Anticancer Potential of Piper nigrum

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Current anticancer therapy suffers from several limitations, including lack of selectivity and multidrug resistance. Natural products represent an excellent opportunity for the identification of new therapeutic options due to their safety, low toxicity, and general availability. Piper nigrum is one of the most popular species in the world, with growing fame as a source of bioactive molecules with pharmacological properties. Undeniable anticancer properties are reportetd for different Piper nigrum constituents, such as its main alkaloid piperine.

Keywords: Piper nigrum ; piperine ; cancer therapy ; anticancer mechanisms

1. Introduction

Nowadays, cancer represents one of the biggest challenges that must be handled as a multifaceted global health issue. In fact, cancer is still the second leading cause of death worldwide and was responsible for 9.6 million deaths in 2018 [1]. Notwithstanding advances in the knowledge of cancer, supported by cutting-edge research and advanced technologies for its diagnosis and treatment, the discovery of new therapeutic agents is a hot topic in cancer research. The major disadvantages of conventional chemotherapy are the recurrence of cancer, drug resistance, and toxic effects on non-targeted tissues. Moreover, side effects can restrain the use of anticancer drugs and thus impair a patient's quality of life [2]. Numerous medicinal plants and isolated phytochemicals have gained immense attention due to their ability to target heterogeneous populations of cancer cells and regulate key signaling pathways involved in cancer development at different stages and their wide safety profile [3].

Black pepper (*Piper nigrum* L. family Piperaceae) is one of the most used household spices in the world, with its characteristic biting quality. The use of black pepper is not limited to culinary purposes, and it is also used as a preservative, an insecticide, and medication [4]. *Piper nigrum* is a perennial climbing herb native to the Malabar Coast of India. The herb grows up to a height of 10 m by means of its aerial roots. The black pepper fruits, which are obtained from dried green unripe drupe, and seeds have been extensively used in folk medicine to treat conditions ranging from gastrointestinal diseases to epilepsy [5]. The medical properties of pepper are mainly imputable to the alkaloid piperine. Piperine exerts anti-inflammatory, neuroprotective, immunomodulatory, cardioprotective, and anticancer effects [6][7]. Moreover, piperine is well-known to influence the bioavailability of drugs and nutrients, increasing their intestinal absorption and regulating their metabolism and transport, thus representing a bioenhancer [7].

2. Anticancer Activity of *Piper nigrum* Extracts

Piper nigrum extracts could offer an interesting synergy of its single bioactive constituents, achieving anticancer activity through complementary mechanisms. Extracts from different parts of the plant, including roots, seeds, and fruits, have been explored. Different preparations of the extract from the same part of the plant give rise to different and surprising effects (Table 1), discussed hereunder.

Table 1. In vitro and in vivo anticancer effects of *Piper nigrum* extracts.

***Piper nigrum* Extracts**	**Experimental Model**	**IC_{50} [a] or EC_{50} [b] (Time of Treatment)**	**Anticancer Effects and Molecular Targets**	**Reference**

Seeds' ethanolic extract (50% ethanol)	Colorectal cancer cells (HCT-116, HCT-15, HT-29)	IC_{50}: HCT-116: 4.0 (24 h) 3.1 (48 h) 3.4 (72 h) µg/mL HCT-15: 3.2 (24 h) 2.9 (48 h) 1.9 (72 h) µg/mL HT-29: 7.9 (24 h) 6.1 (48 h) 7.4 (72 h) µg/mL	↑ tumor cell death	[8]
Seeds' extract, SnO_2 nanoparticles	Colorectal cancer cells (HCT-116) and lung cancer cells (A549)	IC_{50}: HCT-116: 165 µM A549: 135 µM	↑ ROS [c]	[9]
Fruits' ethanolic extract	Vitro: Breast cancer cells (MCF-7) and colon cancer cells (HT-29) (1–1000 µg/mL) Vivo: Ehrlich ascites carcinoma-bearing male Balb/c mice (intraperitoneal injection (i.p.), 100 mg/kg/day in saline containing 1% Tween 80, for 9 days)	EC_{50}: MCF-7: 27.1 µg/mL (24 h) HT-29: 80.5 µg/mL (24 h)	Vitro: ↑ tumor cell death ↓ tumor cell proliferation ↑ ROS, ↑ DNA damage Vivo: ↓ tumor growth, ↑ mice survival ↑ apoptosis cell-cycle arrest at G1/S (↑ Bax, p53; ↓Bcl-xL, cyclin A) ↑ oxidative stress (↑ lipid peroxidation, protein carbonylation, GR [d], SOD [e], CAT [f])	[10]
Supercritical fluid extract (SFE) of fruits' ethanolic extract	Vitro: Breast cancer cells (MCF-7) (1–1000 µg/mL) Vivo: Ehrlich ascites carcinoma-bearing male Balb/c mice (i.p., 10 or 100 mg/kg/day in saline containing 1% Tween 80, for 9 days)	EC_{50}: 14.40 µg/mL (72 h) IC_{50}: 27.8 µg/mL (24 h)	Vitro: ↑ apoptosis Silico (docking study): Piperine interaction with CDK2 [g], ATP binding site; cyclin A binding site and Bcl-xL binding site. Vivo: ↓ tumor growth, ↑ mice survival ↑ apoptosis cell-cycle arrest at G2/M (↑ Bax, p53; ↓Bcl-xL, ↓cyclin A, ↓CDK2)	[11][12]

