Asian Journal of Chemistry; Vol. 33, No. 9 (2021), 2093-2098

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2021.23294

Antibacterial Activity against *Enterococcus faecium*, *Enterococcus faecalis* and Inhibitory Activity of Monoamine Oxidase A & B by *Persea americana* "Avocado" Seed Extracts

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Received: 6 May 2021; *Accepted*: 22 June 2021; *Published online*: 20 August 2021; AJC-20470

This study reported various *Persia americana* seed extracts for the presence and total content of phytochemicals, antibacterial activity and inhibitory activity against monoamine oxidase (MAO) A & B enzymes. Phytochemical studies showed that phenols, flavonoids and tannins were found in all the polar solvents. The highest antibacterial activity was exhibited against Gram-positive bacteria by acetone extract on *Enterococcus faecium* and methanol extract on *Enterococcus faecalis*. From the monoamine inhibition experiments, a significant inhibitory activity was observed from ethanolic extracts against MAO-A and from *n*-hexane extracts against MAO-B. This study demonstrates the antimicrobial activity of *P. americana* seed extracts against enterococci bacteria, *E. faecium* and *E. faecalis*. This finding offers hope of a potential new antibacterial compound in the treatment of these multidrug resistant bacteria.

Keywords: Antibacterial activity, *Persia americana, Enterococcus faecalis, Enterococcus faecium.*

INTRODUCTION

Since the discovery of antibiotics, they have served multiple therapeutic roles in medical practice and have become the mainstay of modern medicine. These roles, which include the prevention and treatment of human and animal infections have however, led to the irrational use of the antibiotics and ultimately development of resistance by bacteria. Several studies have reported an overall decrease in antibiotic effectiveness after a period of use due to the development of resistance to antibiotics that were initially potent against the bacteria [1,2]. The United States Centre for Disease Control and prevention (US CDC) estimates that microorganisms with antibiotic resistance are responsible for more than 2 million infections, which cause 23,000 deaths each year in U.S.A. [3]. World Health Organization (WHO) issued guidelines on the rational use of antibiotics and published a list of bacteria, for which new antibacterial drugs are needed to tackle the spread and effects of antibiotic resistance [4]. This emphasizes the global need for newer antibiotics.

The use of plant extracts with known antimicrobial activity can be of great value in therapeutic treatments. The avocado seed has been of great medicinal interest in Mexican traditional medicine [5]. It has been reported that Mexicans use it to treat monorrhagia, hypertension, stomach-ache, bronchitis, diarrhoea and diabetes [5]. Research conducted using the avocado seed extracts has shown antibacterial activity against methicillin resistant *Staphylococcus aureus* [6] and *Listeria monocytogenes* [7]. In addition, it was found to display activity against *Mycobacterium tuberculosis* H37R, Mycobacterium non-tuberculosis such as *Mycobacterium fortuitum*, *Mycobacterium avium*, *Mycobacterium smegmatis* and *Mycobacterium abscessus* [8]. Furthermore, avocado seeds were found to possess antiparasitic activity against *Entamoeba histolytica* strain HM1-IMSS, *Giardia lamblia* strain IMSS: 0989:1 and *Trichomonas vaginalis* GT9; antifungal activity against *Candida albicans* [9] and *Cryptococcus neoformans* [6]; and antiviral activity directed against *in vitro* replication of *Herpes simplex virus* 1 (HSV-1), *Adenovirus* type 3 [10]. The extracts of *Persia americana* seed have demonstrated a broad spectrum of activity against medically important microorganisms.

Present study reports on the significant findings of investigations against *Enterococcus faecalis* and *Enterococcus faecium*. These two organisms are responsible for a variety of infections such as urinary tract infections, intra-abdominal, pelvic, soft tissue infections, bacteraemia and endocarditis [11]. They have demonstrated resistance to antibiotics leading to their appearance in the list of priority organisms by WHO [12].

The discovery of the inhibition of monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B) for the treatment of neurodegenerative diseases was a conceptual breakthrough, which has now been firmly established [13]. The MAO isoforms appear to be predominantly responsible for dopamine metabolism in the basal ganglia, thus, inhibition of this enzyme in the brain may conserve the depleted supply of dopamine [14]. Because of the safety considerations associated with irreversible MAO-B inhibitors, such as the requirement of *de novo* synthesis of the MAO-B protein for enzyme activity to return, there is the need for development of specific, reversible MAO-B inhibitors [13,14]. Considering the multipurpose medicinal use of avocado seed, the various extracts were tested for inhibition against MAO-A and MAO-B activities, since this could lead to the discovery of new therapeutically useful neuroprotective agents.

