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Averrhoa bilimbi: A Prospective Source of Bioactive Compounds against Antimicrobial and Cytotoxic Activities

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Averrhoa bilimbi fruit has been studied to find out a possible source of bioactive compounds against antimicrobial and cytotoxic activity. To achieve this goal, total phenolic content and total flavonoid content, antioxidant, antimicrobial and cytotoxic activities of five different fractionates such as methanolic crude, petroleum ether, chloroform, ethyl acetate and water fraction of *A. bilimbi* were investigated by UV-vis spectrophotometry, disc diffusion and brine shrimp lethality test methods simultaneously. Other phytochemicals *viz*. carbohydrate, tannins, polyphenols, glycosides, *etc*. were also screened out by conventional test methods concurrently. Total phenolic contents and total flavonoid contents were found to be ranging from 3.807-262.129 mg GAE g^{-1} and 67.848-1159.061 mg CE g^{-1}, respectively, in all the analyzed fractions. DPPH free radical scavenging activity revealed distinctive IC_{50} value of 5.63 and 10.26 μg mL^{-1} in the chloroform and ethyl acetate extractives. Fractionates of petroleum ether and methanolic crude also presented strong cytotoxic potential with the LD_{50} values of 8.89 and 20.3 μg mL^{-1} in the brine shrimp lethality bioassay test. Petroleum ether and ethyl acetate fractions exhibited good antimicrobial activity with the zone of inhibition against *Bacillus cereus* (11 mm and 14 mm) and *Escherichia coli* (7 mm and 9 mm). Finally, it has been recommended that *A. bilmbi* fruit might be an effective source of bioactive compounds against antimicrobial and cytotoxic activity as well as antioxidant with potent radical scavenging activity.

Keywords: *Averrhoa bilimbi*, Phytochemicals, Flavonoid, Phenolic compounds, Total antioxidant activity.

INTRODUCTION

Plant derived remedies still plays a vital role in the primary health care in the world including in Bangladesh. In way of life, traditional familiarity of plants and their properties has achieved significant interest within the scientific civic in recent years. About 80% of the world's occupants' nuisance is treated by therapeutic herbal drug for their primary health care according to World Health Organization (WHO) survey [1]. A careful evaluation must be needed of plant materials composition and biological activity prior to use to encourage as a possible source of antimicrobial, antioxidant and/or cytotoxic agents [2].

Averrhoa bilimbi is a long-lived, multipurpose tropical plant which is a comestible and attractive fruit from the oxalidaceae family. It grows in Indonesia and cultivated or found in Philippines, Sri Lanka, India, Bangladesh, Myanmar and Malaysia. The fruit and leaf of the plant are used to treat traditionally for inflammatory conditions such as hepatitis, fever, diarrhoea, itches, swelling, pimples, hypertensions, ulcer, syphilis, skin eruptions, coughing, *etc*. [3]. Mokhtar and Aziz [4] reported that *A. bilimbi* fruit is also used for obesity in India. Different extracts of *A. bilimbi* fruit were established to demonstrate the antifungal action against *A. niger* and *C. albicans* [5]. The methanolic extract presented cytotoxic activity against MCF-7 human breast cancer cell lines [6]. Several researchers from Malaysia [7,8] have reported that *A. bilimbi* is as an important source of constituents responsible for antioxidant property, cytotoxicity and antimicrobial activity.

Phytochemical analysis of *A. bilimbi* fruit extract have revealed conflicting data on the existence of alkaloids, tannins, glycosides, saponins and steroids [9]. The phytochemicals

existent in the fruits of *A. bilimbi* by biochemical assessments investigated in this study to validate scientifically its medicinal properties. Total phenolic content, total flavonoid, total antioxidant and DPPH free radical scavenging activities were also studied to develop the antioxidant profile of different fractions.

A quantitative evaluation of macro and micronutrients of the fruit of *Averrhoa bilimbi* has been reported earlier [10]. It is recommended that *A. bilimbi* could be a potential source of many micro nutrients such as Ca, Fe, Zn, Mn, *etc*. Herein, the antioxidant, cytotoxic and antimicrobial profiles of methanolic crude extract of *A. bilimbi* and its further partitioned fractions including petroleum ether, chloroform, ethyl acetate and water fraction are reported, which might accelerate further research for the isolation of bioactive compounds against different ailments.

