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Antimicrobial and Proximate Evaluative Studies of *Persea americana* (Mill.) Seeds

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Abstract

Background: The pulp of *Persea americana* (Mill.) fruit (avocado) has been reported to have several health benefits; however, the seed of the fruit is often discarded.

Objective: This study evaluated the proximate parameters of *P. americana* Mill. seeds, as well as the chemical constituents and antibacterial activity of its ethyl acetate seed extract, to elucidate the nutritional and medicinal potentials of the seeds.

Methods: Seeds from ripe avocado fruits were chopped into pieces, air-dried and pulverized into powder with the aid of a kitchen blender. Proximate analyses of the powdered seeds were carried out as described by Association of Official Analytical Chemists (AOAC), while the antimicrobial studies of the ethyl acetate seed extract were evaluated against clinical isolates (*E. coli, S. aureus, P. aeruginosa, K. aerogenes,* and *B. subtilis*) using agar-well diffusion and agar dilution methods. Chemical constituents of the ethyl acetate seed extract of the avocado seeds were elucidated using gas chromatography and mass spectroscopy technique (GC-MS).

Results: Proximate analysis of 1.0 g of powdered seeds revealed the presence of carbohydrate (76.91% ± 0.2682), crude protein (1.82% ± 0.0727), fat (6.23% ± 0.2333), fibre (2.53% ± 0.3179), moisture content (9.46% ± 0.036) and ash value (2.98% ± 0.5198) The ethyl acetate seed extract was oily and dark brown with a yield of 5.36% w/w. Antimicrobial activity of the ethyl acetate extract against test microorganisms showed inhibition zone diameters ranging from 20 to 42 mm. at concentrations of 100-400 mg/mL of crude extract. Minimum inhibitory concentration range was 6.25-25 mg/mL of extract against test bacteria. GC-MS analysis of the ethyl acetate extract indicated the presence of 19 chemical components with ethanethioamide (23.54%), aziridine (16.21%), bicyclo-(3,1,1)-cis-vaccenic acid (15.51%) and propane nitrile (8.86%) as the most prominent known antimicrobial principles.

Conclusion: The study showed that the powdered seeds of *P. americana* has carbohydrates, crude fibre, protein and fat. The ethyl acetate extract possesses antimicrobial constituents, thus justifying the possible use of these seeds in food and medicine for the benefit of man.

Keywords: *Persea americana* seeds, antimicrobial, proximate, GC-MS constituents

INTRODUCTION

Plant parts (seeds, leaves, bark, fruits and stems) contain bioactive constituents (Egbuonu et al., 2018). These confer such plant parts with nutritive and antimicrobial properties which could contribute to the management of diseases. However, proper assessment of the possible dietary and therapeutic potentials of

such plant parts are required for informed use in the enhancement of both human and life stocks (Egbuonu et al., 2018).

Avocado plant (*Persea americana*), belonging to the family of Lauraceae and genus Persea, bears fruit commonly known as avocado pear or alligator pear that contains the avocado pear seed. They are cultivated in tropical and mediterranean climates of many countries. It is a tree that grows to 20 m (66 ft), with alternately arranged leaves. Panicles of flowers with deciduous bracts arise from new growth or the axils of leaves. The flowers are inconspicuous, greenish-yellow and about 5-10 mm wide (Silva and Ledesma, 2014).

As subtropical species, avocados need a climate without frost and with little wind. High winds reduce the humidity, dehydrate the flowers and affect pollination. The trees also need well-aerated soils and abundant ground or surface water not including rainfall and this depends on where it is grown (Carr, 2013). Depending on the variety, avocados fruits have green, brown, purplish or black skin when ripe and may be pear-shaped, egg-shaped or spherical (Egbuonu et al., 2018).