EXPERIMENTAL

The seed of *P. americana* Mill. (avocado) was obtained from the University of Pretoria Agricultural Farm, Pretoria, South Africa in winter June 2016. The collected seeds were dried for three weeks at room temperature while protecting them from direct sunlight. They were afterwards powdered using Retsch mils SM 100, (Germany) and stored away from light.

Preparation of extracts: The serial exhaustive extraction method was used to prepare the extracts using four solvents; hexane, acetone, ethanol and methanol in order of polarity as previously described by Olivier *et al.* [15]. Briefly; 500 g of dried powder of avocado seed was macerated at room temperature with 5000 mL of solvent for 24 h on an orbital shaker (Labotec 262, South Africa, ENF-280C/FE, USA) at 150 rpm. The process was repeated three times using the same volume of fresh solvent with the same plant material. The mixture was allowed to settle down before being filtered using Whatman No. 1 filter paper. The solvent was then evaporated using a rotary evaporator (Buchi R-200, Labotec, South Africa) under reduced pressure at between 20 and 40 ºC. The remaining solvent extract mixture was transferred into a pre-weighed beaker and left in a fume hood to dry until the extract was free of solvent. The extracts were re-dissolved in a sterile 2 mL screw cup tube to obtain a concentration of 1 mg/mL using DMSO and distilled water as follows: 10 µL of DMSO (Thermo Scientific, Denmark) was added to 1 mg of hexane extract of *P. americana* and left to dissolve overnight (24 h) at room temperature then adjusted to 1000 µL with distilled water. For acetone, ethanol and methanol extracts, 1 mg of each extract was dissolved overnight (24 h), in 1 mL of distilled water at room temperature.

Screening for phytochemicals

Qualitative analysis: The colorimetric qualitative phytochemical analyses were used to investigate the presence of secondary metabolites of avocado extracts following the standard methods as described below [16-22].

Test for tannins: Little quantity (3 mL) of each extract was dissolved in 3 mL of distilled water in a test tube and two drops of $FeCl_3$ solution was added to the mixture, then the mixture was stirred and formation of a green colour precipitate indicated the presence of tannins.

Test for terpenoids (Salkowski test): A quantity of each extract, 0.8 g was dissolved in 10 mL methanol, mixed thoroughly, filtered and 2 mL of chloroform as well as 3 mL sulphuric acid added to 5 mL of the filtrate. A reddish-brown colour interphase confirmed the presence of terpenoids.

Test for flavonoids: For the confirmation of flavonoids, 0.5 g of each of the extracts was dissolved in 10 mL distilled water and filtered. To a portion of aqueous filtrate of each extract, 5 mL of dilute ammonia solution was added, followed by the addition of 1 mL concentrated H_2SO_4. The presence of flavonoid was confirmed by the appearance of a yellow colour.

Test for alkaloids: A mixture of each extract 0.2 g and 3 mL hexane was shaken thoroughly, filtered and 5 mL of 2% HCl was added to the filtrate. This was heated, filtered again and a few drops of picric acid added. The presence of alkaloids was confirmed by the formation of a yellow colour precipitate.

Test for saponins (Frothing test): Each extract 3 mL was added to 10 mL of distilled water in a test tube. The mixture was shaken vigorously for 5 min and allowed to stand for 30 min. The formation of stable honeycomb froth confirmed the presence of saponins.

Test for steroids: Concentrated sulphuric acid and 2 mL of chloroform were added sidewise to each extract. Formation of red colour in the lower chloroform layer indicated the presence of steroids.

Test for quinones: Each extract was mixed with benzene and filtered. Ammonia solution (10%, 10 mL) was added to the filtrate, shaken vigorously for 30 s and pink, violet, or red colour indicated the presence of quinones in ammonia phase.