EXPERIMENTAL

All the chemicals used for this experiment were of analytical grade. Organic solvents and other chemicals were procured from Sigma (USA) and/or Merck (Germany) without additional refinement. Catechin, DMSO and agar nutrient were purchased from Wako Pure Chemical Industries Ltd., Japan.

Extraction of fruit: *Averrhoa bilimbi* fruits were collected from the residential area (Pachimpara) of Rajshahi University Campus, Bangladesh. The fruits authentication was done by one of the Professors of the Botany Department of Rajshahi University. After collection, the ripe fruits were washed with distilled water and then air dried. The fruit (3 kg) were used for extraction process with methanol (4 L, 95% v/v) in a Soxhlet apparatus for the period of 48 h. Then, the methanolic extract was concentrated by using a rotary vacuum evaporator (Buchi Rotavapor, R-200) and the concentrated part was presented as methanolic crude extract (MC). The methanolic crude extract was then further partitioned into petroleum ether, chloroform, ethyl acetate and water fraction by Kupchan partitioning method.

Analysis of phytochemicals: Screening of phytochemicals of the obtained five different fractions *viz*. methanolic crude, petroleum ether, chloroform, ethyl acetate and water fraction of *Averrhoa bilimbi* has been carried out by the convenient protocols [11,12] in order to examine the presence of various phytoconstituents.

Assay of total phenolic content: Total phenolic content was determined according to the method described by Singleton and Rossi [13]. Typical 300 µL solutions of the fractions were mixed with 2.25 mL Folin-Ciocalteu reagent. The reaction mixture was then kept 5 min at room temperature and 2.25 mL 6% Na_2CO_3 solution was added. Absorbance of the prepared reaction mixture was determined at 760 nm by UV-Vis spectrophotometer (APEL, PD-303S, Japan). Gallic acid was used as standard and all the fractions were performed in triplicates.

From the plot of different absorbance against corresponding concentrations of gallic acid, a regression equation, $y = 0.0147x + 3 \times 10^{-5}$) with $R^2 = 0.9907$ was established. Results were expressed as mg gallic acid equivalent (GAE) per gram of the fraction using the following equation (1).

$$C = \frac{cV}{M} \quad (1)$$

where, C = concentration of total phenolic compound in mg gallic acid equivalent (GAE) per gram of the fraction, c = concentration of gallic acid, V = volume of the fraction and M = weight of the fraction.

Assay of total flavonoid content: The total flavonoid content of the fractions of *A. bilimbi* fruit was determined by aluminium chloride colorimetric assay [14] where catechin was used as reference material. A solution of fraction (500 µL) was mixed with 2.25 mL distilled water and then 150 µL 5% $NaNO_2$ solution was added and incubated for 6 min at room temperature. After that 300 µL of 10% $AlCl_3$ solution was added and kept stand for 5 min. Then, 1 mL of 1 M NaOH was added and the absorbance was taken by using UV-Vis spectrophotometer at 510 nm. All the fractions were triplicated in the same way.

From the plot of absorbance against corresponding concentrations of catechin, a regression equation, $y = 0.0022x + 0.0134$ with $R^2 = 0.9982$ was established. Total flavonoid content of the fractions was measured by using this equation and result was expressed in mg catechin equivalent per gram of the fraction by the following equation:

$$C = \frac{cV}{M} \quad (2)$$

where, C = concentration of total flavonoid content in mg catechin equivalent (CE) per gram of the fraction, c = concentration of catechin, V = volume of the fraction and M = weight of the fraction.

