

Antimicrobial Activity and Phytochemical Screening of Fruit Extracts and Fractions of *Averrhoa bilimbi* L. (*Oxalidaceae*) in Trinidad

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INTRODUCTION

Trinidad and Tobago is home to a wide variety of plants used in traditional medicine to treat a plethora of diseases. The wood sorrel family, *Oxalidaceae*, comprises 601 recognized species distributed in 6 genera, prevalent in both the tropics and subtropics¹. One *Oxalidaceae* genus, *Averrhoa*, contains 5 well-described species; however, the edible fruits of only two species, *Averrhoa carambola* L. (known as star fruit) and *Averrhoa bilimbi* L. (known as cucumber tree), are to date cultivated and studied for their health benefits.

A. bilimbi fruits (ABF), which grow on the tree trunk (Figure 1), were selected for this study based on the traditional medicinal uses such as treatment of diabetes mellitus, management of obesity and various skin conditions². While international reports document antimicrobial and anticancer activities of ABF², there is a lack of scientific validation regarding the chemical composition and biological activities of ABF from the Neotropical areas such as the Caribbean. This study aimed to find active compounds responsible for the antibacterial activities of ABF grown locally in Trinidad, using a combination of chemical and biological techniques.



Figure 1: *Averrhoa bilimbi* fruits growing in clusters on tree trunks.

METHODS

In this study, fruits (both green and yellow) were harvested from a single, field-grown *A. bilimbi* tree in the vicinity of St. Augustine (Trinidad) during the month of November (rainy season characterized by maximum temperatures 28-32°C). A voucher specimen (TRIN 50539) of the mature plant was deposited for future reference at the National Herbarium of Trinidad and Tobago (The UWI, St. Augustine, Trinidad).

The overall methodology used for the extract preparation, fractionation and screening for antimicrobial activity is summarized in Figure 2. The fruits were oven dried, powdered for maceration in methanol (MeOH), filtered and solvent allowed to evaporate. The crude MeOH extract was further fractionated using solvents of increasing polarity: hexane (hex), dichloromethane (DCM), ethyl acetate (EtOAc) and 1-butanol (but) following the procedure by Ebada et al.³ (Figure 3). Antimicrobial activities of all ABF fractions were evaluated *in vitro* against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecium*) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) ATCC reference bacteria. These strains were obtained from the Microbiology Research Group at the Department of Life Sciences (DLS), Faculty of Science and Technology (FST), The UWI. The potency of each fraction was assessed by determining the minimum inhibitory concentration (MIC, the lowest concentration at which there is no visible bacterial growth), and the minimum bactericidal concentration (MBC, the lowest concentration at which bacteria is killed) using the 96 well microtitre format according to CLSI guidelines⁴. Ampicillin (Sigma-Aldrich) and ciprofloxacin (HiMedia) were used as positive controls to monitor the validity and reproducibility of the antimicrobial assays.

The most active fraction was subjected to a preliminary phytochemical screening following the procedures described by Shukla et al.⁵ for alkaloids, steroids, tannins, carbohydrates, flavonoids and proteins, and Hussain and El-Anssary⁶ for saponins, phenolics and triterpenoids. Subsequently, bioassay-guided fractionation was carried out using silica or Sephadex LH-20 column chromatography (CC) and preparative thin-layer chromatography (PTLC). Antibacterial screening against *S. aureus* and *E. coli* was performed at every step of the process to help prioritize subsequent purification and separation. Fractions with similar PTLC profiles and ability to inhibit microbial growth were pooled and separated further. The efficiency of the purification was confirmed by analytical TLC. Retention factor (Rf) values, characteristic of the mobility of the organic compound and calculated as the relative distance travelled by a particular compound with respect to the mobile phase, were determined to help identify the pure compounds.