Fruits' (i) methanol crude extract or (ii) dichloromethane crude extract	Breast cancer cells (MCF-7, MDA-MB-231, MDA-MB-468)	IC_{50} (72 h) methanol crude extract: MCF-7: 20.25 µg/mL MDA-MB-231: 22.37 µg/mL MDA-MB-468: 9.04 µg/mL IC_{50} (72 h) dichloromethane crude extract: MCF-7: 23.46 µg/mL MDA-MB-231: 38.82 µg/mL MDA-MB-468: 7.94 µg/mL	↑ tumor cell death	[13]
Piperine-free *Piper nigrum* fruits' extract (PFPE)	Vitro: Breast cancer cells (MCF-7, MDA-MB-231, MDA-MB-468, ZR-75-1), colorectal cancer cells (HT-29, SW-620), lung cancer cells (H358, A549), neuroblastoma cells (LA-N-5, SK-N-SH). Vivo: Female ICR mice (oral administration (os) 5000 mg/kg b.w. in mixture of distilled water and Tween-80 (4:1 *v*/*v*) for acute oral toxicity studies) or NMU-treated female Sprague-Dawley treated orally with (i) 100 or 200 mg/kg b.w. in mixture of distilled water and Tween-80 (4:1 *v*/*v*) at 14 days after NMU application three times per week up to 76 days, or (ii) 100, 200, or 400 mg/kg b.w. PFPE after the first NMU-induced tumor every two days up to 30 days	IC_{50} (72 h): MCF-7: 7.45 µg/mL MDA-MB-231: 22.67 µg/mL MDA-MB-468: 18.19 µg/mL ZR-75-1: 13.85 µg/mL HT-29: 27.74 µg/mL SW-620: 29.56 µg/mL H358: 34.69 µg/mL A549: 30.77 µg/mL LA-N-5: 111.28 µg/mL SK-N-SH: 21.51 µg/mL	Vitro: ↓ cell proliferation ↑ apoptosis (↑ p53 and cytochrome c; ↓ topoisomerase II) Vivo: ↓ tumor bearing rats ↓ tumor size, ↑ cytochrome c in tumor tissues	[14]

	Vitro: Breast cancer cells (MCF-7)		Vitro: ↓ E-cadherin, c-myc, VEGF [h]	
Piperine-free *Piper nigrum* fruits' extract	Vivo: NMU-treated female Sprague-Dawley rats. PFPE treatment regimen as previously described above		Vivo: ↑ p53 ↓ E-cadherin, MMP [i]-9, MMP-2, c-myc, and VEGF	[15]
Root dried power crude (i) petroleum ether extract, (ii) chloroform extract, (iii) ethylacetate extract	Promyeolocytic leukemia cells (HL60)	IC_{50}: petroleum ether extract (72 h): 11.2 µg/mL chloroform extract (72 h): 9.8 µg/mL ethylacetate extract (72 h): /	↑ tumor cell death	[16]

↑: increase; ↓: decrease; [a] IC_{50}: half maximal inhibitory concentration; [b] EC_{50}: half maximal effective concentration; [c] ROS: reactive oxygen species; [d] GR: glutathione reductase; [e] SOD: superoxide dismutase; [f] CAT: catalase; [g] CDK2: cyclin-dependent kinase 2; [h] VEGF: vascular endothelial growth factor; and [i] MMP: matrix metalloproteinase.

Ethnomedicinal surveys have revealed that seeds and fruits are the most used and studied part of the *Piper nigrum* plant [4].

Different seeds' ethanolic extracts (50, 70, or 100% ethanol) were studied in three colorectal cell lines (Table 1). The highest cytotoxic effect was seen for the 50% seeds' ethanolic extract (EEPN) [8]. The highest biological activity of EEPN was imputable to the highest content of total phenolic compounds extracted. Additionally, EEPN showed antioxidant and anti-inflammatory properties, which were assessed by biochemical assays [8]. No insight into the molecular mechanisms of EEPN's cytotoxicity was provided. Recently, Tammina and colleagues [9] investigated the anticancer activity of a *Piper nigrum* water seeds' extract formulated as SnO_2 nanoparticles in colorectal (HCT-116) and lung (A549) cancer cell lines (Table 1). They demonstrated that higher dose and smaller size nanoparticles generated more reactive oxygen species (ROS) and hence exhibited a higher cytotoxicity compared to larger size nanoparticles [9], underlying the crucial role of formulation in improving the biological activity of *Piper nigrum* preparations.

