# Antioxidant, Antibacterial, and Antiviral Activity of Commercial Products of *Piper guineense* and *Piper borbonense*

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## ABSTRACT

This study aims to analyze the chemical composition of commercial products derived from *Piper guineense* and *Piper borbonense,* and to evaluate their fruit extracts' antioxidant capacity antibacterial, and antiviral potential. The genus *Piper* has a long history of use in traditional medicine and is known for its diverse biological activities that validate its traditional applications. In these experiments, *P. guineense* and *P. borbonense* were identified as sources of natural antioxidants, offering potential protection against diseases associated with reactive oxygen species. Both species also demonstrated significant antibacterial activity against gram-positive *Bacillus subtilis*, highlighting their possible role in food preservation. Notably, *P. borbonense* exhibited antiviral activity against SARS-CoV-2PsV, with a high therapeutic index, suggesting its potential as a therapeutic agent for treating COVID-19.

## INTRODUCTION

In many African countries, herbal medicine and spices are commonly used forms of therapy, and a large proportion of the population relies on traditional healthcare needs. Although modern medicine has become more accessible, traditional medicines continue to be used due to historical, cultural, and economic reasons (Ogbunugafor et al., 2017; Kasilo et al., 2018).

*Piper* species (Piperaceae) are widely distributed throughout tropical regions, ranging from endemic to widespread. In traditional medicine, *Piper* species have been utilized globally to enhance the flavor and taste of food and as natural preservatives and remedies for various ailments, including stomachaches, rheumatoid arthritis, general infections, and as antipyretic and anti-inflammatory agents (Mgbeahuruike et al., 2017; Salehi et al., 2019; Kumar et al., 2020). The medicinal properties of *Piper* species are due to the accumulation of a wide range of natural products, mainly in fruits and other parts of the plant. Several reviews have reported the antimicrobial, antioxidant, antidiabetic, anti-inflammatory, anticancer, and antimutagenic properties (Salehi et al., 2019; Kumar et al., 2020; Zahin et al., 2021). There have been nearly 600 compounds isolated from *Piper* species, including alkaloids/amides, terpenes (essential oils), and polyphenols, among others. The major components of the fruits are piperine (1-piperoylpiperidine), a nitrogenous pungent alkaloid, and essential oils, which contribute to the characteristic aroma and are mainly responsible for its biological activities (Salehi et al., 2019).

Although the genus reportedly contains more than 1000 species (Scott et al., 2008), the most popular and economically important is *Piper nigrum* or black pepper, which is a well-known spice considered "The King of Spices" (Damanhouri and Ahmad, 2014; Abukawsar et al., 2018; Takooree et al., 2019, Kumar, 2020). Spices are well known because they are aromatic or pungent substances that are added to flavor foods and thus are a rich source of numerous phytochemicals, many of which possess significant biological activities (Larson, 1988; Velioglu et al., 1998; Yashin et al., 2017).

*Piper guineense*, also known as Bush pepper, West African black pepper, or guinea pepper, grows widely in the highland forests of West and Central Africa. The main product of commerce is the fruits, but in traditional medicine, leaves, roots, and stem bark are used as condiment/spice or for medicinal purposes to treat bronchitis, catarrh, chest pains, lumbago rheumatism, wounds, stomachaches, and discomfort (Ghana herbal Pharmacopoeia, 2007; Adobo and Iwu, 2020). In addition, the fruits have been reported to have insecticidal, antifungal and antimicrobial, antidiabetic, anti-inflammatory and antioxidant properties (Juliani et al., 2013; Iwu, 2014; Mgbeahuruike et al., 2019; Ojimelukwe, 2021).

*Piper borbonense*, or voatsiperifery pepper, is a wild pepper from Madagascar forests, picked by hand in limited quantities and mainly used and consumed locally (Weil et al., 2014). In recent years, there has been a growing demand in international markets for *P. borbonense* because of its endemic and exotic origin (Razafimandimby et al., 2017). Voatsiperifery is used as a spice and for medicinal purposes, mainly to treat tooth decay and against diseases such as diabetes, malaria, intestinal worms, hypertension, influenza, asthma, cough, and diarrhea (Soidrou et al., 2013).

Numerous metabolic processes in the body produce oxygen free radicals and other reactive oxygen species (ROS) as by-products. Free radicals are molecules that possess one or more unpaired electrons, are unstable, and thus are highly reactive. Two major free radicals are the superoxide anion, which is formed when oxygen accepts an electron, and hydroxyl radical, which is the most potent oxidant known. Free radicals can attack most biological molecules, resulting in the propagation of free radical chain reactions (Betteridge, 2000). Unsurprisingly, organisms have evolved central antioxidant defense mechanisms including the peroxidase, dismutase, and catalase enzymes to protect them from these free radical attacks (Arouma, 1998; Betteridge, 2000; Siefried et al., 2007). Overproduction of these free radicals can cause cellular or oxidative damage leading to aging and many chronic cardiovascular and degenerative diseases (Halliwell, 1994; Aruoma, 1998; Betteridge, 2000; Finkel and Holbrook, 2000). Extensive research has been focused on the role of free radical reactions in human diseases, biology, and deterioration of food (Aruoma, 1998). Antioxidants are compounds that in small amounts can delay or inhibit the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Halliwell, 1994, 1996). There is considerable interest in finding natural antioxidants derived from plants for maintaining and improving human health, and for prolonging the shelf-life and maintaining the quality of lipid-containing foods (Halliwell, 1996). Several studies have demonstrated the role of medicinal plants in preventing damage caused by ROS and are a potential source of antimicrobial agents with low toxicity (Cowan, 1999; Forni, 2019).

Although *Piper nigrum* has been shown to exhibit strong antioxidant properties, little is known about the biological activity and health benefits of the commercial products of other species. The objective of this study is to determine the chemical composition of commercial products of *Piper guineense* and *Piper borbonense*, and to assess the antioxidant capacity and potential antibacterial and antiviral activity of their fruit extracts.

## MATERIALS AND METHODS

*Plant Materials:* Commercial sample of fruits of *Piper nigrum* (PN1) were obtained from McCormick (New Jersey, US). Three commercial samples of *Piper guineense* from Nigeria were obtained from (PG1) Nigeria World Food Store Company, (PG2)

Darmol African Market, and (PG3) Foodsby Testimony. Three commercial samples of *Piper borbonense* from Madagascar were obtained from (PB1) Sama, (PB2) Pili Pili Dock -Madepices, and (PB3) Floribis.

***Chemical analyses***

*GC-MS Analysis:* A qualitative ITEX/GC-MS analysis of the volatile compounds emitted from dried plant material was conducted using an innovative headspace in-tube extraction (ITEX) dynamic headspace gas chromatography-mass spectrometry technique. The volatile compounds from the dried plant material were analyzed using a Shimadzu 2010 Plus gas chromatograph with an AOC-6000 autosampler (Shimadzu, Kyoto, Japan) and a headspace syringe ITEX-IIII equipped with a microtrap (ITEX-2TrapTXTA, Tenax TA 80/100 mesh, Switzerland).

About 100 mg of dried, ground dried fruits were weighed and placed into a 20 mL headspace vial with a sealed cap. They were then continuously agitated in the autosampler's oven at 90°C for 10 minutes.

The analytes were thermally desorbed into the injection port (250°C) of the Shimadzu TQ8040 MS system, with the microtrap subsequently flash-heated using N2. The following conditions were consistently maintained for all samples: 30 extraction strokes, syringe temperature at 80°C, agitation speed at 700 rpm, extraction volume of 1000 µL, extraction speed of 100 µL/s, desorption temperature at 250°C, trap cleaning temperature at 250°C, and trap cleaning time of 300 seconds.