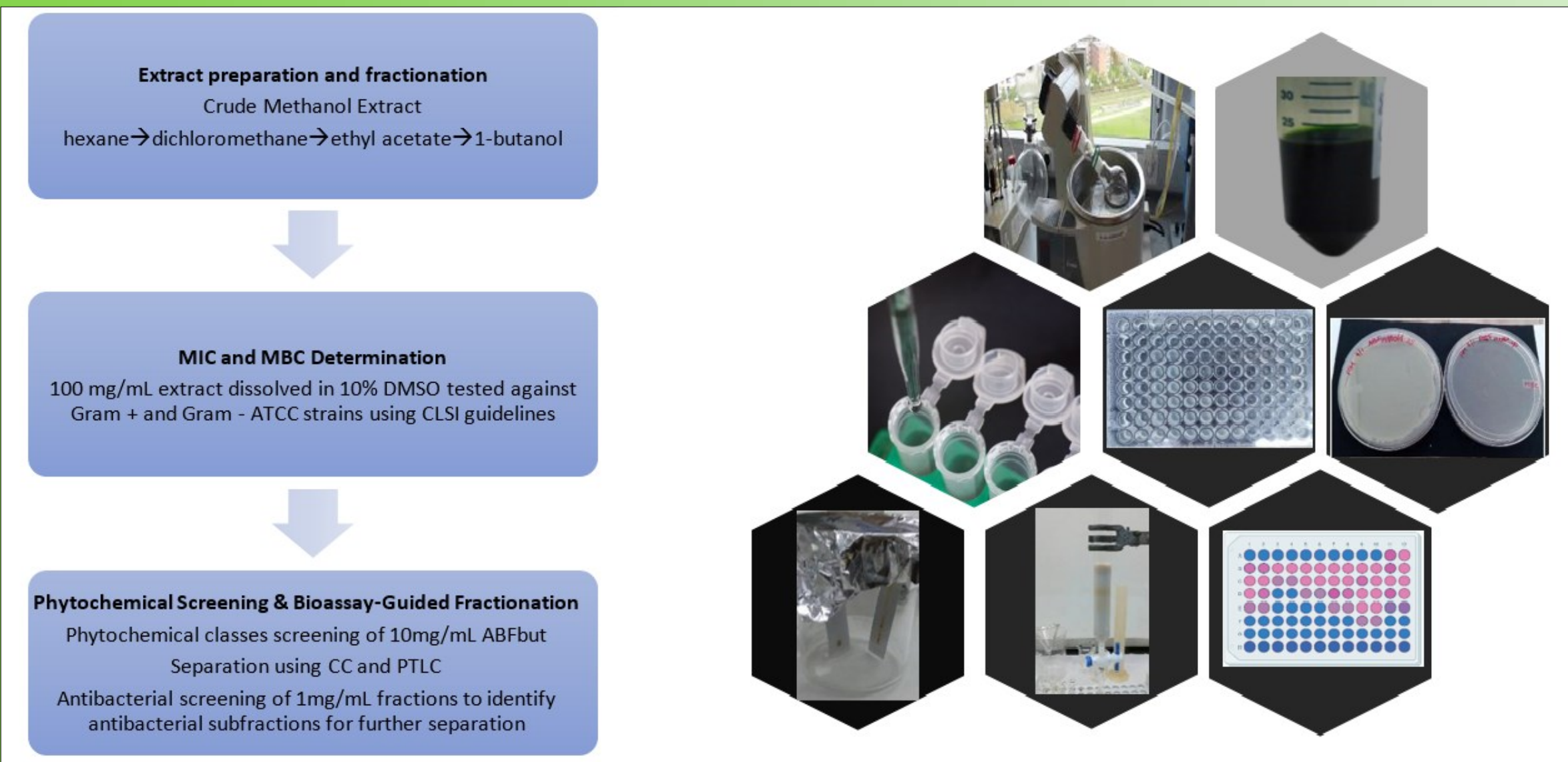


Figure 2: General methodology for extract preparation, fractionation and screening for antimicrobial activity.

