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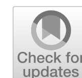

Piper anisum as a promising new source of bioactive metabolites

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Abstract

Piper species are commonly used by indigenous communities to treat several gastrointestinal diseases. In China, they are also used as an active ingredient in formulae to treat cancer. The objective of the study was to perform a large-scale metabolite profiling analysis to identify bioactive compounds in *Piper anisum*. Antioxidant capacity was assessed by the DPPH assay and total phenolics were assessed by Folin–Ciocalteu's method. Antimicrobial activity was assessed against several Gram-positive and Gram-negative bacteria, whereas cytotoxicity was assessed against tumor cell lines MCF-7, HCT116, HepG2 and HL-60, and non-tumor cell line MRC-5. The multiplatform metabolite profiling approach encompassed NMR, GC–MS and LC–MS analyses. *P. anisum* root extract showed the greatest antioxidant capacity and total phenolic content, followed by the stem and leaf extracts. *P. anisum* extracts showed a highly selective antimicrobial profile, being specifically active against *C. albicans* (MIC of 500 μg mL^{-1}). Additionally, the root extract (50 μg mL^{-1}) showed the highest cell inhibition percentages against tumor cell lines MCF-7 (59.5%), HCT116 (49.2%), and HepG2 (61.0%). Forty-eight metabolites were annotated by GC–MS and 27 by LC–MS. These included alkaloids, carbohydrates, fatty acids, hydrocarbons, organic acids, phenolic compounds, and terpenes. Taken together, these results showed that *P. anisum* root extract is a promising source of bioactive compounds.

Keywords Antioxidant capacity · Antimicrobial compounds · Antitumor activity · Bioactive metabolites · Ethnopharmacology · Metabolomics

Introduction

Medicinal plants have been used since ancient times by traditional communities for the treatment of various diseases. During the nineteenth century, we experienced a boost of studies investigating the chemical composition of bioactive

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constituents from plants, which contributed to the development of the first drugs as we know nowadays (Gertsch 2011). Therefore, natural products obtained from plants used by traditional communities play a key role in the discovery of novel bioactive metabolites as raw material for the development of more specific and efficient drug synthesis (Lang et al. 2008).

Piper anisum (Spreng.) Angely, also known as jaborandi, belongs to the Piperaceae family. It is a 2 m tall shrub with pubescent branches and internodes ranging 2.8–6 cm, striate petiole, short sheath and symmetric leaf blade (Christ et al. 2016). *P. anisum* is endemic to the Northeast and Southeast regions of Brazil, where it is used by traditional "quilombola" communities in religious rituals and also as anesthetic and diuretic (Monteiro 2013). Piper species are used by indigenous groups in Central and South America as decoction and infusion to treat stomach ache, rheumatoid arthritis, ulcers, diarrhea, infections, and a wide array of gastrointestinal diseases. Additionally, they are also used for their stomachic, sudorific, tonic, diuretic, and carminative effects (Brú and Guzman 2016; Mgbeahuruike et al. 2017; Ricardo et al. 2017). *P. anisum* is used by Pataxó Indians in Bahia state (Brazil) as a tooth anesthetic (Cunha Lima et al. 2012). *P. amalago* is used by indigenous communities in the Xingu Indigenous Park in Mato Grosso state (Brazil) to treat toothache and inflammations, whereas *P. daguanum* is used by the Ticuna Indians in Amazon forest in Brazil, Colombia, and Peru for the same diseases (Milliken and Albert 1997; Rodrigues et al. 2006). *P. cuyabanum* is used to treat malaria and liver diseases (Ribeiro et al. 2017), whereas *P. arborea* is used by the Yanomami Indians in Amazon state (Brazil) to treat fever (Milliken and Albert 1997; Rodrigues et al. 2006). *P. cavalcanti* is used by indigenous communities in Amazon region of the Rondônia state (Brazil) for gastrointestinal diseases and convulsion, whereas *P. peltatum* is used to treat inflammation, anemia, and microbial infection (Santos et al. 2014). *P. tuberculatum* is used by the Krahô Indians in Tocantins state (Brazil) as hypotensive due to its action on the central nervous system (Rodrigues and Carlini 2005). In China, Piper species are used as active ingredient in formulae to treat cancers (Wang et al. 2014). Additionally, essential oils from Piper species are used as antimicrobial and antiprotozoal agents, as acetylcholinesterase inhibitor, and for their antinociceptive, anti-inflammatory, and cytotoxic activities (da Silva et al. 2017). Terpenoids, polyphenols, and alkaloids are major constituents of Piper species extracts and most likely responsible for their pharmacological properties (Parmar et al. 1998; Perigo et al. 2016; Santana et al. 2016; Setzer et al. 2008; Xiang et al. 2017). Metabolite profiling techniques are important allies for the discovery of novel bioactive metabolites in complex plant extracts. LC–MS/MS-based metabolomics of *P. nigrum* has allowed the identification of several phenolic and polyhydroxy compounds (Gu et al. 2018). *P. cubeba* metabolite profile was assessed by nuclear magnetic resonance (NMR) and showed the presence of alkaloids, amides, terpenes, iridoid glycosides, saponins, phenylpropanoids, flavonoids and phytosterols (Raja Mazlan et al. 2018), whereas GC-MS and HPTLC analysis of *P. betle* extracts revealed the presence of several phenolic compounds, organic and fatty acids, amino acids, and carbohydrates (Karak et al. 2019). This is the first study that reports the chemical composition of *Piper anisum* extracts. Thus, we used large-scale metabolomics to identify antioxidant, antimicrobial, and cytotoxic metabolites present in *P. anisum* extracts.

