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Evaluation of Antimicrobial Activity and Cytotoxicity Effects of Extracts of *Piper nigrum* L. and Piperine

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Abstract: *P. nigrum* L. extracts and the piperine alkaloid have important antimicrobial, anti-inflammatory, and antioxidant properties. Therefore, in this study, we evaluated the antimicrobial activity and cytotoxicity of *P. nigrum* L. extracts and piperine, a compound isolated from the extracts of *P. nigrum* L. Extracts obtained via maceration, soxhlet, and purification steps, in addition to isolated piperine, were used in this study. Spectroscopic methods, such as nuclear magnetic resonance, scanning electron microscopy, X-ray diffraction, thermogravimetry, and differential scanning calorimetry, were used to characterize piperine. In the microbiological analyses, the extract obtained via maceration-derived sample showed high efficiency in inhibiting *Salmonella* spp. (MIC < 100 µg/mL). The extract obtained via a soxhlet-derived sample showed promising inhibitory activity against almost all microorganisms, with negligible inhibition of *Pseudomonas aeruginosa.* Favorable inhibition coefficients were also observed against *Staphylococcus aureus* and *Salmonella* spp. (MIC < 100 µg/mL) for the extract obtained via purification of the steps-derived sample. Piperine showed an excellent inhibition coefficient against most microorganisms, with inactivity only observed against *P. aeruginosa*. Cytotoxicity evaluation assays in cancer cell lines revealed that piperine exhibited inhibitory potential on all tested tumor cell lines, causing a decrease in cell viability and achieving an IC_{50} of less than 30 µg/mL. The analyzed extracts from *P. nigrum* L. seeds showed cytotoxic activity against tumor and non-tumor cell lines.

Keywords: *P. nigrum* L. extracts; piperine; antimicrobial activity; cytotoxicity

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1. Introduction

Piper nigrum L., known as black pepper, belongs to the Piperaceae family and is commonly found on the east coast of India and in tropical regions of Asian countries [1–3]. In many cultures, this spice is used as a condiment. There are also reports that this species is used in herbal Ayurvedic formulations (Trikatu), which are used in traditional Indian medicine to treat a wide range of diseases [4–7].

P. nigrum L. was introduced in Brazil in the 17th century. The Brazilian production of black pepper supplies the international market with three varieties, namely, black, white, and green, with Pará, Espírito Santo, Maranhão, Paraíba, Ceará, Amapá, Bahia, and Minas Gerais being the main states producing these goods [8,9].

Several classes of secondary metabolites, such as lignans, neolignans, flavones, chalcones, and flavanones, are produced by the genus *Piper*. Among these, the most important are

alkaloids and amides, which have excellent biological activities, such as anticancer activity. In the species *P. nigrum* L., only 20% of its compounds have been characterized [10–12].

Amides are the class of compounds most characteristic of the black pepper species. In addition to piperine, many other amides have been isolated from the *Piper* species [13,14]. Studies on the pharmacological effects of piperine have shown that it has antimicrobial, anticancer, anti-inflammatory, insecticidal, analgesic, anticonvulsant, antiulcerogenic, gastroprotective, antioxidant, and antiseptic properties; piperine also makes other bioactive compounds more absorbable and bioavailable [15–17].

Regarding anticancer properties, piperine has been shown to have good activity against sarcoma 180 cancer cells, which affect mesoderm cells, and tumor inhibition in its solid form in 60 female mice [18]. This therapeutic potential was also demonstrated in lung cancer, which alters lipid peroxidation and activates antioxidant protection via enzymes [19,20]. Piperine also exhibits activity in prostate cancer, inhibits the activity of liver enzymes, and increases the effectiveness of docetaxel with no side effects in rats. Activity against breast cancer has also been reported [21,22].

In evaluating antimicrobial and antifungal activities of leaf extracts and essential oils extracted from black pepper (*P. nigrum* L.), significant action has been found against different microorganisms, namely, *Pseudomonas aeruginosa,* and *Candida albicans*, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli* [23–25].

This study was conducted to investigate the biological activities of the macerated black pepper ethanol extract (EEPM), black pepper extract acquired via Soxhlet (EEPS), and extract from the steps of purification (RP), in addition to isolated piperine. The antimicrobial and antifungal properties of piperine were evaluated against *Staphylococcus aureus* (ATCC 0538), *Pseudomonas aeruginosa* (ATCC no code), *Salmonella* spp. (ATCC 4029), *Proteus mirabilis* (ATCC 1353), and *Candida albicans* (ATCC 10635). Further, the cytotoxic properties of the abovementioned extracts and isolated piperine were evaluated in tumor cell lines. Our results demonstrate promising antimicrobial and anticancer activities for black pepper extract and piperine.

2. Results and Discussion

2.1. Extraction Procedures

The methodology of antimicrobial and antifungal biological assays and the performance of toxicity tests were applied to the samples collected during the stages of black pepper extraction (i.e., macerated extract (EEPM), ethanol extract from Soxhlet (EEPS), extract from purification steps (R), and piperine crystals).

2.2. Characterization of Piperine Crystals

2.2.1. Nuclear Magnetic Resonance (NMR)

Analysis of the NMR spectra of piperine can be found in the study by Alves et al. [26] (Figures S1–S3 and Table S1).

2.2.2. Optical Microscopy Analysis

Microscopic examination at 100× magnification reveals the presence of crystals that are large and rectangular, with tapered tips. The crystalline bodies observed in Figure 1B,C are elongated in the form of bars and have considerable thicknesses. The surfaces have a metallic shine and form grooves or striations, such as fissures. Some crystals are dense and overlap one another.

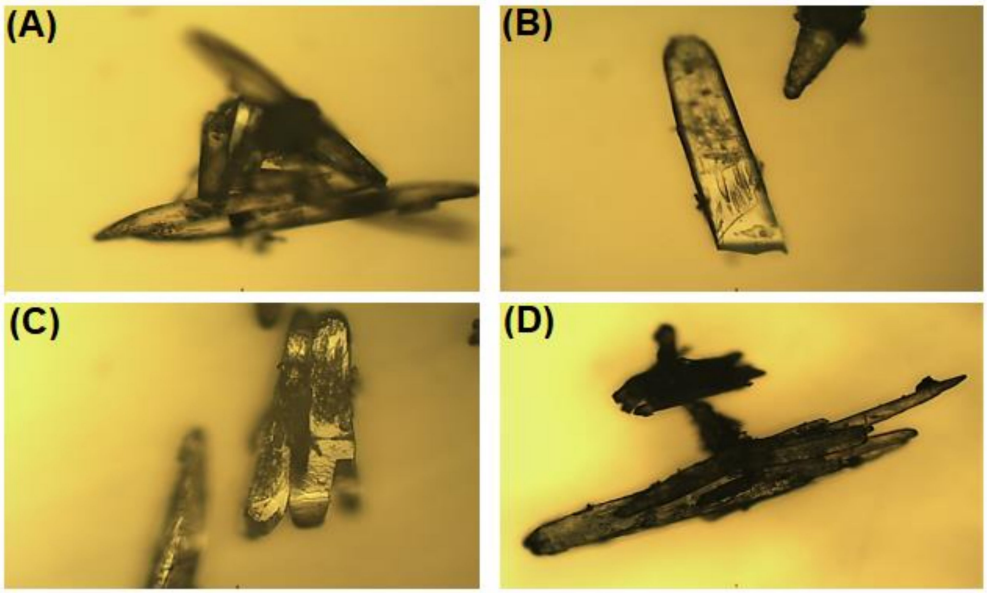

Figure 1. Microscopic analysis of piperine crystals. In (**A**–**D**), we have the representation of the large and rectangular crystals of the piperine.

