresistance and improving therapeutic outcomes. Furthermore, this review highlights the potential benefits of PNE in resource-limited settings, where the cost of cancer treatments often restricts access. However, challenges such as compositional variability, limited bioavailability, and the need for standardization and clinical validation need to be addressed to advance the integration of PNE into basic oncology. By providing a comprehensive analysis of the anticancer mechanisms of PNE and its potential as a cost-effective adjuvant therapy, this review provides new insight into the exploitation of *Piper nigrum* to improve cancer treatment efficacy while reducing side effects. Future research directions are discussed to address current limitations and facilitate clinical translation.

Keywords Anticancer · Apoptosis · Black pepper · Chemotherapy · *Piper nigrum* · Synergistic therapy

Hesti Lina Wiraswati, Daniela Calina and Javad Sharifi-Rad have contributed equally to this work.

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1 Introduction

Cancer is the leading cause of death globally and negatively impacts an individual's quality of life. An estimated 19.3 million new cases contributed to 10.0 million deaths in 2020 [1]. The disease burden in low- and middle-income countries (LMICs) is high, with the number of new cases being 12,122,139 reported in compared to 7,775,879 in high-income countries in 2022. Additionally, LMICs accounted for 6,869,816 deaths compared to 2,834,224 in high-income countries [2]. Several factors contribute to the high proportion of deaths seen in LMICs, including limited resources, limited access to health services, ineffective screening programs, delays in diagnosis, and high treatment costs [3]. Of these the high cost and limited availability of anticancer drugs are the factors that are most likely to influence patients with low-income levels to abandon treatment [3]. In addition, the adverse side effects associated with chemotherapy drugs (e.g., hair loss, diarrhea, fatigue, vomiting, and quality of life reductions) may influence patients to refuse or discontinue treatment [4]. Herbal medicines have been used in traditional treatments for many years [5–9]. The World Health Orgaization (WHO) reported that approximately 80% of people in developing countries rely on herbal medicines for their health needs. Herbal medicine's popularity and acceptance have increased over the years due to perceptions about its safety, lower prices, and increased availability [10]. A study involving hospitals, health centers, and independent medical practices in seven provinces in Indonesia reported that 79.6% of patients experienced improved quality of life following the use of herbal plants as a complementary cancer therapy [11]. Many studies have evaluated herbal drugs [10]. Such studies have reported that these drugs can kill cancer cells, inhibit cancer cell growth, and improve the cell microenvironment and immunity [12]. Several studies have also sought to clarify the mechanisms underpinning the synergistic interactions between herbal and chemotherapy drugs [13]. The genus *Piper* (*Piperaceae*) is widely used in traditional medicines. Globally, 10 species of *Piper* are known to be used as anticancer agents [14]. In India, *Piper longum* L., the root of *Piper boehmeriifolium* Wall, and *Piper sylvaticum* Roxb. are used to treat tumors [15]. *Piper capense* L.f, the seeds of *Piper cubeba* L, the juice of *Piper gibbilimbum* C.DC, and the seeds of *Piper guineense* Schum and Thonn are used as cancer treatments in Cameroon, Morocco, Papua New Guinea, and Nigeria, respectively [16]. The root of the "King of spices," *Piper nigrum*, is used to treat abdominal tumors in Thailand [17]. In China, the fruit of *P. nigrum* is used to treat respiratory and gastric cancer [18]. *Piper nigrum* is also one of the ingredients used in traditional Indian medicine to control tumors [19]. *Piper nigrum* is a tropical woody vine that produces small, round, green fruits that turn red when ripe. The fruit of the plant is commonly referred to as peppercorn. Propagation can be achieved through seeds or stem cuttings. Although the plant requires a considerable amount of time to produce its first flowers and berries, once they appear, it can yield a significant quantity of peppercorns under suitable conditions. Currently, Vietnam, Brazil, and Indonesia are the leading producers of pepper globally, followed by Burkina Faso, India, Sri Lanka, China, Malaysia, and Tajikistan [20]. Due to the prevalence of this plant in numerous tropical regions, its adaptability to a broad range of environmental conditions, and its suitability for cultivation by rural farmers, it is a promising herbal medicine option [20]. This study focused on *P. nigrum*'s phytochemicals in the context of the anticancer activities of its extracts. In vitro and in vivo evaluation studies should be conducted to scientifically prove the safety and efficacy of herbal drugs and meet regulations [21]. Therefore, we included anticancer studies of *P. nigrum* that used both cellular and animal models, as well as clinical studies to assess its potential use in standardized herbal or phytopharmaceutical drugs. The production cost of these drugs is cheaper than pharmaceutical drugs, which are associated with extremely high prices, particularly for pure plant-derived compounds. The production of pure compounds requires lengthy isolation steps, such as extraction, fractionation, and further purification, which, in turn, necessitates large quantities of expensive organic solvents, product development, and the pharmaceutical industry for product development and production [22]. When compared to a pure drug, an extract typically exhibits a higher degree of activity due to synergistic and positive interactions between its components [23]. Additionally, many studies have reported that plant extracts contain substances that inhibit multidrug resistance in cancer [24]. Various compounds can enhance the bioavailability, pharmacokinetic properties, and pharmacodynamic effects of the extracts they are present in [25].

This review provides a novel perspective by emphasizing *Piper nigrum* extract (PNE) synergistic potential with chemotherapy to enhance efficacy and reduce side effects, a focus not comprehensively addressed in recent literature. Unlike other reviews, it examines clinical applicability, bioavailability-enhancing strategies and its economic advantages in resource-limited settings, offering a translational approach to its use as an integrative cancer therapy.

2 Review methodology

This updated review systematically analyzed the anticancer properties of *P. nigrum* (black pepper) extracts by searching multiple electronic databases, including PubMed, MedLine, Scopus, Web of Science, Google Scholar, and Cochrane Library. The search utilized terms such as "*Piper nigrum*," "black pepper," "anticancer properties," "complementary therapy," and "phytochemicals". The literature review for this study covered a time period ranging from 2000 to 2024. This range was chosen to encompass both foundational studies and the most recent advancements in research related to *Piper nigrum* and its anticancer mechanisms. The inclusion criteria focused on peer-reviewed original research articles, reviews, clinical trials, in vitro, in vivo, and in silico studies that investigated the anticancer properties of *Piper nigrum*, reported in English, involving various cancer cell lines, animal models, or human clinical studies, and those that demonstrated outcomes such as apoptosis induction, cell cycle arrest, inhibition of cell proliferation and metastasis, or enhancement of conventional chemotherapeutic efficacy. Exclusion criteria included studies unrelated to cancer, non-peer-reviewed sources such as conference abstracts, editorials, and commentaries, and articles lacking clear methodologies or results regarding the anticancer effects of *Piper nigrum*. The most important and relevant data from these studies have been summarized in comprehensive tables and figures, detailing bioactive compounds, experimental models, doses and observed anticancer activities, to present a clear and systematic understanding of the potential role of PNE in cancer therapy.

3 Bioactive phytochemicals and anticancer potential of different parts of *Piper nigrum*

The anticancer properties of *P. nigrum* are attributed to a diverse range of bioactive compounds. Through the analysis of the plant's chemical composition, researchers have gained insights into its pharmacological mechanisms. Numerous studies have concentrated on the secondary metabolites found in various parts of the plant, including the roots, seeds, as well as dried and fresh fruit. Notably, the fruit is the most commonly utilized part in anticancer research, with both ripe and unripe fruit serving as valuable sources of raw materials for anticancer applications. Deng et al. investigated a piperine-free extract (PFPE) of dried fruits of *P. nigrum* and reported significant cancer prevention effects in NMU-treated Sprague–Dawley rats. PFPE inhibited VEGF expression, suppressed tumor progression, and induced ROS generation. These findings suggest its potential as an angiogenesis inhibitor and ROS activator for cancer therapy [26].

Saetang et al. identified caryophyllene as a key component (25%) in a low-piperine extract of *P. nigrum*. Their study demonstrated enhanced antitumor immunity via Th1/Th2/Treg modulation in NMU-induced mammary tumor models, highlighting its immunomodulatory role [27]. De Souza Grinevicius et al. focused on ethanolic extracts enriched in piperamides (piperine and piperyline). The extracts induced oxidative stress and apoptosis in MCF-7 and HT-29 cells, reduced tumor growth in vivo, and modulated apoptosis markers, including Bax, p53, and Bcl-xL. This dual mechanism highlights its therapeutic potential against both breast and colorectal cancers [28]. Kiranmayee et al. synthesized tin oxide nanoparticles (SnO_2 NPs) using aqueous extracts of *P. nigrum*. The nanoparticles exhibited cytotoxicity against MCF-7 cells and favorable pharmacokinetics in silico, interacting effectively with EGFR. These findings support the application of *P. nigrum*-based nanoparticles in targeted cancer therapy [29]. Buranrat and Boontha demonstrated that ethanolic extracts of *P. nigrum* inhibited MCF-7 proliferation by suppressing cyclin D1 and NF-κB and inducing ROS production and apoptosis. This study highlights its dual role in regulating cell cycle and promoting cancer cell death [30]. Buranrat further explored phenolic and flavonoid-rich extracts, which inhibited HeLa cell proliferation and migration. The extracts induced mitochondrial dysfunction and ROS-mediated apoptosis. This reinforces the importance of phenolic compounds in cancer inhibition [31]. Kanniah et al. synthesized silver nanoparticles (AgNPs) from *P. nigrum* seeds, which showed broad-spectrum cytotoxicity against cancer cell lines, including breast, pancreatic, and ovarian cancers. The integration of nanotechnology with *P. nigrum* extracts broadens their applicability in cancer treatment [32].

Black pepper essential oil (BPEO), rich in terpenes like sabine, D-limonene, and 3-carene, has demonstrated significant anticancer activity, particularly against triple-negative breast cancer. The complementary effects of its bioactive components, including piperine, further enhance its therapeutic value. Research continues to uncover the vast anticancer potential of *P. nigrum* through its ability to target multiple pathways, including oxidative stress, immune modulation, and apoptosis. Detailed experimental data, including IC_{50} values and doses, are presented in Table 1.

Many other biologically important phytochemicals are extracted from *P. nigrum* plants, including alkaloids, amides, propenyphenols, lignans, neolignans, terpenes, steroids, kawapyrones, piperolides, chalcones, dihydrochalcones,

Table 1 Different PNE and their anticancer mechanisms

P. nigrum plant part used	Sample used	Phytochemical/Compounds identified	Experimental model	IC_{50}/Doses	Anticancer activity	Refs.
Dried fruit	Piperine-free *P. nigrum* fruits extract (PFPE)	n.d.	In vitro: Breast cancer cells MCF-7 ZR-75-1 In vivo: NMU-treated female Sprague–Dawley mammary tumor rats	MCF-7 (IC_{50} = 7.45 µg/mL), ZR-75-1 (IC_{50} = 7.45 µg/mL) (Dose = 100, 200, 400 mg/kg BW)	Showed cancer prevention effects through ROS generation in vivo ↓ MMP-2, ↓MMP-9, ↓VEGF in vivo ↓ cancer cells proliferation activity by downregulated trancription factor, c-Myc in vitro	[26]
Dried fruits of black peppercorn	Low piperine fractional *P. nigrum* extract (PFPE)	Fifteen compounds were detected, and caryophyllene is a majority with 25%	In vivo: NMU-Induced Sprague–Dawley mammary tumor rats	(Dose = 200 mg/kg BW)	↓ tumor progression ↑ the antitumor immunity by regulating the Th1/Th2/Treg	[27]
Dried fruits	Ethanolic extract rich in piperamides	Two major compounds of piperamides: piperine and piperyline	In vitro: MCF-7 cells human breast cancer HT-29 human colorectal adenocarcinoma In vivo: Ehrlich ascites carcinoma-bearing mice	MCF-7 human breast cancer cells (IC_{50} = 27.1 µg/mL), HT-29 colorectal adenocarcinoma (IC_{50} = 80.5 µg/mL) (Dose = 100 mg/kg BW)	↓ cell proliferation in MCF-7 and HT-29 cells ↓ Tumor growth in vivo ↑ oxidative stress and apoptosis in vitro and in vivo ↑ Bax, ↑p53, Bcl-xL expression associated with apoptosis Tumor cell death related to oxidative stress in MCF-7	[28]
Dried fruits	Aqueous water	*P. nigrum* tin oxide nanoparticle (SnO_2 NPs)	In vitro: human breast cancer cell MCF-7 In silico: EGFR receptor	Dose = 75–200 µg/mL	The favorable pharmacokinetics pharmacodynamics, and toxicity profiles of three promising compounds in silico Exhibited cytotoxicity against cancer cell in vitro	[29]
Dried unripe fruits of black pepper	Ethanolic extract	Phenolic group	In vitro: Human colorectal cancer cells line HCT-116 HCT-15 HT-29	HCT-116: IC_{50} = 4.0 µg/mL, HCT-15: IC_{50} = 3.2 µg/mL, HT-29: IC_{50} = 7.9 µg/mL	Inhibited cell proliferation	[33]

Table 1 (continued)