Materials and methods

Sample preparation and extraction

Stems, leaves, roots of the species *Piper anisum* were collected in the Sapiranga Reserve located in the Praia do Forte, BA region, at the coordinates of 12° 34′40″ South and 38°1′51″ West. A voucher specimen (ALCB-13535) was deposited at Alexandre Leal Costa herbarium of the Biology Institute, UFBA. Two hundred grams of dried leaves, stems, and roots were extracted by maceration with ethanol for 3 days at room temperature.

DPPH radical scavenging assay

The antioxidant capacity of the extracts was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as described by Santos et al. (2018). Extract concentrations ranged from 10–1000 μg mL^{-1}. Ethanol was used as a blank. Results were expressed as EC_{50}.

Total phenolic content assay

Total phenolic quantification was performed by the Folin–Ciocalteu's method as described in Pereira et al. (2014). The absorbance was read at 725 nm (VersaMax™ Microplate Reader, USA) and the quantification was based on a gallic acid standard curve. Results expressed as mg GAE/g of dry extract.

Antimicrobial activity

The antifungal and antibacterial potential of the extracts were assessed as minimum inhibitory concentration (MIC) as described by D'Sousa Costa et al. (2015). The following microorganisms were used: *Bacillus subtilis*, *B. cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Candida albicans*, and *C. glabrata*. Chloramphenicol (0.19–25 μg mL^{-1}) was

used as positive control for Gram-positive bacteria, gentamicin (0.039–5.0 μg mL^{-1}) for Gram-negative bacteria and ciclopirox olamine (0.39–50.0 μg mL^{-1}) for fungi.

Cytotoxicity assay

Cytotoxicity assay was performed as described in De Lima et al. (2018). We used four tumor cell lines MCF-7 (human breast carcinoma), HCT116 (human colon carcinoma), HepG2 (human hepatocellular carcinoma) and HL-60 (human promyelocytic leukemia), and one non-tumor cell line MRC-5 (human lung fibroblast). The alamarBlue assay was used to assess cell viability (Ansar Ahmed et al. 1994). Extracts were tested at a final concentration of 50 μg mL^{-1} and doxorubicin was used as the positive control.

Metabolite profiling analyses

Nuclear magnetic resonance (NMR) analysis

Extracts were prepared in DMSO-d6 (14.28 mg μL^{-1}) and analyzed using a Varian 500 spectrometer (500 MHz for ^{1}H, Agilent Technologies, Ltd., Santa Clara, CA). 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TMSP) was used as internal standard.

Gas chromatography–mass spectrometry (GC–MS) analysis

GC–MS analyses were performed in the exact conditions as described by Santos et al. (2018). For these analyses, we used the Agilent gas chromatography system (model 7890 A) coupled to a single quadrupole mass spectrometer (model 5975 C inert XL, Agilent Technologies). An aliquot of 150 μL of the stock solution of the extracts (1.0 mg mL^{-1} in acetonitrile) was dried, derivatized (TMS), then diluted in 100 μL heptane, and then analyzed by GC–MS. Methyl tridecanoate was used as internal standard.

Liquid chromatography–mass spectrometry (LC–MS) analysis

LC–MS analyses were performed in the exact conditions as described by Santos et al. (2018). For these analyses, we used the Prominence (Shimadzu Co., Japan) liquid chromatography system coupled to a quadrupole time-of-flight mass spectrometer (microTOF II, Bruker Daltonics, Germany). An aliquot of 500 μL of the stock solution of the extracts (0.5 mg mL^{-1} in acetonitrile) was filtered and directly injected into the equipment.

Data processing and metabolite identification

^{1}H-NMR data were processed using NMRprocflow online software (Deborde et al. 2019). Buckets of 0.005-ppm-width were used to construct the dataset matrix. Data were normalized against the TMSP signal and uploaded to MetaboAnalyst 2.0 to perform ANOVA and multivariate analyses (Xia and Wishart 2011). We used row-wise normalization, log transformation and auto-scaling to allow proper comparison of the features (Ribeiro et al. 2015a, b, c). GC–MS and LC–MS data were processed in XCMS package as described by Santos et al. (2018). For GC–MS, the identification of the metabolites was performed by comparison with the Fiehn RT and NIST libraries, whereas for LC–MS identification we used the Metlin database (http://metlin.scripps.edu). The following adducts were used: $[M+H]^+$, $[M+2H]^{2+}$ and $[M+Na]^+$.

Statistical analysis

Experiments were performed in triplicate. Statistical analysis was performed using IBM® statistics package as described by Santos et al. (2018).