The anticancer activity of a macerated ethanolic extract of *Piper nigrum* fruits was explored in both in vitro and in vivo breast cancer models [10] (Table 1). Treatment with the extract induced intracellular oxidative stress, which was considered the main component responsible for its cytotoxic effects in cancer cells. Since ROS can cause DNA damage, the observed oxidative DNA damage corroborated ROS involvement in the anticancer effects of the extract. These findings were confirmed in vivo, where increased lipid peroxidation and protein carbonylation and an elevated activity of the antioxidant enzymes were recorded (Table 1) [10]. The same research group investigated the anticancer potential of a high-pressure extract from unripe fruits of the black pepper cultivar Bragantina, obtained by supercritical fluid extraction (SFE) [11]. SFE represents an energy-efficient and environmentally friendly extraction technology that helps to overcome the limitation of the poor solubility of molecules such as piperine. The SFE extract showed a higher content of piperine and the highest cytotoxic activity compared to conventional ethanolic extracts [11] (Table 1). A following docking study [12] revealed the interaction of piperine with the ATP binding site of the cell-cycle regulators cyclin-dependent kinase 2 (CDK2) and cyclin A and with the antiapoptotic protein Bcl-xL. In vitro and in vivo studies on the cytotoxic activity of the SFE extract confirmed its ability to arrest the cell cycle in the G2/M phase and its pro-apoptotic effects through CDK2, cyclin A, and Bcl-xL inhibition [12]. Interestingly, treatment with macerated *Piper nigrum* ethanolic fruit extract induced cell-cycle arrest in the G1/S phase [10], whereas the SFE extract induced cell-cycle arrest in the G2/M phase [12]. This is not surprising if we consider that the SFE extract is enriched in piperine, which univocally induces cytostasis in the G2/M phase in breast cancer cells (Table 2). These data suggest that piperine is the main component responsible for the anticancer effects of the SFE extract.

Table 2. In vitro and in vivo anticancer activity of piperine.

Cancer Type	Experimental Models	Piperine	IC_{50} [a]	Anticancer Effects and Molecular Targets	Reference

Breast cancer	Vitro: 4T1 mouse mammary carcinoma cells Vivo: Female BALB/c mice syngeneic to 4T1 cells (4T1 cells transplanted subcutaneously)	Vitro: 35–280 µM Vivo: Intratumoral injection of 2.5 or 5 mg/kg every 3 days 3 times	105 ± 1.08 µM (48 h) 78.52 ± 1.06 µM (72 h)	Vitro: ↑ apoptosis (↑ caspase-3 activity) ↓proliferation (↓ cyclin B1, cell-cycle block in G2/M phase) ↓ migration; ↓ MMP [b]-9 and MMP-13 Vivo: ↓ tumor growth ↓ lung metastasis	[17]
	HER-overexpressing cells: SKBR3 and BT-474 Basal HER-expressing cells: MCF-7 and MDA-MB-231	10–200 µM	SKBR3 50 µM (48 h) MCF-7 > 200 µM (48 h)	↑ apoptosis (↑ caspase-3 activity, cleaved-PARP [c], DNA damage) ↓ HER2 [d] expression ↓ SREBP-1 [e] and fatty acid synthase via ERK1/2 [f] inhibition ↓ MMP-9 via inhibition of Akt and MAPK [g] signaling	[18]
	Vitro: MDA-MB-231, MDA-MB-468, murine 4T1 Vivo: BALB/c female mice orthotopically-inoculated 4T1	Vitro: 25–200 µM Vivo: Oral administration (os) 50 mg/kg/day from day 7 to 21		Vitro: ↓ proliferation (cell-cycle block in G2/M phase) ↓ survivin and p65 phosphorylation Vivo: ↓ tumor growth	[19]
	MDA-MB-231, MDA-MB-468, T-47D, and MCF-7	50–150 µM		↑ apoptosis (↑ Smac/DIABLO [h], cytochrome c; ↓ IAPs [i]) ↓ cell-cycle progression (↑ p21; ↓ CDK [j]4, CDK1, cyclin D3, cyclin B, E2F1 [k], CDC25 C [l]) ↓ mammospheres' growth ↓ MMP-2, MMP-9	[20]

Vitro: Mouse mammary EMT6/P cancer cells Vivo: Balb/C female mice with EMT6/P cells injected subcutaneously in the abdominal area	Vitro: 50–1200 µM Vivo: Intraperitoneal injection (i.p.) 25 mg/kg/day in PBS for 14 days	870 µM (48h)	Vitro: ↑ apoptosis (↑ caspase-3 activity) ↓ VEGF [m] Vivo: ↓ tumor size ↑ apoptosis in tumor tissue ↓ ALT [n], AST [o], creatinine	[21]
MCF-7, T-47D	3–100 µM	MCF-7 37.34 µM (24 h) T-47D 61.05 µM (24 h)	↑ apoptosis (↑ Bax, ↓ Bcl-2) ↓ proliferation (cell-cycle block in G2/M phase)	[22]
MDA-MB-231	20–320 µM	238 µM (72 h)	↓ proliferation	[23]