An Rxi-5Sil MS (DB5 equivalent) column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Shimadzu) was utilized for chromatographic separation of volatile compounds. The column oven temperature program was as follows: starting at 35ºC for 4 minutes, then increasing to 155ºC at a rate of 20ºC/min for 1.25 minutes and finally reaching 250ºC at 10ºC/min with no hold, resulting in a total run time of 20.7 minutes. The injector and interface temperatures were maintained at 250ºC, and the ion source temperature was set at 200ºC. Helium served as the carrier gas at a constant 1 mL/min flow rate. The pressure was set at 47.7 kPa, with a linear velocity of 36 cm/s and a solvent cut time of 3.5 minutes. A split ratio of 1:25 was applied to all samples. The mass spectrometry mode employed was Electron Impact (EI) ionization.

Peak integration and retention index calculation were conducted using the GCMSsolution v4.3© software from Shimadzu Corporation. The volatile compounds were identified by comparing their mass spectra to those in the current literature and screening them against the NIST05.lib, NIST05s.lib, and W10N14.lib mass spectral libraries. Compound identities were further validated by matching the calculated retention indices (based on a C8-C20 alkane series) with published retention indices of plant volatiles (Adams, 2007). The results were expressed as a percentage of the total peak area.

*UHPLC-DAD-QTOF/MS Analysis:* The instrument used for HPLC analysis was Agilent 1290 Infinity II UHPLC (Agilent Technology, Palo Alto, CA, USA) system with UV-DAD interfaced with an Agilent 6546 Quadrupole Time of Flight (QTOF) with dual AJS electrospray ionization (ESI) source. The column used for compound separation was an Acquity UPLC BEH C18 column (50 x 2.1 mm, 1.7 µm) with Acquity UPLC BEH C18 guard column (2.1 x 5 mm, 1.7 µm). Gradient elution of mobile phase A (0.1% TFA in water) and mobile phase B (0.1% TFA in ACN) was used. The elution profile was 30% B for 0.80 min, raised to 60% B from 0.80 to 13.00 min, kept at 60% from 13.00 to 14.00 min, raised to 95% from 14.00 to 15.00 min, kept at 95% from 15.00 to 16.00 min then dropped to initial gradient 30% B from 16.00 to 16.20 min and equilibrated with 30% B for 0.20 min between injections. The flow rate was 0.4 mL/min. The column temperature was constant at 30℃. The injection volume was 2.5 µL and autosampler was set to 4℃. UV-DAD parameters range from 190-600nm; however, max detection of alkaloids occurred at 340nm. MS conditions are as follows: Nitrogen was used as nebulizing and drying gas. The nebulizer was set to 30 psi and the drying gas to 300 ℃ with a flow rate of 12 L/min. The sheath gas was set to 250 ℃ with a flow rate of 10 L/min. Fragmentor voltage was 180V, collision energy was 0, 10, 20, 30V, skimmer at 65V, and Oct 1 RF Vpp at 750V. Positive scan was conducted in the sequence. Data acquisition mass

ranged from 200 to 1500 m/z. Sample and Standard Preparation. OptimaTM LC/MS grade trifluoroacetic acid (TFA), acetonitrile (ACN), water, and ACS grade methanol (MeOH), were purchased from Fisher Scientific (Fair Lawn, NJ). For each sample group, approximately 100 mg of crushed powder was weighed out, extracted with 10 mL of 80:20 MeOH:Water, vortexed for ~10s, then sonicated for 10 minutes. The sample extract was then diluted 20x by spiking 50 µL of sample extract in 950 µL of 80% MeOH in a microfuge tube, centrifugated at 12500 rpm for 10 minutes at 23°C, and the supernatant was injected into the LC directly. All sample groups were analyzed in duplicates. Piperine (Sigma Chemical Company) standard stock solution was prepared by diluting 2.5 mg in 25 mL of 80% MeOH. The standard stock solution was further diluted in 2-fold serial dilutions to make working standard solutions from 100 ug/mL to 0.0244 ug/mL. Quantification of alkaloids were based on the molecular weight ratio of piperine and analytes.

*Extracts preparation:* Twenty mg of powdered fruits were dissolved in 1 mL of water, vortexed, then either boiled for 5 min (boiled) or sonicated for 5 minutes using a Cole- Parmer 08895-04 sonicator (sonicated); or 20 mg were dissolved in 70% ethanol (Sigma Aldrich, St Louis, MO), then sonicated for 5 min (70% ethanol). All samples were centrifuged for 5 minutes at 4°C at the highest rpm (13,000). The supernatant of each solution was then filtered through a polytetrafluorethylene non-polar disposable syringe filter with a pore size of 0.45 µm (Chromafil 0-45/15 MS, Macherey-Nagel GmBH &, KG, Düren, Germany). Extracts were stored in a refrigerator at 4°C until use.

Concentrated extracts were prepared for antibacterial, antiviral, and cytotoxicity assays. 240 mg of powdered material were dissolved in 6 ml of sterile distilled water, vortexed, and then sonicated three times at two-minute intervals in a Cole-Parmer 08895-04 sonicator at 100% sonication in 1200 mL of room temperature distilled water. Samples were vortexed for 10 seconds between intervals. Samples were spun for five minutes at 4 degrees Celsius and 8000 rpm in a Sorvall Biofuge Stratos centrifuge with a Herause #3057 roter. Extracts were filtered and stored as described above.

*Trolox equivalent antioxidant capacity (TEAC) assay:* The TEAC assay was carried out according to Re et al. (1999) to determine the free radical scavenging capacity using the ABTS•+ radical cation. The ABTS•+ radical cation was produced by mixing a stock solution of ABTS (7 mM) and potassium persulfate (2.4 mM) at room temperature and incubating in the dark for 12-16 h before use. The cation radical solution was diluted until it reached an absorbance of 0.7± 0.05 at 734 nm using Trolox as a standard (0-0.64 mg/mL). Results are expressed as mg Trolox Equivalents/mL plant extract (TEAC mg/g DW).

*Total phenolic content (TPC):* Total phenolic content was determined using a modified Folin-Ciocalteu's protocol according to Gao et al. (2000). The absorbance was measured at 765 nm on a Biotek Synergy 4 Spectrophotometer (BioTek Instruments, Winooski, VT). The calibration was established using gallic acid (0-1.5 mg/mL). Total phenolic content was expressed as mg gallic acid Equivalents/mL plant extract (GAE mg/mL).

*Antibacterial activity in liquid culture:* Dehydrated BD Difco™ nutrient agar and BD Difco™ Luria-Bertani (LB) broth (Carolina Biological Supply Co, Burlington, NC.) were prepared and sterilized as described by the manufacturer. For liquid culture experiments, LB broth was prepared at a 4x concentration. *Escherichia coli* K-12 (12-4500) in tryptone agar and *Bacillus subtilis* in nutrient agar were obtained from Carolina Biological Supply Co. (Burlington, NC.) LB/*Piper* media was prepared by mixing one-part sterile 4x LB broth with three parts sterile *Piper* extract. LB control media was prepared by mixing one-part sterile 4x LB broth with three parts sterile distilled water. Fresh overnight LB broth cultures of *E. coli* and *B. subtilis* were incubated overnight at 37 °C and 150 rpm in MaxQ 4450 orbital shaker (Thermo Fisher Scientific). For each bacterium, 600 µL of either LB or LB/*Piper* media were inoculated with 6 µL of overnight culture (for a density of $10^5$ cells/ mL) or with 6 µL of 1x LB broth. Each 600 µL inoculum was divided equally into two

wells of a Corning 96-well flat-bottom tissue culture plate (Thermo Fisher Scientific). Blank wells were set up containing 300 µL uninoculated LB broth and 300 µL uninoculated LB/*Piper* media. Microplates were incubated at 37 °C and 150 rpm in a MaxQ 4450 orbital shaker (Thermo Fisher Scientific). Optical density (OD) was measured at 600 nm every hour for 8 hours using a GenTek Synergy 4 spectrophotometer. Two readings were recorded for each culture with 10 seconds of agitation prior to each reading.