Results and discussion

Ethanolic extract from the roots has the greatest antioxidant capacity and total phenolics content

The damage caused by oxidative stress is known to increase the risk of diseases incidence in humans. The antioxidant defense system usually prevents those damages by inactivating the deleterious reactive oxygen species (ROS). Oxidative damage caused by ROS is closely related to the development of cancer, and contributes to a diverse set of cardiovascular and neurodegenerative diseases (Mandel and Youdim 2004). Thus, it is important to find new antioxidant-like metabolites, especially from natural sources. The EC_{50} values of the extracts ranged from 35.54 to 271.18 μg mL^{-1}, and it showed close dependency with the type of tissue (Table 1).

P. anisum extract from the root had the greatest antioxidant capacity with an EC_{50} of 35.54 μg mL^{-1}, followed by the extract from the stem with an EC_{50} of 180.38 μg mL^{-1}, and the extract from the leaf with an EC_{50} of 271.18 μg mL^{-1}.

Antioxidants are substances that, at low concentrations, counteract free radicals. Therefore, antioxidant-like molecules prevent the degradation of other compounds and cell structures, thus shielding them against the deleterious effects of ROS (Ndhlala et al. 2010). This protection depends on structural and electronic factors of the antioxidant-like molecules as well as their concentration (Pereira et al. 2014;

Table 1 Antioxidant capacity and total phenolics content of *P. anisum* extracts

Extraction solvent	Part of the plant	Antioxidant capacity (EC_{50}, μg mL^{-1})	Total phenolics (mg GAE g^{-1} of dry weight)
Ethanol	Leaf	271.18 ± 6.37^{c}	27.53 ± 1.29^{a}
	Stem	180.38 ± 6.85^{b}	31.48 ± 1.51^{a}
	Root	35.54 ± 0.70^{a}	49.86 ± 2.33^{b}
Gallic acid		1.26 ± 0.02	–

Different letters show statistical differences among samples by Tukey's HSD ($p < 0.05$)

Santos et al. 2018). Phenolic compounds present an extensive conjugated π-electron system that gives them great antioxidant properties (Pereira et al. 2014; Santos et al. 2018). These compounds play a crucial role in the prevention of cardiovascular diseases, cancer and inflammatory processes (Afolabi et al. 2018; Lim and Loh 2016; Limmongkon et al. 2018; Saleem et al. 2018). For these reasons, we quantified total phenolics in the *P. anisum* extracts. Total phenolics of the extracts ranged from 27.53 to 49.86 mg GAE/g of dry weight, and it showed close dependency with the antioxidant capacity (IC_{50}) (Table 1). *P. anisum* extract from the root had the highest total phenolics content (49.86 GAE/g of dry weight), which justifies its greatest antioxidant capacity. No statistical difference was observed between the phenolics content of extracts obtained from *P. anisum* stem and leaf.

Phenolic compounds encompass simple phenolic acids, such as benzoic acid, 3,4-dihydroxybenzoic acid, gallic acid, and 4-hydroxy-3-methoxybenzoic acid, as well as polyphenols such as coumarins, tannins, lignins, lignans and flavonoids (Dai and Mumper 2010; Goleniowski et al. 2013). Based on the antioxidant capacity and total phenolics results, it could be inferred that the metabolome of the extracts presents a chemical diversity that might reflect on their pharmacological properties.

Antimicrobial activity and cytotoxicity

Plant extracts can provide a large variety of complex and structurally diverse chemical compositions with valuable pharmacological properties such as antimicrobial and cytotoxic activities. The treatment of multiresistant microorganisms has become a global public health concern and plants are important allies in the discovery of new drugs. The tested microorganisms have been selected due to the traditional use of *P. anisum* leaves to treat urinary, gastrointestinal, and pulmonary disorders by indigenous communities in Brazil. *P. anisum* extracts showed a highly selective antimicrobial profile, being specifically active against *C. albicans* (MIC of 500 μg mL^{-1}). Candida infection is the most common hospital-acquired bloodstream infection and usually leads to further complication of the patient status (Diekema et al. 2002; Edmond et al. 1999). Candida species are one of the main agents of opportunistic infections in HIV-infected individuals, due to the lesions in oral mucosa (Vazquez 1999). The diagnosis of such infections is challenged by the diverse array of fungal pathogens, which may act opportunistically (Pfaller and Diekema 2004). Therefore, *P. anisum* might be an important source of bioactive compounds for treatment of Candida infections.

Cancer is one of the leading causes of death worldwide, with global cancer incidence increasing exponentially. 17–20% of the world population is expected to develop cancer during their life time, whereas 9–12% will die from the disease. Lung and breast cancer are the deadliest types, followed by liver, stomach, and cervix cancer (Bray et al. 2018; Milroy 2018; Torre et al. 2015b). Usually, the development of a tumor is caused by uncontrolled cellular growth during abnormal cell division and its prevention and treatment has been a worldwide concern (Torre et al. 2015a). Cancer treatment usually consists of a combination of procedures, such as chemotherapy and/or radiation therapy or even surgery. In the recent years, an increased resistance of cancer cells to chemotherapy has been observed, posing a major clinical obstacle to the success of cancer treatments (Cox et al. 2006; Rivera and Gomez 2010; Szakacs et al. 2006). The cytotoxic activity of *P. anisum* extracts against human breast carcinoma (MCF-7), human colon carcinoma (HCT116), human hepatocellular carcinoma (HepG2), human leukemia (HL-60), and human lung fibroblast (MRC-5) tumor cell lines was evaluated (Table 2).