	DU145, LNCaP, and PC3	20–320 µM	LNCaP 74.4 µM (24 h) DU145 226.6 µM (24 h) PC3 111.0 µM (24 h)	↓ proliferation (cell-cycle block in G0/G1 phase, ↓cyclin D1 and cyclin A; ↑ p21 and p27) ↑ autophagy (↑ LC3B [p]-II and LC3B puncta formation)	[24]
	Vitro: DU145, LNCaP, 22RV1, and PC3 Vivo: Nude mice (LNCaP or DU145 transplanted subcutaneously)	Vitro: 50–200 µM Vivo: I.p., 100 mg/kg/day in vegetable oil for 1 month os 10 mg/kg body weight (b.w.) for 1 month	LCNaP 60 µM (24 h) PC3 75 µM (24 h) 22Rv1 110 µM (24 h) DU145 160 µM (24 h)	Vitro: ↑ apoptosis (↑ caspase-3 activity and cleaved-PARP) ↓ migration (↓ STAT-3 [q] and NF-kB [r]) Vivo: ↓ tumor growth	[25]
Prostate cancer	LNCaP and PC3	5–150 µM	LNCaP 39.91 µM (24 h) PC3 49.45 µM (24 h)	↑ apoptosis ↓ proliferation (cell-cycle block in G0/G1) via voltage-gated K^+ current blockade	[26]
	LNCaP ad PC3	0.1–100 µM	LNCaP 39.91 µM (24 h) PC3 49.45 µM (24 h)	↑ apoptosis ↓ proliferation (cell-cycle block in G1 phase) via voltage-gated K^+ current inhibition	[27]
	DU145	80–320 µM		↑ apoptosis (↑ Bax, ↓ Bcl-2) ↓ proliferation ↓ migration (↓ MMP-9 via inhibition of Akt/mTOR signaling)	[28]

Colon cancer	DLD1	1–200 µM		↓ proliferation	[29]
	HT-29, Caco-2, SW480, HCT-116 (p53+/+), and HCT-116 (p53−/−)	10–150 µM	HT-29 53 ± 1 µM (72 h) Caco-2 54 ± 5 µM (72 h) SW480 126 ± 3 µM (72 h) HCT-116 (p53+/+) 109 ± 9 µM (72 h) HCT-116 (p53−/−) 118 ± 7 µM (72 h)	↑ apoptosis (↑ loss of mitochondrial membrane potential, caspase activity, cleaved-PARP) ↑ ROS [s] ↑ endoplasmic reticulum stress (↑ IRE1α [t], CHOP [u], BiP [v]) ↓ survivin ↓ proliferation (cell-cycle block in G1 phase; ↓ cyclin D1 and cyclin D3, CDK4 and CDK6; ↑ p21 and p27) ↓ colony formation and spheroids' growth	[30]
	HCT6, SW480, and DLD1	20–200 µM		↓ proliferation ↓ migration ↓ Wnt/β-catenin and GSK3β [w]	[31]
	SW480 and HCT-116	25–800 µM		↓ migration and EMT [x] (↓ STAT-3/Snail, ↓ vimentin, ↑ E-cadherin)	[32]
Rectal cancer	HRT-18	10–150 µM		↑ apoptosis ↓ proliferation (block cell-cycle progression) ↑ ROS	[33]

				↑ animal survival	
	Vivo: C57BL/6 Mice lung metastasis from melanoma cells (B16F-10 lateral tail vein injection)	I.p., 200 µmol/kg b.w. in 0.1% gum acacia for 10 days		↓ metastatic lung fibrosis, ↓ uronic acid and hexosamine in lung tissue ↓ serum level of sialic acid and GGT [y]	[34]
	Vivo: Swiss Albino mice benzo(a)pyrene induced lung cancer (os in corn oil 50 mg/kg b.w.)	Os 50 mg/kg b.w. in corn oil: (i) On alternate days for 16 weeks immediate after the first dose of carcinogen; (ii) piperine as (i), but starting from the sixth week of B(a)P till the end of the experiment		↓ lipid peroxidation, protein carbonyls, nucleic acid content, and polyamine synthesis in lung	[35]
Lung cancer	Vivo: Swiss Albino mice benzo(a)pyrene induced lung cancer (os in corn oil 50 mg/kg b.w.)	Os 50 mg/kg b.w. in corn oil for 16 weeks. Treatment: (i) Immediately after the first dose of benzo(a)pyrene; (ii) after the last dose of benzo(a)pyrene		↓ hexose, hexosamine and sialic acid in serum, liver, and lung tissues	[36]
	A549	25–400 µM	122 µM (48 h)	↑ apoptosis (↑ caspase3 and -9 activity, Bax/Bcl-2 ratio, p53 expression) ↓ Proliferation (cell-cycle block in G2/M phase)	[37]
	A549	100–500 µM		↑ apoptosis (↓ c-myc)	[38]
	A549	20–320 µM	198 µM (72 h)	↓ EMT (↓ fibronectin and N-caderin, ↑ E-cadherin) ↓ ERK 1/2 and SMAD [z] 2 ↓ migration (↓ MMP-2)	[23]