Assay of total antioxidant capacity: Total antioxidant capacity was determined by the phosphomolybdenum method in which ascorbic acid used as reference [15]. Typical 300 µL of the extracts in various concentrations (10 µg mL^{-1} to 100 µg mL^{-1}) was treated with 3 mL of reagent solution (0.8 M sulphuric acid, 14 mM sodium phosphate and 4% ammonium molybdate). Then, the reaction mixture was kept under water bath at 95 ºC for 90 min. After cooling the reaction mixture, absorbance was measured at the wavelength 695 nm. The antioxidant capacity was articulated as the number of equivalent of ascorbic acid.

DPPH scavenging assay: DPPH free radical scavenging activity of the fractions was determined with some modifications where ascorbic acid was used as positive standard [16,17]. The test was performed by treating 3 mL of 0.1 mM freshly prepared methanolic solution of DPPH into 1 mL of the extract prepared in various concentrations (10 µg mL^{-1} to 100 µg mL^{-1}). The mixtures were shaken and allowed to stand in dark for 0.5 h for completion of reaction at room temperature. Then, absorbance of the mixture was determined at the wavelength 517 nm against blank methanol. Standard ascorbic acid and all the fractions were performed in triplicates. Corresponding percentage of radical scavenging activity was calculated using the following equation:

$$\text{Radical savenging activity (\%)} = \left\{1 - \left(\frac{A_{sample}}{A_{control}}\right)\right\} \times 100 \quad (3)$$

The IC_{50} values of a fraction were calculated by linear regression equation obtained from the plot of % of radical scavenging activities against respective concentrations.

Cytotoxic activity: The cytotoxic action of the extracted fractions in DMSO was applied to brine shrimp (*Artemia salina*) for 24 h [18,19]. The eggs of *Artemia salina* were collected and hatched in a small partitioned tank containing artificial sea water (3.8% NaCl) with constant oxygen supply at 37 ºC. Each fractions (6 mL) is taken in 200 µL DMSO and adjusted to 5 mL by adding artificial sea water to prepare solution of 600, 300, 150, 75 and 37.5 µg mL^{-1} by serial dilution. Then, the solutions were added to the vials containing 20 living nauplii in 2.5 mL artificial sea water. The living nauplii were counted by the aid of magnifying glass after 24 h observation. Calculation of the % of mortality at each concentration and control was determined as follows:

$$\text{Mortality } (\%) = \left(\frac{\text{Actual number of dead nauplii}}{\text{Total number of nauplii}} \right) \times 100 \quad (4)$$

The LC_{50} value after 24 h was determined by a plot of prohibit against the log of corresponding concentration.

Antimicrobial activity: Antimicrobial activity of the different fractions was determined by disc diffusion method with slight modification [4]. Gram-positive *Bacillus cereus* and Gram-negative *Escherichia coli* bacteria were used to determine the antimicrobial activity. The bacterial strains (obtained from Rajshahi Medical College, Bangladesh) were cultured in nutrient agar and inoculated in 10 mL Luria-Bertani (LB) broth. Then incubated at 37 ºC for 24 h on incubator shaker at 150 rpm and then refrigerated at 4 ºC. Discs with 50 µg of the sample were dried for 3 h in a laminar flow hood. After that discs were placed at the petri dish containing agar nutrient and suspension of bacteria and kept in refrigerator for 4 h observation. The petri dish was then incubated at 37 ºC for 12 h. The results were obtained by measuring inhibition zone diameters and compared to kanamycin (K-30) as reference drug.

RESULTS AND DISCUSSION

Phytochemicals analyses: The presence of medically significant phytochemicals has been identified in the different fractions whose have countless potentialities in drug innovation for numerous diseases. It is seen from Table-1 that phenols, flavonoids, glycosides and steroids are present whilst alkaloids are absent. These phytochemicals could fight against different types of pathogens as well as they are being used as medicine to cure illnesses. The absence of alkaloids in the fractions might be owing to the inappropriate selection of solvent for this group of compounds. Another reason might be region-wise alteration, soil/water quality and the mode of accumulation of phytochemicals in any plants. However, alkaloids were found in ethanolic fraction of this fruit [20].