2.2.3. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

Figure 2 shows the micrographs of the piperine crystals obtained via the SEM analysis. Figure 2A, captured with an image magnification of 60×, displays agglomerated crystal particles with different shapes and irregular geometries. Figure 2B, showing an enlarged region (250× magnification) of the previously analyzed area, emphasizes some bar-shaped crystals with different sizes. Significant surface features can also be visualized, such as cracks and thicker crystals, with a supposed imperfect cubic formation. Figure 2C corresponds to another image magnification at 150× and, in addition to bar-shaped crystals, the figure shows an agglomeration of the crystals that resembles a microneedle flower. In Figure 2D, the micrograph shows the same crystal aggregates. Piperine crystals are generally characterized by micro-needling, and the standard microneedles observed can have relevant characteristics [27].

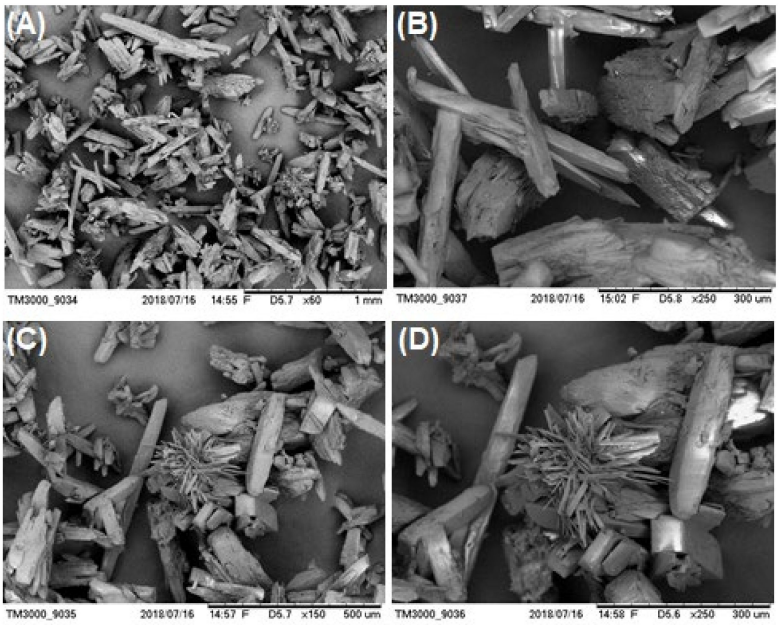

Figure 2. SEM images of pure piperine crystals.

Padalkar and Gaikar [27] stated that this irregular growth is due to surface–solvent interactions. As organic solvents often favor the reduction of interfacial tension, causing the transition from smooth to rough surfaces and rapid structural growth, as evidenced in this study.

In the EDS analysis of the piperine crystal sample, carbon, oxygen, and nitrogen, which are the major components of the piperine molecule, were quantified. A point analysis was performed on four different areas of the sample. EDS analysis confirms the presence of carbon (74 wt.%), oxygen (22.65 wt.%), and nitrogen (3.35 wt.%) in the piperine crystals. Figures 3 and 4 show that there is a carbon (76.2 wt.%) and oxygen (23.8 wt.%) in the whole imaged area, but no nitrogen.

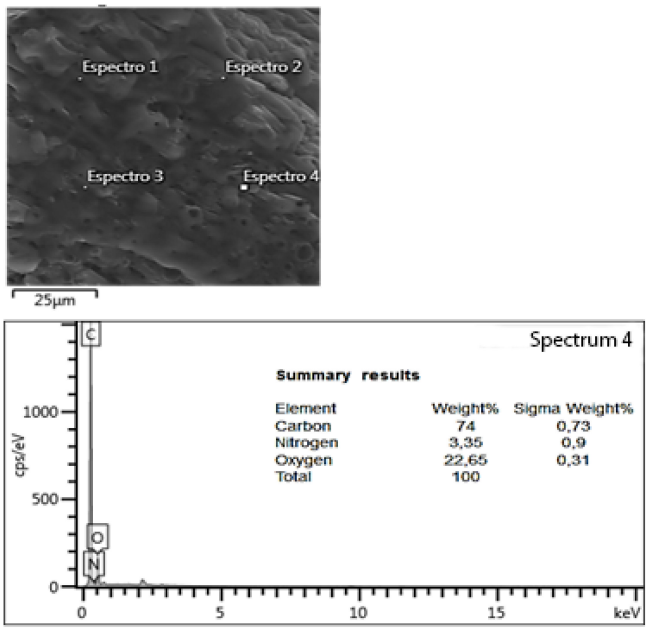

Figure 3. EDS image and corresponding spectrum of area 4 with weight percentages.

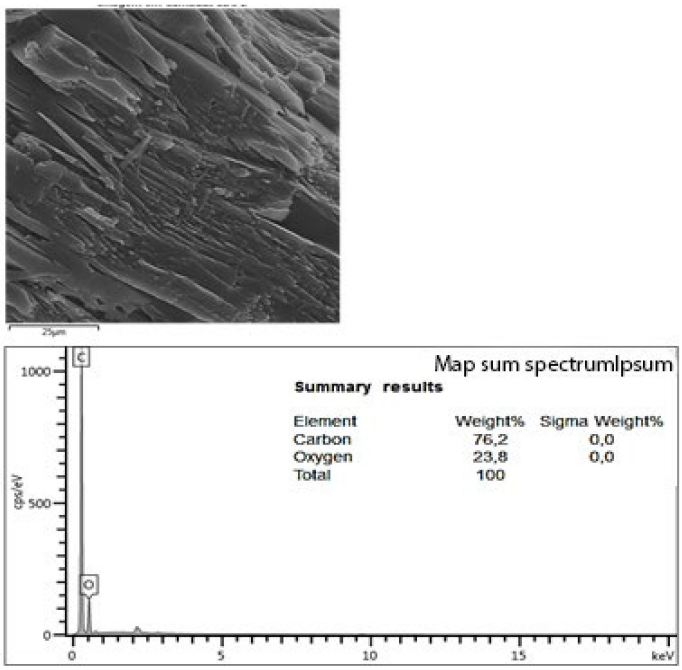

Figure 4. EDS image and corresponding spectrum of the total imaged area with weight percentages.

2.2.4. X-ray Diffraction (XRD)

The diffractogram of the piperine crystal sample (Figure 5) shows regions with high-intensity reflections at 2θ = 14.8465° (d = 5.96 Å; I% = 100) and 2θ = 25.8795° (d = 3.44 Å; I% = 55.57). Medium-intensity reflections can be found at 2θ = 13.0225° (d = 6.79 Å; I% = 22.12), 2θ = 14.2457 (d = 6.21 Å; I% = 30.49), 2θ = 19.7710° (d = 4.49 Å; I% = 26.06), 2θ = 22.3622° (d = 3.97 Å; I% = 33.71), 2θ = 22.6158° (d = 3.93 Å; I% = 35.19), and 2θ = 28.2905° (d = 3.15 Å; I% = 14.08). The remaining reflections are those with the lowest intensities, with d-spacing values of 8.20, 5.65, 5.54, 5.46, 5.25, 4.59, 4.29, 4.16, 4, 10, 3.67, 3.63, 3.52, 2.98, 2.80, 2.73, and 2.47 Å. All reflections identified in the diffraction pattern of the piperine crystal sample are comparable with those reported in the PPC1 standard form (COD no: 00-043-1627) obtained from the X'Pert High Score Plus® program, which identifies piperine as a monoclinic crystal with lattice parameters of a = 8.6950 Å, b = 13.6020 Å, and c = 13.1580 Å. The literature also reveals that when analyzing piperine using XRD, reflections can be detected in a wide range of 2θ angles (i.e., 4–60°) [28,29].

