CHAPTER V

Study of Antimicrobial Property of Wild Fruits

Plants are important sources of natural antimicrobials. Antimicrobials of plant origin are more effective in the treatment of infectious diseases than the synthetic drugs which are often associated with side effects [1]. Polyphenols from plants possess several benefits including antimicrobial properties against pathogenic and spoilage microbes and variations in the chemical compositions of these compounds lead to remarkable differences in their antimicrobial activity [2]. The presence of both antimicrobial and antioxidant properties in a single molecule makes them better suited and more effective as food preservatives. In general, plants have more powerful effect on gram-positive than gram-negative bacteria [3]. Plant-derived antimicrobials have been recognized for centuries, but only scientifically confirmed in the last 30 years [4, 5]. Natural antimicrobials play a significant role in food control by preventing microbial contamination or growth and by extending shelf life due to the removal of undesirable pathogens [6, 7]. Thus, there is an increasing interest in finding natural antimicrobials for application in various food products.

In this study, antibacterial properties of five wild fruits *viz*. *G. sapida*, *O. alismoides, A. dioica, A. bunius* and *E. operculata* from Assam of North East India were investigated against two strains of gram-positive bacteria (*Staphylococcus aureus* MTCC-7443 and *Bacillus cereus* MTCC-430) and two strains of gram-negative bacteria (*Escherichia coli* MTCC-40 and *Proteus vulgaris* MTCC-7299) and the results were reported.

V.1 Materials and Methods

V.1.1 Materials

Mueller-Hinton broth and resazurin were obtained from Hi-Media Laboratories Mumbai, India. Dimethyl sulfoxide (DMSO), amoxicillin and filter paper have been purchased from Merck, Mumbai, India. All other reagents used in the experiments were of analytical grade.

V.1.2 Preparation of fruit extracts

Fresh fruits of five plant species of this study (**Table II.1**) were collected from Chirang and Kokrajhar district of Assam, India. The powdered sample was prepared as per the procedure described in **Section II.2.3** (**Page no. 67**) and extracted with methanol as per the procedure mentioned in the **Section IV.1.2** (**Page no. 90**).

V.1.3 Bacterial strains

The antibacterial activity of methanol extract of each fruit was determined using four bacterial strains causing food poisoning diseases. Two strains of gram-positive bacteria (*Staphylococcus aureus* MTCC-7443 and *Bacillus cereus* MTCC-430) and two strains of gram-negative bacteria (*Escherichia coli* MTCC-40 and *Proteus vulgaris* MTCC-7299) were taken for this study as the phytochemicals present in wild fruits can exhibit antimicrobial activity against some foodborne pathogens responsible for foodborne disease outbreaks [8]. These bacterial strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

V.1.4 Inoculums preparation

Each bacterial strain selected for this study was subcultured overnight at 35°C in Mueller-Hinton agar slants. The bacterial growth was harvested using 5 mL of sterile saline water and its absorbance was adjusted at 570 nm and diluted to attain viable cell count of 10^7 colony-forming unit (CFU)/mL using UV-VIS spectrophotometer (Lambda 35, Perkin Elmer, USA) [9].

V.1.5 Determination of antibacterial activity of fruit extracts

The antimicrobial activity of each fruit extract was determined using Kirby-Bauer disc diffusion method [10]. The methanol extract (100 mg) was dissolved in 2 mL of DMSO and sterilized through membrane filter (0.22 µm). It was then diluted to different concentration (10, 20 and 30 mg/mL) and then 5 µL was loaded over 6 mm diameter of sterilized filter paper discs. After that 10-15 mL of Mueller-Hinton agar medium was poured into sterilized petri-dishes and allowed to solidify for 15 min followed by addition of 100 µL of bacterial culture on top of the plate, spread with sterile L-loop and allowed to dry for few min. The sterile filter paper disc loaded with different concentrations of fruit extract was placed on the top of Mueller-Hinton agar plates and allowed to incubate for 24 h. *E. coli* and *S. aureus* were incubated at 37°C and *P. vulgaris* and *B. cereus* were incubated at 27°C. Filter paper

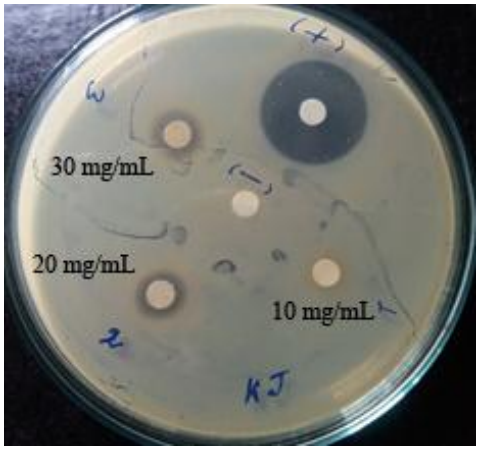
disc loaded with 30 µg/mL of amoxicillin was taken as positive control. Filter paper disc soaked in sterile distilled water and DMSO was taken as negative control. The inhibition zones were measured by vernier calliper (M-532-119, Mitutoyo) and the diameter of inhibitions were expressed in millimetre (mm).