P. nigrum plant part used	Sample used	Phytochemical/Compounds identified	Experimental model	IC_{50}/Doses	Anticancer activity	Refs.
Dried fruits	Methanol extract or dichloromethane extract	Alkaloids group	In vitro: Human Breast cancer cells MCF-7 MDA-MB-231 MDA-MB-468	MCF-7: IC_{50} = 20.25 µg/mL, MDA-MB-231: IC_{50} = 22.37 µg/mL, MDA-MB-468: IC_{50} = 9.04 µg/mL	Inhibited cell proliferation	[34]
Unripe fruit	Ethanolic extract	Alkaloids gropus including piperine as a major constituent	In vitro: PANC-1 human pancreatic cancer	IC_{50} = 54.2 µg/mL	Inhibited proliferation of PANC-1 through G0/G1 arrest ↓ protein levels of cell cycle regulators such as cyclin B1, cyclin D1, survivin, and Forkhead box M1 (FoxM1) ↓ cell migration and invation by decreasing FoxM1 protein level	[35]
Black pepper	Piperine enriched supercritical extract	Piperine is major compound was identified	In vitro: MCF-7 human breast cancer cells In vivo: Balb/c mice-bearing Ehrlich Ascites Carcinoma (EAC) In silico: piperine binding to CDK2-Cyclin A and BCl-xL	IC_{50} = 27.8 µg/mL Dose = 100 mg/kg BW	↓ growth of breast cancer cell line MCF-7 that confirmed by interaction of piperine and protein targets CDK2-Cyclin A and BCl-xL ↑ apoptosis in MCF-7 breast cancer cells Inhibited tumor growth and showed lower levels of CDK2 and Cyclin A ↑ cell cycle arrest in G2/M phase in vivo ↑apoptotic cells and upregulated of pro apoptotic proteins (p53 and Bax) Hydrophobic interactions between piperine and residue Ser5 in CDK2; with residue Lys8 in Cyclin A; and Bcl-xL receptor	[36]

Table 1 (continued)

P. nigrum plant part used	Sample used	Phytochemical/Compounds identified	Experimental model	IC_{50}/Doses	Anticancer activity	Refs.
Dried young fruit	Ethanolic extract	n.d.	In vitro: Human breast cancer cells MCF-7	IC_{50} = 15.6 µg/mL	Stimulated growth inhibition by increasing the G1 phase arrest and inhibiting cyclin D1 and NF-κB Inhibited cell migration and reduced MMP-2, MMP 9, VEGFA, and ICAMP1 genes level Activated the ROS formation, increase caspase-3 activity, and induced breast cancer cell death	[30]
Dried young fruit	Ethanolic extract	Phenolic and flavonoid contents including Piperine	In vitro: HeLa human cervical cancer cells	IC_{50} = 22.71 µg/mL	Inhibited cell ploriferation and induced cell cycle arrest at G0/G1 phase Triggered apoptosis by inhibiting mitochondrial function and amplifying ROS production Suppressed cancer cells migration	[31]
Dried young fruit	Ethanolic extract	n.d.	In vitro: Cholangiocarcinoma (CCA) /bile duct cancer KKU-100 KKU-M452	KKU-100 IC_{50} = 12.76 µg/mL, KKU-M452 IC_{50} = 38.32 µg/mL	Inhibited cell viability and colony formation Decreased cell growth by cell cycle arrest at G0/G1 phase in KKU-100 cells and S to G2/M phase in KKU-M452 cells Induced apoptosis by decreasing mitochondrial function and increasing ROS production Suppressed cell migration	[37]
Black pepper seed	Aqueous extract	PNE silver nanoparticles (AgNPs): nineteen phytochemicals such as piperine, piperanine, ecuramide, pipecolic acid, betaine, salsolinol, hexadecanamide, oleamide, quinine	In vitro: MDA-MB-231 human breast cancer PANC-1 human pancreatic cancer SKOV-3 human ovarian cancer PC-3 human prostate cancer HeLa human servical cancer	MDA-MB-231: IC_{50} = 10 µg/mL PANC-1: IC_{50} = 10 µg/mL, SKOV-3: IC_{50} = 10 µg/mL PC-3: IC_{50} = 10 µg/mL HeLa: IC_{50} = 10 µg/mL	Showed potent cytotoxicity against human cancer cell lines	[32]

Table 1 (continued)

P. nigrum plant part used	Sample used	Phytochemical/Compounds identified	Experimental model	IC_{50}/Doses	Anticancer activity	Refs.
Black pepper seed	Aqueous extract	*P. nigrum* tin oxide nanoparticle (SnO_2 NPs)	In vitro: HCT-116 human colorectal cancer A549 human lung cancer	HCT-116: IC_{50} = 0.165 µg/mL A549: IC_{50} = 0.135 µg/mL	Demonstrated toxicity towards HCT 116 and A549 cells through the generation of ROS	[38]
Black pepper seed	Ethanolic extract	Characterized piperine	In vitro: Human metastatic melanoma SK-MEL 19 cells Intestinal adenocarcinoma malignant ascites AGP-01 Intestinal adenocarcinoma with an inactivated PIWIL1 gene AGP-01 PIWIL1$^{-/-}$ Neoplastic human pulmonary fibroblast cell line MCR5	SK-MEL 19: IC_{50} = 14.94 µg/mL AGP-01: IC_{50} = 13.52 µg/mL AGP-01 PIWIL1$^{-/-}$ IC_{50} = 21.26 µg/mL MCR5: IC_{50} = 14.17 µg/mL	Induced cancer cells toxicity	[39]
Dried fruit	Aqueous extract	*P. nigrum* AgNPS	In vitro: Human hepatocyte carcinoma HepG2	IC_{50} = 4.98 µg/mL	Induced cancer cells toxicity	[40]
Dried fruit	Aqueous extract	*P. nigrum* AgNPs	In vitro: Human breast cancer cells MCF-7 Human larynx carcinoma cancer Hep-2	MCF-7: IC_{50} = 52 µg/mL Hep-2: IC_{50} = 54 µg/mL	Induced cancer cells toxicity	[41]
Dried black pepper	Aqueous extract Methanolic extract	Mainly piperine and alkyl amides	In vitro: Human colon carcinoma HCT-116 Human breast cancer MCF-7 Human glioblastoma SF-268 Human lung carcinoma NCI-H460	HCT-116, MCF-7, SF-268, NCI-H460: IC_{50} = 200 µg/mL	Induced cancer cells toxicity	[42]
Dried fruit	$CHCl_3$ extract	Alkaloids group (piperidine present)	In vitro: Human servical cancer HeLa	IC_{50} = 17.47 µg/mL	Induced cancer cells toxicity	[43]
Dried fruit	Extract of methanol:water 1:1	n.d.	In vivo: DMBA-induced skin tumorigenesis in *Swiss albino* mice	Dose = 150 mg/kg BW	Reduced the number of tumours in vivo test model	[44]
Dried fruit	Ethanolic extract	n.d.	In vitro: Murine Ehrlich Ascites Carcinoma EAC Murine melanoma-B16 Human servical cancer HeLa	Murine Ehrlich Ascites Carcinoma EAC: IC50 = 8 µg/mL, Murine melanoma-B16: IC_{50} = 10 µg/mL, HeLa: IC_{50} = 17 µg/mL	Induced cancer cells toxicity	[45]
Dried fruit	n-hexane, chloroform, methanol and water extracts	Phenolic content	In vitro: Human cervical cancer cell line CaSki	IC_{50} = 36 µg/mL	Induced cancer cells toxicity	[46]

Table 1 (continued)

P. nigrum plant part used	Sample used	Phytochemical/Compounds identified	Experimental model	IC_{50}/Doses	Anticancer activity	Refs.
Dried black pepper	Dichloromethane extract	Piperine free *P. nigrum* extract (PFPE), rich in pipercitine alkaloid and caryophyllene terpene	In vitro: Human cholangiocarcinoma KKU-100 KKU-M213 KKU-M055	KKU 100: IC_{50} = 17.79 µg/mL, KKU M213: IC_{50} = 13.70 µg/mL, KKU M055: IC_{50} = 16.74 µg/mL	Induced cancer cells toxicity	[47]
Dried leaves	Methanolic extract	Major: Tannin, flavonoid, steroid, polyphenol Minor: saponin, terpenoid, triterpenoid, alkaloid	Human lung carcinoma A549	Dose = 200–500 µg/mL	Induced cancer cells toxicity	[48]
Dried root	Extracts: petroleum ether and $CHCl_3$ petroleum ether $CHCl_3$	Alkaloids group	In vitro: Human leukemia cells HL 60	Petroleum ether: IC_{50} = 30 µg/mL, CHCl3: IC_{50} = 11.2 µg/mL, Combined: IC_{50} = 9.8 µg/mL	Induced cancer cells toxicity	[49]

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Table 1 (continued)

P. nigrum plant part used	Sample used	Phytochemical/Compounds identified	Experimental model	IC_{50}/Doses	Anticancer activity	Refs.
Black pepper	Supercritical carbon dioxide extracts rich in piperine (SFE)	Piperine is major compound was identified	In vivo: Balb/c mice bearing-Ehrlich ascites carcinoma cells (EAC) In silico: dGlutathione Peroxidase	Dose = 100 mg/kg BW	Inhibited EAC cells viability Enhanced EAC pro-oxidative status to induced oxidative stress Decreased glutatione peroxidase/GPx activity and GSH depletion The GPx-piperine poses hydrogen and hydrophobic bonds that contribute to inhibitionof GPx	[50]
	n.d	Five lead compounds: Clarkinol A Isodihydrofutoquinol B Burchellin Kadsurin B Lancifolin C	In silico: Five lead compounds to Epidermal growth factor receptor (EGFR)	Binding score: – 7.304 to – 6.342 kcal/mol	Interacted well with EGFR receptor	[51]
	n.d	Campesterol Cholesterol Piperine Linoleic acid	In silico: PPARγ, a glucose metabolism regulator factor that responsible for colorectal cancer	Binding score: Campesterol (– 8.8 kcal/mol), Cholesterol (– 8.2 kcal/mol), Piperine (– 8.6 kcal/mol), Linoleic acid (– 6.2 kcal/mol)	Interacted well with PPARγ target	[52]
	Pure Piperine (97–98%) dissolved in DMSO	Piperine	HT-29 colon carcinoma cells	IC_{50} = 75–150 µM	Inhibited HT-29 colon carcinoma cell proliferation by inducing G1 phase cell cycle arrest Triggered apoptosis by producing hydroxyl radical	[53]
Dried roots	*P. nigrum* in petroleum ether and chloroform extracts	Several alkaloid groups such as: pellitorine, (E)-1-[30,40-(methylenedioxy) cinnamoyl] piperidine, 2,4-tetradecadienoic acid isobutyl amide, piperine, sylvamide, cepharadione A, piperolactam D and paprazine	HL60 (human promyelocytic leukaemia cells)	IC_{50} = 30 µg/mL	Inhibitory effect toward HL60 (human promyelocytic leukaemia cells)	[49]

IC_{50}: half-maximal inhibitory concentration; BW: body weight; PNE: *P. nigrum* extract; AgNPs: silver nanoparticles; PFPE: Piperine-free *P.nigrum* extract; PFPE-CH: Piperine-free *P. nigrum* extract combined with coconut oil and honey ROS; Reactive Oxygen Species: NMU: N-nitroso-N-methylurea; DMBA: 7:12-dimethylbenz[a]anthracene; n.d: not determined

brachyamide, dihydropipericide, 3,4-dihydroxy-6 (N-ethylamine), benzamide, (2E,4E)-N-eicosadienoyl pereridine, N-trans-feruloyltyramine, N-formyl piperidine, guineensine, (2E,4E)-N-5[(4-Hydroxyphenyl)-pentadienoyl] piperidine, (2E,4E)-N-isobutyldecadienamide, (2E,4E)-N-isobutyl-eicosadienamide, (2E,4E,8Z)-N-isobutyl-eicosatrienamide, (2E,4E)-N-isobutyloctadienamide, piperamide, piperamine, piperettine, pipericide, piperine, piperolein, trichostachine, sarmentine, sarmentosine, tricholein, and retrofractamide [54]. Of these, the most prominent alkaloid compounds in *P. nigrum* are piperine and piperidine, which are commonly extracted from the root or fruit. Research on alkaloids has recently gained special attention as several have shown promising anti-inflammatory, antioxidant, and anticancer effects. Ethanolic *P. nigrum* extract has been shown to inhibit tumor growth and enhance the antitumor immune response in murine models of breast cancer and melanoma. Piperine demonstrates anticancer properties against multiple cancer types, including HER overexpressing breast cancer cell lines (SKBR-3 and MCF-7), the MCF-7 cell line, and 7,12-dimethylbenz[a]anthracene (DMBA)-induced carcinogenesis in Syrian golden hamsters. Studies have shown that piperine and piperidine can activate or inhibit several signaling pathways fundamental for cancer regulation, demonstrating their anticancer effects against ovarian, prostate, and lung cancer [55]. Fifteen alkaloid compounds extracted from the dried fruits of *P. nigrum* L. using MeOH, including piperine, piperolein, pipersintenamide, trichostachine, and piperamide, have demonstrated strong cytotoxic activity against a human cervical cancer cell line, Hela, a breast cancer cell line, MCF-7, and the HL-6 human promyelocytic leukemia cell line [56]. *Piper nigrum* extracts (PNE) may be interesting alternatives to their respective bioactive components, as they demonstrate anticancer action via complementary pathways. PNE has been investigated for its anti-inflammatory, immunomodulatory, antioxidant, and anticancer properties [57]. The ethanolic extract of *P. nigrum* contains a substantial amount of piperine and has been found to cause cell cycle arrest [56]. Notably, the raw extract showed greater specificity and was more harmful to cancer cells than the two primary alkaloids, piperine and pellitorine, individually [58]. Black pepper essential oil (BPEO) has demonstrated strong anticancer activity, particularly against triple-negative breast cancer [59]. BPEO-loaded nanoparticles considerably reduce MDA-MB-231 cell migration, invasion, and proliferation [60]. This inhibition is driven by a reduction in the Wnt/β-catenin signaling pathway and GSK-3β activation. Sabine, 3-carene, D-limonene, α-pinene, caryophyllene, β-phellandrene, α-phellandrene, α-thujene, and β-bisabolene are the principal chemical components of BPEO. These substances, in addition to piperine, support BPEO's anticancer characteristics [60].

