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ISOLATION AND CHARACTERIZATION OF SECONDARY METABOLITES OF COMMONLY AVAILABLE *AVERRHOA BILIMBI* L.

SHEIKH ADNAN SAKIB, MM TOWHIDUL ISLAM AND MD ENAMUL HAQUE*

Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka-1000, Bangladesh

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Abstract

Averrhoa bilimbi L., locally known as bilimbi, is a medicinal plant, well-recognized in Bangladesh for its fruit, Carambola. This study aims to identify novel phytochemical compounds responsible for the health benefits of the plant by analyzing its stem bark. The stem barks were collected, dried, and ground to extract the phytochemicals, which were then analyzed using microbiological, chromatographic, and spectroscopy-based methods. The analysis revealed that the extract contained sitosterone and lupeol. Biochemical characterization demonstrated that the extract has potential antibacterial, antifungal, antioxidant, and cytotoxic properties. Further research is needed to characterize these compounds and determine their full spectrum of efficacy.

Introduction

Plants with medicinal properties, have been utilized throughout human history for the treatment of different diseases. Medicinal plants show therapeutic properties and exert beneficial pharmacological effects on the body (Nagmoti *et al.* 2010, Setyawan *et al.* 2021). *Averrhoa bilimbi* (local name bilimbi) is a medicinal plant originally native to the Maluku Islands of Indonesia but has naturalized and is now common throughout Southeast Asia. It has been reported that *Averrhoa bilimbi* has hepatoprotective, antioxidant, anti-fertility, antidiabetic, anticoagulant, and antimicrobial activities. The paste of the pickled bilimbi is to get rid of fever. The fruit helps in stopping bleeding in the stomach, rectum, and eliminating internal hemorrhoids (Nagmoti *et al.* 2010). It also fights against cholesterol and obesity, mumps, pimples, diabetes, rheumatism, syphilis, whooping cough, hypertension, and ulcer as well as can be used as a tonic and laxative (Tan *et al.* 2005). Supporting to this *Averrhoa bilimbi* extract showed hypoglycemic, hypotriglyceridemic, anti-lipid peroxidative, and anti-atherogenic properties in STZ-diabetic rats (Kumar *et al.* 2013).

Phytochemical screening of *Averrhoa bilimbi* extracts yielded flavonoids, saponins, and triterpenoids (Kumar *et al.* 2011). The chloroform and methanol fruit extracts exhibited antibacterial activities (Prastiyanto *et al.* 2020). A study of the aqueous extract of leaves and fruits showed antibacterial activity due to flavonoids (Chau *et al.* 2023). Since this plant has a lot of medicinal properties and contains biologically active compounds, a deeper analysis of the Bangladeshi variant of this plant both phytochemically and biologically may help to identify and isolate novel compounds.

Interestingly, systematic analysis of *A. bilimbi* yielded two triterpenoids botulin and lupeol (Setyawan *et al.* 2021). Moreover, other extracted compounds showed promising antibacterial, antifungal, cytotoxic, and antioxidant activities. Thus, the expected outcome was to purify and

*Author for correspondence: <enamulmd@du.ac.bd>.

characterize compounds from the stem bark extract of *Averrhoa bilimbi* to study their antibacterial, antifungal, cytotoxic, antioxidant, analgesic, and anti-inflammatory activities. It is expected that the characterization of these compounds will help to use these chemicals properly and effectively by different healthcare professionals in Bangladesh.

Materials and Methods

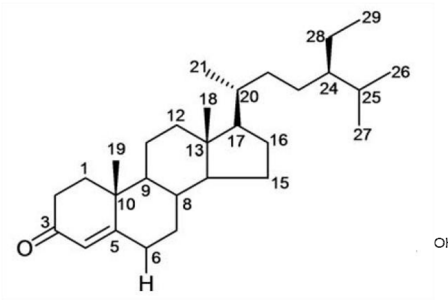
Stem barks from *Averrhoa bilimbi* were collected, dried up, and then grounded. After extraction by appropriate solvents (petroleum ether, ethyl acetate, and methanol), extracts were fractionated by flash column chromatography. Fractionated compounds were detected by various spreading reagents and by the use of UV light on the thin layer chromatographic plates. Then isolation of compounds was done by preparative thin layer chromatography to identify them using IR, ^{1}H NMR, and ^{13}C NMR spectroscopy as well as by comparing with reported values (Chau *et al.* 2023).