Quantitative analysis

Total phenol content determination: A modified Folin-Ciocalteu method was used to determine the quantity of total phenolic components of the extracts of avocado seeds [23]. Standard solutions of gallic acid (20, 40, 60, 80 and 100 µg/mL) were prepared and an aliquot (1 mL) of each of the extracts as well as the standard solutions was added to a 25 mL volumetric flask, containing 9 mL of distilled deionized water to form a reaction mixture. Folin-Ciocalteu's phenol reagent (1 mL) was added to each reaction mixture formed and mixed together. After 5 min, 10 mL of 7 % Na_2CO_3 was added and the solution was made to the mark with deionized H_2O, mixed and allowed to incubate for 90 min at room temperature. The absorbance readings of all the resultant solutions, were measured against the blank at 550 nm using an UV/vis spectrophotometer (CECIL CE 1021, Cecil Instruments Ltd., UK). From the gallic standard curve assisted to calculate the total phenolic content in the seed extracts of *P. americana*, which were expressed as mg Gallic acid equivalence (GAE)/100 g extract. All the samples were analyzed in triplicates.

Total tannins content determination: A modified Folin and Ciocalteu method was also employed in the determination of the quantity of tannins components of the extracts of avocado seeds [23]. Standard solutions of gallic acid (20, 40, 60, 80 and 100 µg/mL) were prepared. An aliquot (0.5 mL) of each of the extracts as well as the standard solutions was added to a 50 mL volumetric flask, to which 2.5 mL Folin-Ciocalteu's phenol reagent and 1 mL 35% Na_2CO_3 solution were added and the solution was made to the mark with distilled water. The solution was mixed thoroughly and kept at room temperature for 30 min to incubate. The absorbance readings of the resultant solutions were then measured against that of the blank with solution at 725 nm using UV/vis spectrophotometer (CECIL CE 1021, Cecil Instruments Limited, UK). All the samples were analyzed in triplicates. The results obtained as the total tannins content in the seed extracts of *P. americana* are expressed as mg of gallic acid equivalence/g extract (mg GAE/g).

Total flavonoids content determination: The aluminium chloride colorimetric assay as described by Tambe & Bhambar [24] was used in measuring the total flavonoid content of avocado seed extracts. An aliquot (1 mL) of each of the extracts as well as the standard solutions of quercetin (20, 40, 60, 80 and 100 mg/L) was added to a 10 mL volumetric flask containing dd H_2O (4 mL) and 0.3 mL of 5 % $NaNO_2$ was added and mixed. After 5 min, 10 % $AlCl_3$ (0.3 mL) was added. At the 6th min, 1 M NaOH (2 mL) was added and the flask filled to the mark with deionized water. The solution was thoroughly mixed and absorbance readings of each of the resultant solutions were measured against prepared reagent blank at 510 nm with an UV-vis spectrophotometer. All the samples were analyzed in triplicates. Total flavonoid content of *P. americana* was calculated from quecertin's standard curve and expressed as mg quercetin equivalence/g extract (mg QE/g).

Screening for antibacterial activity: Kirby Bauer disc diffusion method was used to screen for antibacterial activity. All tests were done in triplicates. Standardized to 0.5 MacFarland, each organism was lawned onto Mueller-Hinton (MH) agar plates (Diagnostic Media Products, South Africa) and Whatman No. 1 filter paper was used to make disks, previously sterilized using an autoclave (HL-300 portable, Huxley). The disks were imbibed using a sterile pipette with 5 drops of extract and placed on inoculated agar. The process was done for each extract obtained using *n*-hexane, acetone, ethanol and methanol as solvents. Disks of vancomycin for *E. faecium* (clinical isolate) and ampicillin for *E. faecalis* (ATCC 51299) were used as control for antibacterial activity. The agar plates were then incubated for overnight (12-24 h). Finally, the agar plates were assessed for zones of inhibition around the discs. The extracts which showed antibacterial activity were considered for minimum inhibitory concentration (MIC) evaluation.

Determination of minimum inhibitory concentration (MIC): The broth microdilution method was used to determine the MIC of the extracts that showed some antibacterial activity following screening. The MIC was determined using the same bacterial strains. The MH broth (50 µL) was pipetted into 96-wells plate (Thermo scientific, Denmark). Thereafter, 50 µL of the extract solution (1 mg/mL) was added to the first well followed by a two-fold serial dilution. An overnight bacterial suspension (50 µL) prepared in MH broth and standardized to 0.5 MacFarland, was added to each well. The 96-well plate was then sealed and incubated for 24 h at 35-36 °C. After incubation, 40 µL of *p*-iodonitrotetrazolium chloride (0.2 mg/mL; Fluka Biochemika GA13931, Germany) was added and incubated for 1 h. The MICs were recorded as the lowest concentrations that inhibited visible bacteria growth.