In Nigeria, avocado seeds are under-utilized and usually discarded during the processing of the pulp. Since the seed accounts for about 13-18% w/w of the fruit, the seed waste poses a severe ecological problem, and it may be of interest to industry as a source of bioactive compounds. Several biological activities of the seed have been reported, such as antioxidants, antihypertensive, larvicidal, hypolipidemic and recently, amoebicidal and giardicidal activities (Rodriguez-Carpena et al., 2011; Bahru et al., 2019; Kupnik et al., 2023). These activities of the seed may be the result of some of its reported constituents; phytosterols, triterpenes, fatty acids and two new glucosides of abscisic acid (Ramos et al., 2004; Padilla-Camberos et al., 2013; Rozan et al., 2022). Additionally, the green peel and thick pale-yellow pulp of the avocado fruit have also been reported to be rich in fatty acid such as linoleic, oleic, palmitic, stearic, linolenic, capric and myristic acids (Nasri et al., 2021; Akusu et al., 2021).

Extensive research work has been done on medicinal properties of avocado seed using extractive solvents like hexane, chloroform, water, acetone and several alcohols (Dabas et al., 2013; Rivai et al., 2019; Soledad et al., 2021). However, there is paucity of data on the ethyl acetate extract. In order to bridge this gap, this study evaluated the proximate parameters of avocado seed (*Persea americana*) powder as well as the chemical constituents and antimicrobial properties of the ethyl acetate seed extract.

MATERIALS AND METHODS

Materials

Ethyl acetate and dimethyl sulphoxide (DMSO) (JHD Science-Tech Co., China), and ciprofloxacin powder (Sigma Aldrich, Germany). Fresh fruits of *Persea americana* were purchased from New Benin Market in Benin City, Edo State, Nigeria between the months of March and April 2021. The fleshy parts of the fruits were removed and their seeds were chopped into pieces and air dried for about 14 days The dried seeds were pulverized using a kitchen blender (Kenwood BL460, England) and the resulting powder was weighed, sealed in air-tight containers and stored at 4°C prior to analysis and extraction.

Methods

Proximate analysis

Moisture content

Exactly 1.0 g of powdered seed (W1) was weighed separately into three different clean dry crucibles of known weight. Each crucible and its contents were weighed (W2) and placed in a hot air oven for 24 hours at a temperature of 105°C, until a constant dry weight was achieved. All crucibles and their contents were removed from the oven, placed in a desiccator containing silica gel, and allowed to cool for 20 minutes. After which, they were each weighed again (W3). The percentage moisture content was then calculated using Equation 1 (AOAC, 2000).

$$\text{Moisture content} = \frac{W2 - W3}{W1} \times 100 \qquad (1)$$

Ash content

Three (3) clean and dried crucibles were individually poured a 1.0 g quantity (X1) of the powdered avocado seeds and weighed (X2). The crucibles with their content were heated in a Gallenkamp muffle furnace at 600°C for 4 hours or until light grey residue was obtained. The furnace was switched off, allowed to cool, and the crucibles were transferred into a desiccator for further cooling. The crucibles were reweighed (X3), and their ash contents were calculated with Equation 2 (AOAC, 2000).

$$\text{Ash content} = \frac{X2 - X3}{X1} \times 100 \qquad (2)$$

Determination of protein content

About 1.0 g of the powdered seeds sample was placed in a digestion flask and 5.0 g of Kjedahl catalyst and 200 mL of concentrated H_2SO_4 were added. A blank solution containing only the powdered seeds was also prepared. The flasks were placed in an inclined position and heated gently until frothing ceased. It was then boiled briskly until the solution cleared. The flasks were cooled and 60 mL of distilled water was added slowly and cautiously. The flask was connected to a digestion bulb on a condenser with the tip of the condenser immersed in standard acid and 5-7 drops of mix indicator in the receiver. The flask was rotated to mix the content thoroughly then heated until its NH_3 content was distilled. The excess standard acid was titrated with standard NaOH solution. The protein content was calculated using Equation 3 (Foss Analytical, 2003; Latimer, 2016).

$$\text{Protein content} = \frac{(\text{A-B}) \times \text{N} \times 14.007 \times 6.25}{\text{Weight of sample}} \times 100 \quad (3)$$

Where A = Volume (mL) of 0.2 N HCl used in sample titration, B = Volume (mL) of 0.2 N HCl used in blank titration, N = Normality of HCl, 14.007 = atomic weight of nitrogen, 6.25 = Protein-nitrogen conversation factor for fish and its by-products.

