Secondary Metabolic Profile as a Tool for Distinction and Characterization of Cultivars of Black Pepper (Piper nigrum L.) Cultivated in Pará State, Brazil

Luccas M. Barata 1, Eloisa H. Andrade 2, Alessandra R. Ramos 3, Oriel F. de Lemos 4, William N. Setzer 5,6,* 1, Kendall G. Byler 7, José Guilherme S. Maia 8, and Joyce Kelly R. da Silva 1,5,* 8

1 Programa de Pós-Graduação em Biotecnologia, Universidade Federal do Pará, Belém, PA 66075-110, Brazil; luccas.miranda2@gmail.com
2 Coordenação de Botânica, Museu Paraense Emílio Goeldi, Belém, PA 66077-830, Brazil; eloisa@museu-goeldi.br
3 Instituto de Estudos em Saúde e Biológicas, Universidade Federal do Sul e Sudeste do Pará, Marabá, PA 68507-590, Brazil; rezendea@unifesspa.edu.br
4 Centro de Pesquisa Agroflorestal da Amazônia Oriental, Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Belém, PA 66095-100, Brazil; oriel.lemos@embrapa.br
5 Aromatic Plant Research Center, 230 N 1200 E, Suite 100, Lehi, UT 84043, USA
6 Department of Chemistry, University of Alabama in Huntsville, Huntsville, AL 35899, USA
7 Department of Biological Sciences, University of Alabama in Huntsville, Huntsville, AL 35899, USA; kgb0011@uah.edu
8 Programa de Pós-Graduação em Química, Universidade Federal do Maranhão, São Luís, MA 65080-905, Brazil; gmaia@ufpa.br

* Correspondence: wsetzer@chemistry.uah.edu (W.N.S.); joycekellys@ufpa.br (J.K.R.d.S.);
Tel.: +1-256-824-6519 (W.N.S.); +55-91-3201-7297 (J.K.R.d.S.)

Abstract: This study evaluated the chemical compositions of the leaves and fruits of eight black pepper cultivars cultivated in Pará State (Amazon, Brazil). Hydrodistillation and gas chromatography–mass spectrometry were employed to extract and analyze the volatile compounds, respectively. Sesquiterpene hydrocarbons were predominant (58.5–90.9%) in the cultivars “Cingapura”, “Equador”, “Guajarrina”, “Iaçar”, “Kottanadan”, and “Clonada”, and “Üthirankota” displayed oxygenated sesquiterpenoids (50.6–75.0%). The multivariate statistical analysis applied using volatile composition grouped the samples into four groups: γ-Elemene, curzerene, and δ-elemene (“Equador”/“Guajarrina”); δ-elemene (“Iaçar”/“Kottanadan”/“Cingapura”, “Iaçar”); elemol (“Clonada”/“Üthirankota”, “Iaçar”/“Kottanadan”, and “β-murolol, bicyclogermacrene, and cubebol (“Bragantina”, “Clonada”), and “Üthirankota” displayed oxygenated sesquiterpenoids (50.6–75.0%). The multivariate statistical analysis applied using volatile composition grouped the samples into four groups: γ-Elemene, curzerene, and δ-elemene (“Equador”/“Guajarrina”); δ-elemene (“Iaçar”/“Kottanadan”/“Cingapura”, “Iaçar”); elemol (“Clonada”/“Üthirankota”, “Iaçar”/“Kottanadan”, and “β-murolol, bicyclogermacrene, and cubebol (“Bragantina”, “Clonada”), and “Üthirankota” displayed oxygenated sesquiterpenoids (50.6–75.0%). The multivariate statistical analysis applied using volatile composition grouped the samples into four groups: γ-Elemene, curzerene, and δ-elemene (“Equador”/“Guajarrina”); δ-elemene (“Iaçar”/“Kottanadan”/“Cingapura”, “Iaçar”); elemol (“Clonada”/“Üthirankota”, “Iaçar”/“Kottanadan”, and “β-murolol, bicyclogermacrene, and cubebol (“Bragantina”, “Clonada”), and “Üthirankota” displayed oxygenated sesquiterpenoids (50.6–75.0%).

Keywords: sesquiterpenes; monoterpenes; phenolic compounds; carotenoids; PAL activity; molecular docking

1. Introduction

The genus Piper is the largest from the Piperaceae, with about 3000 species identified, and is widely distributed in tropical and subtropical regions of the planet, and in Brazil, there are around 44 varieties reported, distributed among 292 species [1,2]. The most representative species of the genus is Piper nigrum L. (black pepper), which is considered
the “King of Spices” and is commonly used as a condiment around the world, but is also applied in traditional medicine in several countries, mainly in Asia, Africa, and South America [3,4]. Black pepper is an aromatic plant usually known for the application of its flavoring agents in the reduction of muscle pain, fevers, conditions, diarrhea, gastric conditions and cholesterol, in addition to acting as an anti-inflammatory, antioxidant, antimicrobial, and repellent agent [5–8].

*Piper* species are distinguished by the presence of nodes in the shoots, inflorescence spikes, and their aromatic smell [9]. Black pepper is a perennial, woody vine that easily grows in shaded places, with indeterminate growth, but some studies report that some plantations limit their height up to 9 m. If the plant touches the ground, the root can come out of the leaf nodes [10]. Cultivars of black pepper present differences in leaf shape, stem, flowers, as well as the fruit size and thickness [11–13]. The species presents several cultivars identified in the literature, with dozens of these cultivars explored in the main production areas of Brazil, such as “Apra”, “Bragantina”, “Cingapura”, “Clonada”, “Equador”, “Gujarina”, “Iacará”, “Kottanadan”, “Kuthiravally”, and “Uthirankota”, among others [13–17].

India, Brazil, Indonesia, Malaysia, Vietnam, China, and Sri Lanka are the largest producers of black pepper [18–20]. In Brazil, their economic value started increasing at the end of World War II [21]. Since then, Brazil became one of the largest producers of black pepper in the world. In 2018, the national production was about 101,000 tons, and the State of Pará, which historically was the most significant national producer, appeared as the second largest producer, with more than 33,000 tons (33% of national production). Occasionally, the State of Espírito Santo was the largest national producer of the year [22]. However, the fluctuations in annual production and product availability, and the occurrence of plant diseases, such as fusariosis (*Fusarium solani* f. sp. *piperis*) and yellow wilt (*Fusarium oxysporum*), have resulted in price instability in the international market of this condiment [21,23,24].

Secondary metabolites are defined as products of metabolism that are non-essential for the survival of a plant, but they play an important role in the interaction with the environment in which the plant is inserted, including protection against phytopathogenic microorganisms [25,26]. They are distributed in different parts of the plant (leaves, roots, fruits, seeds, or flowers), and can be obtained from extracts or in their essential oils (EOs), which have several properties, such as fungicide, bactericide and insecticide, in addition to antioxidant activity [12,27,28].

Some secondary metabolites of black pepper are used to fight infections caused by microorganisms, such as viruses and fungi, that are called phytoalexins [29,30]. Studies report that its fruit extracts have presented high bactéricidal activity against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Bacillus subtilis* [31]. Additionally, the EO of *P. nigrum* exhibited potential usability as a natural antioxidant and food supplement, and presents a pharmacological potential [32].

The metabolomic analysis of plants against these stimuli can help in the identification of secondary metabolites involved in different plant physiological processes, and can be used as biomarkers for the discrimination and characterization of the species [33–36]. In the literature, several studies show the effectiveness of plant extracts against different pathogens. However, more detailed analysis is required to understand compounds that are directly responsible for the antimicrobial activity and their protein–ligand interaction mechanisms [37]. Thus, molecular docking analysis has become an important tool to evaluate small-molecule ligands’ potential interactions with protein targets. The results can lead to the elucidation of biologically active agents’ mechanisms from plant sources that can assist in natural-product drug discovery against different pathogens [38].

The soil-borne pathogens *Fusarium solani* f.sp. *piperis* and *F. oxysporum* are the most notably responsible for the fungal diseases in black pepper that negatively interfere in its fruit production and the plant quality [39,40]. Moreover, they cause significant yield loss due to the severe damage, since they do not present apparent symptoms in the initial phase of infection [41]. Strategies of control have not been totality efficient in treating
and preventing black pepper diseases. In this sense, the metabolomic analysis of black pepper cultivars combined with molecular docking studies is presented in this study. The results can be employed as an alternative in target prediction for developing new natural fungical agents based on their mechanisms of protein–ligand interactions.

Therefore, this study aims to identify the secondary metabolic profile in the P. nigrum cultivars cultivated in the Pará State (Brazil), and to evaluate the potential volatile phytoalexins by molecular docking analysis on Fusarium protein targets. The discrimination and characterization of the cultivars in chemical groups can give more insight into plant–pathogen interactions, so as to develop new strategies in plant disease control.