***Antiviral Activity against SARS-CoV-2 using pseudo viral model***

*Cell Line and Pseudovirus Production:* HeLa cells expressing human angiotensin-converting enzyme 2 (HeLa ACE-2) were obtained from Dr. Dennis Burton at The Scripps Research Institute, La Jolla, CA, USA. Pseudoviruses (PsVs) displaying SARS-CoV-2 K1417N/E484K/N501Y spikes were produced using the method described by Schmidt et al. (2020). The plasmid containing the gene of SARS-CoV-2 spike [pSARS-CoV2-Strunc (with K1417N/E484K/N501Y mutations)], along with pCRV1NHG GagPol and pNanoLuc2AEGFP, were used for PsV production by transfecting 293T cells (ATCC, Manassas, VA, USA) using lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA, USA) as previously described (Alasaidi et al., 2021). The SARS-CoV-2 K1417N/E484K/N501Y PsV collected from cell supernatants was filtered (0.22 µm PVDF filter), aliquoted, and stored at −80°C before titering in a cell-based pseudoviral entry assay and using the TurboLucTM Luciferase One-Step Glow Assay Kit (Thermo Fisher Scientific).

*Cytotoxicity and Antiviral Assay:* HeLa ACE-2 cells were seeded in clear bottom 96-well microplates and treated with various dilutions of each extract in triplicate or different concentrations of Tween 20 (positive control for cytotoxicity). The microplates were then incubated for 72 hours at 37°C, 5% $CO_2$, and 98% humidity. After this incubation, the XTT reagent was added to all wells, the plates were incubated for 1 hour at 37°C, 5% $CO_2$, and 98% humidity, and finally, the absorbance was measured at 450 nm using a Spectramax iD. For the antiviral activity assessment, the same extracts tested in the XTT assay were co-incubated with SARS-CoV-2 K1417N/E484K/N501Y PsV in HeLa ACE-2 cell monolayers seeded in white opaque 96-well microplates and following the same procedure previously described by Alsaidi et al. (2012). The microplates were again incubated for 72 hours under the same conditions. At the end of this incubation, the TurboLuc™ Luciferase One-Step Glow Assay was used to determine the entry percentage of PsVs in the presence of each extract dilution when compared to the virus control. The half-maximal cytotoxic concentration ($CC_{50}$) and half-maximal effective concentration ($EC_{50}$) were determined for each extract using a dose-response-inhibition analysis with GraphPad Prism v9.0.2 software. The therapeutic index ($TI = CC_{50}/EC_{50}$) was calculated for each extract to assess its selective antiviral activity against SARS-CoV-2.

***Cytotoxicity studies in Caco-2 cells***

*Cell culture:* The human colonic adenocarcinoma cell line, Caco-2, was obtained from American Type Culture Collection (ATCC HTB37; Manassas, VA). The cells were cultured in Eagle's Minimum Essential Media with sodium bicarbonate, non-essential amino acids, L-glutamine, and sodium pyruvate (EMEM) (Corning, Manassas, VA) supplemented with 10% fetal calf serum (FCS; Hyclone, Marlborough, MA) and 100 U/ml penicillin and 100 µg/ml streptomycin (Corning Mediatech, Manassas, VA) (10% FCS-EMEM). The cells were cultured at 37°C in a 95% air-5% $CO_2$ humid environment. Extracts as prepared above were further filtered, for sterility purposes, with a Titan3 0.2µm PFTE hydrophobic filter (Thermo Fisher Scientific) which was prewet with 70% ethanol and rinsed with distilled water.

*Cell viability assays:* The CyQuant XTT viability assay (Thermo Fisher Scientific, Waltham, MA) was used to assess the viability of Caco-2 cells after exposure to plant extracts. Caco-2 cells were plated at $1.25 \times 10^4$ cells/well (100µl total volume) of a 96-well tissue culture plate in 10% FCS-EMEM and cultured overnight. The following day, media was removed and replaced with fresh 10% FCS-EMEM

only (cells only) or dilutions of *Piper guineense* (PG), *Piper borbonense* (PB), or *Piper nigrum* (PN) extract stock solution (40mg/ml) in 10% FCS-EMEM to working extract concentrations of 1 mg/ml or 8 mg/ml in 100µl total. The cells were further cultured for 24 hours. After incubation, 70µl of XTT working solution was added to each well and incubated for 4-6 hours at 37°C in a 95% air-5% $CO_2$ humid environment. The absorbance of the samples was measured at both 450nm and 660nm using the Biotek Synergy 4 plate reader (Agilent Technologies, Santa Clara, CA). As per the instructions, specific absorbance for each sample was determined as follows: [Abs450 nm(Test) – Abs450 nm(Blank)] – Abs660 nm(Test). Data is represented as a percent of the control sample (cells only) which is set to 100%. Average and standard error was determined for three independent experiments.

The CyQuant LDH cytotoxicity assay (Thermo Fisher Scientific, Waltham, MA) was used to assess the possible cytotoxic effects of the plant extracts on Caco-2 cells. Caco-2 cells were plated at 1 x $10^4$ cells/well (100µl total volume) of a 96 well tissue culture plate in 10% FCS-EMEM and cultured for 48 hours. Following the initial culture, media was removed and replaced with fresh 1% FCS-EMEM only (cells only) or dilutions of *Piper guineense* (PG), *P. borbonense* (PB), or *P. nigrum* (PN) extract stock solution (40 mg/ml) in 1% FCS-EMEM to working extract concentrations of 1mg/ml to 0.1µg/ml in 100 µl total. Serum levels were reduced to 1% to prevent interfering effects with the assay. Both spontaneous and maximum LDH release was monitored as outlined by the manufacturer. The cells were further cultured for 24 hours. To determine LDH levels, 50 µl of reaction mixture was added to each well and incubated for 30 minutes in the dark at room temperature. 50 µl of stop solution was added to each well and mixed by gentle tapping. The absorbance of the samples was measured at both 490nm and 680nm using the Biotek Synergy 4 plate reader. To correct for background, the absorbance at 680nm was subtracted from the absorbance at 490nm for each well. Last, the percent of cytotoxicity for treatments was determined as outlined in the kit instructions: the percentage of cytotoxicity = [(Extract-treated LDH activity – Spontaneous LDH activity)/(Maximum LDH activity – Spontaneous LDH activity)] x 100%. Average and standard error was determined for 3-4 independent experiments.

## RESULTS AND DISCUSSION

*Volatile oil composition*: The chemical composition of *P. nigrum* essential oil is dominated by alpha pinene (6%), beta pinene (14%), delta 3 carene (8%), limonene (24%), 1,8 cineole (7%) and E-caryophyllene (7%) (Juliani et al., 2013).

The commercial sample used in this study (PN1) was characterized by high levels of delta-3-carene (38.24%), and lower levels of alpha pinene (7.86%), beta pinene (13.34%), limonene/beta phellandrene (26.05%), with low percentages levels of (E) – caryophyllene (5.14 %) (Table 1).