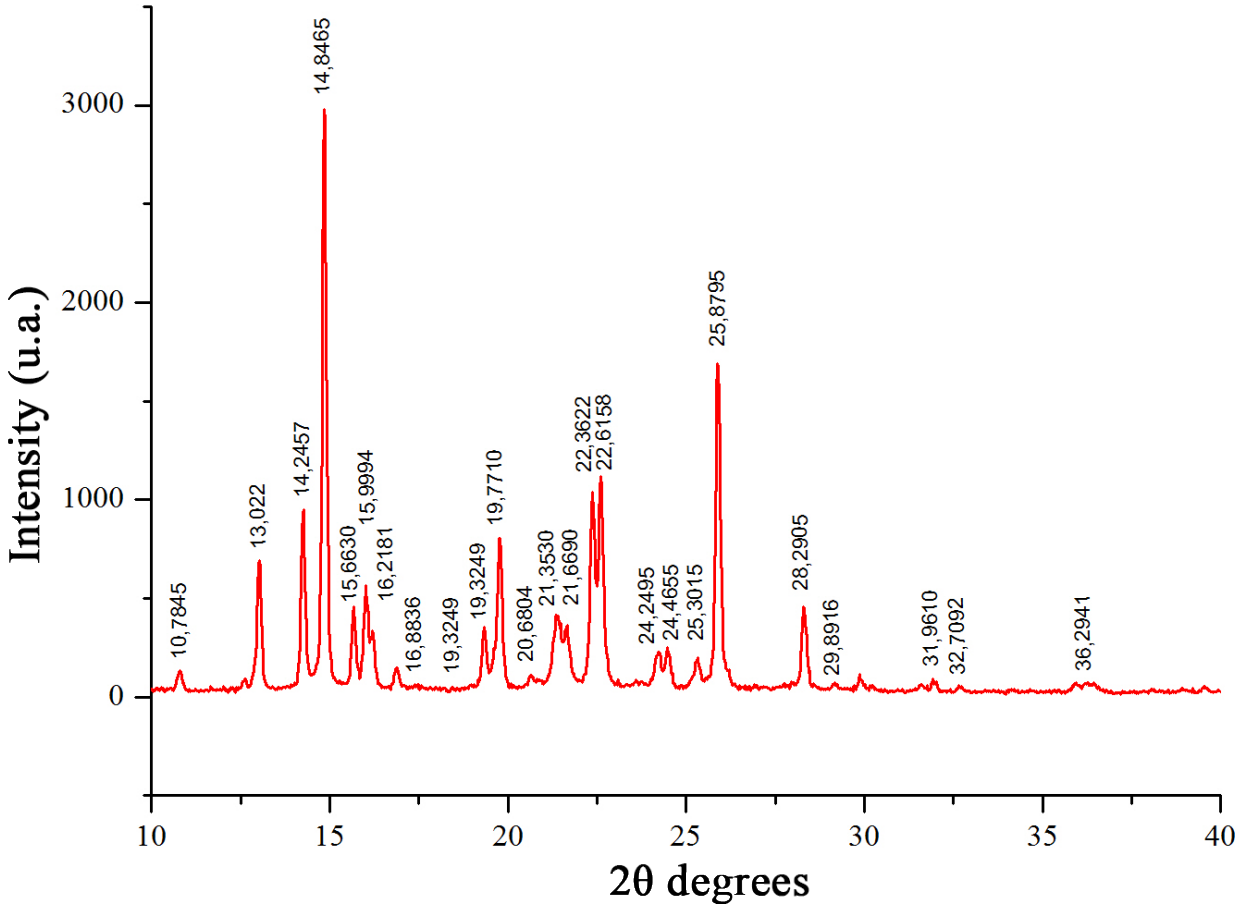

Figure 5. Diffractogram of piperine crystals.

2.2.5. Thermogravimetry (TG) and Differential Scanning Calorimetry (DSC)

Figure 6 shows the TG, DTG (derivative TG), DSC, and DDSC (derivative DSC) curves that reveal the changes caused by heating the piperine crystal sample. Using this data, we calculate temperature ranges where the sample acquires a fixed composition and where it decomposes.

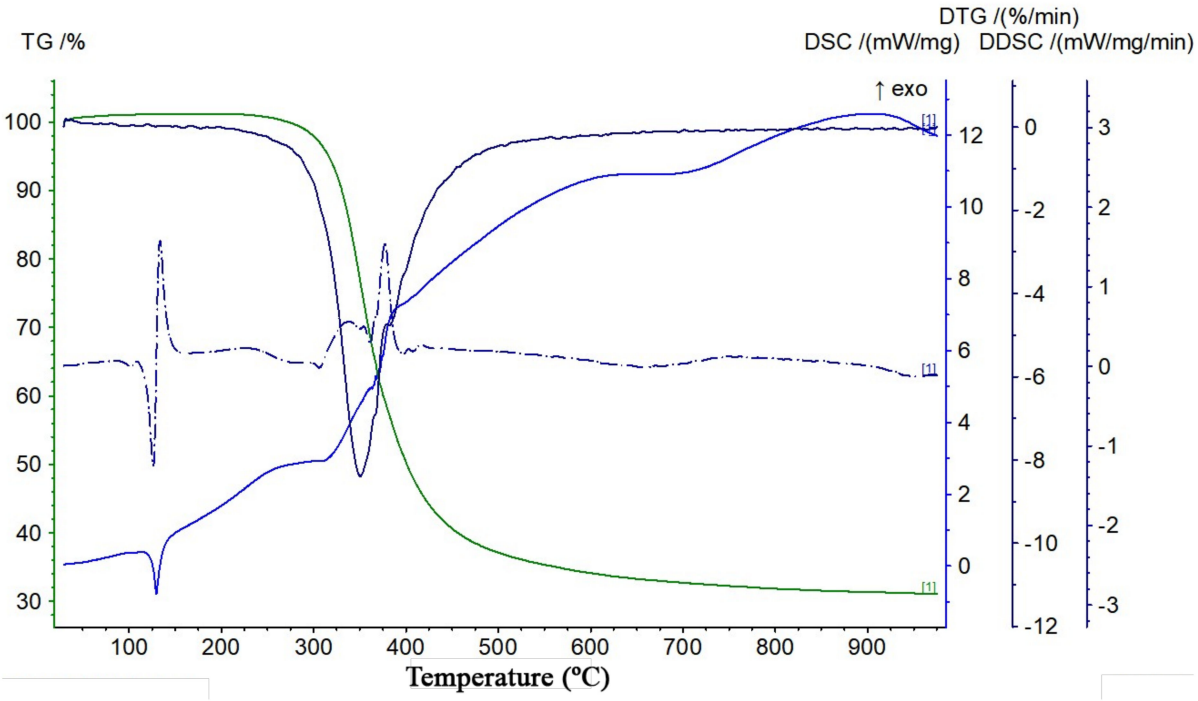

Figure 6. TG, DTG, DSC, and DDSC curves were obtained for the piperine crystal sample.

As shown in Figure 7A, the decomposition of piperine occurs in one step, with a mass loss of approximately 64%. The initial temperature corresponding to the principal mass loss is 225 °C, and the final decomposition temperature reached is 525 °C.