2. Results and Discussion

2.1. Evaluation of Production and Identification of Secondary Metabolites from Cultivars of Black Pepper

2.1.1. Volatile Compounds in the Leaves of Black Pepper

The chemical analysis by gas chromatography–mass spectrometry (GC-MS) resulted in the identification of 114 components (see Supplementary Material, Table S1), representing an average of 98.7% of the total composition. The predominance of sesquiterpene hydrocarbons was observed in the cultivars “Cingapura” (58.48 ± 0.94%), “Equador” (59.35 ± 5.45%), “Guajarina” (71.81 ± 4.47%), “Iaçará” (90.90 ± 1.03%), and “Kottanadan” (74.63 ± 2.38%). On the other hand, the cultivars “Bragantina” (50.55 ± 10.35%), “Clonada” (60.90 ± 5.45%), “Guajarina” (71.81 ± 4.47%), “Iaçará” (90.90 ± 1.03%), and “Kottanadan” (74.63 ± 2.38%) showed an average of 98.7% of the total composition. The predominance of sesquiterpene hydrocarbons was observed in the cultivars “Cingapura” (58.48 ± 0.94%), “Equador” (59.35 ± 5.45%), “Guajarina” (71.81 ± 4.47%), “Iaçará” (90.90 ± 1.03%), and “Kottanadan” (74.63 ± 2.38%). On the other hand, the cultivars “Bragantina” (50.55 ± 10.35%), “Clonada” (60.90 ± 5.45%), “Guajarina” (71.81 ± 4.47%), “Iaçará” (90.90 ± 1.03%), and “Kottanadan” (74.63 ± 2.11%) showed an average of 98.7% of the total composition. The predominance of sesquiterpene hydrocarbons was observed in the cultivars “Cingapura” (58.48 ± 0.94%), “Equador” (59.35 ± 5.45%), “Guajarina” (71.81 ± 4.47%), “Iaçará” (90.90 ± 1.03%), and “Kottanadan” (74.63 ± 2.38%). On the other hand, the cultivars “Bragantina” (50.55 ± 10.35%), “Clonada” (60.90 ± 5.45%), “Guajarina” (71.81 ± 4.47%), “Iaçará” (90.90 ± 1.03%), and “Kottanadan” (74.63 ± 2.38%) showed an average of 98.7% of the total composition. The predominance of sesquiterpene hydrocarbons was observed in the cultivars “Cingapura” (58.48 ± 0.94%), “Equador” (59.35 ± 5.45%), “Guajarina” (71.81 ± 4.47%), “Iaçará” (90.90 ± 1.03%), and “Kottanadan” (74.63 ± 2.38%).

The “Bragantina” cultivar showed a predominance of oxygenated sesquiterpenoids (50.55 ± 10.35%), followed by sesquiterpenoid hydrocarbons (34.62 ± 12.97%). The major components were α-muurolol (20.63 ± 2.81%) (Figure 1A), bicyclogermacrene (7.55 ± 4.95%), cubebol (6.49 ± 2.11%) and δ-cadinene (6.04 ± 1.77%). In another study, the major compounds identified in the cultivar “Bragantina” were cubenol (14.55–21.53%), bicyclogermacrene (7.55 ± 4.95%), cubebol (6.49 ± 2.11%) and δ-cadinene (6.04 ± 1.77%). In another study, the major compounds identified in the cultivar “Bragantina” were cubenol (14.55–21.53%), bicyclogermacrene (7.55 ± 4.95%), cubebol (6.49 ± 2.11%) and δ-cadinene (6.04 ± 1.77%). In another study, the major compounds identified in the cultivar “Bragantina” were cubenol (14.55–21.53%), bicyclogermacrene (7.55 ± 4.95%), cubebol (6.49 ± 2.11%) and δ-cadinene (6.04 ± 1.77%). In another study, the major compounds identified in the cultivar “Bragantina” were cubenol (14.55–21.53%), bicyclogermacrene (7.55 ± 4.95%), cubebol (6.49 ± 2.11%) and δ-cadinene (6.04 ± 1.77%).

In the “Cingapura” cultivar, sesquiterpene hydrocarbons (58.48 ± 0.94%) were predominant, followed by oxygenated sesquiterpenoids (20.36 ± 0.80%). The major components identified were δ-elemene (37.17 ± 1.90%) (Figure 1B), linalool (6.75 ± 0.37%), spathulenol (5.85 ± 0.48%), (E)-β-caryophyllene (5.72 ± 0.93%), and muurola-4,10(14)-dien-1-β-ol (4.83 ± 0.64%). “Iaçará” and “Kottanadan” cultivars displayed similar chemical profiles with quantitative differences. The main compounds were δ-elemene (50.89 ± 1.64% and 32.1% ± 7.44%), β-elemene (9.92 ± 0.58% and 6.10 ± 1.32%), viridiflorene (8.87 ± 0.84% and 12.42 ± 2.28%), and β-selinene (8.17 ± 0.57% and 7.54 ± 2.59%) for the “Iaçará” and “Kottanadan” cultivars, respectively. In black pepper, δ-elemene (53.3%) was previously reported as the main component in the leaves of “Cingapura” cultivar. The other main constituents identified in the cultivar were (E)-β-caryophyllene (4.7%), and

Figure 1. The main volatile compounds found in the leaves of black pepper cultivars. (A) α-muurolol; (B) δ-elemene; (C) elemol; (D) γ-elemene.

In the “Cingapura” cultivar, sesquiterpene hydrocarbons (58.48 ± 0.94%) were predominant, followed by oxygenated sesquiterpenoids (20.36 ± 0.80%). The major components identified were δ-elemene (37.17 ± 1.90%) (Figure 1B), linalool (6.75 ± 0.37%), spathulenol (5.85 ± 0.48%), (E)-β-caryophyllene (5.72 ± 0.93%), and muurola-4,10(14)-dien-1-β-ol (4.83 ± 0.64%). “Iaçará” and “Kottanadan” cultivars displayed similar chemical profiles with quantitative differences. The main compounds were δ-elemene (50.89 ± 1.64% and 32.1% ± 7.44%), β-elemene (9.92 ± 0.58% and 6.10 ± 1.32%), viridiflorene (8.87 ± 0.84% and 12.42 ± 2.28%), and β-selinene (8.17 ± 0.57% and 7.54 ± 2.59%) for the “Iaçará” and “Kottanadan” cultivars, respectively. In black pepper, δ-elemene (53.3%) was previously reported as the main component in the leaves of “Cingapura” cultivar. The other main constituents identified in the cultivar were (E)-β-caryophyllene (4.7%), and...
black pepper fruits are related to these compounds, with variations only in their percentages among the cultivars [72,73].

AB C

“Equador” and “Guajarina” cultivars presented the same chemical profile, only with quantitative differences. The main compounds were γ-elemene (38.33 ± 3.80% and 44.36 ± 4.06%) (Figure 1D), curzerene (23.21 ± 4.96% and 23.39 ± 4.45%), and δ-elemene (6.93 ± 0.77% and 13.84 ± 0.80%), respectively. Several Piper species present γ-elemene as one of the main components in their EOs, such as P. gaudichaudianum Kunth (5.4%) [41], P. marginatum (3.75%) [64], P. nemorense (6.8%) [48], and P. vicosanum (14.16%) [65].

Curzerene was widely found in “Equador” (23.21 ± 4.96%) and “Guajarina” (23.39 ± 4.45%) cultivars, and is considered a derivative of furanodiene, which is produced from a reversible thermal rearrangement known as the Cope rearrangement [66]. It is a type of reaction characterized by the substitution of alkenes when the compound is found at high temperatures [67], which suggests that curzerene may have been produced during the essential oil extraction as well as during the gas chromatographic analysis [68].

In Piper species, this oxygenated sesquiterpenoid can be found in P. dilatatum (13.8–28.7%) [54] and P. hispidum (4.9–12.9%) [59,69]. Another well-known species that presents curzerene as one of the main components is Eugenia uniflora, commonly known as “pitangá”, an arboreous plant widely distributed in Brazil and other South American countries and possesses antifungal activity against Candida spp., and its EO presents antiproliferative and cytotoxic effects against some cancer strains [70,71].