The chemical profile of *P. borbonense*, *P. guineense* showed a similar profile to that of *P. nigrum* (Table 1). The percentages of alpha pinene were similar for *P. borbonense* (10.27 – 15.59%) and for *P. guineense* (7.21 – 16.24%). The levels of beta pinene showed a similar profile for *P. borbonense* (6.87 – 19.9%) and with higher level for *P. guineense* (24.13 – 29.0%). Alpha phellandrene was not detected in *P. nigrum*, while higher levels were observed in *P. borbonense* (10.57 – 33.17), and lower in *P. guineense* (4.5 – 14.74%) (Table 1). The percentages of limonene/beta phellandrene were higher in *P. borbonense* (20.8 – 24.14%) and lower in *P. guineense* (9.98 – 16.97%). The (E) caryophyllene levels were as low in *P. nigrum* (5.14%), with slightly higher percentages in *P. borbonense* (4.27-7.31%), and *P. guineense* (2.48-9.31%) (Table 1). One sample of *P. guineense* (PG1) showed high levels of viridifloral (9.8%). Although the three species showed a similar volatile profile, previously Juliani et al, (2013) reported high levels of linalool in *P. guineense* from Liberia (66.7-70.2%).

Table 1. Chemical composition of the essential oils in seven commercial samples of *Piper. Piper nigrum* (PN1) McCormick, *P. guineense* (PG1) Nigeria World Food Store Company, (PG2) Darmol African Market and (PG3) Foodsby Testimony, *P. borbonense* (PB1) Sama, (PB2) Pili Pili Dock -Madepices, and (PB3) Floribis.

| N | | Component | RT | PN1 | PG1 | PG2 | PG3 | PB1 | PB2 | PB3 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 928 | alpha-Thujene | 7.62 | 0.34 | 0.10 | 0.21 | 0.11 | 0.45 | 0.62 | 0.22 |
| 2 | 937 | alpha-Pinene | 7.72 | **7.86** | **7.21** | **10.30** | **16.24** | **13.16** | **10.27** | **15.59** |
| 3 | 954 | Camphene | 7.93 | 0.12 | 1.07 | 0.76 | 0.21 | 0.48 | 0.33 | 0.10 |
| 4 | 976 | Sabinene | 8.18 | 2.50 | 2.30 | 4.76 | 1.45 | 1.07 | 0.72 | 3.57 |
| 5 | 982 | beta-Pinene | 8.25 | **13.34** | **24.13** | **29.00** | **24.17** | **12.67** | **6.87** | **19.90** |
| 6 | 989 | Myrcene | 8.33 | 2.21 | 1.33 | 1.67 | 1.33 | 0.81 | 0.88 | 1.01 |
| 7 | 1001 | delta-2-Carene | 8.47 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 8 | 1009 | alpha-Phellandrene | 8.53 | 0.00 | **10.79** | **14.74** | 4.50 | **21.67** | **33.17** | **10.57** |
| 9 | 1012 | delta-3-Carene | 8.57 | **38.24** | 2.53 | 2.82 | 3.63 | **5.93** | **7.30** | 4.60 |
| 10 | 1020 | alpha-Terpinene | 8.65 | 0.13 | 0.22 | 0.17 | 0.33 | 0.03 | 0.03 | 0.00 |
| 11 | 1028 | para-Cymene | 8.72 | 1.43 | 1.33 | 3.23 | 0.88 | 2.61 | 1.81 | 1.40 |
| 12 | 1034 | Limonene + beta phellandrene | 8.77 | **26.05** | **9.98** | **16.97** | **11.45** | **20.08** | **22.12** | **24.14** |
| 13 | 1048 | beta-Z-Ocimene | 8.90 | 0.03 | 1.26 | 0.27 | 0.40 | 0.24 | 0.38 | 0.31 |
| 14 | 1058 | beta-E-Ocimene | 9.00 | 0.05 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 |
| 15 | 1063 | gamma-Terpinene | 9.05 | 0.14 | 0.32 | 0.29 | 0.51 | 0.08 | 0.09 | 0.06 |
| 16 | 1076 | Sabinene hydrate | 9.17 | 0.00 | 0.02 | 0.01 | 0.00 | 0.10 | 0.11 | 0.05 |
| 17 | 1087 | para Menthadiene* | 9.27 | 0.33 | 0.00 | 0.00 | 0.00 | 0.01 | 0.02 | 0.00 |
| 18 | 1092 | Terpinolene | 9.31 | 0.77 | 3.97 | 0.57 | 1.53 | 0.35 | 0.16 | 0.14 |
| 19 | 1101 | Linalool | 9.39 | 0.00 | 0.63 | 0.05 | 1.22 | 0.16 | 0.18 | 0.17 |
| 20 | 1161 | Borneol | 9.89 | 0.00 | 0.23 | 0.14 | 0.00 | 0.06 | 0.12 | 0.00 |
| 21 | 1266 | Carvenone* | 10.73 | 0.00 | 0.00 | 0.00 | 0.00 | 0.05 | 0.17 | 0.00 |
| 22 | 1348 | delta-Elemene | 11.32 | 0.19 | 0.08 | 0.28 | 0.00 | 0.14 | 0.30 | 0.00 |
| 23 | 1361 | alpha-Cubebene | 11.41 | 0.02 | 0.34 | 0.49 | 0.38 | 0.31 | 0.23 | 0.02 |
| 24 | 1393 | beta-Cubebene | 11.64 | 0.84 | **6.42** | 2.90 | 4.09 | 1.41 | 0.85 | 1.09 |
| 25 | 1403 | (Z)-Caryophyllene | 11.71 | 0.05 | 2.60 | 1.27 | 2.93 | 0.79 | 0.87 | 0.29 |
| 26 | 1429 | beta-Gurjunene | 11.88 | 0.00 | 0.39 | 0.19 | 3.13 | 0.07 | 0.01 | 0.29 |
| 27 | 1443 | (E) – Caryophyllene | 11.97 | **5.14** | **5.02** | 2.48 | **9.31** | **7.31** | 4.27 | **7.14** |
| 28 | 1453 | Muurola,3,5diene | 12.04 | 0.00 | 1.81 | 0.65 | 0.33 | 0.56 | 0.68 | 0.16 |
| 29 | 1454 | Farnesene | 12.10 | 0.00 | 0.17 | 0.00 | 0.11 | 0.00 | 0.00 | 0.02 |
| 30 | 1469 | 159-161-105-119-91 | 12.15 | 0.00 | 0.35 | 0.51 | 0.07 | 0.11 | 0.26 | 0.03 |
| 31 | 1478 | gamma-Curcumene | 12.20 | 0.13 | 0.99 | 0.46 | 1.28 | 0.52 | 0.26 | 1.09 |
| 32 | 1491 | Germacrene D | 12.29 | 0.00 | 0.23 | 0.05 | 0.37 | 0.32 | 0.22 | 0.19 |
| 33 | 1502 | gamma Amorphene+ Viridiflorene | 12.36 | 0.00 | **9.80** | 2.92 | 4.56 | 4.33 | 3.41 | 2.76 |
| 34 | 1513 | Germacrene A | 12.44 | 0.04 | 1.87 | 1.28 | 5.25 | 2.27 | 2.11 | 2.70 |
| 35 | 1534 | gamma Cadinene | 12.55 | 0.02 | 2.18 | 0.35 | 0.18 | 0.60 | 0.47 | 0.16 |
| | | Total analyzed | | 99.98 | 99.68 | 99.81 | 99.96 | 98.72 | 99.28 | 97.77 |

Notes: Bolded relative percentages values higher than 5%. * Tentative identification

*Piperinoid composition:* Piperlonguminine and dihydropiperlonguminine are marker components that are used to identify each *Piper* species. Dihydropiperlonguminine was only found in *P. guineense*, with amounts ranging from 0.44 to 1.35 mg/g. Piperlonguminine was found in *P. borbonense* and *P. nigrum*, with the former showing the highest amounts (0.63 to 1.69 mg/g), and the latter with the lowest amounts (0.43 mg/g) (Table 2).