4 Mechanisms underlying anticancer activity

4.1 Suppresses cancer cell growth

Cancer involves dysregulated cell division, and inducing cell cycle arrest is a key strategy to inhibit tumor growth [61–63]. Most in vitro, in vivo, and in silico studies have shown that *P. nigrum* extracts (PNE) inhibit cancer cell proliferation (Fig. 1).

In vitro experiments have shown that PNE results in anti-proliferation activity in the breast cancer models MCF-7, 4T1, MDA-MB-231, MDA-MB-468, and ZR-75-1; the HeLa and CaSki cervical cancer cell lines; colorectal cancer HT-29, SW-620, HCT-116, and HCT-15 cells; pancreatic cancer PANC-1 cells; melanoma B16-F10 and B16 cell lines; neuroblastoma SK-N-SH and LA-N-5 cells; lung carcinoma H-358, A-549, and NCI-H460 cell lines; cholangiocarcinoma (CCA) KKU-100, KKU-M452, KKU-M213, and KKU-M055 cells; fibrocarcinoma L-929 cells; ovarian cancer SKOV-3 cells; human prostate cancer PC-3 cells; human hepatocyte carcinoma HepG2 cell line; larynx carcinoma cancer Hep-2 cell line; glioblastoma SF-268 cells; murine Ehrlich ascites carcinoma (EAC); and human leukemia HL 60 cells (Table 1). In general, PNE demonstrates strong toxicity against various cancer cells. Dehydrogenase enzyme activity in the active mitochondria of cancer cells decreases in the presence of PNE (Table 1). Slowed cell growth has shown to result in the inhibition of cell colony formation in HT-29, B16-F10, 4T1, MCF-7, HT-29, KKU-100, and KKU-M452 cells and reduced tumor sizes in HT-29 nude mouse xenografts, murine models of 4T1 breast cancer and B16-F10 melanoma, N-nitroso-N-methylurea (NMU) treated Sprague–Dawley mammary tumor rats, EAC, and reduced DMBA-induced skin tumorigenesis in Swiss albino mice [26, 28, 36, 37, 44, 64–66].

4.2 Induction of cell cycle arrest

Disruption of the cell cycle is a hallmark of cancer, and inducing cell cycle arrest is a pivotal strategy employed by anticancer agents to halt uncontrolled tumor cell proliferation and promote therapeutic efficacy [67, 68]. PNE's ability to cause cell cycle arrest has been noted in different cell types and among different tumor behaviors (Fig. 1). For example, in mice

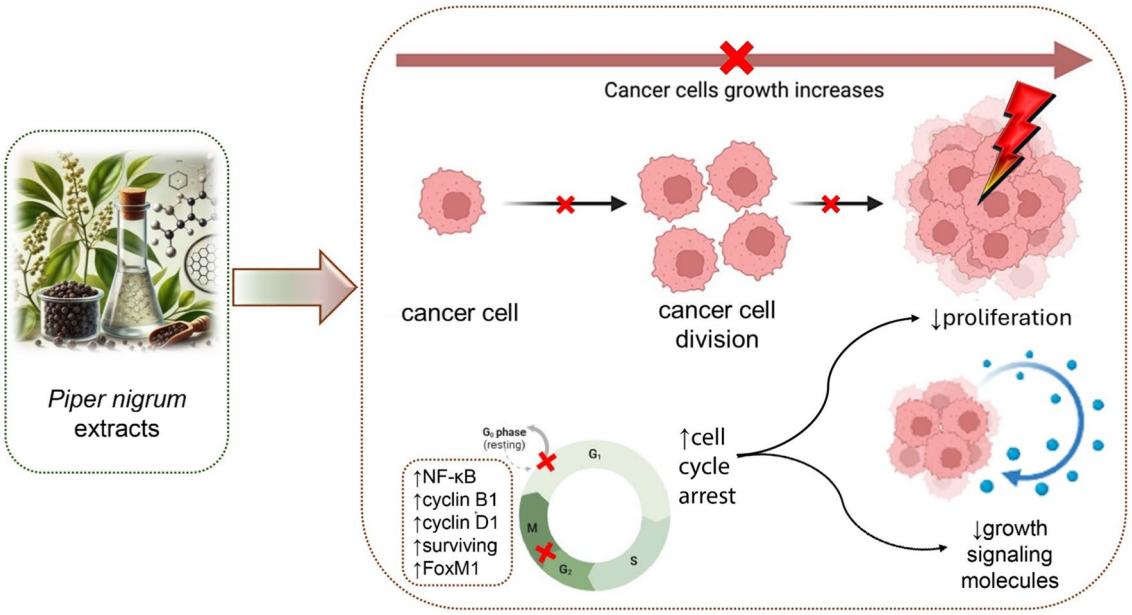

Fig. 1 Mechanism of PNE in inducing cell cycle arrest and reducing tumor growth. PNE inhibit cancer cell proliferation by inducing cell cycle arrest at critical phases (e.g., G_0/G_1), reducing cell division, and downregulating growth-promoting signaling pathways. These extracts regulate key proteins, including NF-κB, cyclin B1, cyclin D1, survivin, and FoxM1, leading to decreased cancer cell division, reduced signaling for proliferation, and suppression of tumor progression. FoxM1: Forkhead Box M1; NF-κB: Nuclear Factor Kappa-light-chain-enhancer of activated B cells

carcinoma and KKU-M452 CCA cell models, PNE results in S to G2/M phase arrest, while in MCF-7 breast cancer, HeLa cervical cancer, and CCA KKU-100 CCA cells, it results in G0/G1 phase arrest [30, 37]. G1 phase arrest has been linked to the downregulation of cell cycle regulators, such as nuclear factor κB (NF-κB), cyclin B1, cyclin D1, survivin, and Forkhead box protein M1 (FoxM1) (Table 1). NF-κB is a pro-inflammatory protein that modulates gene expression, including genes involved in cancer cell growth and progression via cyclin D1 [69]. Cyclin D1, in conjunction with CDK4/6, facilitates the integration of mitogenic signals that control the G1 cell cycle checkpoint in solid tumors [70]. Cyclin B1 is primarily associated with the G2/M phase. High cyclin B1 expression has been observed in benign tumors and is associated with tumor grade and poor survival in patients with solid tumors [71, 72]. Survivin is an apoptosis inhibitor protein that regulates cell division and spindle formation. FoxM1 is a critical proliferation-associated transcription factor that plays a fundamental role in cyclin B1, cyclin D1, and surviving expression. It acts as an upstream regulator of the G1 to S phase transition and the G2 to M phase progression [73]. The overexpression of survivin or FoxM1 is commonly associated with an aggressive cancer phenotype, poor prognosis, and drug resistance in many types of cancer [74]. An in silico study showed that the primary compounds of PNE are potential novel inhibitors of the epidermal growth factor receptor and peroxisome proliferator-activated receptor gamma, which are the receptors involved in G1 cell cycle progression [75]. Various PNE solvents exhibit distinct impacts on the proliferation inhibition pathway of MCF-7 breast cancer cells. For instance, as shown in Table 1, the ethanol extract obtained from young, dried fruit causes cell cycle arrest in the G1 phase. Conversely, the piperine-enriched supercritical extract induces cell cycle arrest in the G2/M phase by lowering the levels of CDK2 and cyclin A both in vitro and in vivo. The findings from in silico studies indicate that piperine exhibits a high affinity for the cyclin A binding site of CDK2, suggesting it may inhibit the cell cycle [30, 36]. In CCA cells, PNE treatment blocks the G0/G1 cell cycle phase in KKU-100 cells, and the S to G2/M phase in KKU-M452 cells [37]. Based on this evidence, it appears that PNE inhibits the progression of cell cycle regulators, leading to a reduction in cancer cell growth through distinct signaling pathways depending on the type of extract and plant part used.

4.3 Apoptosis induction

Apoptosis, a programmed cell death mechanism fundamental for maintaining cellular homeostasis, is a key anticancer strategy targeted by bioactive compounds [76, 77]. In vitro and in vivo studies have shown that PNE induces apoptosis in various cancers, including HT-29 human colorectal cancer, B16-F10 melanoma, MCF-7, and 4T1 breast cancer, HeLa

cervical cancer, CCA KKU-100 and KKU-M452 cells, and EAC (Table 1 and Fig. 1). In MCF-7 cells, PNE induces apoptosis by upregulating p53 and cyt C and increasing caspase-3 activity and oxidative stress generation [28, 30]. In HeLa and CCA cells, PNE induces apoptosis by inhibiting mitochondrial function and amplifying ROS production [31]. Apoptosis induction is associated with increased Bax and p53 levels and reduced Bcl-xL expression in EAC-bearing mice [28, 36]. Therefore, it was concluded that the primary molecular mechanism of *P. nigrum* compounds is inducing apoptosis by activating ROS formation, which leads to oxidative stress generation. Researchers typically use PNE to provoke apoptosis in cancer cells (Table 1). PFPE and rich piperine extract of PNE exhibit comparable apoptotic activity [36, 66]. This suggests that other active compounds also contribute to inducing cancer cell death in addition to piperine. Grinevicius et al. reported that piperamide-rich PNE exhibits apoptosis-inducing activity both in vitro and in vivo [28]. The ability of other extracts to induce apoptosis in cancer cells has yet to be explored. Further evaluation of these extracts, including methanol, dichloromethane, water, $CHCl_3$, and n-hexane, is necessary, given that these extracts exhibit notable cytotoxicity towards cancer cells (Table 1 and Fig. 2).

4.4 Pro-oxidant activity and oxidative stress generation of PNE

Some studies have reported that oxidative stress may be involved in modulating cancer cell growth, either by suppressing growth or inducing cell death. (Table 1) PNE has been shown to generate ROS in MCF-7 breast cancer cells and HT-29 colorectal adenocarcinoma cells, leading to reduced cell proliferation in both cell types and inducing apoptosis in MCF-7 cells [28]. PNE-synthesized nanoparticles have also demonstrated ROS generation in HCT-116 colorectal cancer and A549 lung cancer cells [38]. PNE has exhibited pro-oxidant activity in 4T1, B16-F10, CCA, and HeLa cervical cancer cells, resulting in mitochondrial dysfunction, which was evaluated by measuring cellular oxidative stress using 2′,7′-dichlorodihydrofluorescein diacetate [31, 37, 65]. In vitro findings have aligned with previous research involving mice models of breast tumors treated with NMU, where PNE elevated lipid peroxidation product, malondialdehyde (MDA) levels. These increased levels were associated with apoptosis induction, as demonstrated by the increased protein levels of p53, Bax, and cytochrome c [26]. Another in vivo report demonstrated that PNE pro-oxidant activity in NMU-induced breast

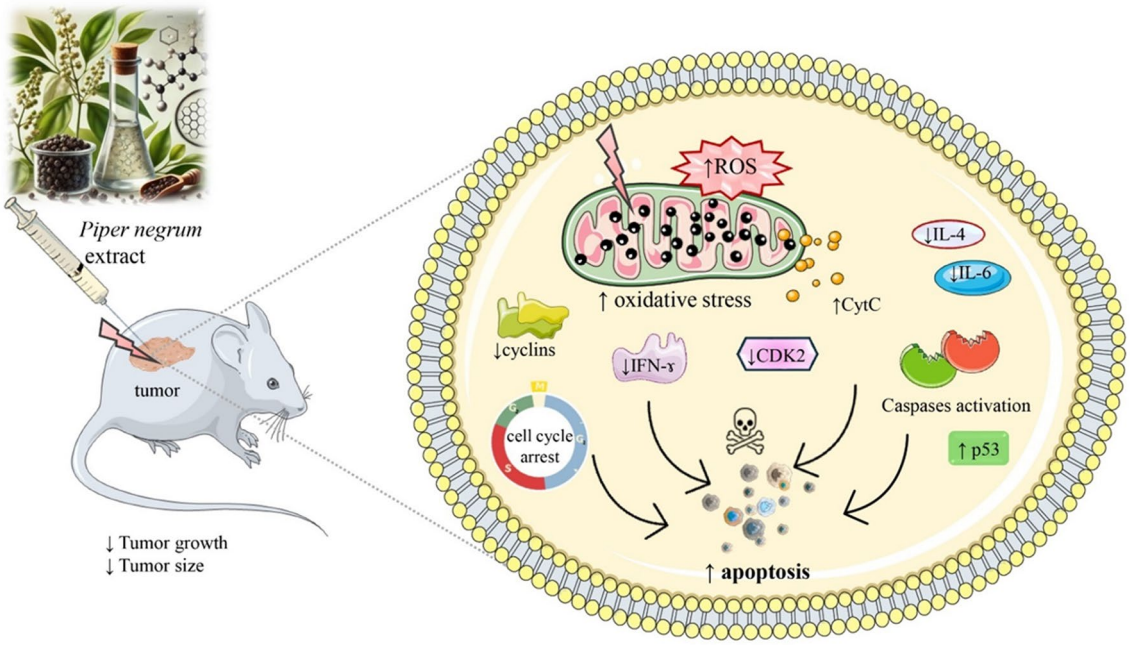

Fig. 2 Apoptosis molecular mechanisms of *Piper nigrum* extract (PNE). PNE administration leads to decreased tumor size and growth, primarily through the induction of oxidative stress via increased ROS levels, resulting in mitochondrial dysfunction and CytC release. This process activates caspases and p53, promoting apoptosis. PNE also causes cell cycle arrest by reducing the expression of cyclins and CDK2. Additionally, PNE modulates inflammatory responses by lowering IL-4, IL-6, and IFN-γ levels, contributing to its anticancer effects. CDK2: Cyclin-dependent kinase 2, a cell cycle regulatory protein; CytC: Cytochrome c, released from mitochondria during apoptosis; IFN-γ: Interferon-gamma, a cytokine involved in immune regulation; IL-4: Interleukin-4, an anti-inflammatory cytokine; IL-6: Interleukin-6, a pro-inflammatory cytokine; p53: A tumor suppressor protein involved in apoptosis regulation; PNE: *Piper nigrum* extract; ROS: Reactive oxygen species, molecules causing oxidative stress; ↓decrease, ↑increase

cancer rat models resulted in increased thiobarbituric acid reactive substances (TBARS) levels or in EAC-bearing mice by increasing lipid peroxidation, carbonyl protein content, and the activity of antioxidant enzymes, such as glutathione reductase, superoxide dismutase, and catalase [28]. TBARS is a widely recognized method for assessing lipid peroxidation and oxidative stress in biological fluids and has been employed to predict the survival prospects of patients who have not received chemotherapy [78].