After that, several properties of the extracts (antibacterial, antifungal, cytotoxic, and antioxidant activity) were studied. The antimicrobial activity was assessed by disc diffusion method (Mokhtar *et al.* 2016), cytotoxic activity was tested by brine shrimp lethality assay (Ali *et al.* 2013, Chowdhury *et al.* 2012), and antioxidant activity was followed by DPPH assay (Utami *et al.* 2019). Moreover, the biological activities of the different plant extracts as well as isolated pure compounds were also studied. The MIC, $LC_{50,}$ and LD_{50} values were also determined.

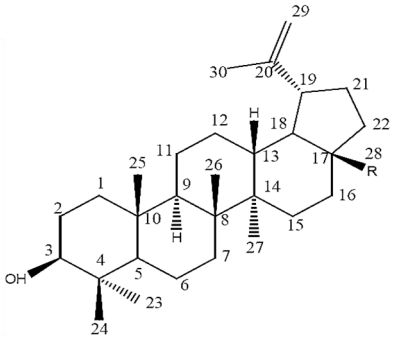
Results and Discussion

The ^{1}H-NMR of compound 1 was recorded and its absorption frequencies were identified by comparing the reported value of a known compound (Aung *et al.* 2020). Compound 1 was isolated from fraction no.4 of ethyl acetate extract of *Averrhoa bilimbi* as white crystals. Spraying the chromatographic plate with vanillin sulfuric acid followed by heating at 110^{o}C for several mins gave a dark-colored spot.

The ^{1}H spectrum of compound 1 showed a one-proton singlet at δ_H 5.70. This significant downfield signal of the olefinic proton at δ_H 5.71 was typical for H-4 of a steroidal nucleus containing a ketone group at the C-3 position. It also displayed two three-proton singlets δ_H at 1.024 and 0.696 assignable for the methyl group at C-18 and C-19, respectively. In addition, two three-proton doublets at δ_H 0.791 (J =7.6 Hz) and 0.813 (J = 7.2 Hz) could be ascribed to the two methyl groups at C-26 and C-27, respectively. Another three-proton doublet at δ_H 0.912 (J = 6.4 Hz) could be attributable to C-21. These ^{1}H NMR spectral features were characteristics of the steroidal carbon skeleton of sitosterone. On this basis and compared with published data, compound 1 was identified as sitosterone (Aung *et al.* 2020).



Compound 1



Compound 2

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound 2 showed one triplet of one proton intensity at δ_H 3.70 typical for H-3. The spectrum displayed two singlets of δ_H 4.668 (one proton each) assignable to protons at C-29. Doublet at δ_H 2.35 assignable to proton at C-19. The spectrum displayed seven singlets at δ_H 0.967, 0.771, 0.830, 1.006, 0.950, 0.794, and 1.642 (3H each) assignable to protons of methyl groups at C-4 (H3-23, H3-24), C-10 (H3-25), C-8 (H3- 26), C-14 (H3-27), C-17 (H3-28), and C-20 (H3-30), respectively. By comparing the ^{1}H NMR data of compound 2 with that of previously published data, it was confirmed as lupeol (Somwong *et al.* 2021).

The antimicrobial activities of the *Averrhoa bilimbi* extracts were examined in the present study (Tables 1 and 2). The zones of inhibition produced by the petroleum ether, ethyl acetate, and methanol extracts of *Averrhoa bilimbi* ranged from 07 mm to 11 mm. However, the methanol extract did not show sensitivity against any bacterial strain used.