2.1.2. Volatile Compounds in the Fruits of Black Pepper

GC-MS analysis identified 104 components, representing 99.45% of the total composition (see Supplementary Material, Table S2). Monoterpene hydrocarbons (average of 76.1%) were predominant in all cultivars, such as α-pinene (6.91–10.67%), β-pinene (22.61–35.05%) and limonene (21.0–31.77%). The presence of α-pinene, β-pinene, and limonene (Figure 2) in the chemical composition can be found in most cultivars of black pepper [72]. The literature widely reports the influence of terpene levels on the aroma and flavor of black pepper fruits. Despite different geographical regions, the pungency and flavor of black pepper fruits are related to these compounds, with variations only in their percentages among the cultivars [72,73].

![Figure 2. Main volatile compounds found in the fruits of all cultivars of black pepper. (A) α-pinene; (B) β-pinene; (C) limonene.](image-url)

The EO chemical composition of black pepper cultivars can present quantitative and qualitative differences according to their geographic occurrence. For example, in cultivars from India (“Indigenous” and “Kerala”), quantitative variations in amounts of α-pinene (7.3–16.7%), β-pinene (13.2–13.6%) and limonene (15.2–16.2%), δ-3-carene (9.2–32.6%), α-phellandrene (2.9–8.9%), and (E)-β-caryophyllene (10.7–18.4%) were observed [74]. Fruits
from “Sreekara”, “Kuching”, and “Vellanamban” cultivars, also found in India, present α-pinene (1.7–5.5%), β-pinene (3.9–11.2%) and limonene (8.3–22.1%) in their EO composition. However, they also display other majorities, such as (E)-β-caryophyllene (16.8–39.1%), sabinene (4.3–18.8%), myrcene (2.0–9.6%), α-phellandrene (3.0–7.7%, only in “Sreekara” and “Kuching” cultivars), δ-3-carene (5.1–11.1%, only in “Sreekara” and “Vellanamban” cultivars), α-cubebene (4.8–6.1%, only in the “Vellanamban” cultivar), and even the oxygenated sesquiterpene elemol (4.2–10.5, only in the “Vellanamban” cultivar) [75]. In Cameroon, the EO of black pepper presented δ-3-carene (18.5%), limonene (14.7%), (E)-β-caryophyllene (12.8%), sabinene (11.2%), α-pinene (5.6%), and β-pinene (6.7%), while the EO of black pepper from S. Tomé e Príncipe displayed limonene (18.8%), (E)-β-caryophyllene (15.4%), sabinene (16.5%) and β-pinene (10.7%) as majorities [76,77].

2.1.3. Hierarchical Cluster Analysis (HCA)

Multivariate analysis based on the leaf volatiles classified the cultivars into four groups (Figure 3). Group I exhibited a similarity level of 99.2% between the cultivars “Equador” and “Guajarina”, and presented high levels of γ-elemene (38.33–44.36%), curzerene (23.21–23.39%) and δ-elemene (6.83–13.84%). This group displayed a similarity of 53.6% with group II. High concentrations of δ-elemene (30.11–50.89%) characterized the group II with a similarity level of 92.3% among the cultivars “Iaçarã”, “Kottanadan”, and “Cingapura”.

![Figure 3. Hierarchical cluster analysis (HCA) analysis from volatile concentrates of black pepper. Different colors in the groups indicate the potential degrees of susceptibility of the cultivars to Fusarium oxysporum (yellow wilt disease). entsprechend the susceptibility levels: dark red, susceptible; yellow, medium tolerance; green, tolerant.](https://example.com/figure3.png)

In addition, cultivars “Iaçarã” and “Kottanadan” presented significant amounts of viridiflorene (8.87–12.42%), β-selinene (7.54–8.17%) and β-elemene (6.10–9.92%). In contrast, the cultivar “Cingapura” exhibited spathulenol (5.85%) and (E)-β-caryophyllene (5.72%) as the majority. This group showed a similarity of 45.9% with group III. The cultivars “Clonada” and “Uthirankota” were grouped in Group III with a similarity level of 98.1% and high content of elemol (40.55–49.78%), and presented a similarity of 43.9% with group IV. Finally, group IV separated only the “Bragantina” cultivar from the others, due to its composition rich in α-muurolol (20.63%), bicyclogermacrene (7.55%), and cubebol (6.49%). Regarding volatiles from the fruits, two clusters were identified with a high similarity between them (~93.5%), displaying no significant variation among the cultivars.
The use of metabolic profiling as a tool for the discrimination of cultivars of several crops has been reported in the literature [78,79]. Principal component analysis (PCA) and HCA techniques classified the EOs from five varieties of *P. betle* into three groups. Group I, which englobes “Misti” and “BARI Paan 3” varieties, presented chavicol (3.97–11.95%), (E)-β-caryophyllene (4.24–4.69%), and valencene (3.02–3.11%). Group II was composed only of the variety “Bangla” due to the presence of eugenyl acetate (1.50%) and α-humulene (1.35%) in its composition. The varieties “Sanchi” and “Khasia” were separated in Group III, which was characterized by γ-muurolene (1.74–4.49%) and (E)-β-caryophyllene (1.74–4.93%) in their compositions [80].

PCA analysis of volatile profiles was able to distinguish fourteen *Citrus* varieties as tolerant and moderately tolerant from those susceptible to *Candidatus Liberibacter asiaticus* (CLas). Tolerant and moderately tolerant cultivars showed higher amounts of volatiles than susceptible varieties. These compounds include aldehydes (undecanal, neral, geranial, and citronellal) and some monoterpenes such as linalool (0.1–68.3%), d-limonene (0.3–55.3%), myrcene (0.1–8.5%), α- (0.2–3.1%) and β-phellandrene (0.1–11.4%) [81].

Previous studies reported the different degrees of the susceptibility of black pepper cultivars to *F. oxysporum* (yellow wilt) [24,82,83]. Our results indicate a possible correlation between resistance to yellow wilt and the volatile composition of the leaves. “Iaçará”, “Kottanadan”, and “Cingapura” (group II) are highly resistant, while “Bragantina” (group IV) and “Uthirankota” (group III) present medium resistance, and “Guajarina” (group I) are more susceptible. Consequently, the cultivar “Equador” may be classified as susceptible due to its high similarity with the cultivar “Guajarina” (99.2%). Furthermore, the variety “Clonada” may have a medium resistance due to its proximity to the cultivar “Uthirankota” (98.1%) (see Figure 3).

In comparison to the fruits, the volatile profiles of leaves functioned as suitable biomarkers for the discrimination of cultivars, and their resistance or susceptibility to yellow wilt. However, further experiments with *Fusarium* inoculation given to each variety are required to confirm this relationship. Moreover, an antimicrobial assay with the main compounds is recommended for understanding the role of specific compounds in each cultivar’s plant defense mechanism.

### 2.1.4. Total Phenolics Determination

The total phenolic contents in the *P. nigrum* extracts showed great variation in the leaves (44.75 ± 1.60 to 140.53 ± 14.01 mg GAE·g⁻¹) (Figure 4A). However, in the fruits we noticed only small changes in the “Cingapura” and “Uthirankota” cultivars (Figure 4B). According to the literature, these variations can be occasioned by several factors, biotic and abiotic, such as plant defense, senescence, plant development, and interactions with the environment in which they are inserted [84].

![Figure 4](image_url)

**Figure 4.** Total phenolic compounds produced in black pepper cultivars. (A) Leaves; (B) Fruits. a–d Different letters represent a statistically significant difference by Tukey’s test (*p* < 0.05).
The levels of total phenolics were higher in the leaves in comparison to the fruits. These differences can be related to the harvesting and processing steps involved in sundrying, which alter the interaction between the enzymes related to phenolic production in the black pepper fruits [85]. In addition, the content of phenolic compounds can be considered a useful parameter for determining plants’ biological functions [86]. Six black pepper varieties (“Sreekara”, “Subhakara”, “IISR Malabar Excel”, “Panniyur-1”, “Panchami” and “IISR Thevam”), and other Piper species (P. chaba Hunter, P. longum L., and P. colubrinum Link) were extracted with n-hexane, chloroform, methanol, and water. Methanol extracts from “IISR Malabar Excel” and “Panchami” varieties, and a chloroform extract of P. colubrinum displayed higher phenolic contents, with values of 50.85, 38.74, and 100.6 mg GAE·g⁻¹ of the extract [87]. Therefore, our findings suggest that black pepper cultivars cultivated in the Pará State, Brazil have high phenolic contents compared to other cultivars from other regions of the world, and the extracts may present several biological activities.

Plants that are under biotic or abiotic stresses can exhibit changes in their phenolic contents in the early stages of development [88]. Black pepper, after infection with F. solani f. sp. piperis, showed a significant variation in total phenolics in the leaves and roots from the “Cingapura” cultivar (tolerant to Fusarium), which can be attributed to the response to the infection in the early stages, since the first symptoms were noted after two weeks of inoculation [49]. Moreover, the association of the “Bragantina” cultivar of black pepper with arbuscular mycorrhizal fungi (AMF) induced an increase in phenolic content, which was correlated to changes in enzymatic activity linked to metabolic pathways related to plant defense. These findings suggest that AMF can induce a better resistance to pathogens [43].