Piperdardine was found in the highest amount in *P. nigrum* (4.53 mg/g), followed by *P. guineense* PG3 (1.71), and PG2 (0.61); lower levels were observed in PG1 (0.32) and *P. borbonense* samples (0.35 – 0.49 mg/g) (Table 2). Cis-piperettine exhibited a similar trend, with highest amount found in *P. nigrum* (1.5 mg/g), followed by *P. guineense* PG3 and PG2 (0.61 – 0.49), with lower levels found in PG1 (0.24), and *P. borbonense* samples (0.26 – 0.33 mg/g). Piperylin showed a different trend, with *P. guineense* showing the highest variability in its amounts, from the lowest in PG1 (0.82), moderate amounts in PG2 (2.76), and the highest amount in PG3 (5.54 mg/g). *Piper borbonense* showed moderate amounts (2.05 to 1.75), with *P. nigrum* showing lower amounts (1.34 mg/g) (Table 2).

The chemical composition showed a variation in the amount of piperinoids. *Piper nigrum* contained the highest amounts, with 40.54 mg/g, followed by *P. borbonense* (24.23 to 29.29) (Table 2). *Piper guineense* showed higher variabilities, with two samples showing lower levels (5.96 to 7.84 mg/g), with one sample at 30.03 mg/g. Piperine dominated the profile in all species, the highest in *P. nigrum* (32.74 mg/g), followed by *P. borbonense* (20.43 to 25.31mg/g), the lowest amount was found in *P. guineense* samples PG1 and 2 (3.41 – 4.13), with higher amounts in PG3 (20.81 mg/g) (Table 2).

While these results showed low levels of piperine in *P. nigrum*, others have reported higher ranges of piperine in black pepper from 2 to 9% (Ziegenhagen et al., 2021). Juliani and collaborators (2013) reported 1.3% for *P. nigrum*, and a range for *P. guineense* from 0.23 to 1.1 %.

Table 2. Chemical composition of piperine and other non-volatile derivatives in different samples of *Piper. Piper nigrum* (PN1) McCormick, *P. guineense* (PG1) Nigeria World Food Store Company, (PG2) Darmol African Market and (PG3) Foodsby Testimony, *P. borbonense* (PB1) Sama, (PB2) Pili Pili Dock -Madepices, and (PB3) Floribis.

| Components (mg/g of dry weight) | PN1 | PG1 | PG2 | PG3 | PB1 | PB2 | PB3 |
|---|---|---|---|---|---|---|---|
| Piperylin | 1.34 | 0.82 | 2.76 | 5.54 | 1.75 | 2.05 | 2.35 |
| Piperlonguminine | 0.43 | 0 | 0 | 0 | 1.69 | 1.32 | 0.63 |
| Dihydropiperlonguminine | 0 | 0.44 | 0.57 | 1.35 | 0 | 0 | 0 |
| Piperine | **32.74** | 4.13 | 3.41 | **20.81** | **22.66** | **25.31** | **20.43** |
| Piperdardine | 4.53 | 0.32 | 0.61 | 1.71 | 0.35 | 0.35 | 0.49 |
| cis-piperettine | 1.5 | 0.24 | 0.49 | 0.61 | 0.27 | 0.26 | 0.33 |
| Total | 40.54 | 5.96 | 7.84 | 30.03 | 26.72 | 29.29 | 24.23 |

*Antioxidant activity*: The formation of free radicals is a major cause of the oxidation process. Thus, the high potential for scavenging free radicals could inhibit the spreading of oxidation. The ABTS method is one method to determine the antioxidant capacity in natural products. $ABTS^{\bullet+}$ radical is generated by a reaction between ABTS and potassium persulfate, which has a blue/green color, and the disappearance of ABTS•+free radicals calculate the activity. Thus, the antioxidant capacity (Trolox equivalent antioxidant capacity -TEAC) is measured as the ability of plant extracts to decrease the color by reacting directly with the $ABTS^{\bullet+}$radical. The degree of color change is proportional to the concentration of antioxidants.

There is variation in the scavenging activity based on the extraction method used and between species (Figure 1). Higher antioxidant capacity was observed in extracts suspended in water and boiled for 5 min (PB2, 49.34 Trolox Eq mg/g DW) or

extracts prepared with 70% ethanol (PB3, 41.56 Trolox Eq mg/g DW). *Piper borbonense* (PB samples) exhibit higher antioxidant capacity (ranging from 41.56 -49.34 Trolox Eq mg/g DW) than *P. guineense* (ranging from 30.98-37.97 Trolox Eq mg/g DW) and *P. nigrum* (ranging 26.3-33.49 Trolox Eq mg/g DW). *Piper borbonense* (PB2) aqueous extracts boiled exhibited the highest TEAC (49.34 Trolox Eq mg/g DW) (Figure 1).

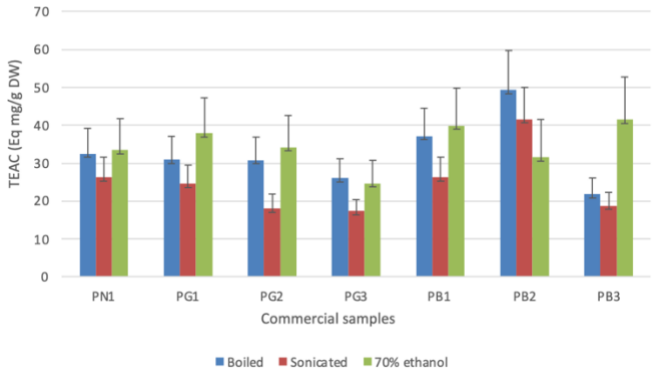


Figure 1. Antioxidant capacity (Trolox Equivalent Antioxidant Activity, mg TEAC/g dry weight) in different *Piper* extracts (boiled, sonicated, and 70% ethanol). *Piper nigrum* (PN1) McCormick, *P. guineense* (PG1) Nigeria World Food Store Company, (PG2) Darmol African Market and (PG3) Foodsby Testimony, *P. borbonense* (PB1) Sama, (PB2) Pili Pili Dock -Madepices, and (PB3) Floribis. Bars indicate the mean value ± SE of at least three independent experiments.

The total phenolic content (TPC) extracted varied among the methods of extraction used, ranging from 6.27 to 17.65 GAE mg/g DW (Figure 2). Ethanol (70%) consistently extracted the highest amount of phenolics in all the samples, while sonicated water solutions extracted the least amount in all the samples. In addition, different commercial samples also accumulated different amounts of phenolics. *Piper guineense* (PB2) showed the highest amount of TPC, ranging from 17.65 (water boiled), 14.75 (water sonicated), and 17.44 (ethanol) GAE mg/g DW.

We observed a positive correlation between antioxidant capacity and total phenolic content, ABTS/TPC in water-boiled extracts (R= 0.9783) and in water-sonicated extracts (R=0.947), indicating that total phenolics are major contributing compounds to the antioxidant capacity of the extracts. However, there was a poor correlation when phenolics were extracted using ethanol, indicating that other compounds might also contribute to the antioxidant properties of these extracts. A positive correlation has previously been reported in fruits and seeds of different species of medicinal plants (Velioglu et al., 1998; Cai et al., 2004; Melo et al., 2020).