Melanoma	SK MEL 28, A375 (human cells), and B16 F0 (murine cells)	75–300 µM	SK MEL 28 221 µM (24 h) 172 µM (48 h) 136 µM (72 h) B16 F0 200 µM (24 h) 155 µM (48 h) 137 µM (72 h) A375 225 µM (24 h) 160 µM (48 h) 100 µM (72 h)	↑ apoptosis (↑ p53; ↓ XIAP [aa], Bid [ab]; ↑ Caspase-3 and cleaved-PARP) ↓ proliferation (cell-cycle block in G1 phase; ↓ cyclin D, E2F1, and Rb [ac] phosphorylation; ↑ p21, ATR [ad], Chk [ae] 1) ↑ ROS ↑ DNA damage (↑ H2AX [af] phosphorylation)	[39]
	Vitro: A375SM (highly metastatic), A375P (moderately metastatic) Vivo: BALB/c nude mice (nu/nu) (A375SM or A375P transplanted subcutaneously)	Vitro: 50–200 µM Vivo: Os 50 or 100 mg/kg b.w. in water 5 times per week for 4 weeks		Vitro: ↑ apoptosis (↑ Bax, cleaved-PARP, caspase-9, ↓ Bcl2) ↑ JNK/p38 MAPK phosphorylation, ↓ ERK1/2 Vivo: ↓ tumor growth ↑ apoptosis (↑ caspase-3) ↓ ERK1/2	[40]
Hepatocellular cancer	Vitro: HepG2 Vivo: Male Wistar rats tumor induced using diethylnitrosamine (DEN, 0.01% of DEN in drinking water for 16 weeks)	Vitro: 5–100 µM Vivo: Os 5 mg/kg b.w. in corn oil for 6 weeks starting from the 10th week of the experimental period	75 µM (24 h) 30 µM (48 h)	↑ apoptosis (↑ cleaved caspase-3 and caspase-9, mitochondrial permeabilization, Bax, cytochrome c release, ↓ Bcl-2) ↓ proliferation ↑ ROS (↓ catalase) ↓ ERK1/2 and SMAD Vivo: ↓ AST, ALP [ag], and ALT ↑ improvement in liver architecture ↓ Ki67	[41]
	HepG2	20–320 µM	214 µM (72 h)	↓ proliferation	[23]

Ovarian	A2780	4–20 µM		↑ apoptosis (↑ cytochrome c release, caspase-3 and caspase-9 activity, cleaved-PARP) ↑ JNK and p38 MAPK phosphorylation	[42]
	OVACAR-3 (ovarian cisplatin-resistant cells)	3.12–200 µM	28 µM (24 h)	↑ apoptosis (↑ caspase-3, caspase-9, and Bax) Cell-cycle block in G2/M phase ↓ migration ↓ MAPK signaling (PI3 K [ah]/Akt/GSK3β)	[43]
Osteosarcoma	HOS and U2OS	25–200 µM	HOS 72 µM (72 h) H2OS 126 µM (72 h)	↓ proliferation (cell-cycle block in G2/M phase, ↓ cyclin B1, ↑ CDK1, Chk2) ↓ Akt, ↑ c-JNK/p38 MAPK phosphorylation ↓ migration (↓ MMP-2 and MMP-9; ↑ TIMP1/2 [ai])	[44]
	U2OS and 143B	50–150 µM		↓ cell proliferation ↑ apoptosis ↓ invasion and angiogenesis (↓ MMP-2 and VEGF) ↓ Wnt/β-catenin and GSK3β (↓ cyclin D1, c-Myc, and COX-2 [aj])	[45]
Fibrosarcoma	HT-1080			↓ MMP-9	[46]
Oral squamous carcinoma	KB	25–300 µM	124 µM (24 h)	↑ apoptosis (↑ caspase-3 activity, loss mitochondrial potential) ↑ ROS ↓ proliferation (cell-cycle arrest in G2/M phase)	[47]

				↑ apoptosis (↑ caspase-3 activity, loss mitochondrial potential)	
Cervical adenocarcinoma	HeLa	10–200 µM		↑ ROS ↑ DNA damage ↓ proliferation (cell-cycle arrest in G2/M phase)	[48]
Leukemia	HL60	10–200 µM	25 µM (24 h)	↑ apoptosis (↑ Bax, ↓ Bcl-2) ↑ autophagy ↓ cell proliferation (cell-cycle arrest in S phase) ↓ migration	[49]

↑: increase; ↓: decrease; [a] IC_{50}: half maximal inhibitory concentration; [b] MMP: matrix metalloproteinase; [c] PARP: poly(ADP-ribose) polymerase; [d] HER2: human epidermal growth factor receptor 2; [e] SREBP-1: sterol regulatory element-binding protein-1; [f] ERK1/2: extracellular signal-regulated kinase 1/2; [g] MAPK: mitogen-activated protein kinase; [h] Smac/DIABLO: second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein with low PI; [i] IAPs: inhibitors of apoptosis proteins; [j] CDK: cyclin-dependent kinase; [k] E2F1: E2F Transcription Factor 1; [l] CDC25C: cell division cycle 25C; [m] VEGF: vascular endothelial growth factor; [n] ALT: alanine transaminase; [o] AST: aspartate transaminase; [p] LC3B: microtubule-associated protein 1A/1B-light chain 3—phosphatidylethanolamine conjugate; [q] STAT-3: signal transducer and activator of transcription 3; [r] NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; [s] ROS: reactive oxygen species; [t] IRE1α: inositol-requiring 1α; [u] CHOP: C/EBP homologous protein; [v] Bip: binding immunoglobulin protein; [w] GSK3β: glycogen synthase kinase 3β; [x] EMT: epithelial mesenchymal transition; [y] GGT: gamma glutamyl traspeptidase; [z] SMAD: small mother against decapentaplegic; [aa] XIAP: human X-linked IAP; [ab] Bid: BH3 interacting domain death agonist; [ac] Rb: retinoblastoma protein; [ad] ATR: ataxia telengectasia and Ras3-related protein; [ae] Chk: checkpoint kinase; [af] H2AX: H2A histone family member X; [ag] ALP: alkaline phosphatase; [ah] PI3K: phosphatidylinositol 3-kinases; [ai] TIMP1/2: inhibitors of metalloproteinase 1/2; and [aj] COX-2: cyclooxygenase-2.