Determination of crude fibre

Exactly 1.0 g (W_0) of sample was weighed into a 1.0 L conical flask. About 100 mL each of water and 1.25% of H_2SO_4 were added and boiled gently for 30 minutes. The content was filtered (Whatman, No. 1) and the residue scrapped back into the flask with a spatula. More quantity of 1.25% NaOH (100 mL) was added and allowed to boil gently for 30 minutes. The content was filtered and the residue washed thoroughly with hot distilled water, then rinsed to neutrality with a 1:1 mixture of ethanol and acetone. The resultant residue was allowed to dry and scrapped into a crucible of known weight and further dried to constant weight at 105°C in an air oven. It was then removed and cooled in a desiccator. The sample was weighed (W_1) and ashed at 300°C for 90 minutes in a Gallenkamp muffle furnace. It was finally cooled in a desiccator and weighed again (W_2). The percentage crude fibre was calculated using Equation 4 (AOAC, 2000).

$$\text{Crude fibre} = \frac{W_1 - W_2}{W_0} \times 100 \quad (4)$$

Determination of crude lipid

Exactly 1.0 g of the sample was homogenized with 16 mL of distilled water, 40 mL of chloroform and 80 mL of methanol at the speed of 9,500 rpm for 1.0 minute at 40°C. Exactly 40 mL of chloroform was added and homogenized for 30 sec. Then 40 mL of distilled water was added and homogenized again for 30 sec. After centrifugation of the homogenate at 2,000 rpm at 40°C for 20 minutes, the supernatant was transferred into a separatory funnel and allowed to separate. The lipid content was determined gravimetrically by measuring triplicate aliquots of the chloroform layer into tared containers, the solvent was evaporated and weighed. The lipid content was then calculated (AOAC, 2000).

Determination of available carbohydrate

The amount of available carbohydrate was determined by difference. The value was gotten by summing up the percentage values of moisture content, ash content, crude fibre, crude protein and crude lipid and subtracting from 100.

Preparation of extract

About 800 g of the powdered seed was macerated with 4.6 L of ethyl acetate for 72 h with stirring at intervals as described by Babaiwa et al., 2022. The mixture was filtered using Whatman filter paper No. 1. The filtrate was concentrated in a rotary evaporator (Stuart, UK) at a speed of 5.0 rpm to obtain a dark brown crude mass extract. The mass was weighed and kept at 4.0 °C in an airtight container, away from light until required for use.

Standardization of test microorganisms

Clinical isolates available in agar slants stored at 4°C at the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Benin, Nigeria were used for the study. These includes; *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis* and, *Klebsiella aerogenes*. Prior to use, test bacteria were sub-cultured from individual slants into sterile nutrient agar plates and incubated for 24 h at 37 °C. After incubation identical colonies were suspended in sterile broth for 12 h and bacterial growth in the suspension was compared to the opacity of a prepared standard barium sulphate (0.36 Normal) solution and adjustment made in the turbidity with sterile water to 0.5 McFarland standard to give an inoculum size of approximately 108 CFU/mL. The adjusted inocula were further diluted with sterile distilled water (1:100)

to give inoculum size of approximately 106 CFU/mL using the M07-A10 approved guideline (CLSI, 2015).

Preparation of antibiotic and extract stock solutions

Stock solution of extract was prepared by dispersion of 1.0 g of extract in 1.0 mL of DMSO solution plus 9.0 mL sterile distilled water to give a 10 mL extract solution containing 100 mg/mL of extract. Stock solution of 10 mL ciprofloxacin was prepared by dispersion 10 mg of ciprofloxacin in 1.0 mL DMSO solution plus 9.0 mL of sterile water to give a final concentration of 1.0 mg/mL.

