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Antioxidant, antibacterial, and anti-SARS-CoV Activity of Commercial Products of *Xylopia (Xylopia aethiopica)*

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ABSTRACT

***Xylopia aethiopica* (Annonaceae) is a spice and medicinal plant that grows wild in many West African countries (from Liberia to Nigeria) and is locally known as Guinea pepper, grains of Selim, hwentia and uda. The dried fruits are used as a flavoring for soups and traditionally in decoctions as an analgesic and anti-inflammatory, as well as a treatment for infections. The medicinal properties of the fruits are associated with the presence of phenolics and essential oil constituents. We studied the total phenols, total flavonoids, and antioxidant activity in different *X. aethiopica* extracts using spectrophotometry. We found variation in total phenolic and flavonoids and antioxidant capacity between different samples and different extraction solvents. Antibacterial activity against *Escherichia coli* and *Bacillus subtilis* were evaluated using a spectrophotometric assay and Kirby-Bauer test. Additionally, a pseudoviral cell-based assay was used to test the antiviral activity against severe acute respiratory syndrome coronaviruses (SARS-CoV-1 and SARS-CoV-2). High total phenolic and flavonoid content was correlated with high antioxidant capacity. Results of antibacterial tests indicated that one *Xylopia* extract potentially has strong antibacterial activity against gram-positive bacteria *B. subtilis.* The pseudoviral assay showed moderate antiviral activity against SARS-CoV-1 and SARS-CoV-2.**

INTRODUCTION

For centuries, African medicinal plants have been used to treat a wide variety of human diseases. Still, to this date, much of the flora have yet to be explored and therefore might be a significant source of novel compounds to treat different pathologies (Iwu, 2014; Adodo and Iwu, 2020). Free radicals and other reactive oxygen species (ROS) are constantly formed in the human body. Free-radical mechanisms have been implicated in the pathology of several human diseases (Aruoma, 1998). Overproduction of ROS can cause tissue injury leading to many chronic diseases (Aruoma, 1998; Betteridge, 2000; Liguori et al., 2018) and aging (Finkel and Holbrook, 2000). Antioxidants act to decrease cell degeneration and death by stabilizing free radicals (which have unpaired electrons), thereby preventing them from reacting with cell membranes and causing damage to proteins, lipids, nucleic acids, carbohydrates, and fatty acids (Gülçin, 2012). Health benefits of medicinal plants have been associated in part with their low toxicity and to the potential effects of their antioxidants on ROS in the body (Forni et al., 2019). Medicinal plants have also been widely studied as

sources of potential antimicrobial agents (Cowan, 1999; Chandra and Kaneria, 2011). Due to the rapid rise in antimicrobial resistance, there is an urgency to renew the arsenal of antimicrobial agents to fight viral and bacterial infections (Chandra et al., 2017). The growing concerns about resistance against conventional antimicrobial agents make the study of potential new antimicrobial agents a priority.

Coronaviruses are a group of RNA viruses that infect various hosts, including humans (Zhu et al., 2020). In 2002, 2012, and 2019, three highly pathogenic viruses SARS-CoV-1, Middle East Respiratory Syndrome Coronavirus (MERS-CoV), and SARS-CoV-2 emerged and caused severe respiratory illness in humans (Zhu et al., 2020). SARS-CoV-2 is a highly contagious virus that emerged in Wuhan, China toward the end of 2019. This virus is responsible for the current pandemic of acute respiratory disease known as Coronavirus diseases 2019 (COVID-19). The COVID-19 pandemic has resulted in more than 107 million infections and over 2.3 million deaths (WHO, 2021). There is an urgent need to identify compounds with selective antiviral activity against SARS-CoV-2 that could be used to prevent and/or treat these infections.

Xylopia aethiopica (Dunal) A. Rich (Annonaceae), is a tree that grows in the forests of tropical and subtropical Africa. It has been introduced into cultivation in many countries, including Ghana (Erhirhie and Moke, 2014; Iwu, 2014; Fetse et al., 2016). The fruits of *X. aethiopica* are used widely in West and Central Africa as both a spice to flavor food and drinks and as traditional medicine (Ayedoun et al., 1996; Fetse et al., 2016, Adodo and Iwu, 2020). Extracts are often obtained through decoction of the dried fruits and used to treat different respiratory, digestive, and inflammatory illnesses and infections, including dysentery and malaria (Fetse et al., 2016). Traditionally, *X. aethiopica* extracts have been used to treat different medical conditions, such as asthma, arthritis, diabetes, and immunological disorders, and linked to oxidative stress due to an imbalance between reactive oxygen species and antioxidant defenses (Sindhi et al., 2013; Iwu 2014). Also, *Xylopia* is used in traditional medicine for managing various ailments including skin infections, candidiasis, dyspepsia, cough, and fever (Burkhill, 1985). Previous studies have investigated the antioxidant potential of *X. aethiopica* extracts as food preservatives and the impact of these extracts on diabetes, radiation, lipids and cholesterol in rats, inflammation in mice, and pain in various animal models (Mohammed and Shahidul, 2017). Many traditional medicinal plants like *X. aethiopica* are chosen for their fruits, which are viewed as beneficial both as flavoring agents and as natural pharmaceuticals (Li et al., 2013).

The aim of this study was: (1) to determine the total phenolic and flavonoid content in five commercial products of *X. aethiopica* fruits; (2) to assess the antioxidant capacity and potential antibacterial properties of their extracts; and (3) explore the potential selective antiviral activity of *Xylopia* extracts against Severe Acute Respiratory Syndrome Coronaviruses 1 and 2 (SARS-CoV-1 and SAR-CoV-2).