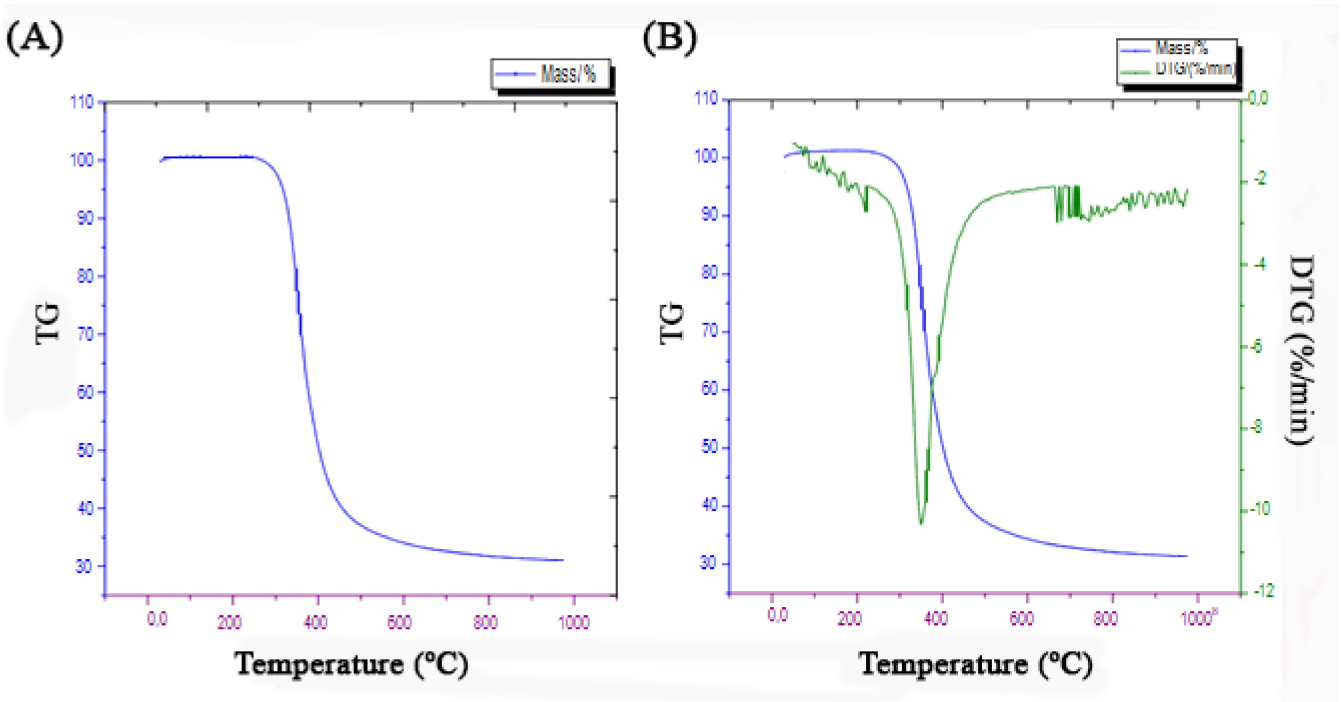

Figure 7. TG (**A**) and DTG (**B**) curves for the piperine crystal sample.

In Figure 7B, which corresponds to the TG derived from the variation in mass as a function of temperature (green line on the graph), the occurrence of a series of secondary or minor reactions close to the primary response during the entire heating process can

be verified. The graph shows that T_{onset} is 300 °C, and that the primary answer has its endothermic point or maximum degradation temperature (T_{peak}) at 349 °C.

Figure 8A shows first-order exothermic events that suggest the presence of crystallization in the sample and second-order events characterized by a variation in heat capacity, albeit one without an enthalpy change, as they do not generate the peaks in the DSC curve that would cause a displacement of the S-shaped baseline. However, as shown in Figure 8B, which corresponds to the derivative of the heat flux (DDSC) as a function of temperature (the blue line in the graph), endothermic events occur that may indicate heat loss. These include mass samping (through water vaporization, volatile components of the reaction, or decomposition) or melting, as well as exothermic events that guarantee the purity of the sample because no peak presents concavity in its formation.

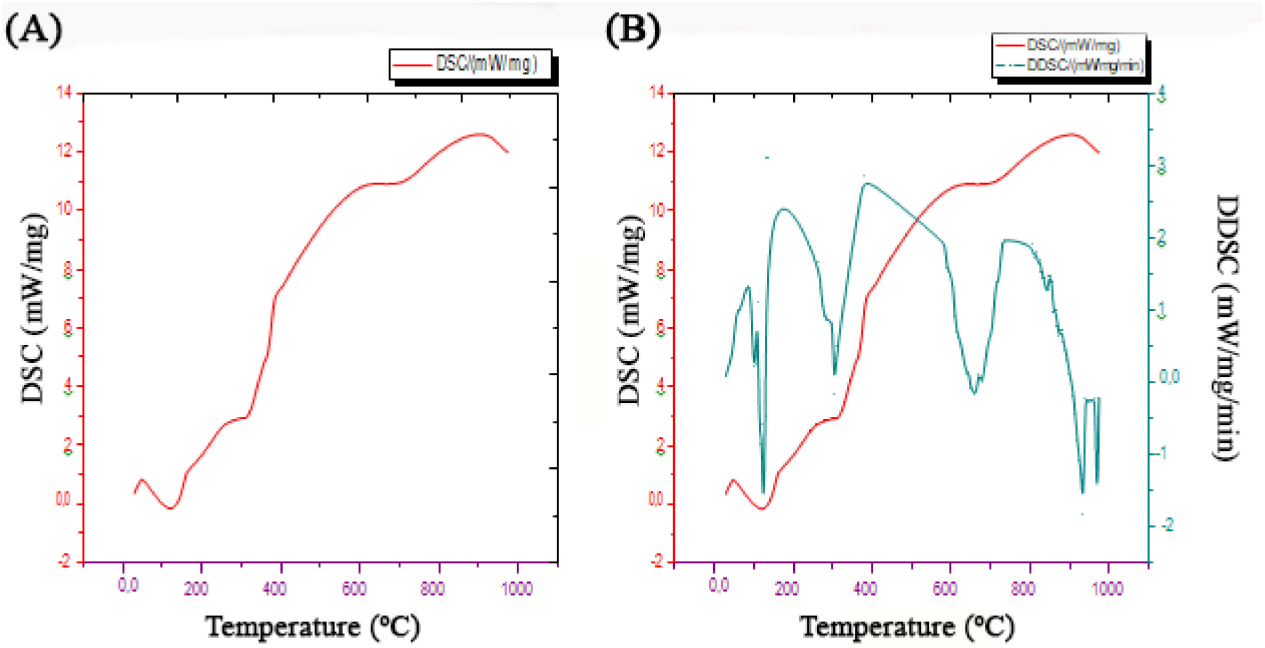

Figure 8. DSC (**A**) and DDSC (**B**) curves for piperine crystal sample.

The first peak at 100 °C (T_{onset}) is typical of a decomposition melt, and is followed by an exothermic peak at around 128 °C. These results corroborate the melting point of crystalline piperine (128–130 °C). The following variations were considered to have similar ratios.

2.3. Biological Assays

2.3.1. Analysis of Antimicrobial and Antifungal Activity

The main chemical constituents of black pepper fruits are lignans, alkaloids, flavonoids, polyphenols (tannins), aromatic compounds (monoterpenes and sesquiterpenes), and amides [30,31]. The antimicrobial and antifungal activities are among the various biological activities of the abovementioned secondary metabolites [32].

As presented in Table 1, the EEPM sample, which corresponds to the black pepper extract obtained via maceration, does not show inhibitory activity against the bacterium *Staphylococcus aureus* as it presents an IC_{50} of 7034435.0, a value which corresponds to a MIC greater than 1000 µg/mL. This sample also shows weak inhibitory activity against *Pseudomonas aeruginosa*, with an IC_{50} of 545.0 (MIC between 500 and 1000 µg/mL). The moderate inhibitory activity is confirmed against *Proteus mirabilis* (IC_{50}: 393.0) and *Candida albicans* (IC_{50}: 287.0) since the MIC is between 100 and 500 µg/mL. However, the extract shows high efficiency in inhibiting *Salmonella* spp., with an IC_{50} of 43.3 following an inhibitory activity (MIC) that is less than 100 µg/mL.

Table 1. Evaluation of antimicrobial and antifungal activities of EEPM, EEPS, RP, and piperine samples of black pepper from the IC_{50} inhibition coefficient for the microorganisms above.