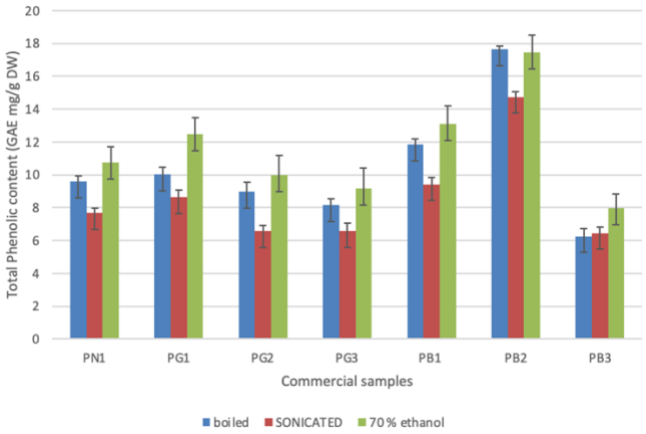


Figure 2. Total phenolic content (Gallic Acid Equivalent, mg GAE/g dry weight) in different *Piper* extracts (boiled, sonicated, and 70% ethanol). *Piper nigrum* (PN1) McCormick, *P. guineense* (PG1) Nigeria World Food Store Company, (PG2) Darmol African Market and (PG3) Foodsby Testimony, *P. borbonense* (PB1) Sama, (PB2) Pili Pili Dock -Madepices, and (PB3) Floribis. Bars indicate the mean value ± SE of at least three independent experiments.

Phenolic compounds are a large class of plant compounds widely distributed in higher plants in various organs such as leaves, fruits, and seeds and play essential roles in plant quality, coloring, flavor, and stress resistance (Larson 1988; Zhang et al., 2022). Phenolic compounds possess an aromatic ring bearing one or more hydroxyl groups, and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular-weight polymer (Bravo, 1998; Balasundram et al., 2006). It has been reported that phenolic compounds exhibit antioxidant properties due to their ability to scavenge free radicals and donate hydrogen atoms or electrons or chelate metal cations (Huang et al., 2005).

Phenolic compounds can be measured using

Folin–Ciocalteu reagent under basic conditions. In a basic medium, the phenolic proton dissociates, forming a phenolate anion. This anion can reduce the Folin–Ciocalteu reagent, leading to the reduction of molybdate and the formation of blue-colored molybdenum oxide. Total phenolic content assay by Folin–Ciocalteu reagent is convenient, simple, and reproducible (Huang et al., 2005).

Because of phenolics' antioxidant properties, various health benefits have been reported, with special reference to aging, cardiovascular diseases, hypertension, diabetes, metabolic syndrome, obesity, and cancer (Pandey and Rizvi, 2009; Durazzo et al., 2019).

*Antibacterial activity:* Each *Piper. spp.* extract was tested for its effect on bacterial growth in liquid microcultures. *E. coli* (gram-negative) and *B. subtilis* (gram-positive) were independently grown in microplate wells in either the presence or absence of 20 mg/mL extract. Growth of bacteria was monitored hourly by spectrophotometry at 600 nm for a total of eight hours. Whereas the growth of *E. coli* appeared not to be inhibited by any of the extract samples, *B. subtilis* microcultures exhibited variable levels of suppression (Figure 3). Relatively higher levels of growth inhibition were observed in the presence of *Piper* extracts PG1, PG2, and PB1. In contrast, more moderate inhibition was seen in the presence of PN1, PG3, and PB2, and results were consistent across three independent trials.

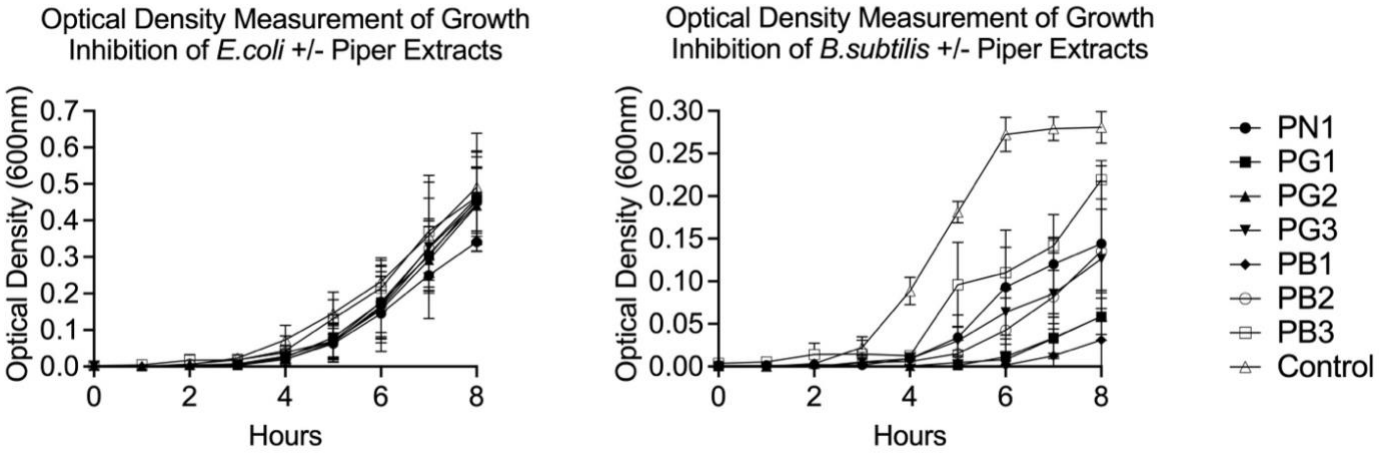


Figure 3. Average measurement of growth by optical density (600 nm) of *E. coli* (left) and of *B.* subtilis (right) in LB +/- *Piper* for *Piper borbonense* (PB1-3), *Piper guineense* (PG1-3), or *Piper nigrum* (PN1) seed extracts. Bars indicate the mean value ± SE of three independent experiments, with two replicates each.

Only one *P. nigrum* extract (PN1) was used in these experiments. While no inhibition of *E. coli* was observed, moderate inhibition of *B. subtilis* was observed. Previous studies reviewed by Abdallah and Abdalla (2018) and Salehi et al. (2019) have shown that *P. nigrum* exerts antimicrobial activity against several gram-positive and gram-negative bacteria. This action is greatly influenced by differential extraction and testing methods. Whereas most previous studies used agar disc-diffusion and well-diffusion methods (Abdallah and Abdalla, 2018), these experiments employed growth in liquid microcultures. In addition, higher levels of bacterial inhibition have been observed when using organic extracts compared to aqueous extracts used here. However, aqueous extracts have also shown antibacterial activity against gram-positive and gram-negative bacteria (Alateeqi et al., 2024). Antibacterial activity of *P. nigrum* has been widely attributed to its relatively high piperine content (Salehi et al., 2019). High levels of piperine in PN1 (Table 1) might be responsible for inhibiting *B. subtilis* growth. Alternatively, as essential oils were expected to be preserved through the sonication

procedure, high levels of delta-3-Carene might also explain the results.