RESULTS

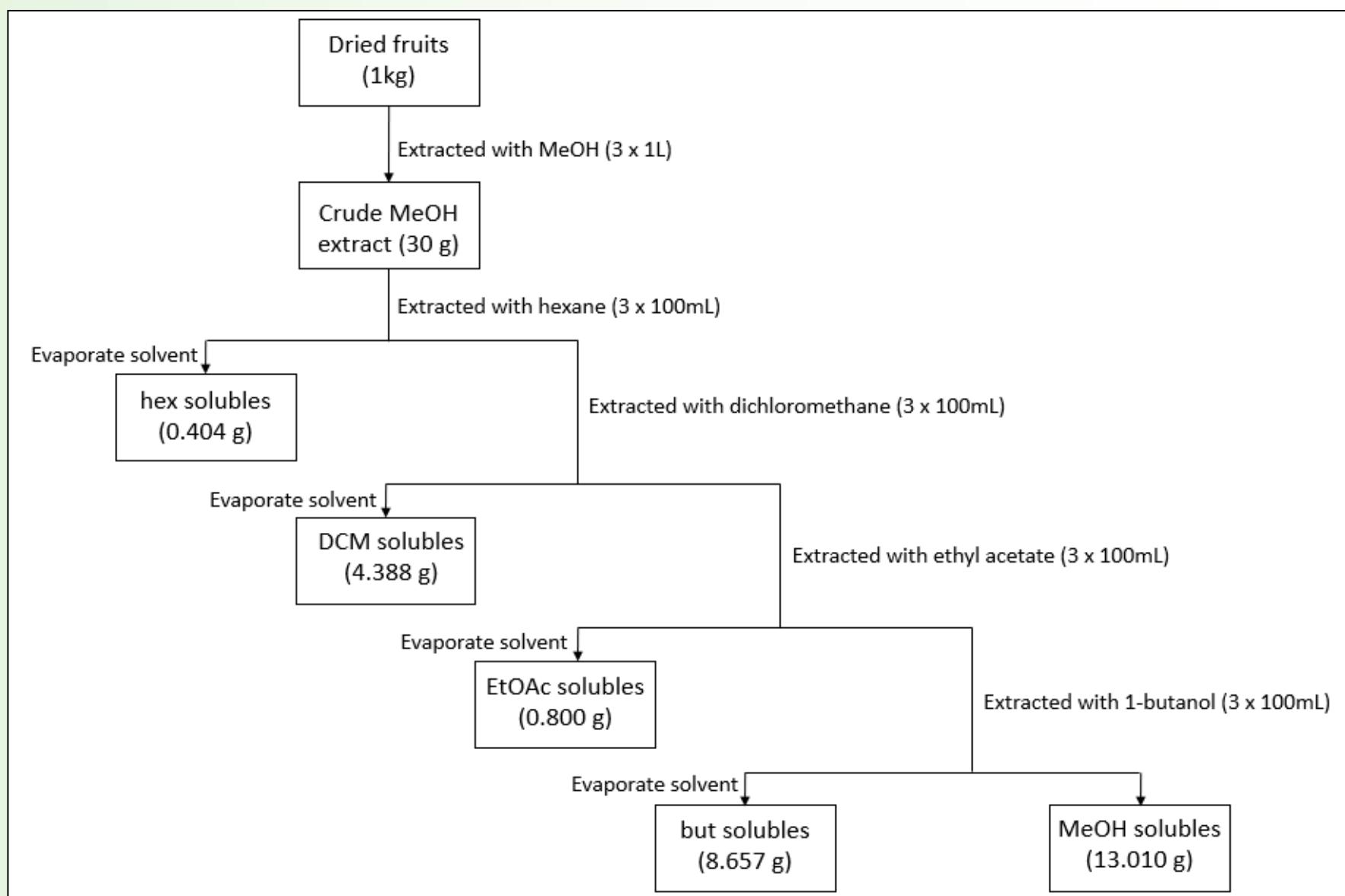


Figure 3: Fractionation of crude methanol extract of *Averrhoa bilimbi* fruits using solvents of increasing polarity: hexane (hex), dichloromethane (DCM), ethyl acetate (EtOAc) and 1-butanol (but). Masses of the dry solvent fractions after solvent evaporation are shown in brackets.