Cytotoxicity of *P. anisum* leaf, stem and root extracts was expressed as cell inhibition percentages. The root extract was by far the most active extract with the highest cell inhibition percentages against cell lines MCF-7 (59.5%), HCT116 (49.2%), HepG2 (61.0%), and MRC-5 (38.6%). The leaf extract was the least active one against tumor cell lines MCF-7 (31.9%) and HepG2 (5.2%), whereas no differences in the cell inhibition percentages between leaf and stem extracts were observed for tumor cell lines HCT116 and MRC-5. Additionally, no differences in the cell inhibition percentages for tumor cell line HL-60 were observed amongst the extracts. Altogether, these results showed that *P. anisum* root extract is a possible source of bioactive metabolites.

Table 2 Cytotoxicity of *P. anisum* leaf, stem and root extracts

Part of the plant	Cell growth inhibition percentage (GI %)				
	MCF-7	HCT116	HepG2	HL-60	MRC-5
Leaf	32.9 ± 2.3	23.9 ± 6.9	5.2 ± 2.9	33.5 ± 2.7	7.9 ± 4.5
Stem	40.0 ± 4.1	23.6 ± 8.4	27.2 ± 7.6	35.9 ± 3.0	4.0 ± 3.9
Root	59.5 ± 4.6	49.2 ± 17.3	61.0 ± 14.6	35.6 ± 2.5	38.6 ± 0.3
DOX	102.0 ± 0.2	102.6 ± 0.6	101.9 ± 0.1	90.2 ± 0.4	105.8 ± 1.1

Metabolite profiling

We used a large-scale metabolomics approach to assess the chemical composition of *P. anisum* extracts, which included nuclear magnetic resonance (NMR), gas and liquid chromatography coupled with mass spectrometry. Together, these analyses permitted us to build a comprehensive depiction of the extract's chemical composition.

Partial least square discriminant analysis (PLS-DA) was applied to the whole NMR data to pinpoint the directions that better explain the variance in the metabolite composition (Fig. 1a). Principal component 1 (PC1) accounted for 53.1% of the total variance, whereas principal component 2 (PC2) accounted for 44.6% of the total variance. The dendrogram (Fig. 1b) is a visual representation of the correlation based on hierarchical clustering of the metabolome composition of each extract within a dataset. It is clear from these analyses that leaf and stem extracts show closer metabolome profile among themselves than to the extract obtained from the roots.

The NMR spectrum of *P. anisum* root extract showed a higher number of signals attributed to aromatic compounds (6–8.5 ppm) as compared to *P. anisum* leaf and stem extracts. However, a higher number of signals attributed to quelated hydroxyls (9–11 ppm) were observed in *P. anisum* leaf extract as compared to root and stem extracts (Supplementary Figs. 1–3). This might indicate that *P. anisum* root extract shows a higher amount of simple phenolics than the leaf and stem extracts, which might contribute to greater antioxidant capacity and higher total phenolic content of the root extract. Nevertheless, it is necessary to perform an in depth analysis of the chemical composition to pinpoint potential antimicrobial and cytotoxic compounds present in the extracts.

Forty-eight metabolites were annotated by GC–MS based upon their low-resolution MS profile. It included carbohydrates and carbohydrates derivatives, fatty acids, organic acids, and phenolic compounds (Table 3). Twenty-eight metabolites were annotated by LC–MS based upon their high-resolution MS profile, which included alkaloids, fatty acid, hydrocarbon and terpenes (Table 4).

Among the carbohydrates and carbohydrate derivatives, we identified allose, fructose, gluconic acid lactone, glucose, lactose, lactulose, leucrose, lyxose,