Of the three *P. guineense* samples tested, PG1 and PG2 exhibited substantial growth inhibition of *B. subtilis* but did not affect the growth of *E. coli.* While previous research on leaf extractions showed inhibition against both gram-positive and gram-negative bacteria (Mgbeahuruike et al., 2018), experiments using seed extracts were more variable. In two separate studies using aqueous *P. guineense* seed extracts (Mgbeahuruike et al., 2018; Eruteya et al., 2017), researchers observed no inhibition against either gram-positive or gram-negative. The inconsistent results presented here with PG1 and PG2 might be explained by differences in extraction preparation, including sonication of crushed *Piper* fruits, and monitoring microculture growth to detect inhibition. Analysis of chemical content indicated that PG1 and PG2 had relatively low levels of piperine, in contrast to PN1 (Table 1). Both, however, contained relatively high levels of beta-pinene and (E)-Caryophyllene, which correlate with the high levels of growth inhibition for *B. subtilis*.

Of the *P. borbonense* extracts, PB1 exhibited stronger inhibition of *B. subtilis* growth than PB2 and PB3, which showed only moderate inhibition (Figure 3). *Piper borbonense* reportedly contain relatively high concentrations of essential oils and low concentrations of piperine (Weil et al., 2017), although all three extracts here contained relatively high piperine levels (Table 2). It is likely that essential oils in PB1, PB2, and PB3 were preserved in the sonication extraction and might account for the inhibition of *B. subtilis* growth. All contained alpha- and beta-pinene, and alpha- and beta- phellandrene (Table 1). Whereas PB1 contained almost twice the level of beta-pinene and (E)-Caryophyllene compared with PB2, both PB1 and PB2 contained higher levels of delta-3-Carene compared with PB3. The differences in essential oil content between these samples might have contributed to the variable inhibition observed. Notably, PB1 and PB2 had relatively high antioxidant capacity compared with the other extracts (Figure 1).

The difference in inhibition levels observed between *E. coli* and *B. subtilis* cultures is likely explained by the physiological differences in the bacterial cell walls. Gram-negative bacteria contain a thick outer lipid membrane that acts as a barrier to entry for large molecules. While the membrane contains channel porins for the entry of small, hydrophilic molecules, these prevent larger molecules from entering (Choi and Lee, 2019). Conversely, gram-positive bacteria have no lipid outer membrane but instead have a relatively thicker layer of peptidoglycan in their cell walls. Antibiotics that target the cell wall disrupt the peptidoglycan cross-linkages, thereby causing gram-positive bacteria to lyse (Kapoor et al., 2017). Though the results of these experiments indicate that the chemical properties of these three *Piper* species might interact with the bacterial cell wall, more research is warranted to investigate this possibility.

As synthetic and chemical products are shown to have potential downsides, natural alternatives for food preservation and antibacterial medicine become more necessary (Rani et al., 2013). Moreover, the cost of these products can be prohibitive. More importantly, microbial resistance to antibiotics has been an ongoing problem and warrants the need for practical solutions. The findings of these studies could provide valuable means for the potential development of natural alternatives to synthetic pharmaceutical products.

*Antiviral activity against SARS-CoV-2 PsV:* The antiviral activity of the extracts against SARS-CoV-2 PsV showed significant variations (Table 3). The PB1, PB2, and PB3 extracts have the best selectivity with TI values above 8. Because of the nature of the pseudoviral assay, using pseudoviral particles that only contain the original viral spike proteins and lack any other SARS-CoV-2 structural component, we can conclude that extracts PB1, PB2, and PB3 interfere with SARS-CoV-2 adsorption or entry to host cells.

Table 3. Antiviral activity of *Piper* extracts against SARS-CoV-2 PsV. *Piper nigrum* (PN1) McCormick, *P. guineense* (PG1) Nigeria World Food Store Company, (PG2) Darmol African Market and (PG3) Foodsby Testimony, *P. borbonense* (PB1) Sama, (PB2) Pili Pili Dock -Madepices, and (PB3) Floribis.

| *Extract Samples* | *Half maximal concentrations (mg/mL)* | | *Therapeutic Index (TI)* |
|---|---|---|---|
| | $CC_{50}$ | $EC_{50}$ | |
| *PN1* | >8 | >8 | - |
| *PG1* | 1.5 | 3.1 | 0.5 |
| *PG2* | 2.9 | 1.9 | 1.5 |
| *PG3* | >8 | 3.7 | >2.1 |
| *PB1* | 12.7 | 1.4 | 9.1 |
| *PB2* | 10.4 | 0.7 | 14.9 |
| *PB3* | 10.7 | 1.3 | 8.2 |

The diverse species of the family *Pipereceae* have shown antiviral properties associated with bioactive compounds such as alkaloids, phenolics, flavonoids, and essential oils. These compounds have shown activity against both DNA and RNA viruses, including herpes simplex virus (HSV) (Nakamura et al., 2022), influenza virus (Mohammed et al., 2020), and dengue virus (Nag and Chowdhury, 2020). The antiviral activity of *Piper* spp. is attributed to several mechanisms, including interference with viral attachment and entry into host cells and modulation of host immune responses against viral infections.

A recent study exploring the molecular docking of secondary metabolites from *Piper nigrum* L. has shown a potential interference with SARS-CoV-2 spike host cell receptor interactions (De Jesus, 2020). Additionally, Alves Borges Leal et al. (2023) have proposed that essential oils from *Piper cernuum* Vell and *Piper rivinoides* Kunth, are potent inhibitors of the heterodimer methyltransferase complex NSP16-NSP10 SARS COV-2 protein.

*Caco-2 cells bioassay*: The assessment of *Piper* extract cytotoxicity on Caco-2 cells was carried out as a precursor to follow-up studies examining the potential anti-inflammatory properties of these extracts. The XTT assay was used to measure the redox potential and, therefore, metabolic health of the cells based on the reduction of a tetrazolium-based substrate. Treatment of Caco-2 cells with 8mg/ml of *P. nigrum* extract resulted in no change in redox activity/cell viability relative to the control condition. Exposure to *P. guineense* resulted in a reduction in redox activity/cell viability between 12-52% while *P. borbonense* treatments reduced viability between 42-65%. For all extracts tested, treatment with 1 mg/ml resulted in an increase of redox activity/cell viability at similar levels relative to untreated Caco-2 cells; an increase of 71% with *P. nigrum*, 51-70% with *P. guineense,* and 29-50% *P. borbonense.*

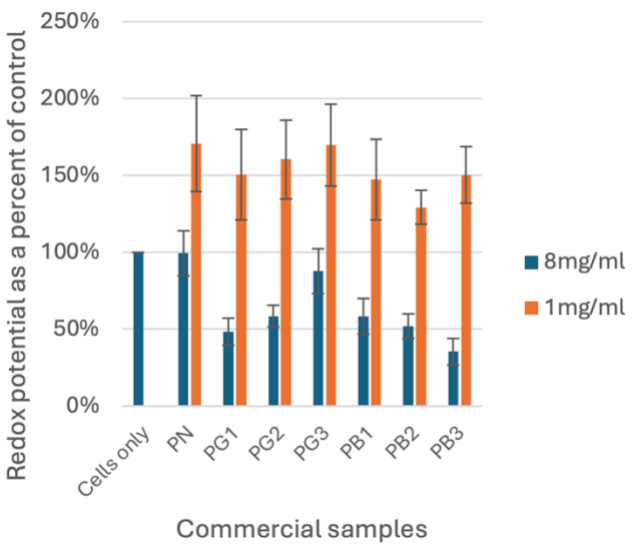


Figure 4. Effects of *Piper* extracts on Caco-2 cells viability as assessed with XTT viability assay. *Piper nigrum* (PN1) McCormick, *P. guineense* (PG1) Nigeria World Food Store Company, (PG2) Darmol African Market and (PG3) Foodsby Testimony, *P. borbonense* (PB1) Sama, (PB2) Pili Pili Dock -Madepices, and (PB3) Floribis. The data shows the average percentage of control (cells + 10% FCS-EMEM) ± Standard Error (SE) of 3 independent experiments.