Table 1: Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs) of solvent fractions of the crude methanol extract of *Averrhoa bilimbi* fruits against reference strains Gram-positive and Gram-negative bacteria

Extract/ Fraction	<i>S. aureus</i> ATCC 12600		<i>E. faecium</i> ATCC 19434		<i>E. coli</i> ATCC 35218		<i>K. pneumoniae</i> ATCC 49474		<i>P. aeruginosa</i> ATCC 27853		<i>S. typhimurium</i> ATCC 14028	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ABFMeOH	25	25	12.5	25	25	25	12.5	25	12.5	25	12.5	25
ABFhex	50	50	50	50	50	50	50	50	25	50	25	25
ABFDCM	25	50	25	50	25	50	50	50	25	50	25	>50
ABFEtOAc	12.5	25	25	25	12.5	25	12.5	12.5	12.5	12.5	25	50
ABFbut	6.25	25	3.13	25	6.25	12.5	6.25	12.5	3.13	12.5	6.25	6.25

*Data are presented as [mg/mL]

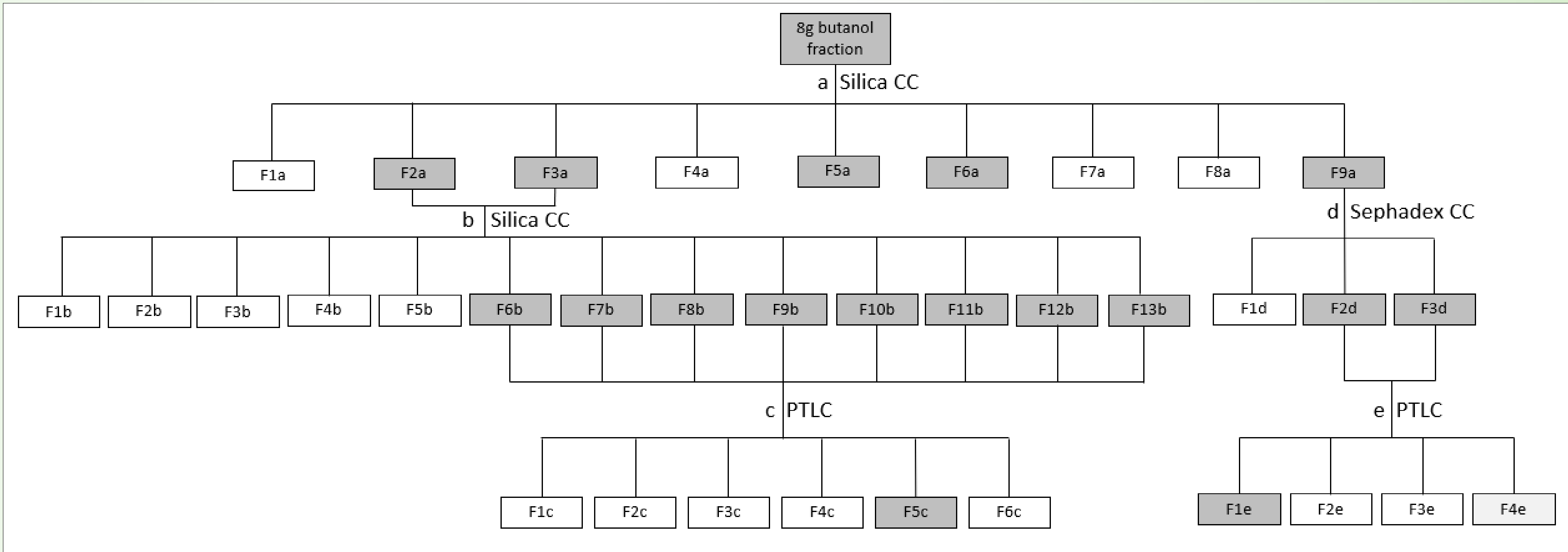


Figure 4: Bioassay-guided fractionation of 1-butanol fraction of *Averrhoa bilimbi* fruits. Fractions were numbered according to the order of elution and the letter indicated the solvent systems used: a) 7DCM: 3MeOH gradient elution to 100% MeOH; b) 7DCM: 3EtOAc: 4MeOH; c) 5EtOAc: 4MeOH: 1H₂O; d) 8EtOAc: 2H₂O; e) 5EtOAc: 5 acetone: 1MeOH: 1H₂O. Fractions with antimicrobial activity (shaded) were pooled and separated further. Fractions F5a and F6a, although antimicrobial, were not processed further since the activity was most likely attributed to oxalic acid (white, needle-like crystals, acidic pH~3). Fractions F5c and F1e were analyzed by analytical TLC.