Total phenolic and flavonoid analyses: Total phenolic and flavonoid contents of different fractions of *A. bilimbi* are shown in Fig. 1. The values of total phenolic content are found to be 125.41 mg GAE g^{-1}, 3.80, 213.69, 262.12 and 81.47 mg GAE g^{-1} in the methanolic crude, petroleum ether, chloroform, ethyl acetate and water fractions, respectively. It is found that ethyl acetate fraction showed the highest (262.129 mg GAE g^{-1}) whilst the petroleum ether fraction showed the lowest (3.807 mg GAE g^{-1}) value. The values of the phenolic content followed the order as: ethyl acetate > chloroform > methanolic crude > water fraction > petroleum ether. The mechanism of antioxidant activity of the polyphenolic compounds has been explained on the basis of its -OH group, hydrophobic benzenoid ring as well as the hydrogen bonds, which have the scavenging power [21-23].

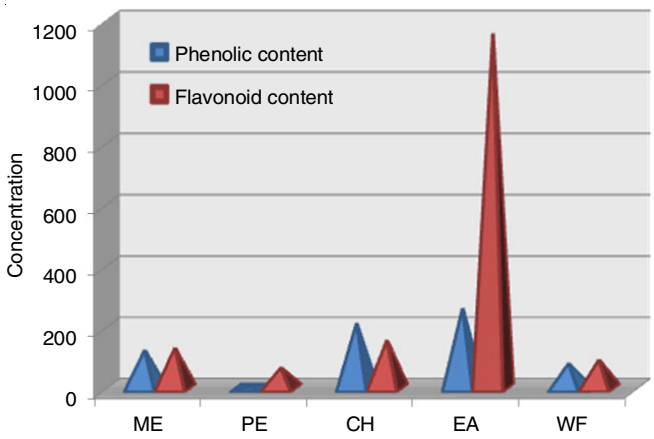

Fig. 1. Total phenolic and flavonoid content of the different fractions of *A. bilimbi*. Here ME, PE, CH, EA and WF means methanolic crude, petroleum ether, chloroform, ethylacetate and water fractions, respectively. The concentration is expressed as mg per gallic acid and/or mg per catechin equivalent

The values of total flavonoid contents are found to be 193.60, 67.84, 157.93, 1159.06 and 132.39 mg CE g^{-1} in the methanolic crude, petroleum ether, chloroform, ethyl acetate and water fractions, respectively (Fig. 1). It has been observed

TABLE-1
PRELIMINARY SCREENING OF PHYTOCONSTITUENTS PRESENT IN DIFFERENT FRACTIONS OF *A. bilimbi*

Photochemical	Test/reagent name	Extracts				
		Methanolic crude	Petroleum ether	Chloroform	Ethyl acetate	Water fraction
Tannin	Ferric chloride/lead acetate test	Present	Present	Absent	Present	Present
Saponins	Frothing test	Present	Present	Absent	Present	Present
Phenol	Ferric chloride/lead acetate test	Present	Present	Present	Present	Present
Flavonoid	Alkaline reagent test	Present	Present	Present	Present	Present
Glycoside	Keller-Kiliani test	Present	Present	Present	Present	Present
Carbohydrate	Fehling's/Benedict's test	Present	Absent	Present	Present	Present
Alkaloids	Dragendorff's test	Absent	Absent	Absent	Absent	Absent
Steroids	Sulphuric acid test	Present	Present	Present	Present	Present

that the ethyl acetate fraction exhibited the highest value of 1159.06 mg CE g^{-1}. Total phenolic content in the ethyl acetate fraction also showed the highest value. The values of the flavonoid content followed the same order as found in the earliest examination: ethyl acetate > chloroform > methanolic crude > water fraction > petroleum ether. From the analyses of total phenolic/flavonoid content, it has been suggested that *A. bilimbi* fruit extract have a lot of polyphenolic compounds that have cytotoxicity as well as radical scavenging property.

Total antioxidant capacity analyses: It is seen from the Fig. 2 that the methanolic crude and water fractions showed the lower antioxidant in comparison to that of the standard ascorbic acid. Conversely, ethyl acetate and chloroform fractions showed the higher antioxidant in comparison to that of the standard ascorbic acid. From this observation, it is seemingly assumed that ethyl acetate and chloroform fractions contain higher amount of polyphenolic compounds than that of the other fractions.