*Cytotoxic effects:* An additional way to assess the cytotoxic effects of the extracts is to examine the release of lactate dehydrogenase (LDH) into the

culture medium, which would indicate damage to the cell membrane. Caco-2 cells were exposed to extract concentrations shown to be cytotoxic in previous studies examining *Piper* species (1000-0.1 µg/ml range) (Salehi et al. 2019). Cytotoxicity percentage was calculated using the suggested formula from the kit manufacturer, where control cells (no treatment) are set to 0% and maximum LDH release (lysed cells) is set to 100%. None of the *Piper* spp. extracts showed a typical dose response when examining cytotoxicity via LDH release. When treated with *P. nigrum* extract, Caco-2 cells showed a cytotoxicity percentage ranging from 1-8% above control samples (data not shown). Extracts from *P. guineense* resulted in Caco-2 cells releasing less LDH (indicating less cell damage) compared to control cells (negative percentage values) or showing a slight increase in LDH released but never more than an 8% increase when compared to control samples. Treatment of Caco-2 cells with *P. borbonense* resulted in cytotoxicity percentages ranging from 33% less than the control samples (PB2 at 1,000µg/ml) to 16% more than control samples (PB1 at 0.1µg/ml).

Further examination of the potential cytotoxic effects of the *Piper* extracts on Caco-2 cells reveals a dose response in cell proliferation/metabolic activity, which was assessed via XTT assay. A high concentration of extracts (8 mg/ml) showed a 42-65% reduction in activity in 5 of the seven treatments (PG1, PG2, all PB samples). When reducing the extract concentration to 1 mg/ml, there was an increase in cell proliferation/metabolic activity relative to untreated controls, with PN increasing by 71%, PG samples by 51-70%, and PB samples by 29-50%.

Another metric related to cytotoxicity examined the release of LDH from Caco-2 cells, indicating compromised membrane integrity, treated with the *Piper* extracts. For these studies, the extract concentrations tested was decreased to 1mg/ml and below to examine the anti-inflammatory effects of these extracts. Working in potentially cytotoxic ranges would not be suitable. The exposure of Caco-2 cells to extract concentrations from 0.1 µg/ml to 1,000 µg/ml did not show any appreciable increase in LDH release relative to untreated control cells, suggesting that utilizing this range of concentrations for future studies would be suitable. A preliminary survey indicated that a pro-inflammatory response where interleukin-1 (IL-1) stimulates interleukin-8 (IL-8) secretion in Caco-2 cells is reduced dose-dependent with the various PG extracts, however, not with the PN extract. Follow-up studies are needed to understand the biological activities of these extracts further.

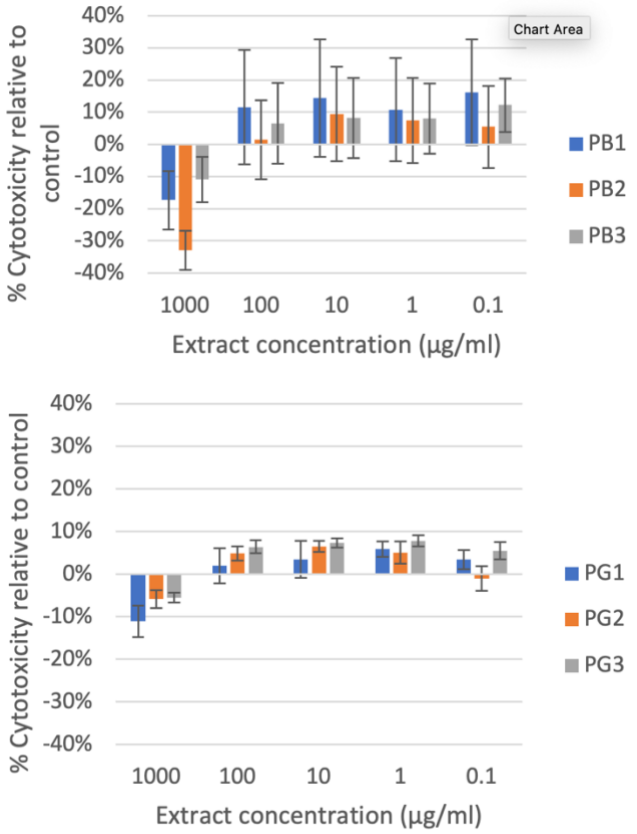


Figure 5. Cytotoxic effects (as percentage) relative to control in *Piper borbonense* (PB1) Sama, (PB2) Pili Pili Dock - Madepices, and (PB3) Floribis (TOP), *Piper guineense* (PG1) Nigeria World Food Store Company, (PG2) Darmol African Market and (PG3) Foodsby Testimony (BOTTOM) extracts on Caco-2 cells as assessed with LDH (lactate dehydrogenase) cytotoxicity assay. The data shows the average percentage of control (cells + 10% FCS-EMEM) ± SE of 3-4 independent experiments.

Prior work by others examining the cytotoxic nature of *Piper* extracts ranges from work involving whole extracts of various plant parts (leaves, fruits/seeds) with different solvents to studies

examining various compound groups of these extracts (essential oil, alkaloids) down to individual compounds (β- Caryophyllene, Phellandrene, piperine, α-pinene, β-pinene) (Salehi et al. 2019). Several studies have examined the cytotoxic impact of *Piper* species on various colon cancer cell lines, and they have shown differing results compared to the current study. Dichloromethane extracts of *P. guineense* showed $IC_{50}$ values for various cancerous cell lines ranging from 3.62-22.96 µg/ml for leaf extracts and 28.16-56.65 µg/ml for seed extracts. Specifically for the colon cancer cell line SW480, the $IC_{50}$ values were highest at 22.96 µg/ml for the leaf extract and 56.65 µg/ml for the seed extracts (Iweala et al. 2015). Ethanolic extracts of *P. nigrum* fruits were shown to have an $EC_{50}$ value of 80.5 µg/ml for cell viability of HT-29 colon carcinoma cells when assessed by MTT assay (Grinevicius et al. 2016). Ethanolic extracts of *P. longum* (50-500 µg/ml) showed selective induction of cell death in colon cancer cell lines (HT-29 and HCT-116) while not inducing apoptosis in a non-cancerous colonic cell line (NCM460) (Ovadje et al. 2014). Essential oil extracts from *Piper aequale*, which contained high levels of α- and β-pinene like our extracts, showed strong cytotoxic activity against HCT-116 colon cancer cell line, with an $IC_{50}$ value at 8.7 µg/ml (DaSilva et al. 2016). These studies show that the *Piper* species extracts examined show cytotoxic activity against colon cancer cells at concentrations of <100 µg/ml which was not observed in our study. This can be explained easiest by the vast diversity of *Piper* species chemotypes and extraction methods employed.

## CONCLUSIONS

The genus *Piper*, which has an extensive record of uses in traditional medicine, has demonstrated various biological effects that support its traditional uses. *Piper guineense* and *P. borbonense* are sources of natural antioxidants that could be used to protect against diseases involving reactive oxygen species. Also, each of these species *P. guineense* and *P. borbonense* exhibited significant inhibition of the gram-positive *B. subtilis* bacteria and could be useful in food conservation. *Piper borbonense* exhibited antiviral activity against SARS-CoV-2PsV with the highest Therapeutic index (above 8), suggesting it may be useful in further studies to determine its potential utility as a therapeutic agent for treating virus diseases.

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