Determination of monoamine oxidase inhibitory activity: The values of IC_{50} for the inhibition of MAO of seed extracts from avocado (*P. americana* Mill.) were measured using the recombinant human MAO-A and MAO-B enzymes from the well-established protocol [25]. The enzyme reactions for each extract were carried out in triplicate in white 96-well microliter plates (Eppendorf) in potassium phosphate buffer (pH 7.4, 100 mM, made isotonic with KCl). MAO-A (0.0075 mg protein/mL) or MAO-B (0.015 mg protein/mL), the test inhibitors (0.003-100 µM) and Kynuramine (50 µM), were all included in the final volume of the reactions which was made up to 200 µL. DMSO was used to prepare the stock solution of the test inhibitors and these were added to the mixture to give a final concentration of 4%.

Reactions were also carried out, in the absence of inhibitors, to serve as negative controls. The initiation of the enzyme reactions occurred with the addition of the MAO enzymes and each reaction mixture was afterwards incubated in a convection oven at 37 ºC for 20 min. The reactions were terminated at endpoint by adding 80 µL NaOH (2N) and the concentration of 4-hydroxyquinoline, the product of kynuramine oxidation by MAO, was measured by fluorescence spectrophotometry (λ_{ex} = 310 nm; λ_{em} = 400 nm) [26].

RESULTS AND DISCUSSION

The qualitative phytochemical screening for the presence of different secondary metabolites showed that all the extracts (hexane, acetone, ethanol and methanol) of *P. americana* seed contain one or more classes of secondary metabolites except for glycosides which tested negative in all the extracts (Table-1).

TABLE-1
QUALITATIVE PHYTOCHEMICAL ANALYSIS ON THE EXTRACTS OF AVOCADO SEEDS

Phytochemicals	Extracts			
	Hexane	Acetone	Ethanol	Methanol
Phenol and tannins	Absent	Present	Present	Present
Flavonoids	Absent	Present	Present	Present
Quinones	Present	Absent	Absent	Absent
Steroids	Absent	Present	Absent	Absent
Glycosides	Absent	Absent	Absent	Absent
Terpenoids	Present	Present	Absent	Present
Saponins	Present	Absent	Present	Present
Alkaloids	Absent	Absent	Absent	Present

The quantitative analysis of secondary metabolites showed that the total phenols content obtained were 0.0430, 0.0096 and 0.0191 mg/mL for acetone, ethanol and methanol extracts, respectively. Meanwhile, the total flavonoids content was found

TABLE-2
P. americana SEED EXTRACT ANTIBACTERIAL ACTIVITY AGAINST TESTED ORGANISMS

Organisms	Extracts				Standards	
	Hexane	Acetone	Ethanol	Methanol	Ampicillin	Vancomycin
E. faecalis	No	Yes	Yes	Yes	Yes	Not tested
E. faecium	No	Yes	Yes	Yes	Not tested	Yes

to be 0.9615×10^{-3}, 0.2375 and 0.4894 mg/mL and total tannins content obtained were 0.0300, 0.0268 and 0.0737 mg/mL for acetone, ethanol and methanol extracts, respectively (Fig. 1).

In the biological studies, the screening revealed that *E. faecium* (clinical isolate) and *E. faecalis* (ATCC 51299) were susceptible to acetone, ethanol and methanol extracts as shown in Table-2. There was no activity observed when using the hexane extract against the same bacterial strains (Table-1). The average MIC determined for the extracts was 0.0022 mg/mL for methanol: 0.0014 mg/mL for ethanol and 0.0009 mg/mL for acetone against *E. faecium* (Table-3). For *E. faecalis,* the average MIC of the extracts was 0.0015 mg/mL for methanol: 0.0026 mg/mL for ethanol and 0.0083 mg/mL for acetone (Table-3). All the standard deviations were ± 0.00 (Table-3). The MIC values of the test organisms revealed that their susceptibility varies according to the polarity of the extracts with *E. faecium* being susceptible to the most polar extract and *E. faecalis* being susceptible to the least polar extract.