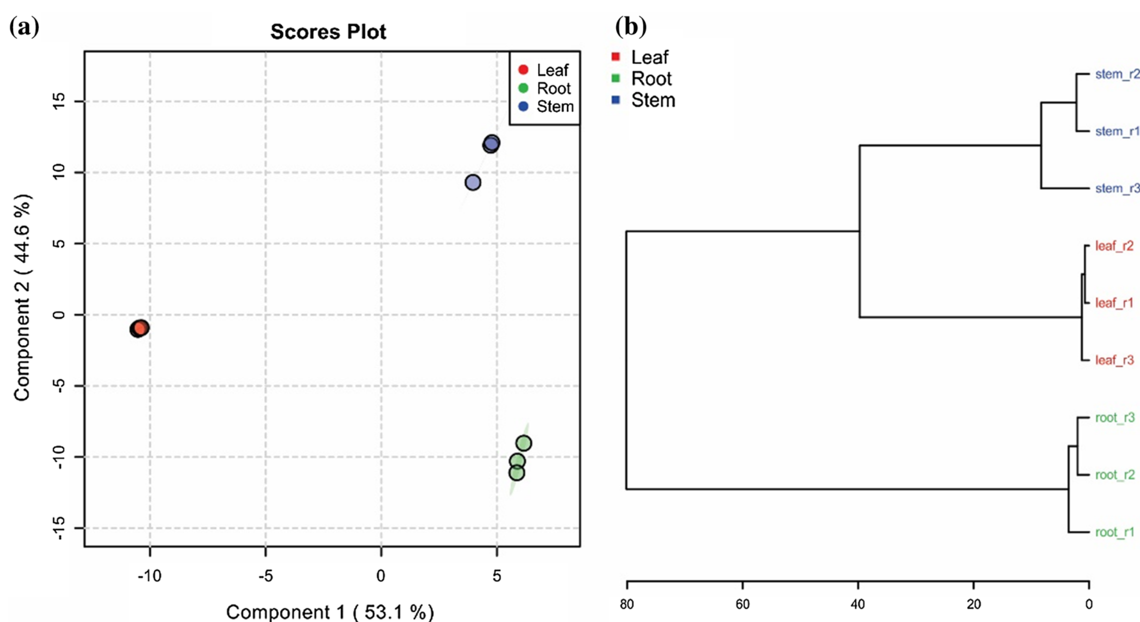

Fig. 1 **a** Partial least square discriminant analysis (PLS–DA) and **b** dendrogram analysis of *P. anisum* extracts based on the NMR profile

Table 3 Metabolites identified by GC–MS

Class	Metabolite	Molecular mass	RT (min)	Formula	Leaf	Stem	Root
Carbohydrates	Allose	180.06	16.85	$C_6H_{12}O_6$	3.31	4.61	4.58
	Fructose	180.06	16.67	$C_6H_{12}O_6$	5.81	6.81	6.68
	Glucose	180.06	16.93	$C_6H_{12}O_6$	6.26	6.94	6.82
	Lactose	342.12	23.94	$C_{12}H_{22}O_{11}$	3.53	4.13	3.96
	Lactulose	342.12	23.71	$C_{12}H_{22}O_{11}$	4.12	4.3	4.28
	Leucrose	342.12	24.48	$C_{12}H_{22}O_{11}$	3.23	3.36	3.32
	Lyxose	150.05	14.36	$C_5H_{10}O_5$	3.81	4.79	4.28
	Methyl-beta-D-galactopyranoside	194.08	16.14	$C_7H_{14}O_6$	4.04	4.56	4.16
	Ribose	150.05	14.77	$C_5H_{10}O_5$	3.84	4.2	4.07
	Sophorose	342.12	24.74	$C_{12}H_{22}O_{11}$	3.58	3.98	3.8
	Sorbose	180.06	16.14	$C_6H_{12}O_6$	4.05	4.93	4.46
	Sucrose	342.12	23.43	$C_{12}H_{22}O_{11}$	4.06	6.39	5.68
	Talose	180.06	17.12	$C_6H_{12}O_6$	5.49	6.51	6.23
	Threose	120.04	12.08	$C_4H_8O_4$	4.22	4.51	3.69
	Trehalose	342.12	24.2	$C_{12}H_{22}O_{11}$	4.11	4.75	4.57
	D-threitol[a]	122.06	12.49	$C_4H_{10}O_4$	5.48	6.58	5.29
	Gluconic acid lactone[a]	466.21	16.84	$C_{18}H_{42}O_6Si_4$	5.04	6.31	5.98
	Xylitol[a]	152.07	15.01	$C_5H_{12}O_5$	5.87	6.7	5.16
Fatty acids	Arachidic acid	312.30	21.44	$C_{20}H_{40}O_2$	3.01	2.89	3.01
	Capric acid	172.15	11.65	$C_{10}H_{20}O_2$	n.d	n.d	2.92
	Heptadecanoic acid	270.26	18.92	$C_{17}H_{34}O_2$	3.12	3.42	3.69
	Linoleic acid ((9Z,12Z)-octadeca-9,12-dienoic acid)	280.24	19.55	$C_{18}H_{32}O_2$	4.85	4.87	5.6
	Myristic acid	228.21	16.08	$C_{14}H_{28}O_2$	3.31	3.97	4.22
	Oleic acid	282.26	19.59	$C_{18}H_{34}O_2$	4.83	5.05	5.6
	Palmitoleic acid ((9Z)-Hexadec-9-enoic acid)	254.22	17.84	$C_{16}H_{30}O_2$	3.06	3.4	3.6
Organic acids	Benzoic acid	122.04	8.86	$C_7H_6O_2$	3.6	3.59	3.67
	Glyceric acid	106.03	10.14	$C_3H_6O_4$	3.35	3.34	3.71
	Glycolic acid	76.02	6.41	$C_2H_4O_3$	2.85	2.96	3.11
	L-(+)lactic acid	90.03	6.2	$C_3H_6O_3$	3.08	2.82	3.25
	Maleamic acid	115.03	13	$C_4H_5NO_3$	3.4	3.8	3.84
	Mucic acid	210.04	17.96	$C_6H_{10}O_8$	n.d	2.73	2.75
	Phosphoric acid	97.98	9.38	H_3O_4P	3.07	3.59	4.23
	Quinic acid	192.06	16.52	$C_7H_{12}O_6$	n.d	3.06	2.94
Flavanoids and simple phenolics	(−)Epicatechin	290.08	24.94	$C_{15}H_{14}O_6$	4.04	4.04	4
	3,4-Dihydroxybenzoic acid	154.03	15.93	$C_7H_6O_4$	n.d	n.d	3.09
	3,4-Dihydroxymandelic acid	184.04	16.99	$C_8H_8O_5$	n.d	3.18	4.11
	4-Hydroxy-3-methoxybenzoic acid	152.15	15.3	$C_8H_8O_3$	n.d	n.d	3.22
	Hydroquinone	110.04	10.99	$C_6H_6O_2$	n.d	3.22	3.76
	p-cresol	108.06	7.54	C_7H_8O	3.09	3.13	3.22
	Resorcinol	110.04	11	$C_6H_6O_2$	n.d	3.12	3.65
Others	1-Hexadecanol	242.26	17.2	$C_{16}H_{34}O$	3.48	3.78	4.02
	Allo-inositol	180.06	16.56	$C_6H_{12}O_6$	n.d	n.d	4.22
	Alpha-D-glucosamine 1-phosphate	259.05	15.77	$C_6H_{14}NO_8P$	3.58	4.1	3.96
	Benzoin	212.08	15.84	$C_{14}H_{12}O_2$	3.07	3.84	5.52
	DL-dihydrosphingosine	302.31	21.98	$C_{18}H_{39}NO_2$	3.25	n.d	3.56
	Glycerol	92.05	9.35	$C_3H_8O_3$	5.08	6.98	6.22
	L-allothreonine	119.06	9.65	$C_4H_9NO_3$	4.09	4.73	3.95
	Phytol	296.31	19.23	$C_{20}H_{40}O$	5.19	5.32	4.37