MATERIALS AND METHODS

Plant Materials: Five commercial products of dried *Xylopia aethiopica* fruits from Ghana were obtained from the following companies: Goldleaf Hydroponics, Bloomington, IN (#1); Ghana, (local farmers) Accra, Ghana (#2); Hwenteea-Flobico, Accra, Ghana (#3); Real Taste Foods UDA, Basmalah Africa, GA, (two different samples from the latter source were tested under the codes UDA-A (#4) and UDA-B (#5)).

Extraction: Twenty mg of powdered material were dissolved in 1 mL of: i) 80-50% Ethanol (Sigma Aldrich, St Louis, MO), ii) Dimethyl Sulfoxide (DMSO, Sigma Aldrich), or iii) distilled H_2O. The samples were sonicated for 5 seconds at 30% intensity using a Bransonic® Ultrasonic bath. Plant extracts were then filtered through a 0.45 µm syringe filter (Thermo Fisher Scientific, Waltham, MA) and stored at 4°C. Distilled water solutions were placed in a boiling water bath for 10- or 30-minutes decoction. Samples were allowed to cool and then centrifuged for 5 minutes. The supernatant was then filtered as above and stored at 4°C.

Total phenolic content: Total phenolic content was determined using a modified Folin-Ciocalteu's protocol (Gao et al., 2000). Absorbance was measured at 760 nm on a Biotek Synergy 4 Spectrophotometer (BioTek Instruments, Winooski, VT) using gallic acid as a standard. Total phenolic content (TPC) was expressed as mg gallic acid equivalents/mL plant extract (GAE mg/mL).

Total flavonoids content: Total flavonoids were extracted with 0.5M $NaNO_2$ (Sigma Aldrich), 0.3M $AlCl_3 \cdot 6H_2O$ (Sigma Aldrich), and 1M NaOH (Sigma Aldrich), then measured at 506 nm according to Saeed et al., (2012). The standard curve for total flavonoids was made using rutin standard solution. Results were expressed as mg of rutin equivalents/mL plant extract (RAE mg/mL).

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 $ABTS^{\bullet+}$ radical cation. The $ABTS^{\bullet+}$ 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 an absorbance at 734 nm reached 0.7± 0.05. Results are expressed as mg Trolox equivalents/mL plant extract (TEAC mg/mL).

2,2- Diphenyl-1-picrylhydrazyl (DPPH) assay: Radical scavenging activity of plant extracts against stable $DPPH^{\bullet}$ (2,2-Diphenyl-1-picrylhydrazyl hydrate) was determined spectrophotometrically (Brand-Williams et al., 1995). When $DPPH^{\bullet}$ reacts with an antioxidant compound, it is reduced. The changes in color were measured using a spectrophotometer at 517 nm. The results were expressed in percentage (%) of DPPH scavenging activity. The correlation between antioxidant capacity and total phenolic content was analyzed using a linear regression, and the correlation coefficient (R^2) was calculated.

Liquid Chromatography: A reversed phase high performance liquid chromatographic (RP-HPLC) method was used for determination of chlorogenic acid, caffeic acid, ferulic acid, and gallic acid. Liquid chromatographic separation was achieved using an Agilent 1200 HPLC system and a Phenomenex® Prodigy™ 4.6X 250mm 5µm (part number: H19-316755) column. The mobile phase solvent consisted of (A) 0.1% TFA and (B) methanol, and the flow rate was maintained at 1 mL/minute, with operating temperature of 40 °C, and detection wavelength of 280 nm. Empower[3] software was used to control all parameters of HPLC.

Gas Chromatography: The chemical composition of the essential oils from different commercial samples were determined following a modification of the method develop by Hwang et al. (2020), using a gas chromatograph (GC-2010 Plus) coupled to a mass spectrometer (MS) (Gas Chromatograph Mass Spectrometer GCMS-TQ8040 System) equipped with Solid Phase Micro Extraction (SPME) automatic injector (Instrument from Shimadzu Corporation). The SPME fiber (Polydimethylsiloxane, PDMS, thickness 30 micrometers) was allowed to extract volatiles for 5 mins at room temperature, no agitation, with a desorption time of 2 mins at 220°C (Inlet temperature).

Preparation of media for bacterial cultures: For bacterial experiments, 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 10x concentration.

Antibacterial activity using disc-diffusion method: Antibacterial activity of *Xylopia* extract #1 (Goldleaf Hydroponics, Bloomington, IN) against *E. coli* and *B. subtilis* was tested using the Kirby-Bauer disc-diffusion method (Biemer, 1973). To prepare *E. coli* and *B. subtilis* lawns, an individual colony from a stock nutrient agar plate was used to inoculate an overnight LB broth culture. Liquid cultures were incubated overnight at 37 °C and 150 rpm in MaxQ 4450 orbital shaker (Thermofisher Scientific, Waltham, MA). Freshly grown bacteria (100-200 µL) were plated onto nutrient agar and spread using a Bel-Art™ SP Scienceware bacterial cell spreader (Thermofisher Scientific). Discs were applied and lawns were incubated at 37 °C overnight. Zones of inhibition were measured in mm. Antibiotic positive control discs (Carolina Biological Supply Co.) contained neomycin (30 µg). LB broth was used to dilute the *Xylopia* extract for disc preparation. *Xylopia* discs were prepared by applying 20 µL of *Xylopia* extract #1 (Goldleaf Hydroponics extracted with water) to a 6 mm GE Healthcare Whatman™ Antibiotic Assay Disc (Thermofisher Scientific). Discs were air dried prior to use.