Samples	Values of IC_{50} (μg/mL) ± DP				
	Staphylococcus aureus	*Salmonella* sp.	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Candida albicans*
EEPM	7034435.0 [d] ± 0.17	43.3 [a] ± 0.17	393.0 [b] ± 0.16	545.0 [c] ± 0.07	287.0 [b] ± 0.11
EEPS	0.99 [a] ± 0.06	195.0 [b] ± 0.28	27.0 [a] ± 0.27	-	62.10 [a] ± 0.13
R	32.2 [a] ± 0.15	11.0 [a] ± 0.26	325.0 [b] ± 0.24	-	899.0 [c] ± 0.20
piperine	59.0 [a] ± 0.11	45.0 [a] ± 0.23	359.0 [b] ± 0.22	-	136.0 [b] ± 0.12

EEPM—macerated extract; EEPS—ethanol extract from Soxhlet; R—extract from purification steps; IC50—inhibitory concentration coefficient 50%; DP—standard deviation; MIC—minimum inhibitory concentration; [a]—high antimicrobial and antifungal activity MIC < 100 μg/mL; [b]—moderate activity, MIC between 100 and 500 μg/mL; [c]—weak activity, MIC between 500 and 1000 μg/mL; [d]—sample considered inactive, MIC > 1000 μg/mL.

The low activity of the EEPM sample against the microorganisms *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Candida albicans* is attributed to the possible loss of essential components during extraction. Molecules, such as monoterpenes and sesquiterpenes, correspond to some of the chemical constituents elucidated in the fruits and the volatile oil of black pepper. These components are potentially active against bacteria and fungi [33,34]. However, for *Salmonella* spp., the results are promising and demonstrate the possible actions of the other metabolites present. In the phytochemical analysis of the EEPM sample, catechin tannins, catechins, and alkaloids are regarded as constituents with good antimicrobial activity [35–37].

The EEPS sample, obtained via Soxhlet extraction, showed promising inhibitory activity against *Staphylococcus aureus*—IC_{50}: 0.99, *Salmonella* spp.—IC_{50}: 195.0, *Proteus mirabilis*—IC_{50}: 27.0, and *Candida albicans*—IC_{50}: 62.1. This suggests that other components have volatilized during the extraction process of the EEPM sample. In the Soxhlet procedure, the extraction time was slightly shorter than that of the maceration, and the influence of the increase in temperature may have promoted the optimization of the method to obtain new components. The analysis corroborates the results by Patani et al., who described the excellent activity of a black pepper extract, obtained via Soxhlet extraction for 24 h, against *Staphylococcus aureus* and *Salmonella typhi* bacteria. However, the EEPS sample shows no significant inhibition against *Pseudomonas aeruginosa*, corroborating the results of Saeed et al., who tested extracts of the fruit [38,39]. In the phytochemical analysis of the EEPS sample, alkaloids, anthocyanins, anthocyanidins, leucoanthocyanidins, catechins, tannins, and flavonoids exhibit antioxidant and antimicrobial activities [40,41].

In the RP sample, the material collected from the purification of the EEPS extract, which corresponds to the process in which the precipitation of tannins and other phenolic materials occurs, shows potent inhibitory activity against *Staphylococcus aureus* (IC_{50}: 32.2) and *Salmonella* spp. (IC_{50}: 11.0). However, the RP sample shows moderate activity against *Proteus mirabilis* (IC_{50}: 325.0) and weak activities against *Candida albicans* (IC_{50}: 899.0). Phytochemical analysis reveals the presence of phenols, catechins, tannins, anthocyanins, anthocyanidins, and flavanones. According to the literature, many bacteria and fungi, including *Staphylococcus aureus*, are sensitive to tannins at minimal concentrations. Furthermore, the active phenolic compounds in the RP sample are known to promote ATP synthase inhibition, which explains why they are potent antimicrobials. According to Rabelo et al., Gram-negative microorganisms are more resistant to the action of certain antimicrobials because their cell walls are protected by a layer of lipopolysaccharides, which may explain the difficulty of inhibiting *Proteus mirabilis*. Anthocyanins and anthocyanidins also contribute to antioxidant and antimicrobial activities [40,41].

The piperine crystal sample shows excellent inhibition against most microorganisms, displaying high activity for *Salmonella* spp. (IC_{50}: 45) and *Staphylococcus aureus* (IC_{50}: 59.0), followed by moderate inhibition for *Candida albicans* (IC_{50}: 136.0) and weak activity

for *Proteus mirabilis* (IC_{50}: 359.0). Inactivity is observed towards *Pseudomonas aeruginosa*. Piperine is known to be a potent antimicrobial [42]. Many researchers have evaluated the biological activities of piperine in black pepper. Using an inhibitory concentration of 250 ppm, Patani et al. obtained satisfactory results regarding the inhibition of Gram-negative and Gram-positive bacteria. Aldaly [43] evaluated disc-shaped piperine against Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Candida albicans* and obtained more satisfactory results for the fungus (MIC: 3.125–100 mg/mL). Additionally, there was low inhibition of *Pseudomonas aeruginosa*. This corroborates the results described earlier.

2.3.2. Cytotoxic Analysis of Piperine and Black Pepper Extracts in Tumor Lineages

The cytotoxicity assay (MTT) reveals that piperine has cytotoxic potential, with an IC_{50} that is lower than 30 µg/mL in all tested tumor cell lines. Piperine shows higher activity in the hepatocellular carcinoma lineage (HEP-G2), with an IC_{50} of 14.34 µg/mL. As shown in Table 2, the IC_{50} value against the metastatic melanoma strain (SK-MEL-19) was 16.39 µg/mL. The obtained IC_{50} values against the gastric adenocarcinoma strains (AGP01 and AGP01 *PIWIL1*$^{-/-}$) were 21.57 and 22.39 µg/mL, respectively.

Table 2. Cytotoxic activity of piperine in cell lines after 72 h of exposure.

	Cell Lines IC_{50} (µg/mL) *				
Sample	**AGP01**	**AGP01 *PIWIL1*$^{-/-}$**	**SK-MEL-19**	**HEP-G2**	**MRC5**
Piperine	21.57 (20.14–23.10) R^2 = 0.9918	22.39 (21.33–23.51) R^2 = 0.9929	16.39 (15.06–17.85) R^2 = 0.9930	14.34 (11.66–17.65) R^2 = 0.9701	23.52 (19.07–24.91) R^2 = 0.9829

* Data are presented as IC_{50} values and 95% confidence intervals obtained by non-linear regression for all cell lines from three independent experiments.

It is important to note that when analyzing the activity of piperine in gastric cancer strains (AGP01 and AGP01 *PIWIL1*$^{-/-}$), it is possible to observe that the molecule acts independently of the *PIWIL1* gene, showing very similar IC_{50} values between the two lineages. Both lineages originate from malignant ascites from gastric adenocarcinoma of the intestinal type, with AGP01 *PIWIL1*$^{-/-}$ being genetically modified via the innovative CRISPR/Cas9 technique [44]. This genetic modification leads to the inactivation of the *PIWIL1* gene, which plays a significant role in the process associated with the viability, migration, and invasion of cancer cells.

Therefore, the evaluation and comparison of cytotoxic responses in both lineages (unmodified and modified) provides insights for future studies dedicated to evaluating the possible mechanism of action of piperine against gastric cancer. We also emphasize that both lineages (AGP01 and AGP01 *PIWIL1*$^{-/-}$) reflect a specific cancer profile in the Brazilian Amazon region. Therefore, this study can inspire comparative studies on the relationship between the phenotypic and molecular profiles of cancers in different areas.

From the cytotoxicity results, it is possible to indirectly evaluate the effect of piperine on cell viability (Figure 9). This action is concentration-dependent; that is, when the concentration of the molecule increases, the viability of the cells declines. Piperine causes a decrease in cell viability in all the tumor cell lines tested.