[a]Carbohydrate derivatives

Table 4 Metabolites identified by LC–MS

Class	Metabolite	Leaf	Stem	Root
Alkaloids	3,5-Didecanoylpyridine	4.11	4.89	4.65
	Anopterine	2.66	2.89	4.30
	Leonurine	3.32	3.63	4.05
	Naproanilide	3.66	4.10	4.65
	Obliquine	2.48	3.87	4.24
	Piperolactam D	2.91	4.34	4.47
	Voacamine	3.27	3.27	3.07
Fatty acid derivatives	10*Z*,13*Z*-nonadecadienoic acid	2.66	2.65	2.71
	13,14-Dihydroxy-docosanoic acid	3.22	4.44	n.d
	19-Oxo-22*Z*-octacosenoic acid	2.79	2.89	2.88
	3-Oxotetradecanoic acid glyceride	4.05	4.65	6.00
	N-oleoyl asparagine	3.05	3.71	3.81
	Octadecyl fumarate	3.71	3.66	4.03
Hydrocarbons	1-Methyl-1,3-cyclohexadiene	4.34	4.24	4.30
	(*Z*)-1,5-tridecadiene	3.79	3.66	3.07
	1,3-Octadiene	3.58	3.33	2.91
	6-Ethyl-4-methyl-3*E*,5*E*,7*E*-decatriene	3.49	3.42	n.d
Terpenoids	Pubescenol	4.70	4.56	3.50
Others	(−)-Matairesinol 4′-[apiosyl-(1−>2)-glucoside]	2.24	3.11	3.48
	11-ketorockogenin acetate	3.13	3.97	4.94
	Benzoylagmatine	3.24	3.65	3.23
	C16 sphinganine	2.64	3.00	4.21
	Eruberin C	2.50	3.70	4.06
	Hordatine A	4.12	5.13	6.08
	Khivorin	2.64	3.00	4.21
	N-cis-tetradec-9*Z*-enoyl-L-homoserine lactone	4.27	4.84	4.75
	Xestoaminol C	6.16	6.52	6.78

methyl-beta-D-galactopyranoside, ribose, sophorose, sorbose, sucrose, talose, threose, D-threitol, trehalose, and xylitol (Table 3). In general, stem and root extracts showed the highest levels of carbohydrates and carbohydrates derivatives, except for threose and trehalose. Carbohydrates and its derivatives lack the conjugated π-electron system necessary to allow them to act as antioxidant-like molecules. Thus, carbohydrates scarcely exhibit in vitro antioxidant capacity and are unable to protect cellular structures against possible damages caused by ROS (Hu et al. 2016). Additionally, these metabolites show very weak or no antimicrobial or cytotoxic activities. Despite their weak contribution to the antioxidant, antimicrobial and cytotoxic activities, stem and root extracts showed the greatest cytotoxic activity. Thus, other metabolites are more likely responsible for the observed pharmacological activities of *P. anisum* extracts.

We identified eight fatty acids (capric acid (C10), myristic acid (C14), palmitoleic acid (C16), heptadecanoic acid (C17), linoleic acid (C18), oleic acid (C18), 10Z,13Z-nonadecadienoic acid (C19), and arachidic acid (C20)) and five fatty acid derivatives (13,14-dihydroxy-docosanoic acid, 19-oxo-22Z-octacosenoic acid, 3-oxotetradecanoic acid glyceride, *N*-oleoyl asparagine, and octadecyl fumarate) in *P. anisum* extracts (Tables 3, 4).