2.1.5. Total Carotenoids

The concentration of total carotenoids revealed a great variation among the cultivars (0.21 ± 0.03 to 2.31 ± 0.10 µg·mL⁻¹) (Figure 5). The literature reports a synergistic effect of carotenoids of great importance for the biological properties of plant extracts, which is linked to the production of different classes of compounds such as phenylpropanoids, terpenes, and alkaloids [89].

![Figure 5. Total carotenoids produced in the leaves of black pepper cultivars. a–d Different letters represent a statistically significant difference by Tukey’s test (p < 0.05).](image)

In plants, the carotenoids are involved in different areas, such as antioxidants, accessory pigments that absorb light, and molecules that act as attractants for pollinators and seed dispersers, in addition to the plant response to environmental stresses [90]. Thus, it is possible to measure the impact of the environment on plant metabolism. An increase in carotenoid concentration was observed in the black pepper cultivar “Panniyur 1”, which is tolerant to drought stress, suggesting a relationship between the production of these compounds and the mechanisms of tolerance to stress [91]. Another study showed the
effect of natural growth promoters on the physiological and biochemical development of the cultivar “Panniyur 1” of black pepper, and displayed an increase of 159% in total carotenoids compared with the control group. Therefore, species that have access to a greater amount of the nutrients essential to plant growth also tend to have a higher concentration of carotenoids [92].

2.2. Comparison of Enzymatic Activity In Vitro in the Leaves of Black Pepper Cultivars
Phenylalanine Ammonia-Lyase (PAL) Activity

P. nigrum cultivars exhibited a considerable variation in PAL enzymatic activity, ranging from 20.19 to 57.22 μU·mL⁻¹ (Figure 6). PAL is a key enzyme in the biosynthetic pathway of phenylpropanoids, which displays an essential role in the production of metabolites related to plant defense, such as flavonoids, alkaloids, lignans, lignins, and coumarins [93].

![Figure 6. Enzymatic activity of PAL in the leaves of black pepper cultivars.](image)

Alkaloids are the main class produced by the shikimic acid pathway present in black pepper, especially the piperamides [94]. These metabolites are present in the fruits and seeds of black pepper, and have wide pharmacological applicability, such as in antimetastatic, antidepressant, hepatoprotective and antitumor activities, and they also have neuroprotective effects [95,96]. Alkaloid concentrations and their biological properties are directly related to PAL activity in the plant [97,98].

An increase in PAL activity can result from a sensitive response that occurs in plants when they are infected by pathogens or during an injury [99,100]. Significant changes in PAL activity were observed in the cultivars “Cingapura” and “Trau” of black pepper, two days after infection with Phytophthora capsici. The results showed that PAL causes an immediate response in the production of defense metabolites when the plant is under stress [101]. After 48 h of co-inoculation with two strains of rhizobacteria (Bacillus subtilis and Pseudomonas fluorescens) and two strains of endophytic fungi (Trichoderma viride and Trichoderma asperellum), an increase in PAL activity induced the systemic resistance of black pepper against P. capsici [102]. Therefore, the quantification of PAL activity can be considered an alternative in the evaluation of plant resistance against infection by pathogens.

2.3. Homology Modeling and Molecular Docking to Fusarium

In an attempt to correlate volatile phytoalexins with Fusarium resistance, we have carried out a molecular docking analysis of the major P. nigrum volatile components with Fusarium protein targets that were available from the Protein Data Bank (PDB): F. oxysporum cutinase (PDB 5AJH), F. solani cutinase (PDB 1AGY, 1XZL, and 1XZM), F. oxysporum endoglucanase (PDB 4OVW), F. oxysporum feruloyl esterase (PDB 6FAT), and F. oxysporum xylanase (PDB
In addition, *F. solani* ornithine decarboxylase was prepared by homology modeling using murine ornithine decarboxylase (PDB 70DC) as a template, *F. oxysporum* glucosamine-fructose-6-phosphate aminotransferase was prepared from human glutamine fructose-6-phosphate amidotransferase (PDB 6R4E) as a template, *F. odoratissimum* β-glucosidase was prepared using *Neurospora crassa* β-glucosidase (PDB 5NBS) as the template, *F. oxysporum* guanine nucleotide-binding protein subunit β was prepared using human guanine nucleotide-binding protein subunit α1 (PDB 6CMO) as the template, and *F. vanettenii* thiamine thiazole synthase was prepared using *N. crassa* thiazole synthase (PDB 3JSK) as the template. Docking energies for the essential oil ligands and 10 *Fusarium* target proteins are listed in Supplementary Material, Table S3.

The major components found in the *Fusarium*-resistant *P. nigrum* cultivars (“Cingapura”, “Iaçará”, and “Kottanadan”) were δ-elemene (abundant in all three cultivars), viridiflorene, β-selinene, β-elemene (abundant in “Iaçará” and “Kottanadan”), spathulenol and (E)-β-caryophyllene (abundant in “Cingapura”). δ-Elemene, viridiflorene, β-elemene, and (E)-β-caryophyllene, all showed selective docking (most exothermic docking energies) to *F. oxysporum* endoglucanase. Furthermore, *F. oxysporum* endoglucanase had the lowest docking average energy for the 40 essential oil components, and was the most targeted protein with 13 ligands showing preferential docking to this protein. Endoglucanase is one of the enzymes responsible for breaking down cellulose by hydrolyzing the β-1,4-glycosidic bonds in the cellulose polymer [103]. Thus, the inhibition of this protein target would protect the plant from cellulose degradation by *Fusarium*. Interestingly, the essential oil components all dock into a hydrophobic pocket removed from the site of the co-crystallized ligand (4-(β-D-glucopyranosylxy)-2,2-dihydroxybutyl propanoate) but still in the active canyon of the enzyme (Figure 7).

![Figure 7](image-url). Lowest-energy docked pose of δ-elemene (left stick figure) with *Fusarium oxysporum* endoglucanase (Protein Data Bank, PDB 4OVW). The co-crystallized ligand (right stick figure) is 4-(β-D-glucopyranosylxy)-2,2-dihydroxybutyl propanoate. (A): Solid protein structure. (B): Ribbon structure.

### 3. Materials and Methods

#### 3.1. Plant Material

The leaves and fruits of “Bragantina”, “Cingapura”, “Clonada”, “Equador”, “Guajaria”, “Iaçará”, “Kottanadan”, and “Uthirankota” cultivars of *Piper nigrum* L. (Piperaceae) were provided by EMBRAPA Amazônia Oriental (Brazilian Agricultural Research Corporation). The plants were acclimatized and maintained in a greenhouse with a daily watering regime. Adult leaves were collected during the fruiting stage and stored at −20 °C for further experimentation. For the fruits, the collection time varied from July to October,
according to its ripeness for each cultivar. After harvesting, the fruits were sun-dried for about three days until the humidity was around 13%, and preserved at room temperature for further experimentation.

3.2. Identification of Secondary Metabolites from Cultivars of Black Pepper
3.2.1. Extraction and Analysis of Volatile Organic Compounds (VOCs)

Leaves and fruits were extracted by the simultaneous distillation–extraction process using a Likens–Nickerson apparatus to obtain the volatile concentrate. The solvent of the extraction was \( n \)-pentane (3 mL) for two hours. After extraction, an aliquot (1.0 \( \mu \) L) of the organic phase was injected in a gas chromatography coupled with mass spectrometry (GC-MS) apparatus. The analysis was performed in GC-MS equipment (Shimadzu QP2010 ultra, Shimadzu Corporation, Tokyo, Japan) according to the following settings: Rtx-5MS (30 m \( \times \) 0.25 mm \( \times \) 0.25 \( \mu \)m film thickness), silica capillary column (Restek Corporation, Bellefonte, PA, USA); programmed temperature, 60–240 °C (3 °C/min); injector temperature, 250 °C; carrier gas, helium with linear velocity of 32 cm/s (measured at 100 °C); injection type, split (1.0 \( \mu \)L); split flow was adjusted to yield a 20:1 ratio; septum sweep was a constant 10 mL/min; EIMS, electron energy, 70 eV; temperature of the ion source and connection parts, 200 °C. A homologous series of \( n \)-alkanes (C8–C32, Sigma-Aldrich, Milwaukee, WI, USA) was applied to calculate the retention index (RI) [104], which were used in conjunction with the mass spectra to identify the compounds found in the libraries of Adams and FFNSC2 [105,106]. The compound percentages are based on peak integrations without standardization.