Antimicrobial activity of *P. americana* seeds extract

Antimicrobial susceptibility test was carried out using agar well diffusion method (Murray et al., 2009). Suspensions of the various test organisms were prepared by measuring 1.0 mL sterile water into small test tubes using a micropipette and individual test organisms were picked from the culture plate with the aid of a wire loop and inoculated into the sterile water in the test tube. Tubes with turbidity comparable with 0.5 mL MacFarland Turbidity Standard (prepared by adding 0.5 mL 1.0% barium chloride to 99.5 mL 1.0% sulphuric acid) were considered as standard inoculum size. Two hundred microlitre (200 µL) of standard inoculum size of each test microorganism was mixed thoroughly with 30 mL of sterile Mueller Hinton agar and poured into a sterile plate to set. A sterile cork borer (10 mm in diameter) was used to bore four wells in each plate. The agar disks were removed and each of the well was sealed with two drops of molten agar. Two wells were filled with 200 µL of 100 mg/mL of ethyl acetate seed extract. Similar procedure was carried out with ciprofloxacin; where 200 µL of stock solution was emptied into each of the remaining two well to give a concentration of 200 µg. in each well All plates were incubated at 37° C for 24 hours.

Minimum inhibitory/bactericidal concentrations (MIC/MBC) determination

Minimum inhibitory concentration (MIC) was determined using agar dilution method as described by Afolayan and Mayer (1997). Serial dilutions of the extract stock solution (100 mg/mL) with molten Mueller Hinton agar to obtain four (4) serial concentrations ranging from 50 - 6.25 mg/mL of extract were carried out. Volumes of extract stock solution ranging from 1.0 - 0.125 mL were incorporated into appropriate volumes of molten agar to give a final volume of 20.0 mL per dilution. Similarly, serial dilution of ciprofloxacin stock solution (1.0 mg/mL) with molten Mueller Hinton agar to obtain four (4) serial concentrations ranging from 0.2 - 0.012 µg/mL of ciprofloxacin was also carried out.

The agar-extract and agar-ciprofloxacin mixtures were casted separately on Petri dishes and allowed to set. The test microorganisms were streaked onto the solidified plates and incubated at 37°C for 24 hours. After incubation all plates were visually examined for growth. The minimum inhibitory concentration (MIC) was the lowest concentration of the extract or antimicrobial agent that inhibited any visible growth of the test micro-organisms after the incubation period. Minimum bactericidal concentration (MBC) was determined by swabbing the inoculated spots in the MIC plates where there was no growth and inoculating onto a fresh plate with no extract or standard antimicrobial agent. Following incubation under standard conditions all plates were visually examined for growth. The minimum bactericidal concentration (MBC), is the lowest concentration of an antimicrobial agent, expressed in mg/L, mg/mL or µg/mL which was able to rendered a given inoculum of bacterial cell incapable of growth/reproduction on subculture into fresh culture medium

Gas chromatography - mass spectrometry analysis

The ethyl acetate extracts obtained from the seeds of *P. americana* was analyzed by GC-MS using a capillary column with specification: DB-5ms (5% phenyl methisiloxane as stationary phase), 0.25×30 mm (internal diameter) and 1.0 µm (film thickness) in gas chromatograph (GC-MS QP2010SE, Shimadzu, Japan) and Network Mass Selective Director. The carrier gas was helium with a constant flow rate of 3.22 mL/min. The inlet temperature was maintained at 250°C and the oven temperature was initially kept at 60°C for 3.4 min, then ramped at 12°C/min to 240°C. The temperature was gradually increased from 60°C/min to 290°C and held isothermally for 2 minutes. An amount of 1.0 µL of the sample solutions was injected in the split mode with split ratio 15:1. Mass spectra were obtained by electron ionization at 70eV over the scan range m/z 1428. **Statistical analysis**

Data was reported as mean from triplicate determination for proximate and inhibition zone diameter values. The compounds were identified by comparison of their mass spectra with those of the NIST/EPA/NIH Mass Spectral Library (NIST, 2005). Compounds with best percentage similarity index were tentatively denoted as the queried compound.