Sriwiriyajan and colleagues [13] explored the cytotoxic activity of methanol and dichloromethane crude extracts of *Piper nigrum* fruits in different breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-468). Both extracts promoted cancer cell death. The calculated half maximal inhibitory concentration (IC_{50}) (Table 1) indicated a different sensitivity to *Piper nigrum* among the three cell lines, probably dependent on their different p53 status [13]. Of note, both *Piper nigrum* crude extracts showed less marked cytotoxic effects in normal breast cells (MCF-12A), suggesting selectivity towards breast cancer cells. Interestingly, the extracts were even more cytotoxic than the two main alkaloids of *Piper nigrum* piperine and pellitorine (tested up to 20 µg/mL) in MDA-MB-468 cells. Chromatographic separation was performed to further understand which fraction, and thus which bioactive compounds, were responsible for the cytotoxic activity of the dichloromethane *Piper nigrum* extract, characterized by the best anticancer activity. Surprisingly, the isolated fractions of alkaloids without piperine, named DE and DF, showed the best IC_{50} value and significant pro-apoptotic activity [13]. However, the DE fraction lost partial selectivity versus cancer cells, as demonstrated by the extract, with a comparable induction of cell death in normal and cancer cells, while DF maintained a selective anticancer effect (IC_{50}: 6.51 µg/mL in MCF-7 cells versus 20.66 µg/mL in MCF-12A).

Motivated by the anticancer activity of DE and DF piperine-free fractions, the same research group further investigated the antitumor effects of a piperine-free *Piper nigrum* extract (PFPE) [14]. The antiproliferative effects of PFPE were explored in breast, colorectal, lung, and neuroblastoma cancer cell lines (Table 1). The greatest cytotoxic effect was recorded in MCF-7 cells, where the pro-apoptotic activity of the extract was demonstrated through the p53 and cytochrome c increase, together with the induction of DNA damage via topoisomerase II downregulation (Table 1). Of note, the PFPE extract

displayed a less marked antiproliferative effect in non-transformed breast cells, with a selective index (SI) value of 6.22 when compared to MCF-12A [14], thus exhibiting not only the highest anticancer effects, but also the best selectivity, in this cancer model (Table 1). The anticancer effects of PFPE were additionally investigated in Sprague-Dawley rats treated intraperitoneally with 50 mg/kg N-nitrosomethylurea (NMU)—a reliable carcinogen-alkylating agent—at 50, 80, and 110 days of rats' age. Two different PFPE treatment regimens were administered orally to test its protective effects against NMU-induced mammary tumors in cancer initiation and post-initiation stages [14] (Table 1). In the first treatment regimen, rats were exposed to 100 or 200 mg/kg body weight (b.w.) piperine at 14 days after NMU application three times per week up to 76 days. At the end of treatment, the incidence of rats bearing tumors was 100% in the control and vehicle groups, and 20% and 10% in PFPE-orally-treated rats at 100 and 200 mg/kg b.w., respectively. In the second treatment regimen, rats were administered PFPE (100, 200, or 400 mg/kg b.w.) every two days up to 30 days after the first NMU-induced tumor was detected. Treatment with the extract significantly reduced the growth rate of tumors compared to control and vehicle groups and induced apoptosis in tumor tissues [14].

A following study [15] investigated the mechanisms underpinning the anticancer effects of PFPE in the same in vitro and in vivo breast cancer models used in [14] (Table 1). PFPE controlled the tumor size and inhibited cancer cell proliferation through the downregulation of c-myc and the upregulation of p53. Moreover, the extract had a pro-apoptotic effect mediated by PFPE pro-oxidant activity. The later stages of tumorigenesis were counteracted through (1) angiogenesis inhibition through vascular endothelial growth factor (VEGF) downregulation and (2) migration/invasion reduction via downregulation of the matrix metalloproteinase (MMP)-2 and -9 [15] (Table 1). Surprisingly, the study recorded a reduction of E-cadherin, which, in tumors, is usually associated with metastasization and cancer recurrence [50]. However, no invasion or metastasization was recorded in the study reported above [15], suggesting that the downregulation of E-cadherin induced by PFPE alone is not able to stimulate invasion and metastasis.

Of note, the role of the vehicle Tween 80 was assessed to clarify its contribution in the anticancer mechanisms evoked by PFPE [15]. Tween 80 helped to dissolve PFPE and to contrast the pungent taste, favoring oral administration in rats. No significant toxic or anticancer effects were recorded after treatment with the vehicle alone. However, Tween 80 may enhance drug uptake, increasing the cell membrane permeability thanks to its chemico-physical characteristics of nonionic surface-active detergent [15]. Therefore, the contribution of the vehicle may not be disregarded in the overall biological effects of the extract.