TABLE-3
STANDARD DEVIATION AND AVERAGE OF MIC CONCENTRATION FOUND FOR *E. faecium* AND *E. faecalis*

Organisms	Extracts	Average (mg/mL)	Standard deviation (±)
E. faecium	Methanol	0.0022	0.0006
	Ethanol	0.0014	0.0008
	Acetone	0.0009	0.0000
E. faecalis	Methanol	0.0015	0.0005
	Ethanol	0.0026	0.0006
	Acetone	0.0083	0.0061

The evaluation of monoamine oxidase inhibition shows that the ethanolic Avocado extract exhibited the highest potency inhibition for MAO A with an IC_{50} ± StDev (µM) of 0.88047 ± 0.04567, followed by the acetone extract with an IC_{50} ± StDev (µM) of 1.29400 ± 0.06022, hexane extract with an IC50 ± StDev (µM) of 1.92867 ± 0.12815 and methanolic extract with an IC_{50} ± StDev (µM) of 3.01267 ± 0.13659 exhibiting the least potency inhibition for MAO-A. The *n*-hexane avocado extract exhibited the highest potency inhibition for MAO-B with an IC_{50} ± StDev (µM) of 0.30430 ± 0.00990, followed by the ethanolic extract with an IC_{50} ± StDev (µM) of 1.44000 ± 0.01980, acetone extract with an IC_{50} ± StDev (µM) of 1.43467 ± 0.26167 and methanolic extract with an IC_{50} ± StDev (µM) of 3.42500 ± 0.30654 exhibiting the least potency inhibition for MAO-B (Table-4).

TABLE-4
EVALUATION OF MAO-A AND MAO-B INHIBITION

Compounds (µM)	MAO-A: IC_{50} ± StDev (µM)	MAO-B: IC_{50} ± StDev (µM)
M1 Avocado-Acetone	1.29400 ± 0.06022	1.43467 ± 0.26167
M1 Avocado-Acetone	1.29400 ± 0.06022	1.43467 ± 0.26167
M2 Avocado-Ethanol	0.88047 ± 0.04567	1.44000 ± 0.01980
M3 Avocado-Hexane	1.92867 ± 0.12815	0.30430 ± 0.00990
M4 Avocado-Methanol	3.01267 ± 0.13659	3.42500 ± 0.30654

IC_{50}: Half maximal inhibitory concentration

Phytochemical analysis of the avocado seed extracts revealed the presence of secondary metabolites such as phenols, tannins, flavonoids, quinones, steroids, terpenoids, saponins and alkaloids. These metabolites found in the plant extracts might be directly linked to the biological activity exhibited by the plant. In 2016, Segovia *et al*. [27] extracted phenols derivatives using the ultrasound power (0-104 W) extractive method in water between 20 and 60 ºC It was revealed that the tempe-

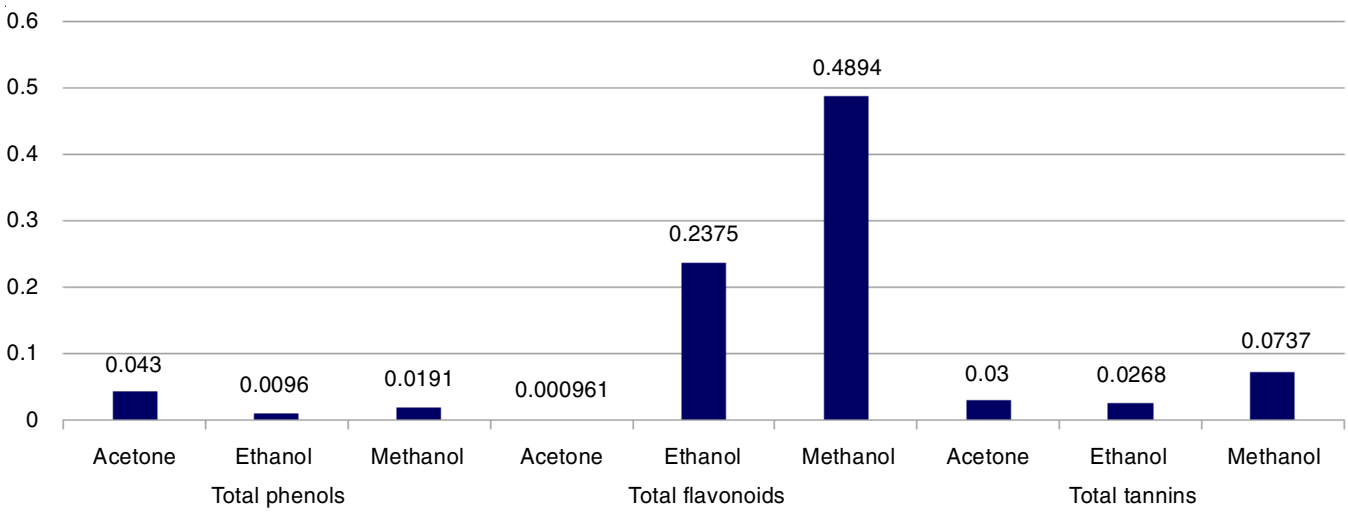

Fig. 1. Total phenols, flavonoids and tannins content results of acetone, ethanol and methanol extracts from avocado seed material