3.2.2. Extracts Preparation

Samples (2.0 g) of leaves and fruits of \( P. \) nigrum were extracted by percolation (96 h) with 100 mL of methanol. Every 48 h, the samples were placed on ultrasound for 10 min. At the end of 96 h, the solvent was evaporated. The extracts were used for Folin–Ciocalteu total phenolic and total carotenoids determination [107–109].

3.2.3. Total Phenolics Determination

The extracts were solubilized in methanol (20 mg\( \cdot \)mL\(^{-1}\)), followed by dilution in distilled water. Then, an aliquot of 500 \( \mu \)L of extract was added to 250 \( \mu \)L of Folin–Ciocalteu reagent (1 N) and 1250 \( \mu \)L of sodium carbonate (75 g\( \cdot \)L\(^{-1} \)). The reaction was kept in the dark and after 30 min, the absorbance of the mixture was read at 760 nm using a UV-visible spectrophotometer (Amersham Biosciences, Little Chalfont, UK). The experimental calibration curve was prepared using gallic acid (Sigma Aldrich, St. Louis, MO, USA) at concentrations of 0.5 to 10.0 mg\( \cdot \)L\(^{-1} \), and the content of total phenolics was expressed as gallic acid equivalents (GAE) in milligrams per gram of extract (mg GAE\( \cdot \)g\(^{-1} \)).

3.2.4. Total Carotenoids

An aliquot of each extract was prepared in ethanol at the concentration of 1 mg\( \cdot \)mL\(^{-1} \). The absorbance (\( Abs \)) of the samples was read at 470, 648, and 664 nm using a UV-visible spectrophotometer (Amersham Biosciences, Little Chalfont, UK). Ethanol was used as a blank. The pigment contents (chlorophyll \( a \)—Chl\(_{A}\), chlorophyll \( b \)—Chl\(_{B}\), and total carotenoids—\( Car_{\text{total}} \)) were calculated using Equations (1)–(3). The carotenoid content was expressed in \( \mu \)g of extract per mL of ethanol (\( \mu \)g\( \cdot \)mL\(^{-1} \)).

\[
Chl_{A} = (13.36 \ast Abs_{664}) - (5.19 \ast Abs_{648})
\]

\[
Chl_{B} = (27.43 \ast Abs_{648}) - (8.12 \ast Abs_{664})
\]

\[
Car_{\text{total}} = \frac{((1000 \ast Abs_{470}) - (2.13 \ast Chl_{A}) - (97.64 \ast Chl_{B}))}{209}
\]
3.3. Determination of In Vitro Enzymatic Activity from Leaves of Black Pepper Cultivars
Phenylalanine Ammonia-Lyase (PAL) Activity

Frozen fresh leaves of each cultivar were pulverized in liquid nitrogen. Each sample (100 mg) was homogenized in sodium borate buffer (0.3 mM, pH 8.8), 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, St. Louis, MO, USA), 1 mM dithiothreitol (DTT) (Sigma Aldrich, St. Louis, MO, USA), and 5% polyvinylpolypyrrolidone (PVP) (Sigma Aldrich, St. Louis, MO, USA), totaling 1 mL of extraction buffer. The samples were centrifuged at 12,000 × g (15 min at 4 °C), and then an aliquot of supernatant (0.5 mL) was mixed with 1.0 mL of reaction buffer composed of sodium borate buffer (0.3 mM, pH 8.8), and 0.03 mM L-phenylalanine (Sigma Aldrich, St. Louis, MO, USA). After 15 min at room temperature, the absorbance was measured at 290 nm using a UV-visible spectrophotometer (Amersham Biosciences, Little Chalfont, UK). The specific molar extinction coefficient of (E)-cinnamic acid (9630 mol·L⁻¹·cm⁻¹) was used to determine the PAL activity based on its formation from the substrate, L-phenylalanine [110,111].

3.4. Computational Methods
3.4.1. Homology Modeling

Homology models for each of the *Fusarium* targets were generated using three-dimensional crystal structures from the Protein Data Bank (PDB, http://www.rcsb.org/) using FASTA sequences obtained from the National Center for Biotechnology Information (NCBI) protein database (https://www.ncbi.nlm.nih.gov/protein) (see Table 1). Protein sequences in the NCBI GenBank with high sequence similarity to structures in the PDB were identified using the PSI-BLAST utility with the BLOSUM80 scoring matrix. Structures with both high similarity to the selected sequence, in addition to a good overlap with the active sites for the protein, were selected for single-reference homology modeling. The protein sequence was then aligned to its respective structural template using the BLOSUM62 substitution matrix. As necessary, either the FASTA sequence or the reference sequence was extended with gaps. Protein structures were generated by the alignment of atomic coordinates of the peptide sequence with those of the template backbone. Side-chain orientations were carried out using the AMBER14:EHT force field with reaction field solvation [112–114]. The protein structure with the lowest deviation from the template backbone was selected and optimized using a constrained minimization. The sequence of each structure was aligned to its respective template and the protein backbone was created and superposed to the reference structures using the protein alignment tool in MOE 2019.01 (Chemical Computing Group, Montreal, QC, Canada).

### Table 1. Structure templates from PDB used for homology modeling for the *Fusarium* enzymes.

| Target                                      | GenBank Accession Number | PDB Entry | Sequence Identity | Expectation Value |
|----------------------------------------------|--------------------------|-----------|-------------------|-------------------|
| *F. solani* ornithine decarboxylase          | ABC47117.1               | 4ZGY      | 51.23%            | 4 × 10⁻¹⁰⁸        |
| *F. oxysporum* glucoseamine fructose-6-phosphate aminotransferase | EWY94476               | 6R4E      | 61.11%            | 0.0               |
| *F. odoratissimum* NRRL 54006 β-glucosidase  | XP_031065848             | 5NBS      | 65.47%            | 0.0               |
| *F. vanettenii* thiamine thiazole synthase   | C7Z8P6.1                 | 3JSK      | 77.4%             | 1 × 10⁻¹⁶⁷        |
| *F. oxysporum* guanine nucleotide-binding protein, beta subunit | RKL24731               | 6CMO      | 66.57%            | 2 × 10⁻¹⁶⁵        |

3.4.2. Molecular Docking

Molecular docking was carried out on 39 *P. nigrum* essential oil major components with 7 *Fusarium* protein targets from the Protein Data Bank (PDB, http://www.rcsb.org/): *F. oxysporum* cutinase (PDB 5AJH), *F. solani* cutinase (PDB 1AGY, 1XZL, and 1XZM), *F. oxysporum* endoglucanase (PDB 4OVW), *F. oxysporum* feruloyl esterase (PDB 6FAT), and *F. oxysporum* xylanase (PDB 5JRM). In addition, homology models of *F. solani* ornithine decarboxylase, *F. vanettenii* thiamine thiazole synthase, *F. oxysporum* glucoseamine-fructose-6-phosphate aminotransferase, *F. odoratissimum* β-glucosidase, and *F. oxysporum* guanine...
nucleotide-binding protein subunit β, were also screened. Structures of the ligands were generated using Spartan '18 for Windows version 1.4.4 (Wavefunction, Inc., Irvine, CA, USA). Conformational analyses and geometry optimizations were carried out using the MMFF force field [115]. The ligands were screened using Molegro Virtual Docker version 6.0.1 (Molegro ApS, Aarhus, Denmark) [116]. Solvent molecules and co-crystallized ligands were removed from the protein structure prior to docking. A binding site sphere large enough to accommodate the binding cavity was positioned on the binding site of each protein. Protonation states of the homology model residues at neutral pH were assigned. The protein structure was used as a rigid model (i.e., no relaxation of the protein structure was performed). The assignment of charges was based on standard defaults of the Molegro Virtual Docker program. The ligands were used as flexible models in both the docking and succeeding optimization schemes. Various orientations of the ligands with the protein were searched and ranked based on their re-rank docking scores. For each docking run the number of iterations for the docking procedure was set to a maximum of 1500, with a maximum population size of 50, and a total of 100 runs per ligand. A threshold of 1.00 Å RMSD was set for multiple poses. The poses generated for each ligand were then sorted by the calculated re-rank docking scores.

3.5. Statistical Analysis

The multivariate analysis was performed using the Minitab® software, version 18.1 (Minitab Inc, State College, PA, USA), using as variables the chemical components that are exhibited at least once in the composition of the cultivars and can reach above 1%. The data matrix was standardized by subtracting the mean from the individual value of each compound and then subtracting it by the standard deviation. The Euclidian distance, complete linkage, and absolute correlation coefficient distance were selected to obtain the similarity of the cultivars for the hierarchical cluster analysis (HCA). For total phenolics determination, total carotenoids, and PAL activity, in each sample, three independent tests were performed, and the results were expressed as mean ± standard deviation. The data were submitted to a normality test for validation (ANOVA), and subsequently, the Tukey’s test was applied with a 5% probability level (p < 0.05), using GraphPad Prism 6.0 software (GraphPad Software, Inc. San Diego, CA, USA).