The three extracts (i.e., EEPM, EEPS, and RP) from *P. nigrum* L., through the evaluation of cytotoxicity (MTT), exhibit cytotoxic activity against tumor and non-tumor cells. This activity follows a pattern where the macerated solution extract (EEPM) is the most active, with an IC_{50} in the range of 13.8–21.3 µg/mL, followed by the Soxhlet solution extract (EEPS) and the retained extract (RP), with IC_{50} values in the ranges of 13.2–25.0 and 27.5–47.4 µg/mL, respectively.

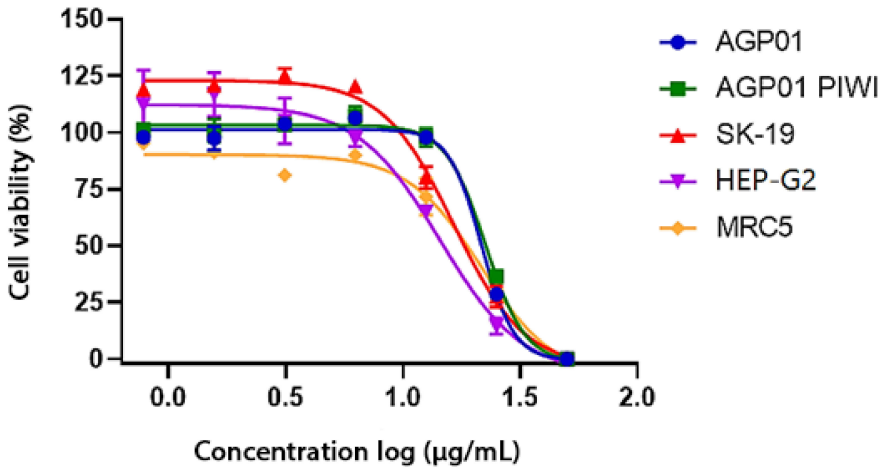

Figure 9. Percentage of cell proliferation after 72 h of treatment with piperine in different cell lines. Each point is equivalent to the average of three replicates.

None of the extracts show any selectivity toward tumor cells. The average inhibitory concentration values in non-tumor cells (MRC5) are similar to or even lower than those in tumor cells (Table 3).

Table 3. Cytotoxic activity of macerated (EEPM), Soxhlet (EEPS), and retained (RP) extracts in cell lines after 72 h of exposure.

Sample	Cell Lines IC_{50} (µg/mL) *			
	AGP01	**AGP01 *PIWIL1*$^{-/-}$**	**SK-MEL-19**	**MRC5**
EEPM	13.82 (12.97–14.73) R^2 = 0.9891	21.26 (18.66–24.21) R^2 = 0.9595	14.94 (12.7–17.57) R^2 = 0.9731	14.17 (11.29–17.79) R^2 = 0.9600
EEPS	19.57 (18.47–20.73) R^2 = 0.9936	24.53 (22.43–25.79) R^2 = 0.9647	25.00 (19.00–32.90) R^2 = 0.9507	13.19 (12.02–14.48) R^2 = 0.9718
RP	43.77 (41.81–45.83) R^2 = 0.9927	35.59 (27.8–45.56) R^2 = 0.9553	47.35 (42.45–52.81) R^2 = 0.9659	27.5 (24.68–30.63) R^2 = 0.9706

* Data are presented as IC50 values and 95% confidence intervals obtained by non-linear regression for all cell lines from three independent experiments.

The number of studies on the biological properties of natural products is increasing and has revealed prominent therapeutic activities related to different diseases [43,44]. An essential axis of research is the evaluation of the anticancer activity. The components of the *P. nigrum* L. species (i.e., extracts and isolated piperine) that were evaluated in this study showed significant cytotoxicity against tumor cells [45].

Based on phytochemical isolation, piperine is the primary alkaloid in *P. nigrum* L. [45]. Furthermore, the biological activities of this compound have been reported, such as anti-inflammatory [46], antimalarial [47], antioxidant [43], and anticancer activities [48]. The results presented in this study show marked cytotoxic and antiproliferative activities of piperine in different models of tumor cells, with IC_{50} values lower than 30 µg/mL, in agreement with the study by Paarakh et al. (piperine in tumor cells, with an IC_{50} of 61.94 µg/mL) [18]. The obtained results showed that the mechanism of action of piperine is related to some common pathways in these tumor types. This is because neoplastic cells have several well-characterized molecular alterations that lead to the malignant phenotype developing [49,50].

Despite not showing a pronounced selectivity for tumor cells, the piperine molecule still shows promise for in-depth investigations regarding its cellular mechanism of action. This is because of its ability to regulate other pathways related to carcinogenesis, such as

invasion, migration, and cell cycle. Furthermore, based on the current results, studies on the structure and molecular interactions of piperine can be conducted to obtain possible analogs with excellent selectivity [51].

The results of piperine cytotoxicity in the two gastric adenocarcinoma models (AGP01 and AGP01 *PIWIL1*$^{-/-}$) are very similar, showing that the presence or absence of the *PIWIL1* gene does not influence its activity. This indicates that the mechanism of action of piperine probably does not involve the interaction of piperine with the *PIWIL1* gene, and may also indicate an excellent scenario for use in cases of gastric cancer where this gene is highly expressed. However, more research is needed to understand the mechanism and relationship between piperine and the *PIWIL1* gene [51].

This study makes an essential contribution to the analysis of the biological properties of the *P. nigrum* L. extracts and isolated piperine compounds. To date, there are very few studies reported on these molecules in gastric cancer and melanoma models. Therefore, such molecules are promising candidates for testing in future studies to elucidate their mechanisms of action and may represent a new approach to investigate therapeutic potential, especially for anticancer drugs.

In this paper, novel studies were conducted on the biological activity (i.e., antimicrobial and cytotoxic) of isolated piperine and *P. nigrum* L. extracts. This study was conducted differently compared to the previous studies conducted on the *Piper nigrum* species [52–54]. The cytotoxic activities of the EEPM, EEPS, and RP extracts observed in this study are consistent with those reported by Grinevicius et al., who showed a cytotoxic activity of the ethanolic extract of *P. nigrum* L. in mammary carcinoma strains (MCF-7) and colorectal strains (HT-29), with an IC_{50} of 27.1 and 80.5 µg/mL, respectively [55–57]. In this study, the extracts showed better activity, with lower IC_{50} values. Despite the significant action on the tumor cell models studied, it can be observed that among the three extracts (EEPM, EEPS, and RP), there is a significant variation in the values of the mean inhibitory concentration (IC_{50}). These variations are possible due to the different extraction methods employed because the phytochemical content can differ significantly and present different concentrations of specific secondary metabolites, as evidenced by Pengkumsri et al. [58]. Since the macerated, Soxhlet, and retained extracts all have different activities against tumor lines, there may be differences in the concentration and structure of secondary metabolites (alkaloid class), which are found in large amounts in the P. nigrum L. species [49,50].

In previous studies conducted with the same extracts, where phytochemical prospection was applied using the precipitation method in test tubes, catechins and alkaloids were detected in the EEPM extract. Catechins, anthocyanins, leucoanthocyanidins, catechins (catechin tannins), flavonoids, and alkaloids were observed in the EEPS extract. The retained extract had phenols, catechins, catechins (catechin tannins), anthocyanins, anthocyanidins, flavonoids, and alkaloids.

Piperine is considered the only alkaloid identified and quantified. Other alkaloid species are not identifiable in the samples. After analyzing the probable synergistic effect of piperine with those components, the mechanism of action of the EEPM extract is increased, promoting an increase in cytotoxic activity. There may have been a decrease in the number of alkaloids in the EEPS extract because the Soxhlet extraction technique requires that the solution be heated in order to facilitate the evaporation of volatile substances. The RP sample, a residue from the purification step of the EEPS extract, would consequently show a significant drop in the proportion of alkaloids and other components [55,59].