PNE's pro-oxidant activity against cancer cells shows promise for the development of complementary medicines. Many pro-oxidant agent have been employed as anticancer drug, such as mitomycin C, doxorubicin, and geldanamycin, due to their ability to trigger oxidative stress. Drug toxicity has been linked to ROS generation, which, in turn, can cause oxidative stress or result in the modification of cellular components, which depletes cellular thiols, ultimately leading to the depletion of antioxidant defenses [79]. Drug-protein interaction studies have demonstrated that interactions between ligands and oxidative stress-related enzymes, such as cytochrome p450 reductase (CYP450R), glutathione peroxidases (GPX), superoxide dismutases (SODs), and apoptosis-inducing factor (AIF), are associated with toxicity against cancer cells [80–82]. Some active compounds of PNE have been reported to display good binding to oxidative stress-related enzymes. For example, GPx-piperine contains hydrogen bonds and is hydrophobic, which contributes to GPx inhibition, thereby increasing the pro-oxidant status that triggers oxidative stress [50]. This finding is in line with our in silico study that showed that the compounds of PNE play a primary role in targeting monoamine oxidase B (MAO B), which was confirmed by SwissTargetPrediction and a molecular docking study [83]. However, docking studies involving PNE active compounds against the enzymes involved in oxidative stress remain limited. Therefore, future research should involve in silico studies to elucidate the mechanisms behind this at the molecular level.

4.5 Metastases inhibition

Some studies have demonstrated an antimetastatic effect of PNE on cancer cell lines or cancer models. At a dose of 15.67 mg/kg, PNE decreased the occurrence of macrometastases in 4T1 model BALB/cAnNCrl mice [65]. Pancreatic cancer metastases overexpress FoxM1. PNE inhibits PANC-1 cell migration and suppresses the protein levels of FoxM1 [35]. PNE ethanol extract also inhibits the migration of the CCA/bile duct cancer KKU-100 and KKU-M452 cell lines [37]. Furthermore, PNE inhibits MCF-7 cell migration by reducing matrix metalloproteinase MMP 9 expression, as well as MMP 2, MMP 9, VEGFA, and ICAMP1 gene expression [30]. PFPE was found to inhibit metastases by down-regulating estrogen receptor, E-cadherin (E-cad), MMP-9, MMP-2, c-Myc, and vascular endothelial growth factor (VEGF) levels in breast cancer rats, as well as protein levels of E-cad, c-Myc, and VEGF in MCF-7 cells [26].

4.6 Modulation of inflammatory factors

Chronic inflammation is a key contributor to the development and progression of various chronic diseases, including cancer, where it plays a pivotal role in tumor initiation, progression, and metastasis, making it an essential target for cancer prevention strategies [84]. PNE have shown potent anti-inflammatory properties by modulating cytokine levels in both in vitro and in vivo cancer models [85]. Studies have demonstrated that PNE reduces the expression of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), which are often associated with tumor-promoting inflammation. Simultaneously, PNE increases levels of anti-inflammatory cytokines, such as interleukin-10 (IL-10), contributing to an anti-tumor microenvironment. For example, in NMU-treated Sprague–Dawley mammary tumor models, PNE significantly reduced IL-4 and IL-6 levels while inhibiting tumor growth through oxidative stress induction [65]. Moreover, PNE's ability to modulate inflammatory pathways not only suppresses tumor progression but also enhances the efficacy of concurrent chemotherapeutic agents [65]. These findings highlight the dual role of PNE in targeting both inflammation and cancer cell proliferation, making it a promising candidate for integrative cancer therapies.

5 Synergistic effects of PNE with conventional therapies or other bioactive compounds

The combination of seeds from *Alpinia galanga, P. nigrum, Citrus aurantifolia, Themeda triandra*, and *Cannabis sativa* has demonstrated antiproliferative and antimigration activity against human colorectal and lung cancer cell lines [86]. The combination of *P. nigrum* seeds and the root bark of *Azadirachta indica* has shown similar actions against the epidermoid carcinoma A 431 cell line [87]. Regarding the molecular mechanism of action, the combinations of *P. nigrum* fruit and dried leaves of *Andrographis paniculata*, dried leaves of *Ziziphus spina-christi,* and dried leaves of *Gymnanthemum*

extensum and the combination of the plant extract of *Ocimum sanctum* and *P. nigrum* have been found to cause apoptosis in human breast cancer MCF-7 [88, 89]. Some combinations also exhibit immunomodulatory activity. For example, the combination of the seeds of black pepper and cardamom enhances the cytotoxic capacity and exhibits a synergistic stimulatory impact on the cytotoxic activity of Natural Killer cells against YAC-1 tumor cells [90]. A combination of PFPE and turmeric has been found to significantly boost IL-10 levels but not IL-4, IFN-γ, or IL-6 levels in NMU-induced mammary rats [25]. Several studies have investigated the combination of PNE with established cancer drugs, such as doxorubicin and paclitaxel. PFPE was found to suppress cancer-related cytokines when combined with doxorubicin and diminished the systemic immune response side effects associated with doxorubicin in NMU-induced mammary tumors in female Sprague Dawley rats [27]. The combination of dried black pepper and doxorubicin demonstrated synergistic activity, enhanced the efficacy and antigenotoxic effect of doxorubicin, and provided protection against the toxic and genotoxic effects induced by doxorubicin in hamster ovary (CHO-K1) cells [91]. A combination of *P. nigrum* stems and paclitaxel improves the anticancer effect of paclitaxel in apoptotic cervical cancer cells [92]. Piperine significantly suppresses the growth of androgen-dependent and androgen-independent prostate cancer cells [93]. Makhov et al. noted enhanced anticancer activity associated with the co-administration of piperine and docetaxel for human prostate cancer treatment [94]. Additionally, piperine induces DNA damage and apoptosis in tumor cells and is a potential therapeutic agent for osteosarcoma treatment. Piperine also stimulates antioxidative protective enzyme activity and decreases lipid peroxidation, resulting in lung cancer reduction. Therefore, piperine exhibits potential anticancer activities [95]. However, limited studies have investigated the antitumor potential of piperine and BPEO, with most using animal models. Therefore, future studies should focus on the bioactivity of BPEO in clinical investigations with humans. The use of *P. nigrum* as a dietary supplement, namely a low piperine fractional *P. nigrum* extract (PFPE) combined with cold-pressed coconut oil and honey (PFPE-CH) in distilled water, was studied to assess its potential to reduce tumor risk and mitigate chemotherapy side effects during breast cancer treatment. At 5000 mg/kg, PFPE-CH caused no toxicity or deleterious effects in rats after 14 days, while a dose of 86 mg/kg body weight/day for six months did not impair kidney or liver function. In rats with breast tumors, PFPE-CH reduced the tumor incidence by up to 71.4%, led to improved immune responses, and reduced chemotherapy-induced toxicity while maintaining doxorubicin's anticancer activity [25]. The effect of PFPE-CH on tumor formation, reactive oxygen species (ROS), and cancer-related cytokines has also been evaluated. Cytokines regulate the critical immunological cells involved in cancer genesis and progression, making them viable therapeutic targets. Some findings suggest that the anticancer effects of *P. nigrum* could be mediated by enabling synergy between the numerous active components acting in complementary pathways [25]. The exploration of alternative sources of piperine could be beneficial for the large-scale production and development of new pharmaceuticals [96]. Endophytic fungi isolated from *P. nigrum* or *P. longum* could be a potential source of piperine. These fungi, such as *Colletotrichum gloeosporioides* and *Periconia* sp, can produce piperine under suitable conditions, such as liquid cultures [97]. Optimizing their growing conditions may enable the large-scale biosynthesis of piperine. Piperine is also well-known for its potential to enhance the bioavailability of various natural compounds, including resveratrol [98]. This effect is achieved through the inhibition of cytochrome P450 enzymes and P-glycoprotein, which reduces the metabolism and efflux of these compounds. The co-administration of piperine or PNE with resveratrol has demonstrated a synergistic effect, improving the pharmacokinetics and therapeutic efficacy of resveratrol. This synergy enhances anticancer activity by promoting apoptosis induction, inhibiting cancer cell proliferation, and modulating key inflammatory pathways. These bioavailability-enhancing properties highlight the potential of PNE and piperine as adjuvants in combination therapies involving other natural compounds. Current combination therapies that combine PNE with another plant extract or cancer drug are shown in Table 2.

6 Challenges, limitations and comparative advantages of *Piper nigrum* extracts as potential adjuvant in oncology

Despite the promising anticancer potential of *Piper nigrum* extracts (PNE), several challenges hinder their clinical application. Variability in the phytochemical composition of PNE is a major concern, as it depends on factors like plant origin, harvesting time, extraction methods, and environmental conditions. This variability complicates standardization and makes it difficult to ensure consistent efficacy and safety in preclinical and clinical studies. For instance, variations in piperine and other alkaloid concentrations have been shown to impact anticancer effects. Additionally, the lack of standardized extraction and purification processes poses challenges in determining optimal dosages for therapeutic use. Piperine, the primary bioactive compound in PNE, exhibits low bioavailability due to poor solubility, rapid metabolism,

Table 2 Combination treatment of PNE and chemotherapy drugs or other compounds

Combination	Extract type	Experimental model	Dose/Concentration	Anticancer activity	References
Seed of *A. galanga*, *P. nigrum*, *C. aurantifolia*, *T. triandra*, and *C. sativa*	Ethanolic extract	In vitro: Human colorectal cancer cells (SW620, HCT116), human lung cancer cells (A549, NCI-H460)	IC_{50} = 12.15–19.72 µg/mL	Exhibited antiproliferative and antimigration activities	[86]
Seed of *P. nigrum* and root bark of *A. indica*	Combination of methanolic extracts	In vitro: Epidermoid carcinoma cell line (A431)	IC_{50} = 208 µg/mL	Inhibited cancer cell proliferation	[87]
Dried fruits of *P. nigrum* and doxorubicin	Piperine-Free *P. nigrum* extract (PFPE) + doxorubicin	In vivo: NMU-induced mammary tumor in female Sprague Dawley rats	Dose = 200 mg/kg BW	PFPE did not affect doxorubicin's anticancer properties; suppressed cancer-related cytokines; reduced immune side effects	[27]
Dried fruits of *P. nigrum* and extracts from: *A. paniculata*, *Z. spina-christi*, *G. extensum*	PFPE + dichloromethane extracts	In vitro: colorectal adenocarcinoma (HT-29), colorectal cancer (SW620), breast cancer (MCF-7), ovarian cancer (A2780)	IC_{50} = 8.82–21.12 µg/mL	Antagonism with *A. paniculata* (HT-29, SW620, MCF-7); Synergy with *Z. spina-christi* in MCF-7; Apoptosis induction	[88]
Plant extract of *O. sanctum* and *P. nigrum*	Combination of aqueous extracts	In vitro: human breast cancer (MCF-7); In silico: ADMET analysis	IC_{50} = 78.61 µg/mL (combination)	Synergistic inhibition of cell proliferation, Apoptosis induction, ADMET properties for eugenol and piperine	[89]
Dried black pepper and doxorubicin	Ethanolic extract + doxorubicin	In vitro: Hamster ovary (CHO-K1) cells	IC_{50} = 8.5 µg/mL	Synergistic activity with doxorubicin; Enhanced efficacy and antigenotoxic effect; protection against genotoxicity	[91]
Stem of *P. nigrum* and paclitaxel	Ethanolic extract + paclitaxel	In vitro: paclitaxel-resistant cervical cancer cells (HeLa/PTX)	IC_{50} = 5.61–33.2 µM	Improved anticancer effect; enhanced apoptosis in resistant cancer cells	[92]
Black peppercorn of *P. nigrum* with turmeric or doxorubicin	PFPE + turmeric; PFPE + doxorubicin; PFPE + turmeric + doxorubicin	In vivo: NMU-induced mammary tumors in rats	PFPE-CH Dose = 100 mg/kg BW Turmeric Dose = 25 mg/kg BW	PFPE + turmeric/doxorubicin increased oxidative stress (TBARS); inhibited tumor growth; PFPE + turmeric enhanced IL-10	[25]