The petroleum ether extract showed moderate activity against Gram-positive bacteria like- *Sarcinana lutea, Staphylococcus aureus, Bacillus subtilis,* and all Gram-negative bacteria, whereas, weak activity was found against *Bacillus cereus, and Bacillus megaterium.* Similarly, ethyl acetate extract exhibited moderate activity against Gram-positive bacteria: *Sarcinana lutea* and Gram-negative bacteria: *Vibrio mimicus, Klebsiella* sp., *Pseudomonas* sp., *Salmonella paratyphi, Shigella boydii.* But *Bacillus megaterium, and Staphylococcus aureus* were found to be insensitive. A weak activity was found against the Gram-positive bacteria: *Bacillus cereus, Bacillus subtilis, and* Gram-negative bacteria *Salmonella typhi, Vibrio cholera, Escherichia coli, Vibrio parahemolyticus*, and *Shigella dysenteriae.*

Table 1. Determination of zone of inhibition (mm) after 24 hrs of incubation of the different fractions of *Averrhoa bilimbi* against Gram-positive and Gram-negative bacteria.

	PEE (400 µg/disc)	EAE (400 µg/disc)	ME (400 µg/disc)	Ciprofloxacin (10 µg/disc)
Gram positive bacteria				
Bacillus cereus	8	7	8	44
Sarcinana lutea	9	10	9	43
Staphylococcus aureus	10	--	9	43
Bacillusmegaterium	8	--	8	44
Bacillus subtilis	10	7.5	10	44
Gram negative bacteria				
Vibrio mimicus	10	11	10	43
Salmonella typhi	9	8	9	43
Salmonella paratyphi	9	9	9	43
Shigella boydii	9	9	9	44
Shigelladysenteriae	9	8	9	44
Pseudomonas sp	9	10	9	44
Escherichia coli	10	8	9	44
Vibrioparahemolyticus	9	8	9	43
Klebsiella sp.	9	9	9	44
Vibrio cholerae	9	8	10	44

Table 2. Determination of zone of inhibition (mm) after 24 hrs of incubation of the Sub fraction F4-A1 and F4-A2 of *Averrhoa bilimbi* against Gram-positive and Gram-negative bacteria.

	F4-A1 extract (400 µg/disc)	F4-A2 extract (400 µg/disc)	Ciprofloxacin (10 µg/disc)
Gram positive bacteria			
Bacillus cereus	4	7	44
Sarcinana lutea	5	--	43
Staphylococcus aureus	8	--	43
Bacillus megaterium	6	7	44
Bacillus subtilis	4	--	44
Gram negative bacteria			
Vibrio mimicus	6	--	43
Salmonella typhi	4	--	43
Salmonella paratyphi	7	--	43
Shigella boydii	3	7	44
Shigella dysenteriae	4	8	44
Pseudomonas sp.	5	7	44
Escherichia coli	5	8	44
Vibrio parahemolyticus	6	7	43
Klebsiella sp.	5	--	44
Vibrio cholerae	4	--	44

Here "--" "means no zone of inhibition, PEE = Petroleum ether extract, EAE= Ethyl acetate extract, ME = Methanol extract.

The methanolic extract showed moderate activity against Gram-positive bacteria *Sarcinana lutea, Staphylococcus aureus, Bacillus subtilis, and* against all Gram-negative bacteria. A weak activity was found against Gram-positive bacteria *Bacillus cereus, Bacillus megaterium.* Sub-fraction F_4-A1 extract showed weak activity against all Gram-positive and-negative bacteria. Similarly, Sub fraction F_4-A2 extract showed weak activity against Gram-positive bacteria like- *Bacillus cereus, Bacillus megaterium*, and against Gram-negative bacteria – *Shigella boydii, Vibrio parahemolyticus, Pseudomonas sp, Escherichia coli, Shigella dysenteriae.* However, *Sarcinana lutea, Staphylococcus aureus, Bacillus subtilis, Vibrio mimicus, Salmonella typhi, Salmonella paratyphi, Vibrio cholera, Klebsiella sp.* showed no sensitivity.