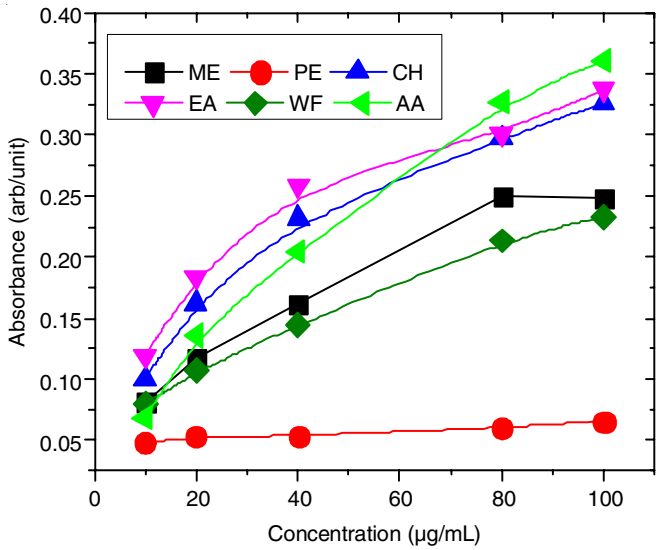

Fig. 2. Total antioxidant capacity of the different fractions of *A. bilimbi*. Here ME, PE, CH, EA, and WF means methanolic crude, petroleum ether, chloroform, ethyl acetate and water fractions, respectively. Ascorbic acid has been used as standard

DPPH free radical scavenging analyses: Plants are the natural sources with high excellence and extent of antioxidants that could scavenge free radicals [24]. DPPH free radical scavenging activity of the different fractions of *A. bilimbi* is shown in Fig. 3. The corresponding IC_{50} values calculated from the regression equation are shown in Fig. 4. It is universal fact that extracts with the highest scavenging activity would exhibit the lowest IC_{50} values and *vice-versa*. The second most activity in comparison to that of standard was found to be in chloroform (5.63 µg mL^{-1}) extract and followed by the ethyl acetate (10.26 µg mL^{-1}), water (20.82 µg mL^{-1}), methanolic crude (25.23 µg mL^{-1}) and petroleum ether (212.95 µg mL^{-1}) fraction. Almost similar results have been reported compared with BHT as standard [16]. From the outcome of total phenolic contents, total flavonoid contents, total antioxidant and DPPH free radical scavenging activity, it could be recommended that *A. bilimbi* might be a good source of antioxidant. It is clear that all the

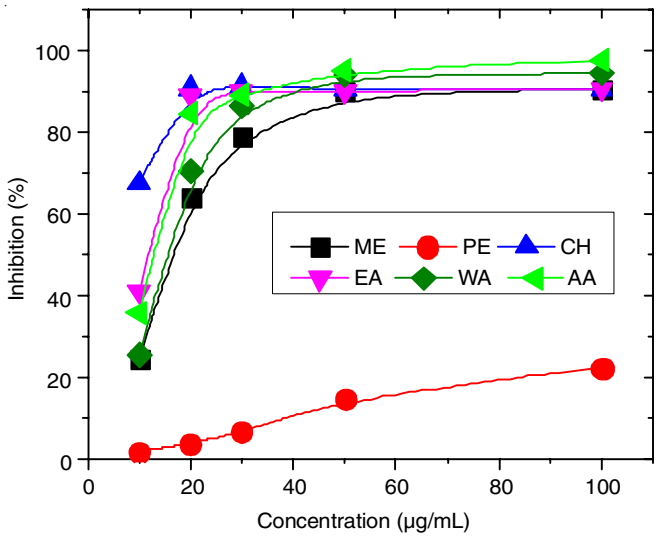

Fig. 3. DPPH radical scavenging activity of the different fractions of *A. bilimbi*. Here ME, PE, CH, EA, and WF means methanolic crude, petroleum ether, chloroform, ethylacetate and water fractions, respectively. Ascorbic acid has been used as standard