Table 2: Classes of bioactive compounds present in 1-butanol fraction of *Averrhoa bilimbi* fruits

Test*	Class of compounds	ABFbut
Folin	Phenolics	+
AlCl ₃	Flavonoids	+
FeCl ₃	Tannins	+
Dragendorff	Alkaloids	+
Fehling	Carbohydrates	+
Foam	Saponins	–
Salkowski	Steroids	–
Ninhydrin	Proteins/ amino acids	–
TCA	Triterpenoids	–

* test-specific reagents used for each class of compounds as described by Shukla et al.⁵ and Hussain and El-Anssary⁶.

+/- indicates presence/absence of compounds respectively

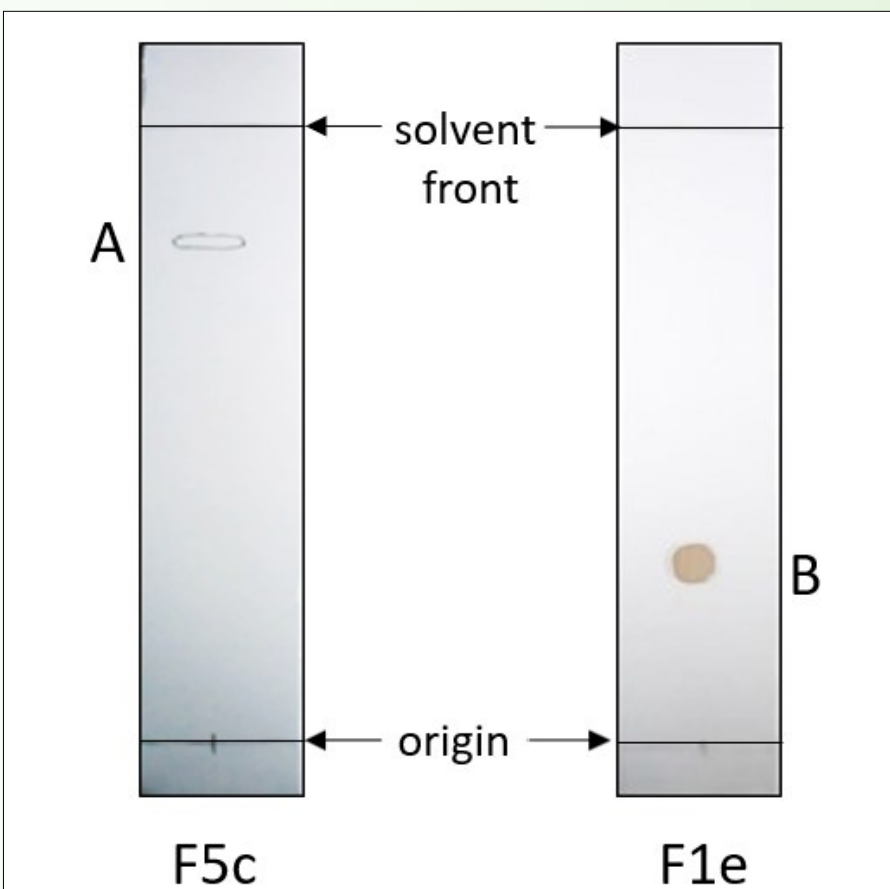


Figure 5: Compounds A and B confirmed as single spots by analytical TLC in fractions F5c and F1e respectively. The solvent systems used were: Compound A - 5EtOAc: 4MeOH: 1H₂O (Rf = 0.8) and Compound B - 5EtOAc: 5 acetone: 1MeOH: 1H₂O (Rf = 0.3).