4. Conclusions

The present study detailed the qualitative and quantitative composition of volatile concentrates from the leaves and fruits of black pepper cultivars, which revealed about 218 components (114 in the leaves and 104 in the fruits). HCA analysis allowed the chemical discrimination of the cultivars based on the biomarkers identified in the aroma of the leaves. Differences among the cultivars were also observed in the total phenolics content, PAL activity, and total carotenoids. Although molecular docking was used to evaluate the potential inhibition of Fusarium enzyme targets, and Fusarium endoglucanase was identified as a potential enzyme target, there are several other mechanisms of anti-Fusarium activity that may be important. Nevertheless, it was possible to correlate the metabolomic analysis with the susceptibility degree to yellow wilt of black pepper cultivars available in the State of Pará, Brazil, and the results highlight the importance of secondary metabolites in understanding the interaction of plants with different environments for the development of new biotechnologies.

Supplementary Materials: Supplementary materials can be found at https://www.mdpi.com/1422-0067/22/2/890/s1. Table S1. Volatile chemical composition of the leaves of black pepper cultivars (Mean ± standard deviation - %). Table S2. Identification of chemical composition in the fruits of black pepper cultivars (Mean ± standard deviation - %). Table S3. Docking energies (kJ/mol) for the essential oil ligands and their respective Fusarium target proteins.
Author Contributions: J.K.R.d.S. and A.R.R. participated in study design; L.M.B. conducted the experiments; O.F.d.L. and A.R.R. cultivated and harvested the plant material used in this work; E.H.A. and J.G.S.M. contributed to GC-MS analyses; L.M.B., W.N.S., K.G.B. and J.K.R.d.S. contributed to molecular docking and homology modeling analysis; L.M.B., W.N.S., J.G.S.M. and J.K.R.d.S. edited and approved the final version manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available within the article or supplementary material.

Acknowledgments: The authors are grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES—Coordination for the Improvement of Higher Education Personnel) for providing a scholarship to L.M.B., W.N.S. and J.K.R.S. participated in this work as part of the activities of the Aromatic Plant Research Center (APRC, https://aromaticplant.org/).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

Abs Absorbance
AMF Arbuscular mycorrhizal fungi
ANOVA Analysis of variance
Car total Total carotenoids
Chl A Chlorophyll A
Chl B Chlorophyll B
CLas Candidatus Liberibacter asiaticus
DTT Dithiothreitol
EDTA Ethylenediaminetetraacetic acid
EIMS Electron ionization mass spectrometry
EMBRAPA Brazilian Agricultural Research Corporation
EO Essential oil
GAE Gallic acid equivalents
GC-MS Gas chromatography–mass spectrometry
HCA Hierarchical cluster analysis
NCBI National Center for Biotechnology Information
PAL Phenylalanine ammonia-lyase
PCA Principal component analysis
PDB Protein Data Bank
PVP Polyvinylpolypyrrolidone
RI Retention indices
VOC Volatile organic compounds

References

1. Durant-Archibold, A.A.; Santana, A.I.; Gupta, M.P. Ethnomedical uses and pharmacological activities of most prevalent species of genus Piper in Panama: A review. J. Ethnopharmacol. 2018, 217, 63–82. [CrossRef] [PubMed]
2. Jardim Botânico do Rio de Janeiro Piperaceae in Flora do Brasil 2020: Em Construção. Available online: http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB12735 (accessed on 25 May 2020).
3. Sen, S.; Gode, A.; Ramanujam, S.; Ravikanth, G.; Aravind, N.A. Modeling the impact of climate change on wild Piper nigrum (Black Pepper) in Western Ghats, India using ecological niche models. J. Plant Res. 2016, 129, 1033–1040. [CrossRef] [PubMed]
4. Grinevicius, V.M.A.S.; Andrade, K.S.; Ourique, F.; Micke, G.A.; Ferreira, S.R.S.; Pedrosa, R.C. Antitumor activity of conventional and supercritical extracts from Piper nigrum L. cultivar Bragantina through cell cycle arrest and apoptosis induction. J. Supercrit. Fluids 2017, 128, 94–101. [CrossRef]
5. Bagheri, H.; Abdul Manap, M.Y.B.; Solati, Z. Antioxidant activity of Piper nigrum L. essential oil extracted by supercritical CO2 extraction and hydro-distillation. Talanta 2014, 121, 220–228. [CrossRef] [PubMed]
6. Swathy, J.S.; Mishra, P.; Thomas, J.; Mukherjee, A.; Chandrasekaran, N. Antimicrobial potency of high-energy emulsified black pepper oil nanoemulsion against aquaculture pathogen. *Aquaculture* 2018, 491, 210–220. [CrossRef]

7. Duangjai, A.; Ingkaninan, K.; Prapatbut, S.; Limpeanchob, N. Black pepper and piperine reduce cholesterol uptake and enhance translocation of cholesterol transporter proteins. *J. Nat. Med.* 2015, 67, 303–310. [CrossRef]

8. Aziz, D.M.; Hama, J.R.; Alam, S.M. Synthesising a novel derivatives of piperine from black pepper (*Piper nigrum* L.). *J. Food Meas. Charact.* 2015, 9, 324–331. [CrossRef]

9. Da Silva, J.K.; da Trindade, R.; Alves, N.S.; Figueiredo, P.L.; Maia, J.G.S.; Setzer, W.N. Essential Oils from Neotropical *Piper* Species and Their Biological Activities. *Int. J. Mol. Sci.* 2017, 18, 2571. [CrossRef]

10. Abdulazeez, M.A.; Sanj, I.; James, B.D.; Abdullahi, A.S. Black Pepper (*Piper nigrum* L.) Oils; Elsevier Inc.: Amsterdam, The Netherlands, 2016; ISBN 9780124166448.

11. Damanhourí, Z.A. A Review on Therapeutic Potential of *Piper nigrum* L. (Black Pepper): The King of Spices. *Med. Aromat. Plants* 2014, 3, 161. [CrossRef]

12. Abdallah, E.M.; Abdalla, W.E. Black pepper fruit (*Piper nigrum* L.) as antibacterial agent: A mini-review. *J. Bacteriol. Mycol. Open Access* 2018, 6, 141–145. [CrossRef]

13. De Lemos, O.F.; Poltronieri, M.C.; Rodrigues, S.d.M.; de Menezes, I.C.; Mondin, M. Conservação e Melhoramento Genético da pimenteira-do-reino. *Infoteca Cnptia Embrapa Br.* 2011, 1, 45.

14. Nile, S.H.; Nile, A.S.; Keum, Y.S.; Baskar, V.; Ramalingam, S. In vitro and *in planta* nematicidal activity of black pepper (*Piper nigrum* L.) leaf extracts. *Crop Prot.* 2017, 1, 1–7. [CrossRef]

15. Chen, Y.S.; Dayod, M.; Tawan, C.S. Phenetic Analysis of Cultivated Black Pepper (*Piper nigrum* L.) as antibacterial agent: A mini-review. *J. Bacteriol. Mycol. Open Access* 2018, 6, 141–145. [CrossRef]

16. Jaramillo, M.A.; Manos, P.S.; Zimmer, E.A. Phylogenetic relationships of the perianthless Piperales: Reconstructing the evolution of floral development. *Int. J. Plant Sci.* 2004, 165, 403–416. [CrossRef]

17. Carvalho Filgueiras, G.; Kingo, A.; Homma, O.; Souza, M.A.; Santos, D. *Conjuntura do Mercado da Pimenta-do-Reino No Brasil e No Mundo*; Embrapa Amazônia Oriental: Belém, Brazil, 2009.

18. Niles, S.H.; Nile, A.S.; Keum, Y.S.; Baskar, V.; Ramalingam, S. In vitro and *in planta* nematicidal activity of black pepper (*Piper nigrum* L.) leaf extracts. *Crop Prot.* 2017, 1, 1–7. [CrossRef]

19. Chen, Y.S.; Dayod, M.; Tawan, C.S. Phenetic Analysis of Cultivated Black Pepper (*Piper nigrum* L.) as antibacterial agent: A mini-review. *J. Bacteriol. Mycol. Open Access* 2018, 6, 141–145. [CrossRef]

20. Khan, M.; Hanif, M.A.; Rehman, R.; Bhatti, I.A. Black Pepper. *Med. Plants South Asia* 2020, 75–86. [CrossRef]

21. Costa, M.R.T.d.R.; Homma, A.K.O.; Rebello, F.K.; Souza Filho, A.P.d.S.; Fernandes, G.L.d.C.; Baleixe, W. Atividade Agropecuária no Estado do Pará; Embrapa Amazônia Oriental: Belém, Brazil, 2017.