RESULTS

Based on results from this study, the ethyl acetate macerated *P. americana* powdered seed yielded a 5.3628%w/w extract, which was oily in consistency and dark brown in colour. The proximate analysis of the powdered seeds showed that it contains nitrogen-free extracts (76.91%), moisture content (9.46%), and ether extract (6.23%) as well as crude fibre (2.53%), crude protein (1.86%), and ash (2.98%), as shown in Table 1.

Antimicrobial susceptibility test conducted on the extract showed that the ethyl acetate extract of the seed has antimicrobial property, inhibiting all test microorganisms with inhibition zone diameters ranging from 22 - 42 mm at a concentration of equal to or greater than 100 mg. The highest activity was against *Staphylococcus aureus*, while *Bacillus subtilis* was the least susceptible (Table 2). The extract had growth inhibitory activity against *Staphylococcus aureus, Klebsiella aerogenes, Escherichia coli, Pseudomonas aeruginosa*, and *Bacillus subtilis*. Equivalent MIC and MBC values were observed for *Escherichia coli*, in contrast with other bacterial strains that showed varying MIC and MBC values (Table 3).

Results obtained from the GC-MS analysis of the ethyl acetate extract of *P. americana* seed showed the presence of 19 chemical constituents, with 5 major peaks. These included ethanethioamide (23.54%), aziridine (16.21%), 3-cyclohexene-1-ethanol (15.51%), propane nitrile (8.86%) and 7-hexadecene (7.82%) as the most prominent (Figure 1 and Table 4).

Table 1: Proximate parameters of *P. americana* seed

Parameter	Value (%)
Moisture content	9.46 ± 0.0336
Ash value	2.98 ± 0.5198
Crude fibre	2.53 ± 0.3179
Crude protein	1.86 ± 0.0727
Ether extracts	6.23 ± 0.2333
Nitrogen free extracts	76.91 ± 0.2682

Values are mean ± standard deviation

Table 2: Inhibition zone diameters of *P. americana* seed extract and standard antimicrobial agent (mm)

Test micro-organism	Extract (mg/mL)			Ciprofloxacin (µg/mL)
	100	200	400	200
Escherichia coli	27	29	29	32
Klebsiella aerogenes	20	24	28	25
Pseudomonas aeruginosa	34	36	36	32
Staphylococcus aureus	33	35	42	34
Bacillus subtilis	20	24	23	22

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of *P. americana* seed extract and standard antimicrobial agent

Test micro-organism	Extract (mg/mL)		Ciprofloxacin (µg/mL)	
	MIC	MBC	MIC	MBC
Escherichia coli	25	25	< 0.02	0.02
Staphylococcus aureus	12.5	25	< 0.02	0.02
Pseudomonas aeruginosa	6.25	12.5	< 0.02	0.02
Bacillus subtilis	6.25	25	< 0.02	0.02
Klebsiella aerogenes	25	> 50	< 0.02	0.02