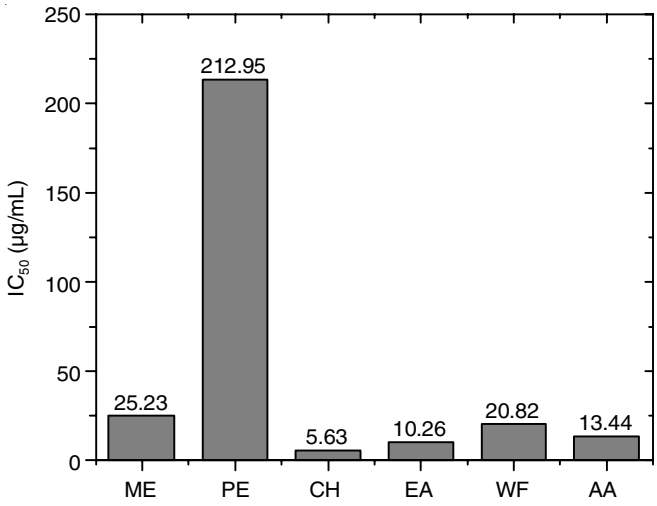

Fig. 4. IC_{50} values of different fractions of *A. bilimbi*. Here ME, PE, CH, EA and WF means methanolic crude, petroleum ether, chloroform, ethylacetate and water fractions, respectively. Ascorbic acid has been used as standard

extractives of *A. bilmibi* possess DPPH radical scavenging activity and the activity increases in the order of: Ascorbic acid > chloroform > ethyl acetate > water fraction > methanolic crude > petroleum ether. From the observation, it is assumed that the chloroform would be a good solvent for separating the polyphenols from *A. bilmibi*, which are potent antioxidant and negatively correlated to IC_{50} of DPPH radical scavenging.

Cytotoxic activity: Cytotoxic activity of the different fractions of *A. bilimbi* fruit has been investigated *in vitro* against the brine shrimp lethality test in which gallic acid has been used as reference. The corresponding LD_{50} values calculated from the linear regression equation are tabulated in Table-2. Comparison results of the different fractions are shown in Fig. 5. The brine shrimp lethality bioassay of petroleum ether exhibited the most characteristic of the lowest LD_{50} value of 8.89 µg mL^{-1} whereas the LD_{50} value of the standard gallic acid showed

TABLE-2
COMPARATIVE LD_{50} VALUE OF DIFFERENT FRACTIONS OF *A. biimbi* AND GALLIC ACID

Fractions	LD_{50} value (μg mL^{-1})	Linear regression equation	R^2
Methanolic crude	20.30	y = 1.156x + 3.4884	0.9690
Petroleum ether	8.89	y = 1.1361x + 3.9217	0.9173
Chloroform	58.00	y = 1.0763x + 3.1019	0.9052
Ethyl acetate	37.50	y = 1.063x + 3.3268	0.9786
Water fraction	54.13	y = 0.9534x + 3.3473	0.8966
Gallic acid	81.48	y = 1.44x +2.239	0.1261

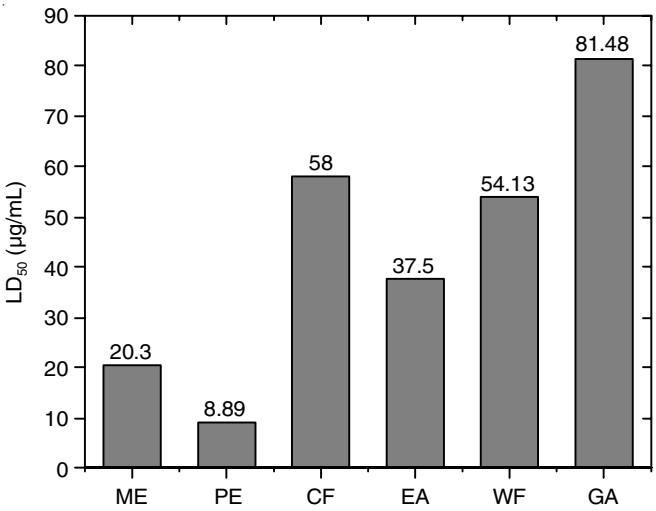

Fig. 5. LD_{50} values of different fractions of *A. bilimbi*. Here ME, PE, CF, EA and WF means methanolic crude, petroleum ether, chloroform, ethylacetate and water fractions, respectively. Gallic acid has been used as standard