22. Instituto Brasileiro de Geografia e Estatística—IBGE Produção Agrícola Municipal. Available online: https://sidra.ibge.gov.br/tabela/1613 (accessed on 25 May 2020).

23. Duarte, M.d.L.R.; Poltronieri, M.C.; Chu, E.Y.; Oliveira, R.F.d.; Lemos, O.F.; Benchimol, R.L.; da Conceição, H.E.O.; de Souza, G.F. *Colecção Plantar: A Cultura da Pimenta-do-Reino No Brasil e No Mundo*; Embrapa Amazônia Oriental: Belém, Brazil, 2006; ISBN 85-7383-380-7.

24. De Castro, G.L.S.; De Lemos, O.F.; Tremacoldi, C.R.; Moraes, F.K.C.; Dos Santos, L.R.R.; Pinheiro, H.A. Susceptibility of *in vitro* black pepper plant to the filtrate from a *Fusarium solani* f. sp. *piperis* culture. *Plant Cell. Tissue Organ Cult.* 2016, 127, 263–268. [CrossRef]

25. Verpoorte, R.; Allermann, A.W. *Metabolic Engineering of Plant Secondary Metabolism*; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; ISBN 0792363604.

26. Yang, L.; Wen, K.S.; Ruan, X.; Zhao, Y.X.; Wei, F.; Wang, Q. Response of plant secondary metabolites to environmental factors. *Molecules* 2018, 23, 762. [CrossRef]

27. Raj Joshi, D.; Adhikari, N.; Raj Joshi, D.; Chandra Shrestha, A. A review on diversified use of the king of spices: *Piper nigrum* (black pepper). *Artic. Int. J. Pharm. Sci. Res.* 2018, 9, 4089–4101. [CrossRef]

28. Takoree, H.; Aumeeruddy, M.Z.; Rengasamy, K.R.R.; Venugopala, K.N.; Jeewon, R.; Zengin, G.; Mahomoodally, M.F. A systematic review on black pepper (*Piper nigrum* L.): From folk uses to pharmacological applications. *Crit. Rev. Food Sci. Nutr.* 2019, 59, S210–S243. [CrossRef]

29. Arslan, U.; Ilhan, K.; Karabulut, O.A. Antifungal activity of aqueous extracts of spices against bean rust (*Uromyces appendiculatus*). *Allelopath. J.* 2009, 24, 207–213.

30. Ahmad, A.; Husain, A.; Mugeeb, M.; Khan, S.A.; Alhadrami, H.A.A.; Bhandari, A. Quantification of total phenol, flavonoid content and pharmacognostical evaluation including HPTLC fingerprinting for the standardization of *Piper nigrum* Linn fruits. *Asian Pac. J. Trop. Biomed.* 2015, 5, 101–107. [CrossRef]

31. Gupta, N.; Parashar, P.; Mittal, M.; Mehra, V.; Khatri, M. Antibacterial potential of *Elletaria cardamomum*, *Syzygium aromaticum* and *Piper nigrum*, their synergistic effects and phytochemical determination. *J. Pharm. Res.* 2014, 8, 1091–1097. [CrossRef]

32. Li, Y.X.; Zhang, C.; Pan, S.; Chen, L.; Liu, M.; Yang, K.; Zeng, X.; Tian, J. Analysis of chemical components and biological activities of essential oils from black and white pepper (*Piper nigrum* L.) in five provinces of southern China. *LWT—Food Sci. Technol.* 2020, 117, 108644. [CrossRef]
57. De Abreu, A.M.; Brighente, I.M.C.; Deaguiar, E.M.; Rebelo, R.A. Volatile constituents of Piperaceae from Santa Catarina, Brazil—Essential oil composition of Piper cernuum Vell. and Peperomia emarginella (Sw.) C. DC. J. Essent. Oil Res. 2005, 17, 286–288. [CrossRef]

58. Gasparetto, A.; Bella Cruz, A.; Wagner, T.M.; Bonomini, T.J.; Correa, R.; Malheiro, A. Seasonal variation in the chemical composition, antimicrobial and mutagenic potential of essential oils from Piper cernuum. Ind. Crops Prod. 2017, 95, 256–263. [CrossRef]

59. Pino, J.A.; Marbot, R.; Bello, A.; Urquiola, A. Composition of the essential oil of Piper hispidum sw. from Cuba. J. Essent. Oil Res. 2004, 16, 459–460. [CrossRef]

60. Autran, E.S.; Neves, I.A.; da Silva, C.S.B.; Santos, J.; Lima, R.H.V.; da Silva, J.K.R.; Maia, J.G.S.; Figueiredo, P.L.B. Seasonal and antioxidant evaluation of the essential oil of Piper umbellatum and Piper rivinoides. J. Essent. Oil Res. 2016, 30, 345–349. [CrossRef] [PubMed]

61. Kaldre, D.; Gleason, J.L. An organocatalytic Cope rearrangement. Angew. Chem.-Int. Ed. 2005, 44, 712–713. [CrossRef]

62. Hamas, A.; Brito, V.; Bittencourt, M.; Silva, L.; Oliveira, F.; Oliveira, R. Essential oils composition of four Piper species from Brazil. J. Essent. Oil Res. 2013, 25, 203–209. [CrossRef]

63. De Souza, S.P.; Valverde, S.S.; Costa, N.F.; Calheiros, A.S.; Lima, K.S.C.; Frutuoso, V.S.; Lima, A.L.S. Chemical composition and antiocceptive activity of the essential oil of Piper mellelomum and Piper rivinoides. J. Med. Plants Res. 2014, 8, 788–793. [CrossRef]

64. Assis, A.; Brito, V.; Bittencourt, M.; Silva, L.; Oliveira, F.; Oliveira, R. Essential oils composition of four Piper species from Brazil. J. Essent. Oil Res. 2013, 25, 203–209. [CrossRef]

65. Hoff Brait, D.R.; Mattos Vaz, M.S.; da Silva Arrigo, J.; Borges de Carvalho, L.N.; Souza de Araújo, F.H.; Vani, J.M.; da Silva Mota, J.; Cardoso, C.A.L.; Oliveira, R.J.; Negrão, F.; et al. Toxicological analysis and anti-inflammatory effects of essential oil from Piper vicosum leaves. Regul. Toxicol. Pharmacol. 2015, 73, 699–705. [CrossRef] [PubMed]

66. Papa, F.; Ricciutelli, M.; Cianfaglione, K.; Maggi, F. Isofurano dione is the main volatile constituent of Smyrnium perfoliatum L. subsp. perfoliatum growing in central Italy. Nat. Prod. Res. 2016, 30, 345–349. [CrossRef] [PubMed]

67. Edelmann, A.; Diewok, J.; Schuster, K.C.; Lendl, B. Rapid method for the discrimination of red wine cultivars based on mid-infrared spectroscopy of phenolic wine extracts. J. Agric. Food Chem. 2001, 49, 1139–1145. [CrossRef]

68. Cano, A.; Medina, A.; Bermejo, A. Bioactive compounds in different citrus varieties. Discrimination among cultivars. J. Food Compos. Anal. 2008, 21, 377–381. [CrossRef]

69. Islam, M.A.; Ryu, K.Y.; Khan, N.; Song, O.Y.; Jeong, J.Y.; Son, J.H.; Jamila, N.; Kim, K.S. Determination of the Volatile Compounds in Five Varieties of Piper betle L. from Bangladesh Using Simultaneous Distillation Extraction and Gas Chromatography/Mass Spectrometry (SDE-GC/MS). Anal. Lett. 2020, 53, 2413–2430. [CrossRef]
81. Hijaz, F.; Nehela, Y.; Killnny, N. Possible role of plant volatiles in tolerance against huanglongbing in citrus. Plant Signal. Behav. 2016, 11, e138193. [CrossRef] [PubMed]

82. Duarte, M.D.L.R.; De Albuquerque, F.C.; Poltronieri, M.C. Phytosanitary conditions of black pepper cultivars crop in Brazil. Int. Pepper News Bull. 2002, 1, 16–22.