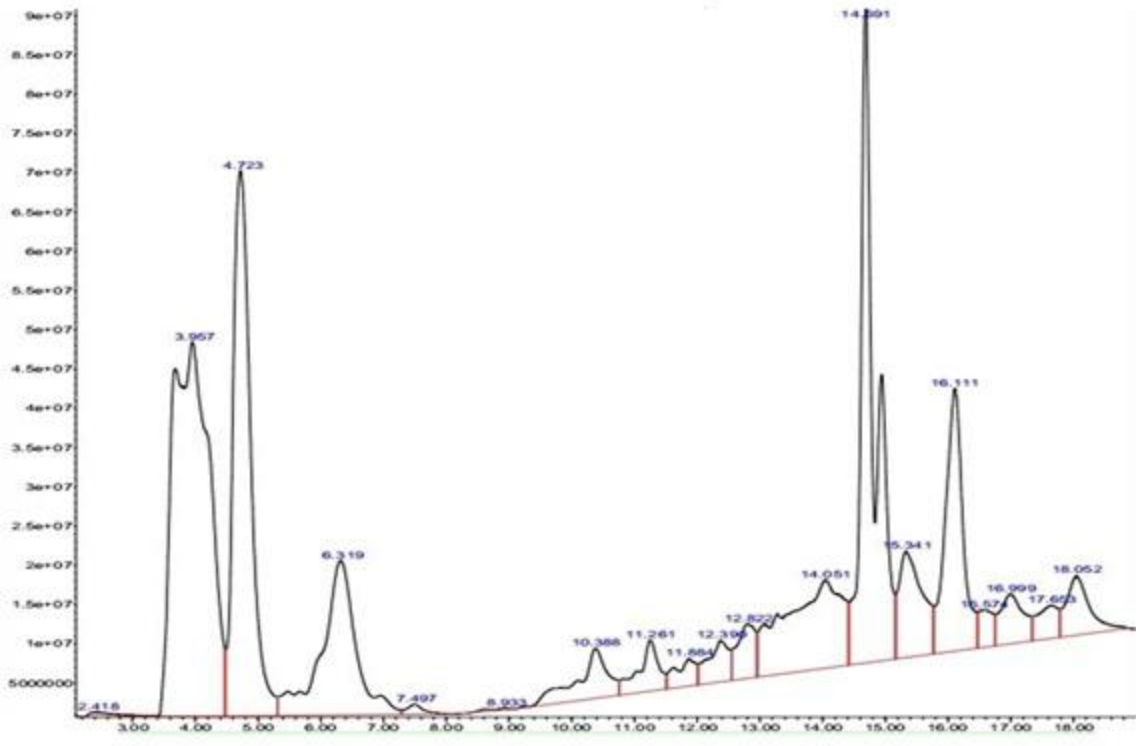

Figure 1: GC-MS chromatogram of the ethyl acetate extract of *Persea americana* seed

Table 4: Chemical components identified by the GC-MS analysis of ethyl acetate extract of *Persea americana* seed

Peak No.	Retention time	Area (%)	Chemical name
1	2.418	0.22	3-(methylthiopropyl-2,2,2-)-trifluoroacetate
2	3.957	23.54	Ethanethioamide
3	4.720	16.21	Aziridine
4	6.322	8.86	Propane nitrile
5	7.498	0.29	Hexanoic acid
6	8.930	0.07	4H-1,2,4-triazole-3-thiol
7	10.388	2.55	1,2,3-propanetriol
8	11.263	1.71	Octadecanoic acid
9	11.883	0.94	Ethyl-9-decenoate
10	12.389	1.47	Phenol
11	12.821	1.63	Trans-sesquisabinene hydrate
12	14.053	15.54	Bicyclo-(3.1.1)-cis-vaccenic acid
13	14.691	6.51	3-cyclohexene-1-ethanol
14	15.342	7.82	7-Hexadecene
15	16.111	6.80	Isoamylpyrazine
16	16.574	0.90	Bicyclo-(2.2.1)-heptane
17	16.999	1.98	Cyclohexane
18	17.650	1.17	1,3-cyclopentadiene
19	18.050	2.34	Cyclopentane ethanol

DISCUSSION

Ethyl acetate extraction of *P. americana* seeds obtained by maceration gave a yield of 5.3628%w/w of the dry powdered seeds. The low yield may be attributed to the method of extraction. Maceration method, as opposed to Soxhlet extraction and steam distillation methods in plant seeds extraction, has been previously documented to account for low extract yields (Ibrahim and Zaini, 2017). However, maceration has a documented advantage of preserving thermolabile phytoconstituents with known antimicrobial activity, hence its choice in this study.

Proximate and nutrient analysis of edible fruit and vegetables play a crucial role in assessing their nutritional significance. As a source of carbohydrate, protein, fiber, and essential micronutrients such as polyphenols, fats, oils, vitamins, and minerals, avocado seed can be invaluable in the production of animal feeds. The seeds are highly consumed in the world due to the presence of unsaturated lipids and its relevance in improving and maintaining a healthy heart and circulatory system (Bahru et al., 2019).