SUMMARY

- All solvent fractions of the crude methanol extract from *Averrhoa bilimbi* fruits (ABF) displayed varying degrees of antibacterial activity.
- The 1-butanol ABF fraction (ABFbut) was confirmed to be:
 - the most potent one against all tested ATCC reference strains bacteria (MICs in the range 3.125 – 6.25 mg/mL);
 - bactericidal (MBC ≤ 4 x MIC) towards all bacterial strains except *E. faecium* (MBC ~ 8x MIC);
 - broad-spectrum, inhibiting both Gram-positive and Gram-negative bacteria.
- Preliminary phytochemical screening revealed the presence of phenolics, flavonoids, tannins, alkaloids and carbohydrates in the ABFbut. These classes of compounds have been previously found to have antimicrobial activities.
- Subsequent bioassay-guided fractionation of the ABFbut was carried out based on:
 - sufficient mass of the material to be assayed (>10 mg);
 - appropriate solvent system;
 - demonstrated antimicrobial activity against *S. aureus* and *E. coli*;
 - purity of the fraction confirmed by TLC.
- Two antimicrobial compounds were confirmed as single spots by analytical TLC: compound A (yellow, crystalline solid) - a flavonoid, and compound B (brown, amorphous solid) - an alkaloid.

CONCLUSIONS AND FUTURE WORK

A. bilimbi fruits are a source of antibacterial compounds with pharmacological potential. Further studies are warranted to elucidate the structures of the two pure active compounds A and B, using IR, 1D and 2D NMR techniques, and to determine their MICs and MBCs against reference bacterial strains and multidrug resistant clinical isolates. Additional studies on safety, mechanism of action and *in vivo* activities of the active compounds A and B would be required to realize their pharmaceutical applications. It is anticipated that this study would promote the wider usage of the edible *A. bilimbi* fruits in Trinidad and Tobago, thus addressing the Sustainable Development Goal 3 to promote good health and wellbeing. This research also contributes to the field of natural products and pharmacognosy in the country.

ACKNOWLEDGEMENTS

This study was made possible by support for research from The UWI Campus Research and Publication (CR&P) Fund (grant CRP.5.OCT18.67) and a postgraduate scholarship from the School for Graduate Studies and Research (SGS&R, The UWI) awarded to F.M. The authors thank Mr. Andrew Mathura for assistance in collecting plant material. The technical support provided by members of the Microbiology Research Group (DLS, FST, The UWI) is greatly appreciated. Special thanks are extended to Dr. Georgette Briggs (Barrett Honors College, Arizona State University, USA) for reviewing and commenting on the abstract.

REFERENCES

- An Online Flora of All Known Plants. Supporting the Global Strategy for Plant Conservation. <http://www.worldfloraonline.org/search?query=Oxalidaceae> (accessed Jul 25, 2021).
- Alhassan, A.M., Ahmed, Q.U. *Averrhoa bilimbi* Linn.: A Review of its Ethnomedicinal Uses, Phytochemistry and Pharmacology. *J Pharm Bioallied Sci* 2016, 4, 265-271.
- Ebada, S.S., Edrada, R.A., Lin, W., Proksch, P. Methods for Isolation, Purification and Structural Elucidation of Bioactive Secondary Metabolites from Marine Invertebrates. *Nature Prot* 2008, 3, 1820-1831.
- Clinical Laboratory and Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 11th ed.; Approved Standard M07; 2019, Wayne, PA: CLSI.
- Shukla, S., Mehta, A., Bajpai, V.K. Phytochemical Screening and Anthelmintic and Antifungal Activities of Leaf Extracts of *Stevia Rebaudiana*. *J Biol Active Prod from Nat* 2013, 3, 56-63.
- Hussein, R.A., El-Anssary, A.A. Plants Secondary Metabolites: The Key Drivers of the Pharmacological Actions of Medicinal Plants. 2018. Herbal Medicine. UK: IntechOpen. doi:10.5772/intechopen.76139. (accessed July 25 2021).