83. Poltronieri, M.C.; de Lemos, O.F. Pimenta-do-Reino: Cultivares; Embrapa Amazônia Oriental: Belém, Brazil, 2014.

84. Cao, Y.; Fang, S.; Fu, X.; Shang, X.; Yang, W. Seasonal Variation in Phenolic Compounds and Antioxidant Activity in Leaves of Cyclocarya paliurus (Batal.) Iljinskaja. Forests 2019, 10, 624. [CrossRef]

85. Gu, F.; Huang, F.; Wu, G.; Zhu, H. Contribution of polyphenol oxidation, chlorophyll and vitamin C degradation to the blackening of Piper nigrum L. Molecules 2018, 23, 370. [CrossRef] [PubMed]

86. Ramilla, L.G.; Kwon, Y.I.; Apostolidis, E.; Shetty, K. Phenolic compounds, antioxidant activity and in vitro inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. Bioreasour. Technol. 2010, 101, 4676–4689. [CrossRef] [PubMed]

87. Sruthi, D.; John Zachariah, T. In vitro antioxidant activity and cytotoxicity of sequential extracts from selected black pepper (Piper nigrum L.) varieties and Piper species. Int. Food Res. J. 2017, 24, 75–85.

88. Tang, C.-S.; Cai, W.-F.; Kohl, K.; Nishimoto, R.K. Plant Stress and Allelopathy. In Allelopathy; Inderjit, Dakshini, K.M.M., Einhellig, F.A., Eds.; American Chemical Society: Washington, DC, USA, 1994; pp. 142–157, ISBN 9780841230613.

89. Misharina, T.A. Antiradical properties of essential oils and extracts from coriander, cardamom, white, red, and black peppers. Appl. Biochem. microbiol. 2016, 52, 79–86. [CrossRef]

90. Havaux, M. Carotenoid oxidation products as stress signals in plants. Plant J. 2013, 79, 597–606. [CrossRef]

91. Vijayakumari, K.; Puthur, J.T. Drought stress responses in tolerant and sensitive varieties of black pepper (Piper nigrum L.). J. Plant. Crop. 2014, 42, 78–85.

92. Muthalagu, A.; Ankegowda, S.J.; Peeran, M.F.; Jagannath, H.; Akshitha, G.; Rajkumar, B.; Chaudhary, N. Effect of Natural Growth Enhancer on Growth, Physiological and Biochemical Attributes in Black Pepper (Piper nigrum L.). Int. J. Curr. Microbiol. Appl. Sci. 2018, Special Issue 6, 2857–2866.

93. Sharma, A.; Shahzad, B.; Rehman, A.; Bhardwaj, R.; Landi, M.; Zheng, B. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. Molecules 2019, 24, 2452. [CrossRef]

94. Mgbarehuruike, E.E.; Yrjönen, T.; Vuorela, H.; Holm, Y. Bioactive compounds from medicinal plants: Focus on Piper species. S. Afr. J. Bot. 2017, 112, 54–69. [CrossRef]

95. Niu, M.; Xu, X.; Shen, Y.; Yao, Y.; Qiao, J.; Zhu, F.; Zeng, L.; Liu, X.; Xu, K. Piperlongumine is a novel nuclear export inhibitor with potent anticancer activity. Chem. Biol. Interact. 2015, 237, 66–72. [CrossRef] [PubMed]

96. Iqbal, G.; Iqbal, A.; Mahboob, A.; M. Farhat, D.; Ahmed, T. Memory Enhancing Effect of Black Pepper in the AlCl3 Induced Neurotoxicity Mouse Model is Mediated through Its Active Component Chavicine. Curr. Pharm. Biotechnol. 2016, 17, 962–973. [CrossRef] [PubMed]

97. De La Rosa, L.A.; Moreno-Escamilla, J.O.; Rodrigo-Garcia, J.; Alvarez-Parrilla, E. Phenolic Compounds; Elsevier Inc.: Amsterdam, The Netherlands, 2019; ISBN 9780128132784.

98. Nabavi, S.M.; Šamec, D.; Tomczyk, M.; Milella, L.; Russo, D.; Habtemariam, S.; Suntar, I.; Rastrelli, L.; Daglia, M.; Xiao, J.; et al. Flavonoid biosynthetic pathways in plants: Versatile targets for metabolic engineering. Biotechnol. Adv. 2020, 38, 107316. [CrossRef] [PubMed]

99. Chen, Z.; Zheng, Z.; Huang, J.; Lai, Z.; Fan, B. Biosynthesis of salicylic acid in plants. Plant Signal. Behav. 2009, 4, 493–496. [CrossRef]

100. Yang, L.; Han, Y.; Li, P.; Li, F.; Ali, S.; Hou, M. Silicon amendment is involved in the induction of plant defense responses to a phloem feeder. Sci. Rep. 2017, 7, 4232. [CrossRef]

101. Anh, T.T.; Linh, T.B.; Phong, N.V.; Bien, T.T.; Tram, T.H.N.; Don, L.D. Expression of Proteins Related to Phytophthora capsici Tolerance in Black Pepper (Piper nigrum L.). Int. J. Agric. Innov. Res. 2018, 6, 75–79.

102. Shobha, M.S.; Lakshmi Devi, N.; Mahadeva Devi, S. Induction of Systemic Resistance by Rhizobacterial and Endophytic Fungi against Foot Rot Disease of Tobacco. Int. J. Agric. Innov. Res. 2019, 78–85.

103. Sulzenbacher, G.; Schülein, M.; Davies, G.J. Structure of the endoglucanase I from Fusarium oxysporum: Native, cellobiose, and 3,4-epoxybutyl β-D-cellobiose-inhibited forms, at 2.3 Å resolution. Biochemistry 1997, 36, 5902–5911. [CrossRef] [PubMed]

104. Van Den Dool, H.; Dec. Kratz, P. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J. Chromatogr. A 1963, 11, 463–471. [CrossRef]

105. Adams, R.P. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed.; Allured Publishing Corporation: Carol Stream, IL, USA, 2007.

106. Mondello, L. Mass Spectra of Flavors and Fragrances of Natural and Synthetic Compounds, 3rd ed.; Wiley: Hoboken, NJ, USA, 2011; ISBN 978-1-119-06984-3.

107. Sousa, C.M.D.M.; Silva, H.R.E.; Vieira, G.M.; Ayres, M.C.C.; Da Costa, C.L.S.; Araújo, D.S.; Cavalcante, L.C.D.; Barros, E.D.S.; Araújo, P.B.D.M.; Brandão, M.S.; et al. Fenóis totais e atividade antioxidante de cinco plantas medicinais. Quim. Nova 2007, 30, 351–355. [CrossRef]
108. Lichtenthaler, H.K.; Buschmann, C. Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. In Handbook of Food Analytical Chemistry; Wrolstad, R.E., Acree, T.E., Decker, E.A., Penner, M.H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Smith, D., Sporns, P., Eds.; John Wiley and Sons, Inc.: New York, NY, USA, 2005; pp. F4.3.1–F4.3.8. [CrossRef]

109. Miazek, K.; Ledakowicz, S. Chlorophyll extraction from leaves, needles and microalgae: A kinetic approach. Int. J. Agric. Biol. Eng. 2013, 6, 107–115. [CrossRef]

110. Dickerson, D.P.; Pascholati, S.F.; Hagerman, A.E.; Butler, L.G.; Nicholson, R.L. Phenylalanine ammonia-lyase and hydroxycinnamate: CoA ligase in maize mesocotyls inoculated with Helminthosporium maydis or Helminthosporium carbonum. Physiol. Plant Pathol. 1984, 25, 111–123. [CrossRef]

111. Vaganan, M.M.; Ravi, I.; Nandakumar, A.; Sarumathi, S.; Sundararaju, P.; Mustaffa, M.M. Phenylpropanoid enzymes, phenolic polymers and metabolites as chemical defenses to infection of Pratylenchus coffeae in roots of resistant and susceptible bananas (Musa spp.). Indian J. Exp. Biol. 2014, 52, 252–260. [PubMed]

112. Gerber, P.R.; Müller, K. MAB, a generally applicable molecular force field for structure modelling in medicinal chemistry. J. Comput. Aided. Mol. Des. 1995, 9, 251–268. [CrossRef] [PubMed]

113. Wang, J.; Cieplak, P.; Kollman, P.A. How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? J. Comput. Chem. 2000, 21, 1049–1074. [CrossRef]

114. Case, D.A.; Darden, T.A.; Cheatham, T.E.; Simmerling, C.L.; Wang, J.; Duke, R.E.; Luo, R.; Crowley, M.; Walker, R.C.; Zhang, W.; et al. Amber 10; University of California: San Francisco, CA, USA, 2008.

115. Halgren, T.A. Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94. J. Comput. Chem. 1996, 17, 490–519. [CrossRef]

116. Thomsen, R.; Christensen, M.H. MolDock: A new technique for high-accuracy molecular docking. J. Med. Chem. 2006, 49, 3315–3321. [CrossRef] [PubMed]