the highest value of 81.48 μg mL^{-1}. The other fractions such as methanolic crude showed the value 20.3 μg mL^{-1} followed by ethyl acetate with 37.5 μg mL^{-1}, water fraction with 54.13 μg mL^{-1} and chloroform with the value of 58 μg mL^{-1}.

From the observation, it is evident that the entire fraction exhibited characteristic cytotoxic activity and this is because of the presence of toxic components or metabolites that are either affected embryonic development or slay of the eggs [25]. Quite different results of *A. bilimbi* fruit and leaf have been reported in which vincristine sulphate was used as standard [26,27]. The dissimilarity findings might be due to the difference mode of bioaccumulation of different bioactive compounds and the other reasons might be of the locality and/or region. However, on the basis of the results it is recommended to be that *A. bilimbi* fruit might be a good natural source of anticancer drug discovery.

Antimicrobial activity: The antimicrobial activities of the different fractions of *A. bilimbi* fruit extract are performed against two pathogens namely *B. cereus* and *E. coli*. The results are shown in Table-3. The zone of inhibition of ethyl acetate fraction was measured to be of 7 mm and 9 mm against *B. cereus* and *E. coli*, respectively. This activity might be due to the presence of polyphenolic compounds in this extract [28]. The zone of inhibition exhibited by the petroleum ether (PE) fraction was found to be of 11 mm and 14 mm against *B. cereus* and *E. coli*, respectively. Although, from the analyses results, the presence of polyphenols in petroleum ether is lower than the other fractions. However, the higher zone of inhibition of petroleum ether might be due to the presence of specific polyphenols, which are responsible to increase the microbial growth. Other fractions such as methanolic crude, chloroform and water fractions did not exhibit considerable zone of inhibition in spite of having high presence of polyphenols assumed from the antioxidant activity test. The low/absence of zone of inhibition might be due to the low concentration (50 μg $disc^{-1}$) of the fractions and/or tannins are one of the phytochemicals, which have been revealed to prevent the microbial growth [29]. The confrontation of the organisms to the extract could be attributed to the outer membrane in the cell wall of the bacteria which prohibited the penetration of hydrophilic compounds into the cells [30].

Conclusion

Finally, it is apparent that *A. bilimbi* fruit might be a potent source of phytochemicals (natural products), antioxidant and cytotoxicity. Ethyl acetate and chloroform fractions exhibited high possibility to contain bioactive compounds responsible for antioxidant activities whereas petroleum ether fraction exhibited high possibility to contain bioactive compounds responsible for cytotoxic activities. The strong cytotoxic properties of the extracts recommended the existence of bioactive principles in the plants. Petroleum ether and ethyl acetate fractions exhibited good antimicrobial activity against *B. cereus* and *E. coli* and are considered to be an excellent source of bioactive compounds responsible for antimicrobial and cytotoxic activity. Furthermore, the bioactivities revealed by the extractives of *A. bilimbi* support the traditional practices of these plants against many diseases.

TABLE-3
ZONE OF INHIBITION OF DIFFERENT FRACTIONS OF *A. bilimbi* WHERE KANAMYCIN WAS USED AS REFERENCE. TWO PATHOGENS: (i) GRAM-POSITIVE *Bacillus cereus* AND (ii) GRAM-NEGATIVE *Escherichia coli* WERE USED IN THIS STUDY

Pathogens	Zone of inhibition (mm)					
	Methanolic crude	Petroleum ether	Chloroform	Ethyl acetate	Water fraction	Kanamycine
Bacillus cereus	–	11	–	7	–	27
Escherichia coli	–	14	–	9	–	31

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CONFLICT OF INTEREST

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

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