The antifungal activity (Table 3) of different extracts of *Averrhoa bilimbi* was performed using the same disc diffusion method, as used for the antibacterial activity test. However, the only difference was that the period of incubation for plant extract was 48 hrs at room temperature. The different extracts showed different antifungal activity by showing promising zones of inhibition against the fungi named *Aspergillus niger.* In this test, Griseofulvin was used as a standard agent that showed antifungal activity with a range of 7 mm to 12 mm.

The per cent mortality of the brine shrimp nauplii was calculated after 24 hrs exposure of shrimps to the sample extracts and the positive control (vincristine sulfate) (Table 4) (Chowdhury *et al.* 2012). The positive control, compared with the negative control (sea water) was lethal, giving significant shrimp mortality. The degree of lethality was directly proportional to the concentration of the extracts ranging from moderate with the lowest concentration (0.781µg/ml) to significant with the highest concentration (400 µg/ml). As expected, it was found that mortality

increases gradually with the increase in the concentration of test samples. The lethal concentration LC50 of the test samples after 24 hrs was obtained by a plot of the percentage of the shrimps killed against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. The LC_{50} values of chloroform soluble crude extract were found to be 1.54 µg/ml.

Table 3. Determination of antifungal activities (mm) after 48 hrs of incubation of different extracts of *Averrhoa bilimbi.*

	PEE (400 µg/disc)	EAE (400 µg/disc)	ME (400 µg/disc)	Griseofulvin (25µg/disc)
Fungi				
Candida albicans	9	11	8	11
Aspergillus niger	--	8	8	12
Saccharomyces cerevacae	9	9	9	--

Table 4. Determination of LC_{50} for plant chloroform soluble crude extract of *Averrhoa bilimbi* and positive control vincristine sulfate using brine shrimp lethality bioassay.

No.	Conc. of experimental compound (µg/ml)	Log C	% of mortality of brine shrimp (Mean)		LC_{50} (µg/ml)	
			Plant extract	Positive control	Plant extract	Positive control
1.	400	2.602	100	100	1.54	3.71
2.	200	2.301	93.33	95		
3.	100	2.000	86.66	90		
4.	50	1.699	83.33	80		
5.	25	1.398	76.66	70		
6.	12.5	1.097	70	65		
7.	6.25	0.796	66.66	53		
8.	3.125	0.495	63.33	47		
9.	1.56	0.194	60	40		
10.	0	-	0	0		

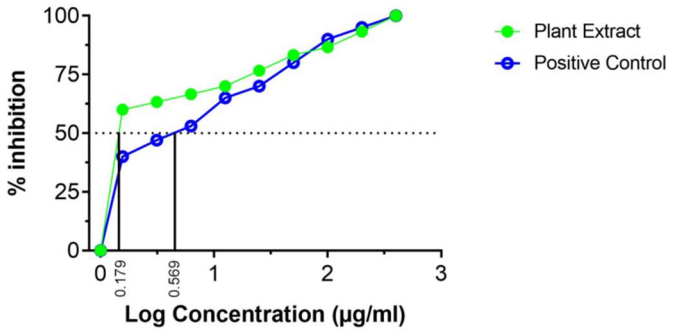


Fig. 1. Identification of LC_{50} value for chloroform soluble crude extract of *Averrhoa bilimbi* (green) and for positive control (vincristine sulfate, blue).

Crude extracts of of *Averrhoa bilimbi* was tested for the free radical scavenging activity by the method of Utami *et al.* (2019). Here, BHT was used as reference standard, which had an IC_{50} value of 18.0 µg/ml. In this investigation, the crude extract showed the free radical scavenging activity with IC_{50} value of 250.0 µg/ml (Table 5).

Table 5. Determination of IC_{50} for plant chloroform soluble crude extract of *Averrhoa bilimbi* and positive control vincristine sulfate using free radical scavenging activity assay.