It is worth mentioning that the content of secondary metabolites can vary and be influenced by factors such as seasonality, with reports on the seasonal variations in the content of all metabolites. Other factors influencing metabolite concentrations are age and variation in plant and fruit development. Younger tissues and fruits generally have higher metabolic rates for sesquiterpene lactones, phenolic acids, alkaloids, and flavonoids.

The selectivity towards tumor cells, in which the three extracts (macerated, Soxhlet, and retained) are not present, can be attributed to the combined effect of different classes of substances. This results in nonspecific activity. Most studies show the cytotoxic activity of

extracts from the Piper genus, but often do not indicate their selectivity. Thus, this study is important because it shows that the IC_{50} values of the three extracts are the same in tumor cells and non-tumor cells. This suggests that the active compounds need to be separated so that more selective molecules can be found [55,60].

3. Materials and Methods

3.1. Plant Material

Black pepper seeds were purchased in the commercial area of the municipality of Abaetetuba-PA and used for pretreatment and extraction techniques.

In this study, two extraction methods were considered to obtain the crude extract of black pepper seeds: extraction by Soxhlet and the maceration route, considering the possibility of extracting different chemical components with the application of other techniques.

First, the black pepper seeds were introduced to the initial drying procedure (40 °C for 72 h) and ground in a laboratory-scale food processor. The raw material was then subjected to extraction.

For extraction via maceration, 200 g of the crushed sample was weighed on an analytical balance and immersed in 1 L of absolute ethyl alcohol. Approximately 30 days after the immersion procedure, the alcoholic solution (EEPM sample) was filtered and stored for future analysis.

Soxhlet extraction was performed using a 40 g sample of black pepper. The volume of solvent (absolute ethyl alcohol) used in the procedure was 250 mL, with an extraction time of 24 h and a temperature of 80 °C. The ethanol extract obtained from the EEPS sample was concentrated using a rotary evaporator and subjected to a purification process based on the procedures described by Ikan [61]. In the purification step, the precipitate formed was collected through simple filtration, and a sample of the retentate (R) was stored for analysis [61,62] (Figure 10).

Piperine crystals were washed with distilled water and ice-cold absolute ethyl ether to remove contaminants. The solution was left for 7 days, and yellow crystals were formed, which were filtered under vacuum conditions. After removing the crystals, the remaining solution (SR) was used for biological tests (Figure 11).

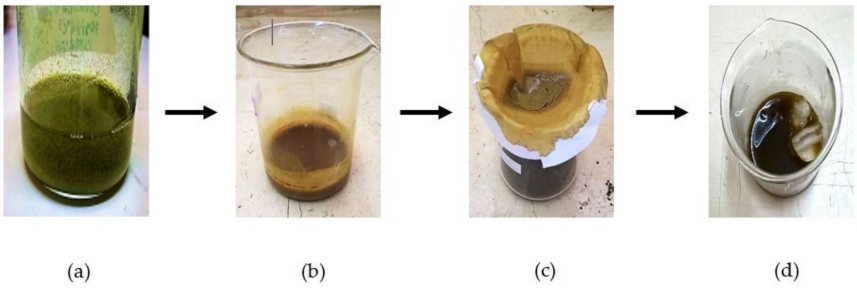

Figure 10. (**a**) Ethanol extract obtained via Soxhlet; (**b**) black pepper extract from the purification process; (**c**) filtration of tannins and precipitated phenolic compounds, and (**d**) supernatant, with the addition of solid distilled water for the precipitation of piperine crystals.

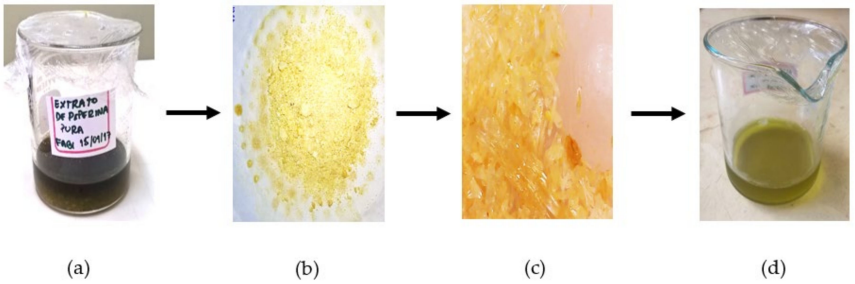

Figure 11. (**a**) Supernatant solution with the piperine crystals; (**b**) solid obtained via vacuum filtration; (**c**) purified piperine crystals, and (**d**) remaining solution (SR).

3.2. Characterization of Piperine Crystals

3.2.1. Optical Microscopic Analysis

Piperine crystal micrographs were taken at the Laboratório de Microscopia (LABMEV/PRODERNA/ITEC/UFPA, Belém, PA, Brazil) using Nikon microscopy (Melville, NY, USA) (ECLIPSE LV150/LV150A).

3.2.2. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

The SEM images of the piperine crystals were taken using a scanning electron microscope, model TM 3000 Hitachi, Chiyoda, Japan. The TESCAN S8000(model VEGA TC) EDS detector was used for semi-quantitative analysis of piperine crystals.

3.2.3. X-ray Diffraction (XRD)

For the analysis of piperine crystals, the sample was initially pulverized and analyzed using X-ray diffraction. A BRUKER model D2 PHASER diffractometer, equipped with a goniometer (θ/θ), radius:141.1 nm, and a copper anode ceramic X-ray tube (Cu-Kα1) was used. The emission line characteristic was 1.540598 Å/8.047 keV, with a maximum power of 300 W (30 kV × 10 mA). The detector was a 1D Lynxeye with a 5° 2θ aperture and 192 channels. The phases and unit cell parameters were identified using the X'Pert High Score Plus program. Sample preparation and XRD analysis were performed at the Instituto de Geociências da UFPA Laboratório de Mineralogia, Geoquímica e Aplicações (LAMIGA-UFPA).

3.3. Biological Assays

3.3.1. Antimicrobial

Antimicrobial activity evaluations were performed using samples collected during the different stages of black pepper extraction (macerated extract–EEPM, ethanol extract–EEPS, retained—RP, and isolated piperine).

Bacterial and Fungal Strains

The strains used with ATCC standards were purified and stored in Eppendorf tubes containing a freezing medium. The bacteria used were *Staphylococcus aureus* (ATCC 0538), *Pseudomonas aeruginosa* (ATCC no code), *Salmonella* sp. (ATCC 4029), *Proteus mirabilis* (ATCC 1353), and *Candida albicans* (ATCC 10635).

Preparation of Media and Standardization of Inoculum

For the cultivation of new microorganisms for the test, the bacteria were prepared and diluted in Muller–Hinton agar (MHA), whereas the fungi were diluted in Sabouraud dextrose agar (SDA). They were left in an oven at 30 °C for a period of 24–48 h for growth. The microorganisms were then immersed in a sterile saline solution with turbidity adjusted to 2 (McFarland standard), corresponding to approximately 6×10^8 CFU/mL. Lastly, they were cultivated on Sabouraud agar (fungi) or Muller–Hinton culture media (bacteria) [61,62].

Sample Microdilution Method for Determining the Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal and Antifungal Concentrations

Approximately 1 mg of each sample (EEPS, EEPM, RP, piperine) was weighed on an analytical balance for solubilization in 1000 µL of absolute methyl alcohol and stored in 1.5 mL Eppendorf tubes. Absolute methyl alcohol was used as a positive control due to its use to dilute the samples. Then, microdilution procedures were conducted to determine the minimum inhibitory concentration (MIC), using the following concentrations: 500, 250, 125, 62.5, 31.25, and 15.625 µg/mL per well, and 10 µL aliquots were deposited on 96-well plates in triplicates [63]. Then, 180 µL of the broth was deposited on 96-well plates. Subsequently, diluted strain suspensions (10 µL) were added to the wells, making up 200 µL in each well.