The antioxidant capacity fatty acids increases with the number of double bonds in their structure due to the π-electron system (Richard et al. 2008). Thus, higher levels of the unsaturated fatty acids linoleic acid ((9Z,12Z)-octadeca-9,12-dienoic acid), oleic acid, palmitoleic acid, 10Z,13Z-nonadecadienoic acid, 19-oxo-22Z-octacosenoic acid, and *N*-oleoyl asparagine might contribute to the greatest antioxidant capacity of *P. anisum* stem and root extracts.

Many fatty acids display important pharmacological properties, such as antimicrobial and cytotoxic activities (Chen et al. 2016; Đurđević et al. 2018; Fajriah et al. 2017; Karthikeyan et al. 2014; Kitahara et al. 2004; McGaw et al. 2002; Watanabe et al. 2019). For example, myristic acid, palmitoleic acid, and oleic acid have been shown to have selective antimicrobial activity, whereas caprylic acid, capric acid, and lauric acid displayed non-selective antimicrobial activity against *Staphylococcus aureus* (Watanabe et al. 2019). Palmitic acid (C16), linoleic acid (C18), oleic acid

(C18), and stearic acid (C20) were identified as the main components of *Annona squamosa* Linn. seed oil, which showed antitumor activity inhibiting the growth of H22 tumor cells in mice (Chen et al. 2016). Furthermore, linoleic acid and oleic acids show potent antitumor and anti-inflammatory activities. Fatty acids acts on the cell membrane, disturbing the oxidative phosphorylation (Yoon et al. 2018).

One flavonoid and six phenolic compounds were identified in *P. anisum* extracts: (−)epicatechin, 3,4-dihydroxybenzoic acid (DHBA), 3,4-dihydroxymandelic acid (DHMA), 4-hydroxy-3-methoxybenzoic acid (HMBA), hydroquinone, p-cresol, and resorcinol (Fig. 2a, and Table 4). Phenolic compounds act as potent antioxidant-like molecules due to the stabilization of the unpaired electron, as outcome of ROS action, by electron delocalization in the aromatic ring (Fig. 2b).

In general, higher levels of the phenolic compounds were found in the root extract, followed by the stem extract. p-Cresol was the only phenolic compound identified in the leaf extract, whereas DHBA and HMBA were exclusively identified in the root extract. This explains the higher antioxidant capacity and total phenolic content of the root extract as compared to the leaf and stem extracts. Besides their role as antioxidant-like molecules, phenolic compounds have shown to possess antimicrobial and cytotoxic properties (Cüce et al. 2017; Ibrahim et al. 2019; Mrkonjić et al. 2017). (−)-epicatechin showed strong to moderate antimicrobial activity against Gram (+) and Gram (−) bacteria and four fungal strains, including *C. albicans*. Additionally, it showed a dose-dependent cytotoxic effect against HepG2, MCF7 and HCT cell lines (Ibrahim et al. 2019). HMBA was identified as the main component of *Hypericum scabrum* L. flower extracts, which exhibited strong cytotoxic activity against several cancer cell lines. Vanillic acid along with two other hydroxylated benzoic acid derivatives (gallic and syringic acids) was identified in *Phoenix dactylifera* L. extracts, which exhibited antibacterial properties and cytotoxic activity against HeLa cell line (Kchaou et al. 2016). DHBA and HMBA were identified in *Pinus coulteri* extract obtained from its needles. This extract showed moderate antimicrobial activity (Merah et al. 2018). Moreover, resorcinol and its derivatives are widely known for their wide range of pharmacological activity (Luís et al. 2016).

Alkaloids are well-known nitrogen-containing natural bioactive compounds (de Ávila et al. 2018; Shang et al. 2018; Zhou et al. 2018). Seven alkaloids were identified in *P. anisum* extracts: 3,5-didecanoylpyridine, anopterine, naproanilide, obliquine, pilosine, piperolactam D, and voacamine (Table 4). 3,5-didecanoylpyridine was first isolated from the Asian medicinal plant *Houttuynia cordata* and possess antiviral, antitumor, antimicrobial, and antioxidant properties (Bauer et al. 1996; Kim et al. 2001). Furthermore, this alkaloid showed cyclooxygenase inhibitory (Bauer et al. 1996), and anti-platelet and cytotoxic activities (Jong and Jean 1993). Piperolactam D was first isolated from ethanolic extracts of *P. attenuatum* and *P. boehmerifolium* (Desai et al. 1990), but it is also found in *P. longum* (Desai et al. 1988). Piperolactam D belongs to the aristolactam alkaloid class commonly found in Piper species (Chen et al. 2004; De Oliveira Chaves et al. 2006; Desai et al. 1988, 1990; Ee et al. 2008; Erik Olsen et al. 1993), which possess potent