Antibacterial activity in liquid culture: LB/*Xylopi*a media was prepared by mixing one part sterile 10x LB broth with nine parts sterile *Xylopia* extract #1 (Goldleaf Hydroponics prepared with water). LB control media was prepared by mixing one part sterile 10x LB broth with nine 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 (Thermofisher Scientific). For each bacterium, 400 µL of either LB or LB/*Xylopia* media were inoculated with 4 µL of overnight culture (for a density of 10^5 cells/ mL) or with 4 µL of 1x LB broth. Each 400 µL inoculum was divided equally into two wells of a Corning 96-well flat-bottom tissue culture plate (Thermofisher Scientific). Blank wells were set up containing 200 µL uninoculated LB broth and 200 µL uninoculated LB/*Xylopia* media. Microplates were incubated at 37 °C and 150 rpm in a MaxQ 4450 orbital shaker (Thermofisher Scientific). Optical density (OD) was measured at 600 nm every hour for 7 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: SARS-CoV-1 and SARS-CoV-2 pseudoviruses (PsV) were generated using the procedure described by Robbiani et al., 2020. pSARS-CoV1-Strunc, pSARS-CoV2-Strunc, pCRV1NHG GagPol and pNanoLuc2AEGFP plasmids were kindly provided by Dr. Theodora Hatziioannou and Dr. Paul Bieniasz (Rockefeller University). 293T cells (ATCC, Manassas, VA) were transfected with pCRV1NHG GagPol, pNanoLuc2AEGFP and pSARS-CoV1-Strunc or pSARS-CoV2-Strunc. Six hours after transfection, the cells were gently washed twice with D-PBS (ThermoFisher Scientific, Waltham, MA) and fresh DMEM (ThermoFisher Scientific) with 10% FBS (ThermoFisher Scientific) and Penicillin+Streptomycin (ThermoFisher Scientific) was added. The supernatants containing SARS-CoV-1 PsV or SARS-CoV-2 PsV were collected 48 h after transfection, filtered through a 0.22 µm pore size PVDF filter, aliquoted and stored at −80 °C. The "infectivity" of SARS-CoV-1 PsV and SARS-CoV-2 PsV was determined by titration on HeLa ACE2 cells using the TurboLuc™ Luciferase One-Step Glow Assay Kit as described by the manufacturer (ThermoFisher Scientific). This assay allowed us to estimate luciferase activity in those cells in which the pseudoviruses successfully delivered the reporter genome and therefore the enzyme was expressed.

To determine selective antiviral activity against SARS-CoV-1 and SARS-CoV-2, we first evaluated the cytotoxicity of *Xylopia aethiopica* extracts Goldleaf Hydroponics (#1); Ghana (#2), Hwenteea-Flobico (#3), Real Taste Foods UDA-A (#4) and UDA-B (#5) as well as caffeic acid and chlorogenic acid. A series of dilutions of each extract, caffeic acid and chlorogenic acid were added in triplicate to HeLa ACE-2 cells seeded in clear bottom 96-well microplates. Tween 20 (Sigma Aldrich) was used as a positive control for cytotoxicity. The plates were incubated at 37ºC, 5% CO_2, and 98% humidity for 72 h, at which time the XTT colorimetric assay was performed, following the procedure described by Melo et.al., 2020. The XTT assay allowed us to estimate cell viability and obtain the half maximal cytotoxic concentration (CC_{50}) for each extract and compound tested.

The TurboLuc One Step assay was used to test the antiviral activity of *Xylopia aethiopica* extracts Goldleaf Hydroponics (#1); Ghana (#2), Hwenteea-Flobico (#3), Real Taste Foods UDA-A (#4) and UDA-B (#5), caffeic acid and chlorogenic acid against SARS-CoV-1 PsV and SARS-CoV-2 PsV. We also evaluated the combination of equipotential concentrations of caffeic acid and chlorogenic acid. The same dilutions of extracts or compounds tested in the XTT assay were preincubated with SARS-CoV-1 PsV or SARS-CoV-2 PsV at 37ºC, 5% CO_2 and 98% humidity for 30 minutes. We also included a virus control consisting in cell culture medium and pseudovirus only. After this brief incubation, all dilutions and controls were transferred to 96-well plates containing HeLa ACE2 cells. All extracts and compounds dilutions, as well as all controls, were tested in triplicates. The plates were incubated at 37ºC, 5% CO_2, and 98% humidity for 72 h, at which time the TurboLuc™ Luciferase One-Step Glow Assay was performed. The assay estimated the percentage of pseudoviral entry at each extract or compound dilution versus the virus control. This procedure allowed us to estimate the half maximal effective concentration (EC_{50}) of the extracts or compounds against each pseudovirus.

The raw data obtained in the XTT and TurboLuc One Step assays was transferred to GraphPad Prism software version 9.0.0 (San Diego, CA) to perform the data analysis and obtain the CC_{50} and EC_{50} values. The therapeutic index (TI) was calculated using the CC_{50}/EC_{50} ratio. The percentage of pseudoviral entry inhibition was also used to analyze the effect of the combination of caffeic acid and chlorogenic acid. For this purpose, CalcuSyn software (Biosoft, Cambridge) was used to estimate the combination index (CI) values (Chou, 2006).

Statistical Analyses: The Friedman test was used for overall comparison between solvents for the extraction of phenolic compounds and pairwise comparisons were performed using Dunns test ($p<0.05$). Correlation between total phenolics and antioxidant activity were calculated using GraphPad Prism Software Version 9.0.0.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content. High variation of total phenolic content (TPC) was observed among the samples and the solvent used (Figure 1). Boiling water consistently extracted the highest amount of phenolics in all the samples while ethanol solutions extracted the least amounts. Different commercial samples accumulated different amount of phenolics. Samples UDA-A and UDA-B showed the highest amount of phenolics (12.802 GAE mg/g DW) while Ghana showed the least amount (7.129 GAE mg/g DW). Total flavonoids content (TFC) in the extracts varied widely between samples and solvents used (Figure 1B). DMSO extracted the highest amounts of flavonoids, and ethanol solvent the least. The *Xylopia* samples from Real Taste Foods UDA-A and UDA-B were significantly higher values for other samples and the distilled water decoctions were again the most effective extraction method for flavonoids.