For the minimal inhibitory concentration assays, 180 µL of the Müller Hinton broth + 10 µL of the bacterial suspension was used as a negative control in 6 wells (lane 1),

and 180 µL of broth was added to another 6 wells (lanes 1 and 2). As positive controls, 10 µL of bacterial suspension and 10 µL of gentamicin (G) were used. All procedures were performed in triplicates, and the arrangement of the solutions on the plates is shown in Figure 12. After inoculation, plates were incubated in a bacteriological oven at 37 °C for 24 h. After incubation, an absorbance reader with an ELISA device at 570 nm was used to read the microplates visually.

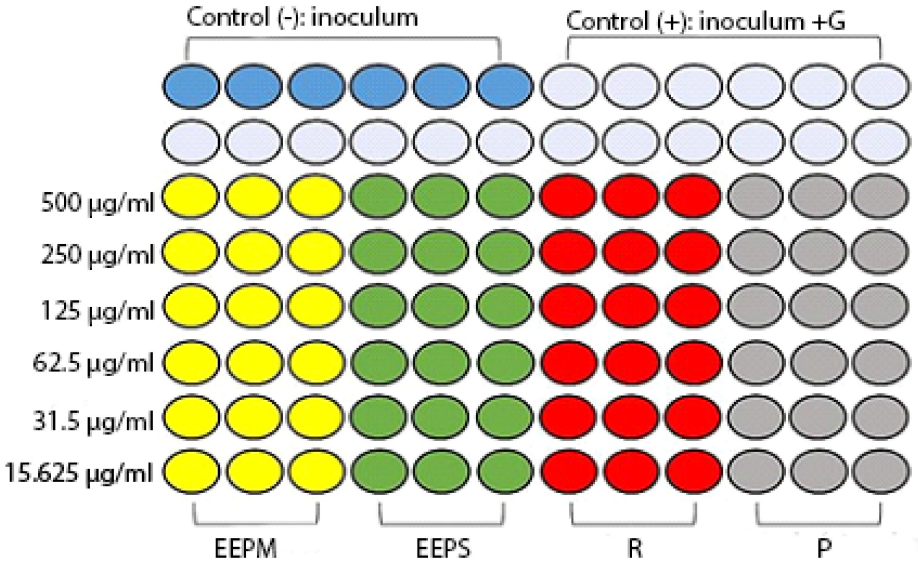

Figure 12. Arrangement of samples and inoculum solutions in control plates (96 wells) for the biological tests on the microorganisms mentioned above.

The following parameters were adopted to classify the antibacterial and antifungal activities of the samples: (1) samples with high antibacterial and antifungal activity had a minimum inhibitory concentration (MIC) less than 100 µg/mL; (2) the samples were considered moderate when the MIC was between 100 and 500 µg/mL; (3) MIC between 500 and 1000 µg/mL was considered weak; and (4) samples with MIC greater than 1000 µg/mL were considered inactive [64].

3.4. Cell Culture

The cell lines used in this study are human metastatic melanoma (SK-MEL 19), intestinal adenocarcinoma (AGP-01, malignant ascites), intestinal adenocarcinoma with an inactivated *PIWIL1* gene (AGP-01 *PIWIL1*$^{-/-}$) [44], and the non-neoplastic human pulmonary fibroblast cell line (MRC5).

Cells were cultivated in adherent monolayer cultures in Dulbecco's modified Eagle's medium (DMEM, Gibco®, Madrid, Spain), supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL, Gibco®), and maintained at 37 °C in a 5% carbon dioxide atmosphere.

3.5. Cytotoxicity Assay

The cytotoxic activities of isolated piperine and the *P. nigrum* L. extracts were evaluated on all of the above-mentioned lineages using the MTT colorimetric assay. All samples presented for examination with an assay were dissolved in Dimethyl sulfoxide (DMSO). The MTT colorimetric assay is based on the metabolically active cells converting the yellow-colored salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into formazan, a purple chromogenic product [65,66]. Cells were seeded in 96-well plates at a density of 10^3 cells/well for 24 h to allow adhesion to the plate. The treatment was performed at a concentration of 0.3125–20 µg/mL for piperine and 1.5625–100 µg/mL for the extracts at an incubation temperature of 37 °C for 72 h. The negative control was

untreated, and the experiments were performed in triplicate. After treatment, 100 µL of the MTT solution (5 mg/mL stock solution, diluted 1:10 in the DMEM medium) was added to each plate well and incubated at 37 °C for 3 h. Absorbance was measured using a microplate spectrophotometer at 570 nm (SYNERGY/HT microplate reader).

3.6. Data Analysis

A sigmoidal dose–response equation (nonlinear regression) was used to determine the half-maximal inhibitory concentration (IC_{50}) and its respective confidence intervals (95% CI).

4. Conclusions

This paper investigated the biological potential of *P. nigrum* using extracts and piperine, a compound isolated from this plant species. We evaluated the antimicrobial potential and cytotoxicity of the extracts and piperine. Microscopic examination of the obtained crystals revealed elongated crystals in the form of bars with considerable thicknesses. From the micrographs of piperine obtained through SEM analysis, a series of agglomerated crystals with different shapes and irregular geometries was observed. EDS analysis of the crystal sample quantified carbon and oxygen within the ranges of 81.92–74 wt.% and 22.65–18.08 wt.%, respectively. However, the wt.% of nitrogen (3.35 wt.%.) was found to be low when spot analysis was conducted. From the diffractogram of the piperine crystal sample, all XRD reflections were comparable with those in the data reported in the literature. Evaluation of the antimicrobial activities of the samples (EEPS, EEPM, RP, and isolated piperine) revealed that the most promising results were achieved with the EEPS and piperine samples, since they were active against almost all the microorganisms analyzed. The EEPS sample showed low inhibitory activity solely against the bacterium *Salmonella* spp., whereas piperine demonstrated low potential only against the microorganism *Proteus mirabilis*. The analyzed extracts (EEPS, RP, and EEPM) were demonstrated by the MTT method to have cytotoxic activity on tumor and non-tumor cell lines. Piperine also showed inhibition potential on all tested tumor lines, causing a decrease in cell viability and achieving an IC_{50} of less than 30 µg/mL. This paper provides relevant information on the cytotoxicity of black pepper and piperine extracts, which showed promising antimicrobial and anticancer activities. We highlighted the importance of this alkaloid's performance for developing research in the fight against cancer and several diseases caused by microbiological agents.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10010021/s1, Figure S1: olecular structure of Piperine. Figure S2: ^{13}C NMR spectrum obtained from analysis of piperine crystals. Figure S3: ^{1}H NMR spectrum obtained from analysis of piperine crystals.; Table S1: NMR data (δC and δH) experimental (Exp.) in ppm for piperine.

Author Contributions: Conceptualization, F.S.A. and J.N.C..; methodology, F.S.A. and J.N.C.; software, F.S.A.; validation, F.S.A., M.L.d.C. and I.N.d.F.R.; formal analysis, D.L.d.N.B., R.N.Q., G.V.d.S. (Glauce Vasconcelos da Silva) and G.V.d.S. (Gleice Vasconcelos da Silva) investigation, F.S.A. and J.N.C.; resources, I.N.d.F.R.; data curation, F.S.A. and J.N.C.; writing—original draft preparation, F.S.A., J.N.C. and M.F.D.; writing—review and editing, F.S.A., J.N.C.; visualization, J.N.C., A.S.K., J.d.A.R.d.R. and D.d.S.B.B.; supervision, F.S.A. and J.N.C.; project administration, J.d.A.R.d.R. and D.d.S.B.B.; funding acquisition, J.d.A.R.d.R. and D.d.S.B.B. All authors have read and agreed to the published version of the manuscript.

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