IC_{50}: half-maximal inhibitory concentration; PFPE: Piperine-free *P. nigrum extract*; PTX: paclitaxel; NK: natural killer; ADMET: absorption: distribution: metabolism: excretion: and toxicity; NMU: N-nitroso-N-methylurea; BPE: black pepper extract

and quick clearance from the body. Advanced delivery systems such as nanoparticles and liposomes have shown promise in enhancing its bioavailability; however, these approaches are not yet fully validated in clinical settings. For example, studies have demonstrated improvements in piperine's pharmacokinetics using these technologies, but their therapeutic potential in humans remains unexplored. Another limitation is the scarcity of clinical evidence. Preclinical studies have demonstrated apoptosis induction, inhibition of metastasis, and enhanced efficacy of chemotherapeutic agents, but the lack of well-designed clinical trials restricts the translation of these findings into clinical practice. For example, PNE's ability to reduce tumor progression in rodent models requires further validation in human trials to establish safety and efficacy profiles. Concerns regarding potential toxicity and safety are also significant. Piperine has been associated with gastrointestinal irritation, hepatotoxicity, and interactions with drug-metabolizing enzymes, potentially altering the pharmacokinetics of co-administered medications. For instance, its interactions with cytochrome P450 enzymes underscore the need for comprehensive toxicological studies, particularly in cancer patients undergoing complex treatment regimens. PNE's interactions with cytochrome P450 enzymes further complicate its use in oncology. These interactions can either enhance or inhibit the metabolism of other drugs, increasing the risk of adverse effects or reduced therapeutic efficacy. This is particularly problematic for cancer patients on polypharmacy, highlighting the need for careful evaluation of drug interactions before considering PNE in combination therapies. Regulatory challenges and quality control issues also hinder the integration of PNE into mainstream oncology. The lack of established guidelines for standardizing PNE production and certification affects consistency and quality, making it difficult to develop reliable formulations for clinical use. Furthermore, the precise mechanisms underlying PNE's anticancer effects and its potential synergistic interactions with other therapies are not fully understood. For example, while piperine has shown promising synergy with chemotherapeutic agents like doxorubicin, optimizing these combinations requires further research. Finally, the integration of PNE into complementary and alternative medicine (CAM) practices is underdeveloped. Despite its historical use in traditional medicine, the absence of standardized protocols and guidelines limits its application alongside conventional cancer treatments. In summary, PNE holds significant promise as a complementary anticancer therapy, but challenges such as variability in composition, low bioavailability, limited clinical evidence, toxicity concerns, and regulatory hurdles must be addressed. Addressing these limitations through rigorous research, standardization, and clinical validation is crucial to realizing the full potential of PNE in oncology.The anticancer potential of PNE positions it as a promising candidate in integrative cancer therapies, yet its contextualization alongside existing treatments provides deeper insight into its utility. Compared to conventional chemotherapeutic agents such as doxorubicin or paclitaxel, PNE offers unique advantages. For instance, standard chemotherapy is associated with severe side effects, such as cardiotoxicity and immune suppression, and PNE has been shown to mitigate these effects when used as an adjunct. In NMU-induced mammary tumor models, Piperine-free *P. nigrum* extracts (PFPE) reduced the systemic immune suppression caused by doxorubicin without compromising its efficacy, indicating its immunomodulatory potential [27]. Cost-wise, PNE exhibits greater affordability than synthetic chemotherapeutic agents, particularly in low- and middle-income countries (LMICs), where the high cost of treatment often limits access. The production of herbal extracts like PNE relies on relatively simple processing techniques compared to the complex synthesis pathways for drugs like docetaxel. This makes PNE not only cost-effective but also sustainable for resource-constrained settings. Regarding efficacy, PNE shows comparable cytotoxicity against various cancer cell lines with IC_{50} values often aligning with those of synthetic drugs. For example, PNE's IC_{50} values range from 4 µg/mL to 52 µg/mL across colorectal, breast, and cervical cancer models, rivaling first-line chemotherapeutic agents. Moreover, its synergistic potential when combined with established drugs like paclitaxel enhances apoptosis and overcomes drug resistance in cervical cancer cells [92]. In terms of integration, the versatility of PNE as a dietary supplement, nanoparticle formulation, or combined therapy enhances its adaptability. However, challenges such as variability in phytochemical content and limited clinical trials necessitate further research. Nonetheless, the dual role of PNE in reducing side effects and augmenting anticancer efficacy underscores its value in integrative cancer treatment regimens.

7 Concluding remarks

In the context of anticancer research, alkaloids represent the primary phytochemical components found in *P. nigrum* extracts (PNE). PNE has demonstrated the ability to inhibit the proliferation of various cancer cells, induce cell cycle arrest, and suppress the expression of critical cell cycle proteins such as cyclin, NF-κB, FoxM1, and survivin. Furthermore, PNE has been shown to generate oxidative stress, contributing to cell death in cancer cells. Its compounds interact with oxidative stress-related enzymes, including CYP450R, GPX, SODs, AIF, and MAO B, suggesting its potential as an oxidative stress

modulator. In addition to its antiproliferative effects, PNE has exhibited antimetastatic activity by inhibiting the expression of MMP and VEGF, which are key regulators of tumor invasion and angiogenesis. Studies have also highlighted its synergistic effects when combined with plant extracts such as *A. galanga, C. aurantifolia, T. triandra, C. sativa, A. indica, A. paniculata, Z. spina-christi, G. extensum*, and *O. sanctum*. These combinations enhance its ability to inhibit cell proliferation, induce apoptosis, and modulate the immune response. Furthermore, synergistic interactions between PNE and established anticancer drugs like doxorubicin and paclitaxel have shown promise in enhancing therapeutic efficacy while potentially reducing the side effects of chemotherapy. Compared to isolated compounds, PNE offers unique advantages due to the synergistic interactions among its bioactive constituents. These benefits include cost-effectiveness, broad-spectrum anticancer activity, and potential to address drug resistance. However, challenges such as limited bioavailability, variability in composition, and a lack of comprehensive clinical evidence remain. Advanced drug delivery systems, including nanoparticles and liposomal formulations, present opportunities to improve PNE's bioavailability and therapeutic application. Future research should prioritize standardizing extraction methods, conducting well-designed clinical trials, and elucidating molecular mechanisms to facilitate the integration of PNE into clinical oncology. This review highlights the potential of PNE as a complementary cancer therapy, with the ability to improve treatment outcomes, minimize adverse effects, and provide a cost-effective option for patients, particularly in resource-constrained settings.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Cytotoxic and anti-inflammatory activities of extracts of Pra-Sa-Ka-Phrao remedy and its plant components

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Original Article

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Cytotoxic and anti-inflammatory activities of extracts of Pra-Sa-Ka-Phrao remedy and its plant components

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ABSTRACT

Background: Pra-Sa-Ka-Phrao (PSKP) is a traditional Thai remedy recognized in the National List of Essential Herbal Medicines of Thailand. It is traditionally used for treating flatulence and colic pain. It consists of eight components derived from plants with *Ocimum tenuiflorum* leaves as a main ingredient. However, there are no reports regarding the biological activities and chemical composition of PSKP. **Objectives:** This study aimed to investigate cytotoxic activities, using cell lines related to the gastrointestinal (GI) system, and anti-inflammatory activities, along with chemical composition, of the PSKP complete formula (PSKPC) and a modified incomplete formula (PSKPIC). **Materials and Methods:** Two extraction processes were used: maceration in 95% ethanol and decoction. The anti-inflammatory effect was determined by an assay for inhibitory effect on nitric oxide production in RAW 264.7 cells, and the cytotoxic activity was evaluated, using the sulforhodamine B assay, against five types of GI cancer cell lines, including gastric carcinoma (KATO III), hepatocellular carcinoma (Hep G2), colorectal adenocarcinoma (SW480 and LS 174T), cholangiocarcinoma (KKU-M156). The chemical composition was analyzed by gas chromatography–mass spectrometry. **Results:** The ethanolic extract of PSKPC (PSKPCE) demonstrated cytotoxic activities against all tested cancer cell lines, and had no effect against the normal cell line. In addition, it also demonstrated an anti-inflammatory effect. The ethanolic extract of PSKPIC (PSKPICE) showed lower cytotoxic and anti-inflammatory activities than PSKPCE. Caryophyllene and *trans*-geranylgeraniol were determined as the main active ingredients of PSKPCE, whereas PSKPICE exhibited higher concentrations of linolenic acid and methyl eugenol. **Conclusion:** The results demonstrated that PSKPCE, which includes *Citrus hystrix* DC. and *Piper retrofractum* Vahl, exhibited higher activity compared to PSKPICE. These findings support the traditional use of PSKP and highlight its potential as a natural source of cytotoxic and anti-inflammatory agents, particularly for GI conditions.

Key words: anti-inflammation, *Citrus hystrix*, cytotoxic, gas chromatography-mass spectrometry, Pra-Sa-Ka-Phrao, traditional Thai medicine

INTRODUCTION

Cancer encompasses a range of diseases that are characterized by uncontrolled cell proliferation and may metastasize to surrounding tissues. It remains a leading cause of global mortality, with approximately 10 million deaths recorded in 2020. Among the most prevalent cancers are lung, colorectal, liver, stomach, and breast cancers.[1] Chronic inflammation has been implicated in carcinogenesis, primarily through oxidative stress-induced cellular damage through reactive oxygen species.[2-4] Natural products derived from medicinal plants offer a promising avenue for drug discovery.[5]

Pra-Sa-Ka-Phrao (PSKP), a traditional Thai remedy listed in the National List of Essential Herbal Medicines of Thailand, has been traditionally employed for treating flatulence and dyspepsia. According to traditional Thai medicine, pain and dyspepsia result from an imbalance in the wind element, which can be modulated by spicy-tasting herbs that enhance circulation. PSKP consists of eight medicinal components, including the leaf of *Ocimum tenuiflorum* L. (OT, holy basil), the peel of *Citrus hystrix* DC. (CH, kaffir lime), the root of *Glycyrrhiza glabra* L. (GG, licorice), the oleo-gum resin of *Ferula assa-foetida* L. (FA, asafoetida), the fruit of *Piper nigrum* L. (PN, black pepper), the rhizome of *Zingiber officinale* Roscoe (ZO, ginger), the fruit of *Piper retrofractum* Vahl (PR, long pepper), and the bulb of *Allium sativum* L. (AS, garlic), with OT comprising 50% of the formulation.

Previous studies have demonstrated that individual components of PSKP exhibit anti-inflammatory, cytotoxic, and anti-genotoxic activities. OT has shown significant anti-inflammatory properties[6-8] while CH and GG have been reported to possess anticancer effects.[9-11] In addition, FA, PN, ZO, PR, and AS have demonstrated anti-inflammatory and cytotoxic activities.[12-24] Despite these findings, the biological activity of the complete PSKP formulation (PSKPC), particularly its cytotoxic effects on gastrointestinal (GI) cancer cells and its anti-inflammatory potential relevant to pain, has not been comprehensively evaluated.

Moreover, due to regulatory constraints imposed by the Thai Food and Drug Administration (FDA), CH, FA, and PR are not included in the approved list of plants for food supplements. To facilitate further development into an acceptable food supplement, a modified PSKP incomplete formulation (PSKPIC) has been devised, omitting these restricted ingredients. If PSKPIC demonstrates cytotoxic and anti-inflammatory properties, it may serve as a viable candidate for GI tract disease prevention and treatment, and within regulatory compliance.

This study aims to compare the biological activities of PSKPC and PSKPIC, focusing on cytotoxic effects against GI tract cancer cells and anti-inflammatory properties relevant to colic pain. In addition, chemical composition analysis using gas chromatography-mass spectrometry (GC-MS) has been conducted to suggest potential active compounds. The findings of this study may contribute to the advancement of PSKPIC as a scientifically validated herbal remedy for GI tract diseases, including inflammation and cancer, aligning with regulatory standards for food supplement development.

MATERIALS AND METHODS

Plant Materials

Plant materials were sourced from China and Thailand in 2019. Voucher specimens were deposited at the Southern Centre of Thai Medicinal Plants, Prince of Songkla University, Thailand. The information and the proportion of plant components used in PSKPC and PSKPIC, in accordance with the PSKPC remedy, are provided in Table 1. Samples were cleaned, sliced, oven-dried at 50°C, and ground into powder for further analysis.

Extraction Method

Maceration method

Dried plant materials were macerated in 95% ethanol at room temperature for 3 days. Filtrates (Whatman No.1) were concentrated via rotary evaporation (45°C) and vacuum-dried. The extraction was repeated twice using the same procedure.

Decoction method

Water extracts were prepared by boiling dried plant materials in water (15 min) and filtering (3×1 L). Filtrates (Whatman No.1) were concentrated to 1 L, then freeze-dried using a lyophilizer.