Antioxidant capacity. The most common and reliable methods involve the spectrophotometric determination of the disappearance of free radicals $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. $ABTS^{\bullet+}$ radical was generated by reaction between ABTS and potassium persulfate, which has a blue/green color. The antioxidant capacity (TEAC) was measured as the ability of plant extracts to decrease the color reacting directly with the $ABTS^{\bullet+}$radical. The degree of color change is proportional to the concentration of antioxidants. Highest antioxidant capacity was observed in the Real Taste Foods UDA-A sample extracted with the distilled water boiled for 10 minutes (15.415 TEAC mg/g DW). There was also great variation between the samples and the solvents, with Real Taste Foods UDA-A and UDA-B samples consistently exhibiting higher values (Figure 1C). The extraction method with the highest TEAC values were the distilled water solutions treated in boiling water baths of 10 and 30 minutes duration.

In addition, the antioxidant capacity was evaluated using DPPH radical. DPPH radical is a stable organic free radical that has a deep purple color. The assay is based on measurement of the reducing ability of the antioxidant toward $DPPH^{\bullet}$.

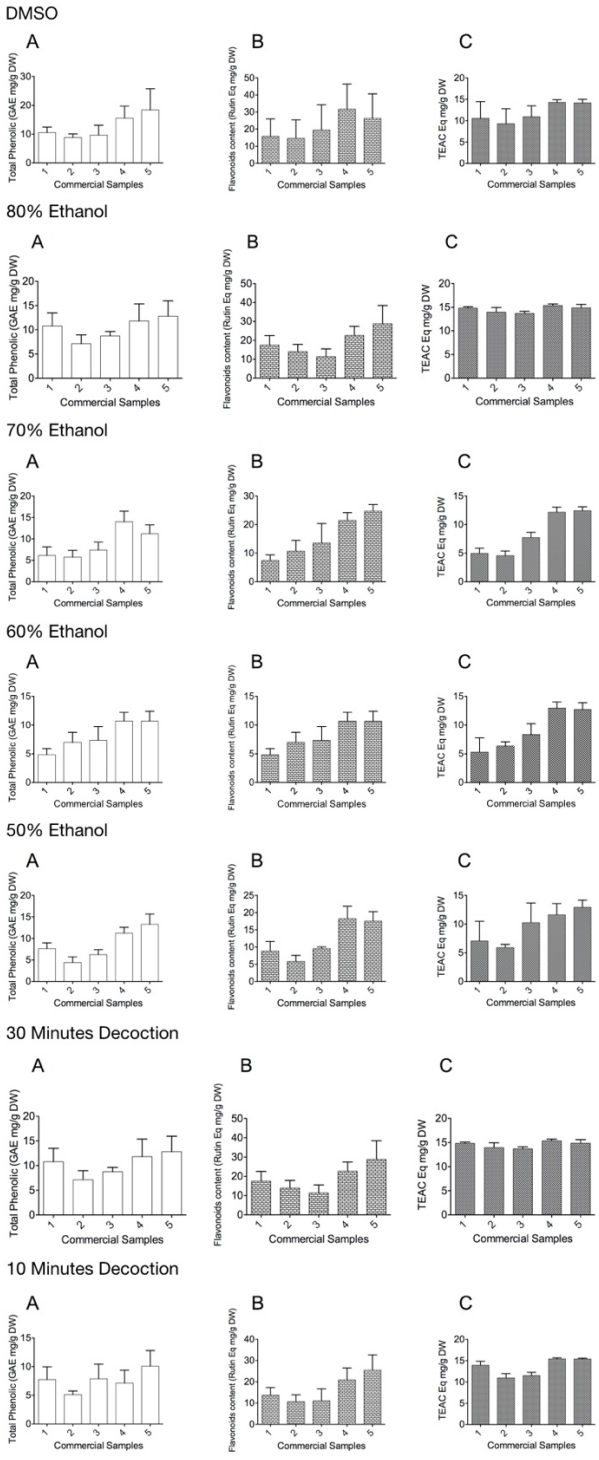

Figure 1. Mean values for the total phenolic (A) and flavonoid (B) content and antioxidant activity (C) of *Xylopia aethiopica* extracts: Goldleaf Hydroponics (1), Ghana (2), Hwenteea-Flobico (3), Real Taste Foods UDA-A(4) and UDA -B (5) Bars indicate the mean value ± SE of three independent experiments.

No significant differences in antioxidant capacity were observed among the samples or solvent extractions used in the DPPH assay (Figure 2). These data suggest that the ABTS assay might be more useful than the DPPH assay for detecting antioxidant capacity in a variety of foods (Floegel et al., 2011; Melo et al., 2020).

Variation among commercial samples on total phenols, flavonoids, and antioxidants shows the same pattern and could be associated with different growing conditions, processing or storage. The correlation between TPC and TFC (R^2= 0.6506) indicates that flavonoids are an important component of the phenolics and the correlation between TEAC and TPC (R^2= 0.4964) was lower. The poor correlation between TEAC and TPC suggests that, in addition to the phenolics, other molecules that are also contributing to the antioxidant capacity.

Liquid Chromatography: Using extracts UDA-A (#4) and UDA-B (#5), HPLC was applied to identify the presence of phenolic compounds that could be associated and possibly responsible for the moderate antiviral activity observed against SARS- CoV-1 and SARS-CoV-2 PsVs. No gallic acid was found after inspecting the chromatograms; however, phenolic compounds, including chlorogenic acid and caffeic acid, were detected in extracts UDA-A (#4) and UDA-B (#5). Since all extract samples were heated during preparation, we suspect that by-products like isomers for either caffeic acid or chlorogenic acid were formed and produced peaks eluding at RT 13.2 and 17.0 minutes, respectively. Furthermore, a phenolic degradant, protocatechuic aldehyde (RT 15.7min), was formed either from caffeic acid or chlorogenic acid (Li et al., 2004) and was present in both UDA-A (#4) and UDA-B (#5).