Higher moisture contents up to 15.10 ± 0.14% have been documented for avocado seed by some studies compared to the 9.46% obtained in our investigation ((Egbuonu et al., 2018; Ejiofor et al., 2018). This difference in moisture content may be due to the length of our drying and storage periods subjected to the seeds used in the study. Total ash content is an important parameter that represents the amount of total minerals present in a biomass. High ash content of plant samples diminishes their inclusion in human food and animal feeds (Liu, 2019). Some previous studies carried out on *P. americana* seeds documented ash contents of 2.40 ± 0.19 and 2.26 ± 0.23, values slightly less than the value (2.98 ± 0.5198) gotten from our study (Arukwe et al., 2012; Ayoola et al., 2012).

The presence of growth inhibitory zones (20 - 42 mm) on seeded agar plate at extract concentrations ranging from 100 - 400 mg/mL as seen in this study was indicative of the antimicrobial activity of *P. americana* seed extract. This is in line with a previous study which considered a crude plant extract as having antimicrobial activity at inhibition zone diameters greater than 10.0 mm at a concentration of 100 mg/well (Usman et al., 2005). Additionally, it has been documented that the seed of *P. americana* demonstrated antioxidant activity and antimicrobial activity against *Bacillus spp*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas spp* and *Listeria monocytogenes* (Rodriguez-Carpena et al., 2011; Salinas-Salazar et al., 2017; Sudhasupriya et al., 2017).

The antimicrobial activity observed in this extract may be partly due to the presence of some chemical compounds elucidated from the GC-MS analysis. Ethanethioamide, which was the most abundant compound in our study, has been previously synthesized and screened for its antibacterial activity against various Gram-positive (*Staphylococcus aureus*, including linezolid-resistant strain; *Enterococcus faecalis, Enterococcus faecium, Streptococcus pneumoniae, Streptococcus pyogenes* and *Staphylococcus epidermidis*) and Gram-negative (*Haemophilus influenzae*) bacteria (Deshmukh and Jain, 2017).

In the study, where linezolid, levofloxacin and vancomycin were used as controls, ethanethioamide showed activity similar or superior to linezolid. Aziridine was another compound identified in this study and it has been documented to have bactericidal effects at MIC values of 16 - 32 µg/mL against a wide array of Gram positive and Gram-negative bacteria (Kowalczyk et al., 2018). Similarly, isoamylpyrazine, identified in the seeds extract, is a derivative of pyrazine which have been documented to have several uses in medicine as antibacterial and antiviral agent (Hu et al., 2023). Cyclohexane derivative in the seed extract was found to be 3-cylohexene-1-ethanol (6.51%). This compound has been reported to possess pronounced antimicrobial and antifungal properties (Mammadova, 2021).

Vaccenic acid derivative which accounted for 15.54% of the chemical constituent identified in the ethyl acetate extract of *P. americana* seed, has been previously reported to possess antibiofilm and antibacterial activity against *Pseudomonas aeruginosa* (Yazici, 2024). This finding suggests that vaccenic acid could be a potential therapeutic agent against *Pseudomonas aeruginosa* infections, especially those involving pyocyanin mediated virulence and biofilm formation. Exploring the mechanism of action of vaccenic acid and its potential synergy with known antimicrobial agent could be the area of focus in future studies.

CONCLUSION

The presence of carbohydrate, crude fiber, protein and fat in the seeds of *Persea americana*, as shown in our study, is indicative of the nutritive potential of the seed and thus a reason for its use as a source of food for both humans and livestock. The GC-MS analysis results of the ethyl acetate seed extract have shown that *Persea americana* contains chemical compounds with documented antimicrobial activity, justifying the observed inhibitory activity of the extract against test bacteria and its use in the ethnomedicinal management of infectious diseases

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