In Vitro Assay for Cytotoxic Activity

Human cell lines

Five types of cancer cells, including gastric carcinoma (KATO III: ATCC® HTB-103), hepatocellular carcinoma (Hep G2: ATCC® HB-8065), colorectal adenocarcinoma (SW480: ATCC® CCL-228 and LS 174T: ATCC® CL-188), and cholangiocarcinoma (KKU-M156, obtained from Khon Kaen University, Thailand. As a non-cancerous control, the human keratinocyte cell line HaCaT (CLS® 300493-SF) was also evaluated.

All cells were kept at 37°C in a humidified incubator containing 5% CO_2. The Institutional Biosafety Committee Thammasat University on the use of cell lines approved this study, and was registered as Process No. 021/2021.

Preparation of sample solution

Crude extract (10 mg) was dissolved in sterile dimethyl sulfoxide (ethanolic extracts) or sterile water (aqueous extracts) to 10 mg/mL. Screening solution was prepared at a final concentration of 50 µg/mL and serial dilutions (200, 100, 20, 2 µg/mL) were prepared for IC_{50} determination.

Sulforhodamine B (SRB) assay

The optimal plating densities for KATO III, Hep G2, KKU-M156, SW480, LS 174T, and HaCaT were 1×10^4, 3×10^3, 2×10^3, 1×10^3, 3×10^3, and 8×10^3 cells/well, respectively. One hundred microliters of cell suspensions were seeded in 96-well plates, incubated at 37°C for 24 h. The cells were then exposed to test samples for 72 h, with solvent controls at ≤1%, and treated with paclitaxel, vincristine, 5-fluorouracil, or cisplatin as positive controls. Cytotoxicity was assessed using the SRB assay,[25,26] a colorimetric method that measures cell proliferation by staining total cellular protein with SRB. Cells were first fixed by adding 100 µL of ice-cold 40% trichloroacetic acid to each well and incubating at 4°C for 1 h. After fixing,

Table 1: Information on plant components of PSKP formulations

Scientific name (Family)	CODE	Voucher specimen number	Part used	Collected from	Proportion of PSKPC* (%w/w)	Proportion of PSKPIC* (%w/w)
Ocimum tenuiflorum L. (LABIATAE)	OT	SKP 095 15 19 01	Leaf	Pathum Thani	50.00	75.00
Citrus hystrix DC. (RUTACEAE)	CH	SKP 166 03 08 01	Peel	Kanchanaburi	22.22	-
Glycyrrhiza glabra L. (LEGUMINOSAE)	GG	SKP 072 07 07 01	Root	China	8.90	13.33
Ferula assa-foetida L. (UMBELLIFERAE)	FA	SKP 199 06 01 01	Oleo-gum resin	China	8.90	-
Piper nigrum L. (PIPERACEAE)	PN	SKP 146 16 14 01	Fruit	Chanthaburi	2.22	3.33
Zingiber officinale Roscoe (ZINGIBERACEAE)	ZO	SKP 206 26 15 01	Rhizome	Nakhon Pathom	2.22	3.33
Piper retrofractum Vahl (PIPERACEAE)	PR	SKP 146 16 03 01	Fruit	Suphan Buri	2.22	-
Allium sativum L. (AMARYLLIDACEAE)	AS	SKP 006 01 19 01	Bulb	Lamphun	2.22	3.33
Sodium chloride	NaCl	-	Crystal	Yasothon	1.10	1.68

*PSKPC: Complete PSKP formulation, PSKPIC: Incomplete PSKP formulation

the plate was washed using water and air-dried. Each well was then treated with 50 µL of SRB stain (1% in acetic acid) for 30 min, followed by rinsing with 1% acetic acid. After drying, 100 µL of 10 mM Tris base was added to each well to solubilize the dye. The optical density was measured at 492 nm. Cytotoxicity was calculated by comparing the treated cells to the control (untreated cells), and IC_{50} values were determined by plotting the percentage of cell survival against sample concentrations. The selectivity index was measured by dividing the IC_{50} value of a test compound in normal cells by the IC_{50} value in cancer cells.

In Vitro Assay for Anti-inflammatory Activity

Cell line

RAW 264.7 (ATCC® TIB71) murine leukemia macrophage cells were cultured in a 5% CO_2 incubator at 37°C.

Preparation of sample solution

The ethanolic extract was prepared at a concentration of 50 mg/mL, and the water extract at 10 mg/mL. The diluted solutions were initially screened at final concentrations of 100 µg/mL and 50 µg/mL. If the percentage of inhibition at these concentrations exceeded 50%, four serial dilutions were made to generate final concentrations ranging from 1 µg/mL to 100 µg/mL of the active extracts.

Determination of lipopolysaccharide (LPS)-induced nitric oxide (NO) production

RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in a 96-well plate and incubated at 37°C for 24 h. Control wells received 100 µL of complete RPMI with 5 ng/mL LPS, while normal wells contained only complete RPMI. Sample solutions (100 µL) were added to the wells and incubated for an additional 24 h. After incubation, 100 µL of the supernatant was transferred to a new 96-well plate, and 100 µL of Griess reagent was added. NO production was quantified by measuring nitrite concentration at 570 nm. The percentage of inhibition and IC_{50} values were calculated.[27]

3-(4,5 dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The MTT colorimetric method was used to assess cytotoxicity.[28] Cells were seeded in 96-well plates and allowed to adhere overnight before treatment with test extracts. Then, the medium was replaced with fresh medium containing the test compounds and incubated for 24 h. After 24 h of incubation, 10 µL of 5 mg/mL MTT solution in PBS was added to each well and incubated for 2 h. The supernatant was removed, and the formed formazan was dissolved in 100 µL of isopropanol with 0.04 M HCl. Absorbance was measured at 570 nm. Samples were considered non-toxic if the cytotoxicity was <30%. Only non-toxic samples were selected for further anti-inflammatory assay evaluation.

Hierarchical Clustering Analysis with Heatmap Visualization

Hierarchical clustering analysis of the resulting data obtained from *in vitro* assays was visualized as a heatmap using MultiExperiment Viewer version 4.9. The cut-off value (IC_{50}) for cytotoxic activity was set at 20 µg/mL. According to National Cancer Institute (NCI) guidelines, plant extracts having IC_{50} less than this value are considered highly cytotoxic.[29] As there are no standardized guidelines for inhibitory activity on NO production, we set the cut-off value for this activity at 30 µg/mL.

GC-MS

The OT and PSKP extracts were analyzed with GC-MS (Agilent 5977B) with an Agilent CP9205 (30 m × 250 µm × 0.25 µm) capillary column. Helium was applied as a carrier gas with a flow rate of 1 mL/min. The column oven was initially set to 50°C, then increased to 220°C at a rate of 5°C/min, and

maintained at 250°C for 15 min. The mass spectrum was recorded between mass values of 30 and 500. Chemical components were identified by the Office of Scientific Instrument and Testing, Prince of Songkla University.

Data and Statistical Analysis

Data obtained from *in vitro* assays were expressed as means ± SEM of three independent experiments, each performed in triplicate. IC_{50} values were calculated using GraphPad Prism 8 (CA, USA).

RESULTS

Cytotoxic Activity

Plants with IC_{50} values below 100 µg/mL were selected for cytotoxicity testing against normal cells (HaCaT). The IC_{50} values and selectivity index were calculated and are summarized in Table 2.

This study showed that the ethanolic extracts of PSKP (PSKPCE) and its plant component extracts exhibited superior cytotoxic activity compared to water extracts. Although the IC_{50} values of both PSKPCE and PSKPICE were higher than the NCI threshold of 20 µg/mL, PSKPCE demonstrated comparatively stronger activity against all tested cancer cell lines, while both extracts showed no cytotoxicity against the normal HaCaT cell line.

Among the ethanolic extracts, ZOE exhibited the strongest and broadest cytotoxic effects, with IC_{50} values below 12 µg/mL across all cancer cell lines except KATO III (26.57 µg/mL). PNE also demonstrated potent activity, especially against LS 174T (7.57 µg/mL) and Hep G2 (10.28 µg/mL), with acceptable selectivity. Similarly, PRE showed efficacy against Hep G2 and LS 174T with IC_{50} values of 14.17 and 18.77 µg/mL, respectively. CHE displayed cytotoxicity, particularly against KATO III, Hep G2, and LS 174T cells. Notably, PSKPCE showed improved cytotoxic activity across all cancer cell lines compared to PSKPICE, with IC_{50} values for HaCaT over 250 µg/mL, indicating good selectivity and safety. These results suggest that including the ethanolic extracts of ZO, PN, PR, and CH in the formulation enhances the bioactivity of PSKPCE.

Table 2: Cytotoxic activities of extracts of PSKP formulations and plant components against five types of cancer cell lines and one type of normal cell line (n=3)

Test sample	IC_{50} (µg/mL) and selectivity index (*in parentheses*)					IC_{50} (µg/mL)
	Hep G2	KKU-M156	SW480	LS 174T	KATO III	HaCaT
OTE	77.72±2.64 (2.62)	71.74±1.39 (2.84)	72.79±1.64 (2.80)	92.67±1.27 (2.20)	57.88±2.64 (3.52)	203.67±2.96
OTW	>50	>50	>50	>50	>50	NT
CHE	27.36±1.73 (2.83)	35.63±1.30 (2.17)	32.06±2.13 (2.41)	27.45±1.90 (2.82)	23.69±2.70 (3.26)	77.30±4.99
CHW	>50	>50	>50	>50	>50	NT
GGE	58.18±4.66 (2.86)	81.77±8.38 (2.04)	58.26±3.70 (2.86)	58.15±7.17 (2.87)	67.45±4.37 (2.47)	166.60±1.90
GGW	>50	>50	>50	>50	>50	NT
FAE	>50	>50	>50	>50	>50	NT
FAW	>50	>50	>50	>50	>50	NT
PNE	10.28±1.39 (3.66)	26.71±1.18 (1.41)	26.08±1.78 (1.44)	7.57±0.72 (4.97)	26.19±0.98 (1.44)	37.61±2.23
PNW	>50	>50	>50	>50	>50	NT
ZOE	9.79±0.57 (3.54)	7.64±0.42 (4.54)	10.46±1.09 (3.31)	11.59±0.50 (2.99)	26.57±0.63 (1.30)	34.64±0.60
ZOW	>50	>50	>50	>50	>50	NT
PRE	14.17±1.81 (5.71)	35.18±1.41 (2.30)	33.23±0.18 (2.44)	18.77±0.67 (4.31)	27.16±0.42 (2.98)	80.95±0.97
PRW	>50	>50	>50	>50	>50	NT
ASE	>50	>50	>50	>50	86.27±2.30 (11.59)	>1000
ASW	>50	>50	>50	>50	>50	NT
PSKPCE	34.89±2.30 (7.61)	54.26±2.74 (4.89)	48.72±3.59 (5.45)	37.41±0.97 (7.10)	30.80±1.99 (8.62)	265.44±5.01
PSKPCW	>50	>50	>50	>50	>50	NT
PSKPICE	59.10±3.84 (6.34)	76.51±4.32 (4.90)	68.80±4.75 (5.44)	58.85±5.70 (6.37)	50.21±2.02 (7.46)	374.61±8.87
PSKPICW	>50	>50	>50	>50	>50	NT
PTX	0.00009 (0.022)	0.00001 (0.200)	0.00069 (0.003)	0.00003 (0.067)	0.0000002 (10.00)	0.000002
VS	0.00615 (0.005)	0.00165 (0.020)	0.00871 (0.004)	0.00234 (0.014)	NT	0.000033
5-FU	NT	NT	NT	NT	0.11768 (0.387)	0.04549
CPT	NT	NT	NT	NT	6.45812±0.009	NT

The endings "E" and "W" in the codes for test samples denote ethanolic and water extracts, respectively. (The codes for plant samples are given in Table 1); PTX: Paclitaxel, VS: Vincristine sulphatem, 5-FU: 5-fluorouracil, CPT: Cisplatin, Hep G2: Hepatocellular carcinoma, KKU-M156: Cholangiocarcinoma, SW480, LS 174T: Colorectal adenocarcinoma, KATO III: Gastric carcinoma, HaCaT: Human keratinocytes cell line, NT: Not tested

Anti-inflammatory Activity

The results of the NO production assay are summarized in Table 3. PSKPCE demonstrated a stronger inhibitory effect on NO production compared to PSKPICE. Among the individual plant extracts, ZOE showed the highest activity, followed by PNE, PRE, and CHE. However, its activity was lower than that of the positive control prednisolone.