Only one study has explored the anticancer potential of a root's extract. Ee and colleagues [16] investigated the cytotoxic activity of three different crude extracts of *Piper nigrum* roots: (i) chloroform; (ii) petroleum ether; and (iii) ethyl acetate followed by ethanol extraction solution (Table 1). The anticancer activity was higher for the chloroform extract (IC_{50}: 9.8 µg/mL) than for the petroleum ether extract (IC_{50}: 11.2 µg/mL), whereas the ethyl acetate extract showed no cytotoxic activity in human promyelocytic leukemia cells [16]. The study analyzed each extract for its alkaloid content. The biological activity of the petroleum ether extract was ascribed to the piperine content and that of the chloroform extract was ascribed to the presence of a mixture of other alkaloids, such as cepharadione, piperlactam, and paprazine [16], for which the anticancer activity has not been assessed in any other studies to date.

Taken together, these results suggest that *Piper nigrum* extracts, where several bioactive molecules coexist, may represent a promising strategy for contrasting cancer in the first and later stages of its development.

3. Other Compounds from *Piper nigrum* with Anticancer Potential

Piper nigrum is a source of bioactive molecules with anticancer potential aside from piperine. After piperine, the most investigated pepper alkaloid is piperlongumine, also named piplartine. Piperlongumine represents the main bioactive constituent of long pepper (*Piper longum* L.) and for this reason, it is not extensively covered in the present review. Although piperlongumine was known over 50 years ago, its anticancer activity was only uncovered in the past decade [51]. Piperlongumine shares various anticancer mechanisms with piperine, including the induction of apoptosis, cell-cycle arrest in G1 or G2/M phases, pro-oxidant activity, and anti-metastatic and anti-angiogenic effects. Moreover, piperlongumine synergizes with traditional anticancer drugs [51][52] and exerts selective cytotoxicity towards cancer cells compared to normal ones [52]. Interestingly, a recent paper showed the synergistic effect of the association of piperine plus piperlongumine in triple-negative breast cancer cell lines (MDA-MB-231 and MCF-7) [53]. The synergistic effects of the combination were recorded at the lower doses of the combination (50 or 100 µM piperine with 5 µM piperlongumine), with a selective anticancer effect towards cancer cells compared to normal cells (MCF-10). The pro-apoptotic effect of the combination was independent of the hormone and p53 status, also showing good cytotoxic activity versus MDA-MB-231, which was poorly affected by the cytotoxic effects of piperine alone [53]. This latter result may be due to the fact that the piperine-induced upregulation of Bcl-2 is counteracted by the piperlongumine-induced reduction of this anti-apoptotic gene in this cell line [53].

Pellitorine represents another bioactive compound isolated from *Piper nigrum* showing anticancer activity. Pellitorine from piper roots exerted cytotoxic effects in breast (MCF-7) and human promyelocytic leukemia (HL60) cells, with IC_{50} values of 1.8 and 13 µg/mL, respectively [54].

Although alkaloids are the main component responsible for *Piper nigrum* anticancer effects, the lignan (−)-kusunokinin, isolated from a piperine-free *Piper nigrum* extract, induced anticancer activity in breast (MCF-7, MDA-MB-468, and MDA-MB-231) and colorectal (SW-620) cancer cells [55]. In those cell lines, kusunokinin induced cell-cycle block in the G2/M phase and apoptosis via i) the activation of both the intrinsic and extrinsic pathway; ii) the upregulation of p53, p21, Bax, cytochrome c, caspase-8, caspase-7, and caspase-3; and iii) the downregulation of Bcl-2 [55]. (−)-Kusunokinin showed partial selectivity towards cancer cells compared to normal mammalian cells [55].

Recently, Rattanaburee and colleagues [56] investigated the potential target responsible for the antiproliferative activity of synthetic (±)-kusunokinin. They concluded that the cytostatic effects of this molecule in breast cancer cells relied on its ability to suppress the colony stimulating factor-1 receptor (CSF1R), whose downregulation then affected Akt and its downstream molecules cyclin D1 and CDK1 [56]. Of note, the affinity of the synthetic (±)-kusunokinin for CSF1R is higher than that of natural (−)-kusunokinin, underlying the importance to use these molecules to improve their affinity for the target. A very recent study investigated, for the first time, the anticancer effects of (−)-kusunokinin in vivo [57]. In female Sprague-Dawly rats, mammary tumors were induced through an intraperitoneal injection of 50 mg/kg NMU. (−)-Kusunokinin (7 or 14 mg/kg injected subcutaneously) significantly suppressed tumor growth and no toxic effects were recorded in any of the analyzed organs (heart, liver, lung, spleen, and kidney) or in hematologic and clinical chemistry parameters [57], suggesting a safe profile of this lignan. Furthermore, the study analyzed the anticancer mechanisms of the molecule in breast tumor tissue of treated rats [55]. (−)-Kusunokinin (14 mg/kg) reduced the levels of signaling proteins, i.e., the proto-oncogene tyrosine-protein kinase Src (c-Src), phosphatidylinositol 3-kinases (PI3K), Akt, and p-ERK1/2 and their downstream targets, such as proteins involved in cell-cycle regulation (c-myc, E2F1, CDK1, and cyclin B1) and cell migration (E-cadherin, MMP-2, and MMP-9) [57].

Taken together, these results suggest that piperine is not the only component responsible for the anticancer activity of *Piper nigrum* and that other alkaloids from this *Piper* species, such as pellitorine, piperlongumine, piperlonguminine, and the lignan (−)-kusunokinin, may represent valuable anticancer strategies.