No.	Concentration (µg/ml)	Absorbance of Tert-butyl-1-hydroxytoluene at 515 nm	% inhibition	IC 50 (µg/ml)	Absorbance Of Extract at 515 nm	% inhibition	IC 50 (µg/ml)
1.	500	0.034	90.17		0.105	69.65	
2.	250	0.065	81.21		0.173	50	
3.	125	0.078	77.46		0.213	38.44	
4.	62.5	0.138	60.12		0.262	24.28	
5.	31.25	0.163	52.89	18	0.302	12.72	250
6.	15.625	0.188	45.71		0.32	7.51	
7.	7.813	0.207	40.17		0.332	4.05	
8.	3.906	0.216	37.57		0.34	1.73	
9.	1.953	0.238	31.21		0.342	1.16	
10.	0.977	0.28	19.08		0.345	0.065	

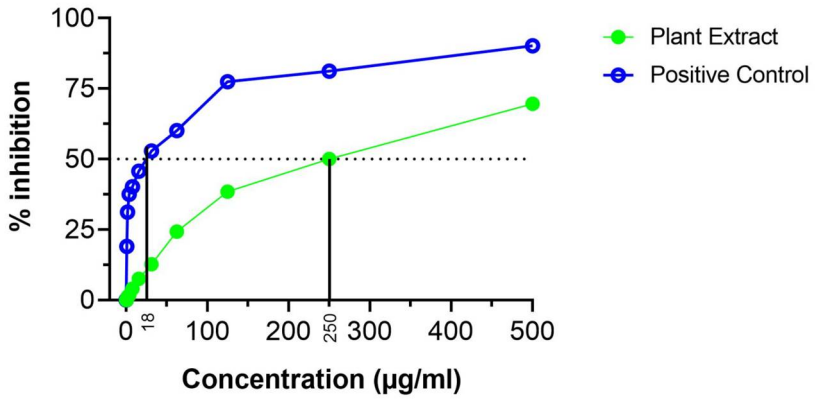


Fig. 2. Comparison ofIC_{50} value for chloroform soluble crude extractof *Averrhoa bilimbi*(green) and for positive control (BHT, Blue).

In conclusion, the bark extracts of *Averrhoa bilimbi* mentioned above have shown significant *in vitro* antibacterial activity compared to the ciprofloxacin standard. It was previously reported that the ethanolic fruit extract of *A. bilimbi* has antibacterial activity, especially for the multi-drug resistant (MDR) strains (Prastiyanto *et al.* 2020). Thus, it suggests that the extracts of *A. bilimbi* have the potential to combat a wide range of microbes that cause common diseases and may lead to the discovery of new, clinically effective antimicrobial compounds. Additionally, when tested for cytotoxicity, the extract of *A. bilimbi* induced *in vivo* lethality and showed bioactive natural compounds that could be used as an antitumor agent. The cytotoxicity exhibited by the test

samples was compared to the positive control vincristine sulfate, indicating that bioactivity-guided investigation can be conducted to identify potent antitumor and pesticidal compounds. This is in agreement with the findings of Ali *et al.* (2013) where they have shown that compared to the positive control, the methanolic fruit extracts of *A. bilimbi* had significantly higher cytotoxic potential (Ali *et al.* 2013). Interestingly, (Aung *et al.* 2020*)* mentioned the role of sitosterone, which is reported in the present study, as a cytotoxic compound after testing it in human cell lines (Aung *et al.* 2020). Moreover, Chowdhury *et al.* 2012 reported that the hydromethanolic extract of *A. bilimbi* fruits has antioxidant capacity. They reported this is due to its high content of total phenol and total flavonoid contents (Chowdhury *et al.* 2012). However, they did not check the bark of *A. bilimbi.* Therefore, as a whole, *A. bilimbi* could be a good source of natural medicine with promising biological activities, including antibacterial, antifungal, antioxidant, and cytotoxic properties. The findings of the present study provide a major platform for more phytochemical and pharmacological research and encourage the cultivation of this highly valuable plant on a large scale to increase the economic status of cultivars in the country and to reduce dependency on allopathy-based medicines. Further research is also necessary to identify other compounds within this plant and to determine its full spectrum of efficacy.

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