Gas Chromatography: Four components having relative percentages of the total aromatic volatiles higher than 5%, dominated the profile of the essential oils of each of the *Xylopia* samples (Table 1). The main aromatic volatiles included β-pinene (39.4-20.9%), α-pinene (23.1-12.6%), sabinene (24.8-5.9%), and 1,8 cineole (8.1-14.2%). Based on the essential oil profile, the essential oils can be organized into three groups. The first one, Goldleaf Hydroponics (#1) and Ghana (#2), was characterized by the highest levels of β-pinene (39.4-37.8%) and α-pinene (23.1-22.7%). The second group, Hwenteea-Flobico (#3), with lower levels of β-pinene (35.6%) and α-pinene (18.2%), and higher levels of sabinene (14%) and 1,8 cineole (10.7%). And, the third group, Real Taste Foods UDA (1, and 2), with the lowest levels of α-pinene (12.6-13.4%) and β-pinene (20.9-23%), and highest levels of sabinene (24.8-24.7%).

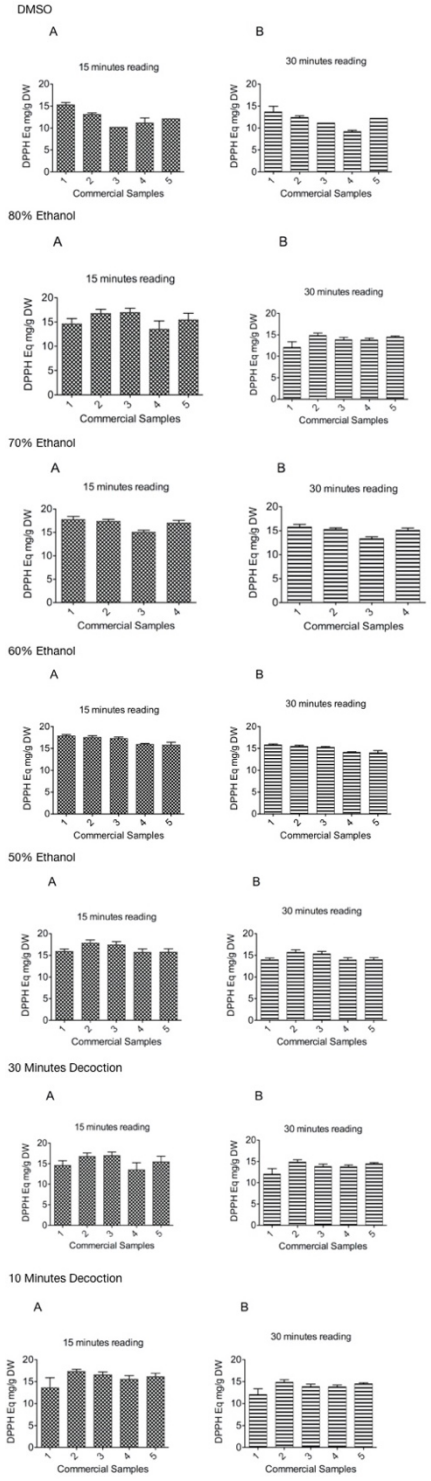

Figure 2. Total antioxidant capacity of *Xylopia aethiopica* extracts (20 mg/mL) determined by DPPH assay. Goldleaf Hydroponics (1), Ghana (2), Hwenteea-Flobico (3), Real Taste Foods UDA-A (4) and UDA -B (5). Bars indicate the mean value ± SE of three independent experiments.

Table 1. Essential oil composition from the whole fruits from five commercial sources of *Xylopia aethiopica*