Clustered Heatmap

The resulting clustered heatmap displays the relationships of extracts of PSKP formulations and plant components (the rows) and biological activities investigated (the columns) as shown in Figure 1. The color key indicates the comparative potency of the activities (IC_{50} μg/mL) based on the cut-off values. Blue represents the low potency, while red represents the high potency; white represents the middle range. As demonstrated by the heatmap, ZOE exhibited the best activity based on overall consideration. The clustering results showed

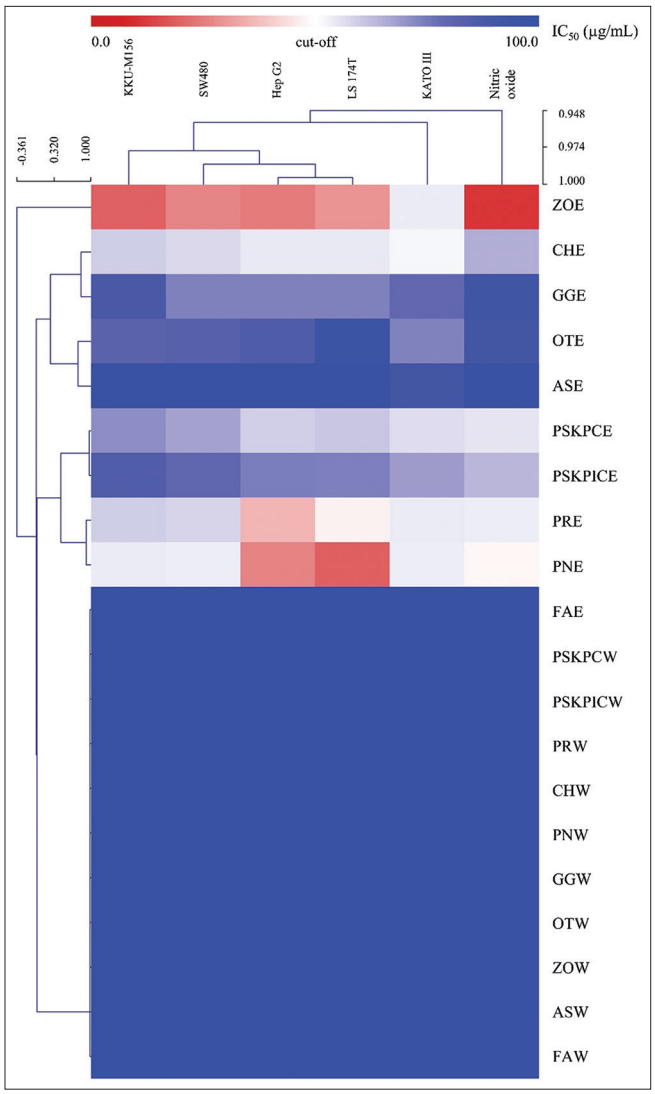

Figure 1: Clustered heatmap of cytotoxic and anti-inflammatory activities of extracts of Pra-Sa-Ka-Phrao formulations and plant components. The columns represent activities investigated; the rows represent extracts

that PRE and PNE had a relatively high degree of similarity in activity profile, as did PSKPCE and PSKPICE. However, PSKPCE displayed stronger potency than PSKPICE for all activities. A correlation was observed between cytotoxic and anti-inflammatory effects. The reduction of NO production at higher concentrations may also reflect cytotoxic effects, which impair the viability of immune cells responsible for inflammatory responses.[2,3]

GC-MS analysis

GC-MS analysis was performed on the ethanol and water extracts of OT (OTE, OTW), PSKPC (PSKPCE, PSKPCW), and PSKPIC (PSKPICE, PSKPICW). The major components identified in each extract are presented in Figures 2-4 and Table 4. In the analysis of essential oil compositions, OTE was found to be primarily composed of linolenic acid (10.11%), eugenol (8.91%), and methyl eugenol (8.71%). In contrast, PSKPCE exhibited a distinct profile, with caryophyllene (8.66%), *trans*-geranylgeraniol (6.45%), and linolenic acid (5.37%) as its most abundant constituents. Similarly, PSKPICE was characterized by a high concentration of caryophyllene (12.28%), followed by linolenic acid (10.42%) and methyl eugenol (8.91%). PN is known as a rich source of caryophyllene and is commonly included in traditional recipes. While basil (*Ocimum* spp.) also contains caryophyllene, OT is specifically noted as not contributing to the high content of this compound in the context of our study. Therefore, it is likely that PN is the main source of caryophyllene in the recipe. These variations in chemical composition highlight the diverse phytochemical profiles among the extracts, suggesting potential differences in their biological activities and applications.

DISCUSSION

This study provides novel evidence supporting the cytotoxic and anti-inflammatory activities of the ethanolic extract of PSKP complete formulation (PSKPCE) and highlights its potential as a candidate for GI disease treatment. In line with the study objective, we found that PSKPCE exhibited significant cytotoxic effects against five GI cancer cell lines, especially gastric carcinoma (KATO III), without harming normal cells. This study suggests its potential for cancer treatment, particularly for stomach-related conditions, which aligns with the traditional use of PSKP in managing gastric discomfort.

While analgesic activity was not directly tested in this study, the observed anti-inflammatory properties of PSKPCE, especially its ability to inhibit NO production, are related to mechanisms involved in pain modulation. Thus, the ability of PSKPCE and its component extracts (notably ZOE, PNE, and PRE) to suppress NO production suggests an analgesic potential. These findings support its traditional use for colic pain and flatulence and warrant further *in vivo* studies to directly evaluate analgesic effects.

The comparison between the complete (PSKPC) and incomplete (PSKPIC) formulations clearly shows that specific ingredients, which are CH, FA, and PR play a key role in enhancing the remedy's biological activity. CH leaves, which are contained in the formula as 22.22%, exhibited cytotoxic

Table 3: Inhibitory effects on LPS-induced NO production of PSKP formulations and plant components in murine macrophage cell line (RAW 264.7) (n=3)

Test sample	% Inhibition of NO production and % cytotoxicity (in parentheses) at various concentrations (μg/mL)				IC_{50} (μg/mL)
	0.01	0.1	1	10	
ZOE	−0.89±2.85	−0.59±3.09	11.51±2.99	62.54±2.25	4.37±0.15
	(0.52±1.48)	(8.49±0.95)	(12.14±1.59)	(36.22±1.63)	
	1	10	50	100	
ZOW	NT	NT	7.12±0.34	8.15±0.74	>100
			(12.50±0.70)	(14.79±1.04)	
OTE	−3.70±2.52	3.67±2.12	27.47±1.60	59.14±0.84	86.31±0.99
	(−10.63±3.43)	(−10.74±2.78)	(−6.21±1.86)	(−5.47±0.17)	
OTW	NT	NT	3.47±0.14	30.22±0.09	>100
			(4.29±0.07)	(15.23±0.12)	
CHE	−0.84±0.88	7.51±2.24	54.97±1.52	85.51±1.25	45.02±1.84
	(−4.95±2.97)	(−1.81±2.96)	(19.37±2.59)	(25.04±3.35)	
CHW	NT	NT	5.57±0.78	8.34±1.44	>100
			(0.76±0.13)	(6.87±0.68)	
GGE	0.90±1.93	3.86±1.54	27.96±1.05	55.42±1.23	89.21±2.43
	(−15.96±2.06)	(−11.09±1.72)	(−6.89±2.92)	(−2.17±2.97)	
GGW	NT	NT	−5.95±1.07	−3.62±0.99	>100
			(−5.69±0.73)	(−5.17±0.37)	
FAE	NT	NT	−3.62±0.41	8.76±0.82	>100
			(8.47±0.39)	(14.40±1.51)	
FAW	NT	NT	−11.62±0.71	−11.80±1.06	>100
			(−3.15±1.05)	(2.93±0.69)	
PNE	4.40±1.30	29.05±2.38	94.10±1.22	97.56±0.23	19.06±1.50
	(−14.31±3.83)	(5.27±4.24)	(26.96±3.67)	(92.20±0.34)	
PNW	NT	NT	−8.53±1.33	−6.91±0.70	>100
			(5.00±0.70)	(14.90±1.42)	
PRE	5.24±1.69	23.65±1.28	75.33±2.59	93.77±1.06	26.02±1.37
	(−2.83±1.69)	(11.24±3.29)	(22.09±2.69)	(55.00±0.71)	
PRW	NT	NT	−14.73±1.68	−7.26±0.99	>100
			(0.56±0.04)	(12.74±0.64)	
ASE	NT	NT	11.63±0.79	33.68±1.23	>100
			(8.19±0.31)	(12.77±1.16)	
ASW	NT	NT	−7.64±0.36	−3.39±1.41	>100
			(−5.99±0.72)	(3.18±0.68)	
PSKPCE	−3.79±2.69	17.35±1.43	71.24±0.40	91.12±0.20	28.78±0.04
	(−2.27±0.56)	(−2.25±1.50)	(10.62±1.31)	(12.62±2.29)	
PSKPCW	NT	NT	7.43±0.29	8.52±0.19	>100
			(1.55±0.03)	(3.68±0.22)	
PSKPICE	−1.09±4.76	13.21±2.98	55.61±0.25	74.53±0.42	42.21±0.36
	(−6.22±0.92)	(−3.64±3.81)	(−2.17±1.74)	(8.16±1.95)	
PSKPICW	NT	NT	7.00±0.49	10.23±0.09	>100
			(−6.06±0.05)	(−3.44±0.08)	
	0.01	0.1	1	10	
Prednisolone	22.90±0.89	29.20±0.53	52.51±0.86	74.23±0.49	0.69±0.01
	(20.17±1.36)	(29.32±1.18)	(30.04±1.96)	(32.31±3.05)	

NT: Not tested, LPS: Lipopolysaccharide, NO: Nitric oxide

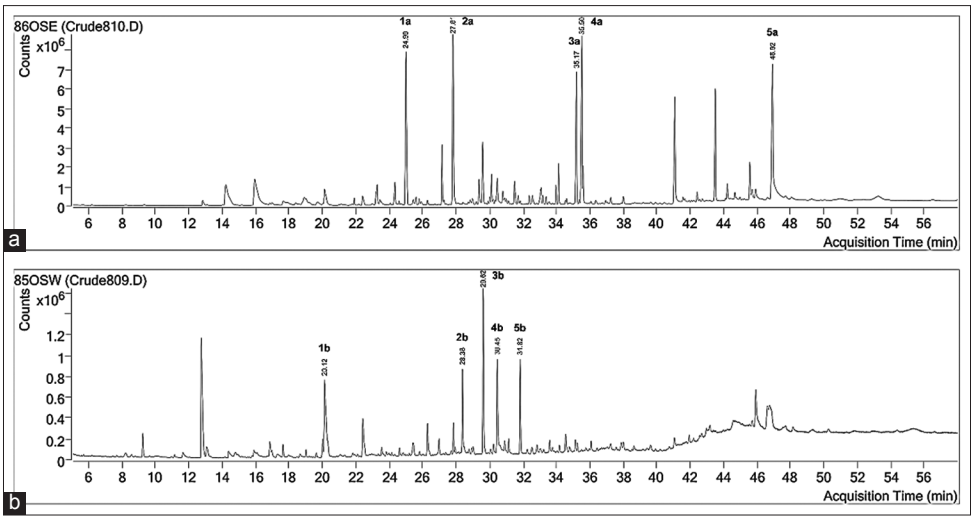

Figure 2: Gas chromatography-mass spectrometry fingerprints of ethanolic (OTE) and water extracts (OTW) of *O. tenuiflorum*: (a) OTE, 1a [methyl eugenol], 2a [eugenol], 3a [ethyl linolenate], 4a [phytol], 5a [linolenic acid]; (b) OTW, 1b [2-acetyloxazole], 2b [2-methoxy-4-vinylphenol], 3b [3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one], 4b [glycerin], 5b [(2E)-3-(2-hydroxyphenyl)-2-propenoic acid]

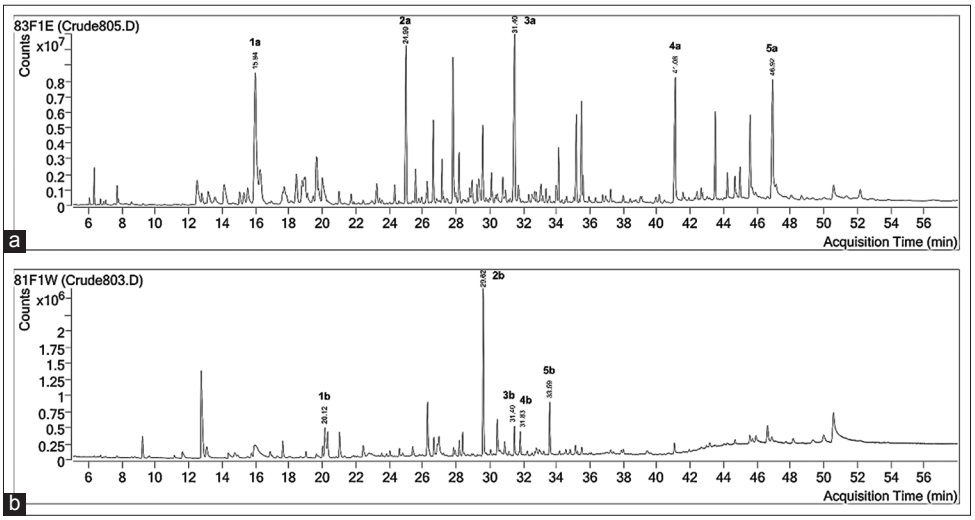

Figure 3: Gas chromatography-mass spectrometry fingerprints of ethanolic (PSKPCE) and water extracts (PSKPCW) of the complete PSKP formulation: (a) PSKPCE, 1a [caryophyllene], 2a [methyl eugenol], 3a [*trans*-geranylgeraniol], 4a [palmitic acid], 5a [linolenic acid]; (b) PSKPCW, 1b [2-acetyloxazole], 2b [3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one], 3b [*trans*-geranylgeraniol], 4b [2,3-dihydrobenzofuran], 5b [5-hydroxymethylfurfural]

effects on the HL60 promyelocytic leukemia cell line and various neuroblastoma cell lines, aligning with its anti-cancer potential observed in this study.[9,30] The presence of CH may enhance the remedy's cytotoxic effects, similar to PR, which showed comparable activity. PSKPICE, demonstrating moderate cytotoxicity and anti-inflammatory effects, lacked the synergistic potency observed in PSKPCE. These findings suggest that the complete formulation (PSKPCE), which includes *C. hystrix*, may improve overall cytotoxic potential, though its activity was not as high as the single extract (CHE).