rature has an impact on the yield of phenols derivatives. For example, it is generally known that the detection of phenolic derivatives such as flavonoids from a plant crude extract indicate the presence of an antioxidant activity [27,28]. According to Minatel *et al.* [29], the antioxidant activity of phenolic derivatives is directly attributed to their ability to scavenge free radical, donating hydrogen atoms, electrons and or chelate metallic cations. In addition, the structure, number and the position of hydroxyl groups as well as the nature of the substitutions on the aromatic ring play an immense role on the antioxidant activity of the phenolic derivatives. The quantitative study revealed that the highest total flavonoid content is found in the methanol extracts followed by ethanol extract and finally acetone extract with 0.4894, 0.2375 and 0.9615 × 10^{-3} mg/mL, respectively. These results agreed with the study reported by Ghasemzadeh *et al.* [30], which concluded that methanol extracted the highest content of total flavonoids. On the other hand, the total phenol contents results indicated that acetone extracted the highest amount followed by methanol and lastly ethanol with 0.0430, 0.0191 and 0.0096 mg/mL, respectively. Generally, tannins are known to exhibit toxic properties and often act as feeding deterrents for herbivores and some like protocatechillic acid and chlorogenic acids are readily oxidized and impart disease resistant properties to plants [31,32]. In this study, methanol extracted the highest content of tannins followed by acetone lastly ethanol with an average concentration of 0.0737, 0.0300 and 0.0268, respectively. The low concentration of tannin content extracted by ethanol agrees with the study conducted by Downey & Hanlin [33], which indicated that acetone could extract tannins in larger amounts than ethanol.

Various parts of *P. americana* Mill. tree have demonstrated antibacterial activity on several microorganisms. According to Gibbons [16], values of MIC below 1 mg/mL for crude extracts are considered significant. The result obtained in this study hence points towards a plausible presence of a very active antibacterial compound against these two organisms in the extracts. Any activity observed following screening of crude extracts is of significance since the concentration of active compound once identified can be adjusted and re-evaluated for better activity. Lubis *et al.* [17] in their study on antimicrobial activity of *P. americana* collected in North Sumatra (Indonesia) found comparable results. These authors also noted moderate activity of *P. americana* peel crude extract obtained using ethanol as solvent, against *E. faecalis*. However, there is no report in literature of previous work on *P. americana* seed extract against *E. faecium* and *E. faecalis*. Hence this first report warrants for further studies to determine the active compound(s) and elucidate the cause for the difference in the extract's activity against the two enterobacteria based on polarity since *E. faecium* and *E. faecalis* are close in their structure and composition.

From the monoamine inhibition data (Table-4), it was clear that the ethanolic and hexane extracts exhibited the most potent inhibitory activity against MAO-A and MAO-B, respectively. The IC_{50} values are significantly lower than the reference inhibitors, toloxatone and lazabemide [34]. These reference inhibitors inhibit MAO-A and MAO-B with IC_{50} values of 3.92 and 0.091 µM, respectively. There may therefore be the need to isolate the phytochemicals responsible for the activity and perform structure activity relationship study while modifying the chemical structure to obtain more potent compounds.

Conclusion

This study showed that with increasing polarity of the solvents, the mass of avocado seed extracts recovered also increases. The methanolic extracts contain the highest concentration of flavonoids and tannins while the acetone extract contains the highest concentration of phenols. Furthermore, the study showed that crude acetone, ethanolic and methanolic *P. americana* seed extracts have compound(s) with antibacterial activity against *E. faecium* and *E. faecalis*. The ethanolic extract exhibited inhibitory activity against MAO-A while the non-polar *n*-hexane extract exhibited inhibitory activity against MAO-B. Further studies will be conducted to isolate and investigate the compounds responsible for the antibacterial and MAO-A and MAO-B inhibitory activities.

ACKNOWLEDGEMENTS

The authors acknowledge the South Africa National Research Fund (NRF) and The Research Development Grant (RDG) for funding the project and financial assistance during this project.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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