RI	Components	Goldleaf Hydroponics		Ghana		Hwenteea-Flobico		Real Taste Foods UDA-A		Real Taste Foods UDA-B	
900	Heptanal	0.1	± 0.0[1]	0		0.03	± 0.0	0		0	
911	α-Thujene	1.9	± 0.0	2.9	± 0.1	1.7	± 0.0	4.	± 0.0	2.7	± 0.1
922	**α–Pinene[2]**	**23.1**	**± 0.1**	**22.7**	**± 0.2**	**18.2**	**± 0.4**	**12.6**	**± 0.2**	**13.4**	**± 0.2**
943	Camphene	0.8	± 0.0	0.9	± 0.0	0.5	± 0.0	0.3	± 0.0	0.4	± 0.0
948	Verbenene	0.3	± 0.0	0.4	± 0.0	0.1	± 0.0	0		0	
971	**Sabinene**	**6.2**	**± 0.1**	**5.9**	**± 0.1**	**14.0**	**± 0.1**	**24.8**	**± 0.7**	**24.7**	**± 0.2**
978	**β-Pinene**	**39.4**	**± 0.0**	**37.8**	**± 0.4**	**35.6**	**± 0.4**	**20.9**	**± 0.3**	**23.0**	**± 0.2**
991	Myrcene	1.8	± 0.0	2.2	± 0.0	2.8	± 0.0	0.6	± 0.0	0.1	± 0.0
996	Menthadiene	0.9	± 0.1	0.9	± 0.0	2.5	± 0.0	0.4	± 0.0	0.02	± 0.0
1006	α - Phellandrene	0		0		0.1	± 0.0	0.2	± 0.0	0.1	± 0.0
1020	α - Terpinene	0		0		0.2	± 0.0	0.7	± 0.0	0.6	± 0.0
1028	para Cymene	1.7	± 0.1	3.2	± 0.0	1.8	± 0.0	1.7	± 0.0	1.4	± 0.0
1034	Limonene	1.2	± 0.0	1.9	± 0.0	0		0		0	
1038	**1,8 Cineole**	**8.1**	**± 0.3**	**8.7**	**± 0.0**	**10.7**	**± 0.1**	**14.4**	**± 0.3**	**14.2**	**± 0.1**
1063	γ - Terpinene	0.1	± 0.0	0.3	± 0.0	0.9	± 0.0	2.2	± 0.0	2.4	± 0.0
1076	cis Sabinene hydrate	0.5	± 0.0	0.4	± 0.0	0.4	± 0.0	1.0	± 0.0	1.2	± 0.0
1085	cis Linalool oxide	0.1	± 0.0	0.1	± 0.0	0		0		0	
1093	Linalool dehydro	0.1	± 0.0	0.1	± 0.0	0.2	± 0.0	0.4	± 0.0	0.4	± 0.0
1100	Linalool	0		0		0.07	± 0.0	0.8	± 0.0	0.6	± 0.0
1120	Chrysanthenone	0.2	± 0.0	0.1	± 0.0	0.1	± 0.0	0		0	
1126	Limonene oxide	0		0.04	± 0.0	0		0		0	
1130	α - campholenal	0.1	± 0.0	0.1	± 0.0	0.2	± 0.0	0.6	± 0.0	0.2	± 0.0
1154	trans Sabinol	2.7	± 0.1	2.2	± 0.1	1.1	± 0.0	0.1	± 0.0	0.2	± 0.0
1175	Pinocarvone	1.8	± 0.1	1.2	± 0.0	0.7	± 0.0	0.06	± 0.0	0.1	± 0.0
1198	Myrtenol	0.1	± 0.0	0.05	± 0.0	0.04	± 0.0	0.3	± 0.0	0.3	± 0.0
1209	Myrtenol	2.8	± 0.0	2	± 0.1	1.2	± 0.1	0.1	± 0.0	0.2	± 0.0
1349	α – Cubebene	0.6	± 0.0	1.1	± 0.1	1.1	± 0.1	3	± 0.2	2.3	± 0.0
1362	α - Copaene	0		0.2	± 0.0	0.1	± 0.0	0.1	± 0.	0.1	± 0.0
1395	β – Cubebene	0.5	± 0.0	2.3	± 0.1	2.1	± 0.1	0.5	± 0.0	0.3	± 0.0
1405	β – Elemene	0.1	± 0.0	0.1	± 0.0	0.3	± 0.1	2.6	± 0.2	2.2	± 0.0
1446	(E) Caryophyllene	1.4	± 0.0	0.1	± 0.0	0.1	± 0.0	1	± 0.1	1	± 0.0
1491	Germacrene D	1.9	± 0.0	0.7	± 0.1	2.4	± 0.1	3.4	± 0.3	4.2	± 0.1
1517	Trans α - Guaiene	0		0		0		0.2	± 0.0	0.2	± 0.0
	Total	**98.0**		**98.3**		**99.1**		**96.9**		**96.5**	

RI, retention indices calculated in a 95% methyl, 5% phenyl silicone column. [1]Mean ± Standard error of three independent replications. [2]Bolded components with relative percentages higher than 5%.

Some of the minor components showed a similar trend to be grouped in clusters. The highest levels of myrcene were found in group two (2.8%), followed by group one (1.8-2.2%), and three (0.6-0.1%). Group one showed high levels of camphene (0.8-0.9%), and verbenene (0.3-0.4%), group two with lower levels (0.5, 0.1%), and group three the lowest levels of camphene (0.3-0.4%), and devoid of verbenene. The monoterpene α-terpinene showed an opposite trend, group one, devoid of it, group two (0.2%), and group three (0.7-0.6%). The sesquiterpene (E)-caryophyllene seemed to be more

variable in group one (1.4-0.1%), 0.1% in group two and 1% in group three. Germacrene D was highest in group three (3.4-4.2%), followed by groups two (2.4%) and one (1.9-0.7%).

Antimicrobial activity: *Xylopia* extracts were tested for antibacterial activity. An initial test showed that only the Goldleaf Hydroponics (#1) extract exhibited significant levels of antimicrobial activity. Thus, a higher concentration of extracts (200 mg DW/mL) was evaluated in further experiments. *E. coli* (gram-negative) and *B. subtilis* (gram-positive) were independently grown in microplate wells in either the presence or absence of Goldleaf Hydroponics (#1) *Xylopia* extracted in distilled water. Bacterial growth was monitored hourly by spectrophotometry for a total of seven hours. Whereas growth of *E. coli* was only slightly suppressed by the presence of the *Xylopia* extract, growth of *B. subtilis* was completely inhibited (Figure 3). Results were consistently observed in three independent trials. Extract in these experiments was at 90% concentration after extraction (180 mg/DW/mL). Additional experiments were performed to determine minimum inhibitory concentration. Results suggest that this variety of *Xylopia* extract can inhibit growth *B. subtilis* at 50% concentration, but not at lower concentrations (data not shown).

In a disc-diffusion assay using the same Goldleaf Hydroponics (#1), *Xylopia* extract did not inhibit *B. subtilis* on agar lawns (data not shown). These results were not consistent with the growth kinetics in liquid culture. It is possible that phytochemicals in *Xylopia* that are active against bacterial growth in liquid culture behave differently in agar media. It should also be considered that whereas growth in culture was monitored for only seven hours, extracts in agar lawns might have lost activity with extended time of incubation.