As a main component in PSKP (containing 50% of the formula), OTE demonstrated lower cytotoxic activity compared to other components such as ZOE and CHE. Although OTE exhibited some activity against KATO III, its IC_{50} was above the NCI threshold for significant cytotoxic activity. Previous studies have also reported the cytotoxicity of leaves and aerial parts of OT against other types of cancer cells, including breast cancer (MCF-7), human gingival cancer (Ca9-22), lung cancer cell (A549), and head-and-neck squamous cell carcinoma cell lines.[8,31-33] For GG root extracts demonstrated limited cytotoxicity against GI cancer cells. However, GG extract has been reported to be active against MCF-7.[34]

For the three plants, PN, ZO, and PR, which are components of the Trikatuk remedy and constitute 6.66% of PSKP, each demonstrated notable cytotoxic activity against all types of GI cancer cells. Despite their relatively small proportion in the complete formula, these plants showed significant effects, particularly ZO, of which the ethanolic extract exhibited the highest cytotoxicity against various cancer cell lines, including cholangioma, liver, rectal, colon and gastric cancer cells. This is the first report demonstrating the high cytotoxic activity of ZO against KATO III. These results are consistent with previous

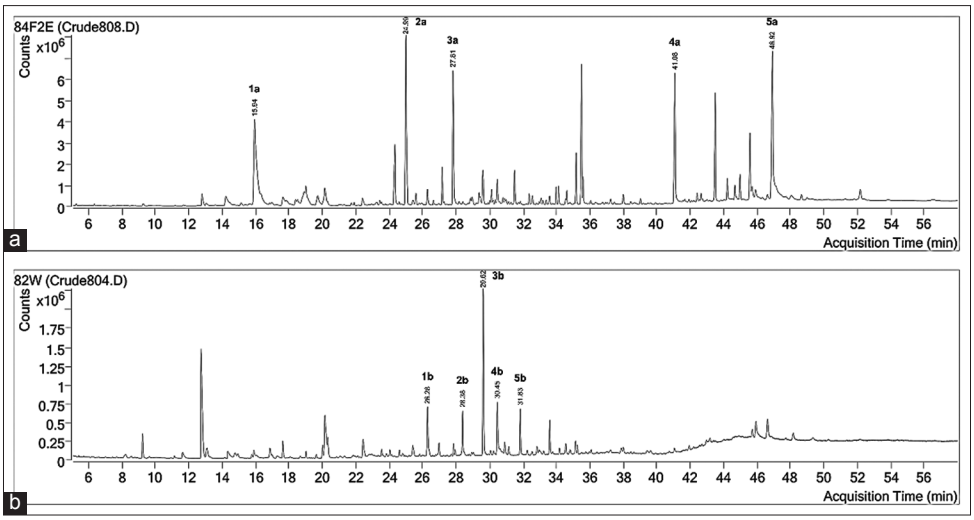

Figure 4: Gas chromatography-mass spectrometry fingerprints of ethanolic (PSKPICE) and water extracts (PSKPICW) of the incomplete PSKP formulation: (a) PSKPICE, 1a [caryophyllene], 2a [methyl eugenol], 3a [eugenol], 4a [palmitic acid], 5a [linolenic acid]; (b) PSKPICW, 1b [dihydroxyacetone], 2b [2-methoxy-4-vinylphenol], 3b [3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one], 4b [glycerin], 5b [2,3-dihydrobenzofuran]

studies where the compound 6-oxo-shogaol in rhizomes of ZO exhibited cytotoxic effects on Hep G2.[35] In contrast, the ethanolic extract of PN showed the highest cytotoxic activity against LS 174T with an IC_{50} value of 7.57 μg/mL, followed by Hep G2 and SW480, with IC_{50} values of 10.28 μg/mL and 26.08 μg/mL, respectively. These findings align with prior research highlighting the anticancer potential of PN seed extracts against GI cancer cells.[15,36] PR also demonstrated cytotoxic effects against multiple GI cancer types. The compound betulinic acid isolated from PR leaves and twigs has been shown to inhibit HCT116 and SW480 cells.[37]

AS, despite demonstrating less cytotoxicity in this study, the roots have been previously reported to have anticancer effects against Hep G2 and colon (Caco-2) cancer cells.[23]

The GC-MS analysis revealed that PSKPCE contained a significant proportion of *trans*-geranylgeraniol (6.45%), a compound not found in PSKPICE. *Trans*-Geranylgeraniol is a diterpenoid alcohol known for its biological activities, including cytotoxic, antimicrobial, and anti-inflammatory properties. In the context of PSKPCE, the likely source of *trans*-geranylgeraniol is CH. Geranylgeraniol has previously been reported for its cytotoxic effects on human tumors,[38] the presence of this compound in PSKPCE may contribute to its enhanced anticancer activity.

For anti-inflammatory activity, PSKPCE demonstrated superior effects compared to PSKPICE. From this study, ZOE showed the highest anti-inflammatory activity, followed by PNE, PRE, and PSKPCE. These results align with previous reports demonstrating that ZO exhibits potent anti-inflammatory activities in both acute and chronic stages, in both *in vitro* and *in vivo* models.[16] In addition, PN fruits have been shown to reduce inducible NO synthase-mediated NO production and inhibit pro-inflammatory cytokines (interleukin [IL]-6, IL-1β, Prostaglandin E2, and tumor necrosis factor-α) in RAW 264.7 cells and anti-inflammatory effects in *in vivo* testing using the carrageenan-induced paw edema model.[14] Similarly, the ethanolic extract of PR flowers exhibited significant anti-inflammatory effects on NO production.[20] The chemical components extracted with ethyl acetate from the fruit of CH also showed anti-inflammatory activity on NO production.[39]

The hierarchical clustering analysis revealed distinct patterns of anti-inflammatory and cytotoxic activities among the plant extracts. The heatmap indicated that water extracts lacked both cytotoxic and anti-inflammatory activity. Among them, ZOE demonstrated a broad spectrum of cytotoxicity across all cancer cell types and strong anti-inflammatory effects. PNE and PRE exhibited selective activity against specific cancer cell lines, such as PNE being particularly potent against colon and liver cancers, and PRE showing activity against colon cancer. These findings support the concept that PSKPCE, due to its diverse plant ingredients, may offer a promising source for developing novel anti-inflammatory and anticancer drugs.

These findings not only validate the traditional use of PSKP for GI conditions but also provide a scientific basis for its further development. PSKP, composed of edible and spice plants, may also contribute to the prevention of inflammation-induced cancers. Many of the plants in PSKP contain volatile oils. For example, CH and FA have been reported to stimulate blood circulation and exhibit antispasmodic effects on the nervous system.[40] Furthermore, GG root contains glycyrrhizin, a triterpenoid saponin, known for its anti-inflammatory and wound-healing properties.[41] These attributes support the traditional use of PSKP for treating flatulence, as the volatile oils in these plants stimulate intestinal movement. Given its anti-inflammatory and anticancer properties, PSKP could be further developed as a functional health food or nutraceutical, contributing to inflammation and cancer prevention.

Although PSKPICE demonstrated lower cytotoxic and anti-inflammatory activities compared to the complete formulation (PSKPCE), it still exhibited moderate biological

Table 4: Chemical compositions of OT, PSKPC and PSKPIC analyzed by using gas chromatography-mass spectrometer

Compound	Formula	RT/min	% of Total					
			OT		PSKPC		PSKPIC	
			EtOH	Water	EtOH	Water	EtOH	Water
Alpha-Terpinene	$C_{10}H_{16}$	6.35	-	-	0.62	-	-	-
Gamma-Terpinene	$C_{10}H_{16}$	7.71	-	-	0.34	-	-	-
Hydroxyacetone	$C_3H_6O_2$	9.24	-	1.33	-	-	-	2.01
cis-Linalool oxide	$C_{10}H_{18}O_2$	12.50	-	-	1.55	-	-	-
trans-Linalool oxide	$C_{10}H_{18}O_2$	13.16	-	-	0.72	-	-	-
Pentadecane	$C_{15}H_{32}$	14.11	3.36	-	1.38	-	-	-
Caryophyllene	$C_{15}H_{24}$	15.94	4.73	-	8.66	-	12.28	-
Glutaric acid	$C_5H_6O_2$	20.02	-	1.04	-	-	-	-
2-Acetyloxazole	$C_5H_5NO_2$	20.12	1.43	9.45	-	3.36	-	-
N-Acetyl-L-proline	$C_7H_{11}NO_3$	22.41	-	3.32	-	-	-	-
Caryophyllene oxide	$C_{15}H_{24}O$	24.34	-	-	-	-	3.28	-
Methyl eugenol	$C_{11}H_{14}O_2$	24.99	8.71	-	5.13	-	8.91	-
Dihydroxyacetone	$C_3H_6O_3$	26.28	-	2.13	-	-	-	4.77
(1*S*,2*R*,5*R*)-2-(2-Hydroxypropan-2-yl)-5-methylcyclohexanol	$C_{10}H_{20}O_2$	26.63	-	-	1.87	-	-	-
(*E*)-2-Propenoic acid, 3-phenyl-, ethyl ester	$C_{11}H_{12}O_2$	27.14	2.18	-	0.91	-	1.28	-
Eugenol	$C_{10}H_{12}O_2$	27.81	8.91	1.22	4.23	-	6.32	-
Cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl-	$C_{10}H_{20}O_2$	28.17	-	-	1.29	-	-	-
2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	28.38	-	4.19	-	-	-	3.31
Ethyl palmitate	$C_{18}H_{36}O_2$	29.58	3.05	-	2.18	-	-	-
3,5-Dihydroxy-6-methyl-2,3-dihydropyran-4-one	$C_6H_8O_4$	29.62	-	8.64	-	11.66	-	12.86
Glycerin	$C_3H_8O_3$	30.45	1.50	6.01	-	-	1.09	4.59
Trans-geranylgeraniol	$C_{20}H_{34}O$	31.40	-	-	6.45	1.88	-	-
Isoaromadendrene epoxide	$C_{15}H_{24}O$	31.48	-	-	-	-	1.44	-
(2*E*)-3-(2-Hydroxyphenyl)-2-propenoic acid	$C_9H_8O_3$	31.82	-	4.49	-	-	-	-
2,3-Dihydrobenzofuran	C_8H_8O	31.83	-	-	-	1.46	-	3.21
5-Hydroxymethylfurfural	$C_6H_6O_3$	33.59	-	-	-	3.37	-	2.44
Phytol, acetate	$C_{22}H_{42}O_2$	33.97	-	-	-	-	0.54	-
Ethyl linoleate	$C_{20}H_{36}O_2$	34.13	1.35	-	-	-	-	-
Ethyl linolenate	$C_{20}H_{34}O_2$	35.17	5.88	-	2.31	-	1.87	-
Phytol	$C_{20}H_{40}O$	35.50	7.63	-	2.49	-	5.26	-
Ethyl p-methoxycinnamate	$C_{12}H_{14}O_3$	35.59	1.36	-	-	-	1.00	-
Palmitic acid	$C_{16}H_{32}O_2$	41.08	5.62	-	4.57	-	6.54	-
Squalene	$C_{30}H_{50}$	43.50	4.84	-	2.16	-	4.24	-
Stearic acid	$C_{18}H_{36}O_2$	44.22	0.75	-	-	-	-	-
Linoleic acid	$C_{18}H_{32}O_2$	45.56	1.66	-	2.56	-	3.12	-
Linolenic acid	$C_{18}H_{30}O_2$	46.92	10.11	-	5.37	-	10.42	-

(-): Not founded or match factor<90.0, OT: *Ocimum tenuiflorum*, PSKPC: Complete PSKP formulation, PSKPIC: Incomplete PSKP formulation

activity against GI cancer cell lines and in NO inhibition assays. Importantly, PSKPICE omits ingredients restricted in food supplements by Thai FDA regulations, making it a legally compliant formulation. These findings suggest that PSKPICE could serve as a functional food or nutraceutical product aimed at managing inflammation-related GI conditions.

CONCLUSION

The ethanolic extract of the complete PSKP formulation (PSKPCE) demonstrated significant biological activity, particularly in its cytotoxic effects against five types of GI cancer cell lines, while sparing normal cells from toxicity. Furthermore, it exhibited anti-inflammatory properties by

effectively inhibiting NO production. These effects were not observed in the incomplete formulation (PSKPICE), highlighting the importance of specific components such as *C. hystrix* and *P. retrofractum*. GC-MS analysis identified caryophyllene and *trans*-geranylgeraniol as the most abundant compounds in the extract. These results suggest that the PSKP, along with its key plant ingredients, holds promise as a multi-functional agent with anti-inflammatory and cytotoxic, particularly for chronic illnesses such as cancer. This study not only supports the traditional use of PSKP but also highlights its possibility for the development of additional products for inclusion in Thailand's National List of Essential Herbal Medicines. Such natural products could serve as alternative healthcare options for the elderly, offering a cost-effective approach to reducing healthcare expenditures. In addition, PSKPICE, while being less potent than PSKPCE, still exhibited cytotoxic and anti-inflammatory effects. Because it excludes ingredients restricted for food supplements by Thai FDA regulations, PSKPICE presents an advantage in terms of legal compliance and product development feasibility. Therefore, PSKPICE could be further explored as a health supplement for GI-related disorders.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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