4. Toxicological Studies

The acute toxicity of piperine was investigated in mice, rats, and hamsters [58]. The lethal dose causing death in 50% of the dosed animals (LD_{50}) values after single intravenous, intraperitoneal, subcutaneous, intragastric, or intramuscular administration to adult male mice were 15.1, 43, 200, 330, and 400 mg/kg b.w., respectively [58]. Lethal dose administration induced animals' death via respiratory paralysis within 3–17 min [58]. With regards to *Piper nigrum* extract's acute toxicity, 5000 mg/kg b.w. os of aqueous extract orally administered to male and female Sprague-Dawley rats did not produce any signs of toxicity [59]. Moreover, acute oral toxicity studies of the piperine-free extract PFPE showed no morbidity or mortality up to 14 days in ICR female mice (5000 mg/kg b.w. per os once) and no tissue damage was recorded [14].

Based on the overall weight of evidence, the non-genotoxic nature of piperine was established. The majority of studies exploring the genotoxic potential of piperine have claimed that it has a non-genotoxic nature, when assessed via tests analyzing different genotoxic endpoints [60][61]. In particular, a recent study investigated the in vitro and in vivo genotoxic potential of piperine using the micronucleus test, which allows both aneugenic and clastogenic effects to be detected. No increase in micronuclei was recorded in vitro or in NMRI BR mice exposed up to the maximum tolerated dose of piperine for 2 days (143.5, 287.0, or 574.0 mg/kg b.w. per day; n = 10 animals/sex/group) [62]. Furthermore, not only this alkaloid has no genotoxic activity, but it was also able to protect from the genotoxicity of other compounds. As an example, piperine inhibited micronuclei formation, chromosomal aberration, or sister chromatid exchanges induced by different agents, such as aflatoxin B1, cyclophosphamide, mitomycin C, or B(a)P [36][38][63][64][65][66][67][68][69]. The antigenotoxic activity of piperine mainly relies on its ability to (i) inhibit phase I enzymes involved in genotoxicants' activation and (ii) induce detoxifying enzymes that contrast carcinogens' activity.

Reproductive toxicity studies are available for piperine [70][71], showing interference with crucial reproductive events. The lowest dose studied (1 mg/kg b.w./day) did not induce any adverse effects on sexual organs and the sperm quality. Doses of 5 mg/kg b.w./day or higher decreased the sexual organs' weight in male animals and reduced the sperm quality [71][72]. In female animals, oral treatment with 10 or 20 mg/kg b.w. piperine per day up to 14 days decreased the mating performance and fertility index and showed anti-implantation activity 5 days post-mating piperine treatment [70].

A further study investigated the toxic effects of piperine on the liver [73]. After the administration of 1.12 mg/kg b.w./day for 23 days, no histopathological lesions were observed.

Conflicting results are available for the immunomodulative potential of piperine. In early studies performed on Swiss male mice gavaged at 1.12–4.5 mg/kg b.w. per days for 5 days, piperine exhibited immunotoxicity [74]. Treatment at the highest tested dose resulted in a significant decrease in the weight of the spleen, thymus, and mesenteric lymph nodes and caused a significant reduction in total leucocytes [74]. At 2.25 and 4.5 mg/kg, piperine inhibited the response of B lymphocytes to the mitogenic stimulus. The lowest dose (1.12 mg/kg) was devoid of immunotoxic effects and was identified as the no observed adverse effect level (NOAEL) for this effect [73]. However, piperine exhibited a protective effect against cadmium-induced immunotoxicity [75].

Recently, the European Food Safety Authority (EFSA) identified the NOAEL of piperine, which is 5 mg/kg b.w. per day based on the most comprehensive study available (90-day dietary toxicity study in rats) [76].

No experimental carcinogenicity studies are available for piperine. However, in silico models predicted a non-carcinogenic effect for piperine [77].

A NOAEL value was established for piperine, as reported above. However, there are conflicting results and missing information, in particular for its reprotoxic effects, that make the NOAEL value uncertain. For this reason and considering that piperine is not genotoxic, an approach based on the Threshold of Toxicological Concern (TTC) has been used. According to its structure, piperine is a Cramer Class III compound [78]. The Cramer Class III TTC threshold was found to be 1.5 µg/kg b.w./day [79].

Although the dietary consumption of black pepper varies considerably within the population, EFSA calculated that the estimated exposure to piperine from natural sources when consuming black pepper as a flavoring ingredient is 6.2 µg/day in Europe and 0.07 µg/day in the USA, based on the maximized survey-derived daily intake [76], which are below the TTC threshold level of 1.5 µg/kg b.w./day (90 µg/day) for Cramer Class III compounds.

In 2016, the Norwegian Scientific Committee for Food Safety (VKM) was required to assess the risk derived from piperine daily intake through food supplements, which was estimated to be 1.5 mg/day by the Norwegian Food Safety Authority. VKM concluded that the daily dose of 1.5 mg piperine in food supplements is unlikely to cause adverse health effects in children, adolescents, or adults, based on the margin of exposure approach (ratio of the NOAEL to the exposure) [79].

Of note, the doses of piperine used in the in vivo anticancer studies are higher than the calculated NOAEL and TTC values. Taken together, data on the putative toxicities of piperine at doses eligible for anticancer activity and after long periods of administration are not exhaustive. A risk/benefit evaluation is still required to figure out its potential use as an anticancer strategy.

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