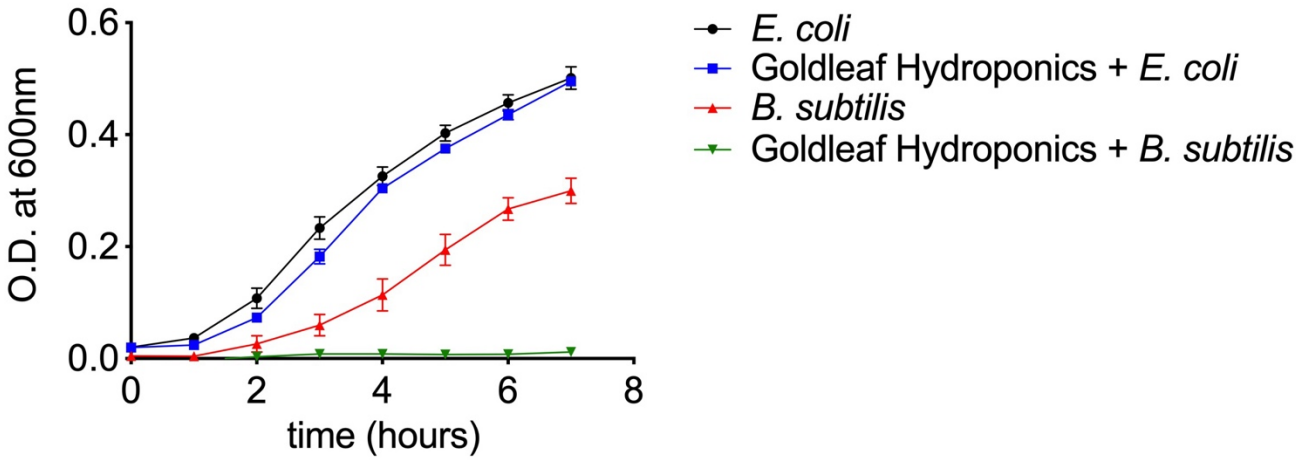

Figure 3. Average growth of *E. coli* and of *B. subtilis* in LB +/- *Xylopia* extract (Goldleaf Hydroponics (#1)). Results shown are the averages of three separate trials of growth from independent cultures. Standard error shown.

Antiviral activity: Using a pseudoviral cell-based assay, we examined the potential antiviral properties of this plant against SARS-CoV-1 and SARS-CoV-2. *Xylopia* extracts UDA-A (sample #4) and UDA-B (sample #5) showed a moderate antiviral activity against the pseudoviruses (Figure 4A and 4C) while other extracts (Goldleaf Hydroponics (#1); Ghana (#2), Hwenteea-Flobico (#3)) didn't show any activity (data not shown). All sample extracts had CC_{50s} higher than 5 mg/mL. The EC_{50} values against SARS-CoV-1 PsV were 3.2 and 1.6 mg/mL for extract UDA-A (#4) and UDA-B (#5), respectively. Additionally, we obtained EC_{50} values against SARS-CoV-2 PsV of 2.8 and 2.7 mg/mL for extracts UDA-A (#4) and UDA-B (#5), respectively. TI values above 10 indicate promising antiviral selectivity. For only UDA products A and B, the TI were calculated and resulted in values between 1.6 and 3.1. These values suggest a low antiviral selectivity.

The detection of chlorogenic acid and caffeic acid in the extracts prompted the evaluation of their antiviral activity against SARS-CoV-1 and 2 using

the pseudoviral system. The antiviral properties of both compounds have been previously reported by different laboratories (Wang et al., 2009; Ikeda et al., 2011; Utsunomiya et al., 2014). Chlorogenic acid, in particular, appears to have a moderate antiviral activity against both pseudoviruses (Figure 4B and 4D) and the combination of both compounds results in additive effect with CI values close to 1 (data not shown). The inhibition by chlorogenic acid is not enough to justify the moderate activity found in the aqueous extracts, suggesting that other compounds in combination with phenols could be responsible for the moderate antiviral activity against SARS-CoV-1 and SARS-CoV-2.