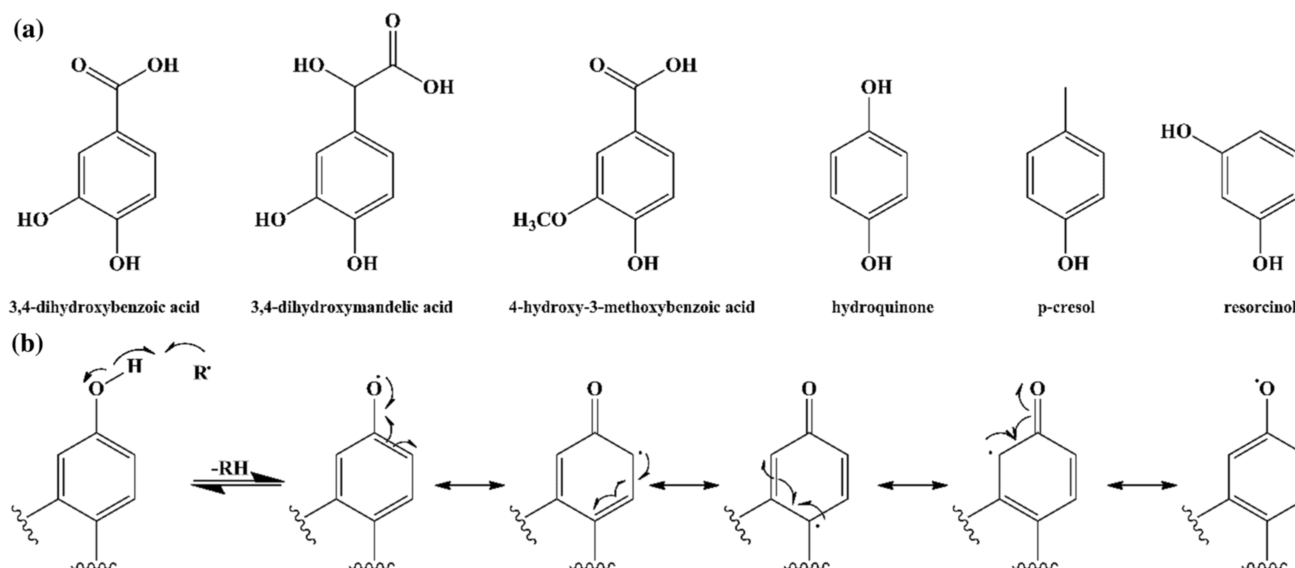

Fig. 2 **a** Phenolic compounds identified in *P. anisum* extracts and **b** the representation of the stabilization of the resulting unpaired electron by resonance in the aromatic ring of phenolic compounds

biological properties, including antimicrobial and cytotoxic activities (Kumar et al. 2003). Higher levels of these alkaloids in stem and root extracts might be casual for greater cytotoxic activity of these extracts.

Organic acids, terpenoids, and metabolites such as eruberin C, hordatine A, phytol, and xestoaminol C may act as antioxidant, antimicrobial, and cytotoxic agents (Tables 3, 4) (Dasyam et al. 2014; Islam et al. 2018; Levrier et al. 2015; Stoessl and Unwin 1970; Valente et al. 2004). The terpenoid pubescenol was obtained from *Euphorbia pubescens* extracts and showed moderate cytotoxicity against several human tumor cells (Valente et al. 2004). The diterpenoid alkaloid anopterine was isolated from *Anopterus macleayanus* and showed potent cytotoxicity against prostate cancer cell lines (Levrier et al. 2015). Hordatine A, a homodimer of the benzofuran p-coumarylagmatine isolated from barley inhibited fungi growth (Stoessl and Unwin 1970). Phytol, a long-chain unsaturated acyclic diterpene alcohol, exhibits a wide range of pharmacological activities. Phytol and its derivatives display strong antimicrobial, antioxidant, antinociceptive, anti-inflammatory, cytotoxic, and immune-modulating properties (Islam et al. 2018). Altogether, our results demonstrate that *P. anisum* might be considered a novel source of bioactive compounds, in which they may act synergistically to confer the broad-spectrum pharmacology of the extracts.

Concluding remarks

The ethnopharmacological use of Piper species by indigenous communities in Central and South America led us to assess the antioxidant, antimicrobial, and cytotoxic activities of *Piper anisum* extracts. The metabolomics approach included NMR, GC– and LC–MS. *P. anisum* root extract showed the most promising bioactivity results, which makes this extract a promising new source of bioactive metabolites. Our results suggest that the metabolomics approach is a robust method of identifying new active compounds from plants used by indigenous communities. We believe that the pharmacological activities might be resulting from the synergistical association of the metabolites presented in the extracts. To the best of our knowledge, this is the first large-scale metabolite profiling study that correlates the chemical composition of *P. anisum* extracts with their antioxidant, antimicrobial, and cytotoxic activities.

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Author contributions PRR designed and supervised all experiments. The extraction, antioxidant activity assay, total phenolic quantification, and antimicrobial activity were performed by DB and PRR. Plant collection and voucher production were performed by PRR, PC, DB, WL and LGF. Cytotoxicity assays were performed by VRS, LSS, DPB, and MBPS. Nuclear magnetic resonance (NMR) analysis was performed by PRR, DB, and MDC. GC–MS and LC–MS analysis were performed by PRR, DB, GABC, PC, LZV, and EP. Data processing and metabolite identification were performed by PRR, and GABC. Statistical analysis was performed by PRR and DB. PRR and DB wrote the manuscript, whereas WL, LGF, DPB, and GABC provided suggestions to the manuscript draft.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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