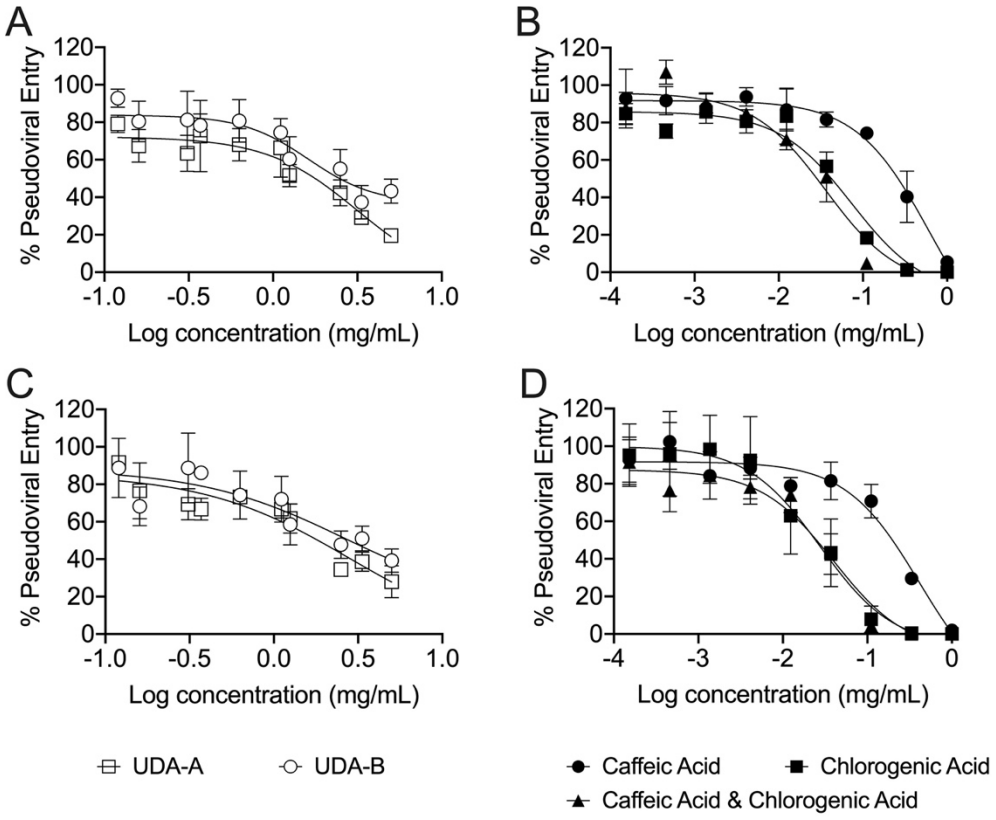

Figure 4. *Xylopia aethiopica* aqueous extracts do not show a strong antiviral activity against SARS-CoV-1 and SARS-CoV-2. Graphs A and C show the activity of extracts UDA-A (#4) and UDA-B (#5) against SARS-CoV-1 and SARS-CoV-2, respectively. Graphs B and D show the activity of extracts caffeic acid, chlorogenic acid, and their combination against SARS-CoV-1 and SARS-CoV-2, respectively. The data show the mean inhibition ± SEM of two independent experiments.

Chlorogenic acid and caffeic acid were detected by HPLC in *Xylopia* extracts. Caffeic Acid (3,4-dihydroxycinnamic) is a phenol that is widely distributed in plants (Magnani et al., 2014; Espíndola et al 2019). This metabolite can be found in coffee drinks, blueberries, apples, wine, and ciders (Magnani et al., 2014). Caffeic acid is widely known for its antioxidant and antibacterial activity *in vitro*. Also, *in vitro* studies have shown anticarcinogenic activity (Magnani et al., 2014; Espíndola et al., 2019). Chlorogenic acid is the ester of caffeic acid and quinic acid and is found in higher plants. This phenol has been reported to exhibit antiviral, antioxidant, anticarcinogenic activity in vitro (Gil and Wianowska, 2017).

Essential oil components that accumulate in *Xylopia* fruits are responsible for the characteristic aroma (Adobo and Iwu, 2020). Essential oils are a complex combination of compounds with potent biological activities, often including antimicrobial, antioxidant, and antiviral (Koroch et al., 2007). Essential oils from the fruits are mixtures of monoterpene hydrocarbons, mainly α–pinene (12.6-23.1%), β-pinene (20.9-39.4%), sabinene (5.9-

24.8%), 1,8 cineole (8.1-14.4%), and sesquiterpene germacrene D (0.7-4.2%). Previous investigations on the essential oils of *X. aethiopica* fruits from various areas revealed that they mainly consist of same range of mono- and sesquiterpenes, among which α-and β-pinene, myrcene, p-cymene, limonene, and 1,8-cineole, germacrene D are the most predominant (Karioti et al., 2004; Juliani et al., 2008; Niamayoua et al., 2014; Hwang et al., 2020). Many essential oils exhibit antioxidant activities, and thus research has been conducted to elucidate the activity of each component (Baratta et al., 1998; Ruberto and Baratta, 2000). Monoterpenes such as sabinene (Ruberto and Baratta, 2000), 1,8 cineole (Ghaffar et al., 2015), α-pinene and lower in β- pinene (Choi et al., 2000), and the sesquiterpene germacrene D (Karioti et al., 2004) have been reported to exhibit high radical-scavenging activity. The antioxidative free radical scavenging activity could be associated with the main components of the oils such as α and β-pinenes; 1,8 cineole and sabinene; and, to some extent, germacrene-D, a 10-membered ring system in which the three double bonds act as electron rich centers (Karioti et al., 2004).

It is likely that one or more components of the *Xylopia* oils are responsible for the strong antibacterial activity against gram-positive *B. subtilis*. The Goldleaf Hydroponics (#1) *Xylopia* extract showed high levels of α and β-pinenes (Table 1), both of which have previously been shown to exhibit antibacterial activity (da Silva et al., 2012; Wang et al., 2012). Prior work showed that *Xylopia* fruit extracts had no activity against *E. coli* (Asekum and Adeniyi, 2004), but that aqueous *Xylopia* extracts in combination with antibiotics exert synergistic effects against a variety of bacterial strains, including *B. subtilis* (Ilusanya, 2012). The results here suggest that essential oil components of the Goldleaf Hydroponics (#1) *Xylopia* fruit extract might be useful in combination with alternative medications for preventing food contamination and for possible treatment of diseases caused by susceptible strains of bacteria.

The UDA samples (A and B), having a characteristic essential oil profile (group 3 with lower levels of α and β pinene, and higher levels of sabinene and germacrene D) (Table 1) are the ones showing the highest antiviral activity. Specifically, these samples showed higher levels in the monoterpenes sabinene and 1,8 cineole and the sesquiterpenes germacrene D, α-cubenene, and β-elemene. Essential oils rich in pinenes isolated from *T. orientalis* and *J. oxycedrus* ssp. *oxycedrus* showed certain activity against SARS-CoV with EC_{50} values of 130 and 270 mg/mL and a SI of 3.8 and 3.7, respectively (Loizzo et al., 2008). Silva et al. (2020), suggested that individual components of *Xylopia* oils are not bioactive components based on their interaction with the SARS-CoV-2 target proteins. They tested different oils including (E, E)-α-farnesene, (E)-β-farnesene, (E, E)-farnesol, sabinene, 1,8 cineole, and (E)-Caryophyllene, among others. They concluded that all compounds had low docking energies and proposed that since essential oils are complex mixtures of compounds, they may need to synergize in order to show some activity. It is important to note that because extracts were prepared by 10 min-decoctions, it is possible that some of the volatile components had evaporated, thus the observed activity could be attributed to the higher molecular weight monoterpenes and sesquiterpenes.

In conclusion, *Xylopia* fruit extracts show antioxidant, antimicrobial and moderate anti SARS-CoV-1 and SAR-CoV-2 activities. This research suggests that the identified components in this study, phenolics and essential oils, can be responsible for the bioactivity of the extracts. However, other unidentified secondary compounds may also contribute to the results observed in this study.

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