V.1.6 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined according to the protocol of Kitzberger *et al*. [11]. MIC is defined as the lowest concentration of the plant extract that inhibits the microbial growth after 24 h of incubation. It was performed using 96-well microtiter-plates methods by serial dilution of factor two. Briefly, 200 µL of each sample (30 mg/mL) were placed in column no. 3 and then 100 µL was transferred to the column no. 4 and the volume was adjusted to 200 µL by adding DMSO (10%) and again 100 µL was transferred in the next column followed by adding DMSO and so on upto column no. 12. Finally 100 µL from column no. 12 was discarded. The column 1 was taken as positive control by adding an antibiotic amoxicillin and in column 2, only the culture medium was added which was taken as negative control. After that 20 µL of microbial suspension was added in each of the well in the plate along with 10 µL of resazurin (0.015%) to observe cell's viability which is initially blue and turns pink when cell grows. The plates were incubated to grow the cells at their respective temperatures (37°C and 27°C) for 24 h. After incubation, the plates were observed by naked eye to observe the colour change from blue to pink and it was considered for MIC which is the last well in the plate before the colour changes to pink.

V.1.7 Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) was determined according to the method of Kitzberger *et al*. [11]. MBC is defined as the minimum amount of plant extract to completely kill the respective microorganism after 24 h of incubation on the freshly inoculated agar plates. Determination of MBC was executed by streaking method which was taken from the two lowest clear inhibition zone of MIC plates and it was again re-cultured on the sterile trypticase soy agar plates by incubating at 35°C for 24 h and observed for bacterial growth in respective concentration.



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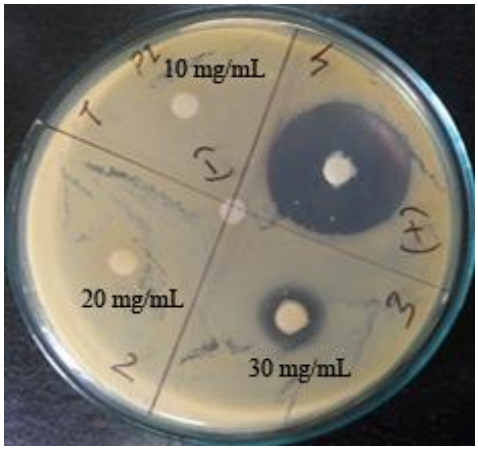

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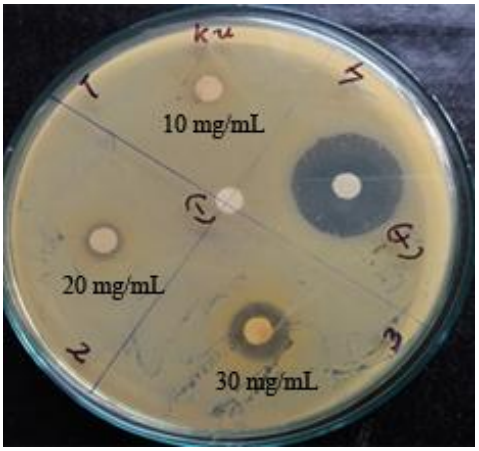

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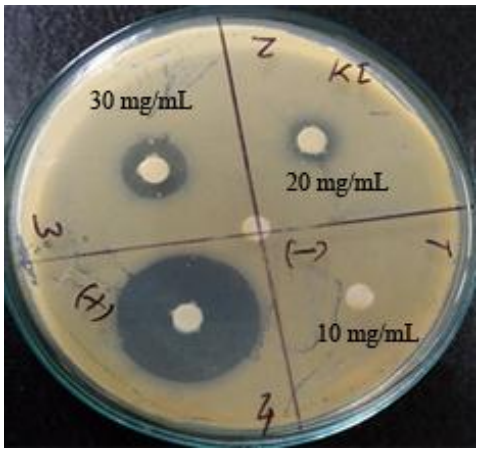

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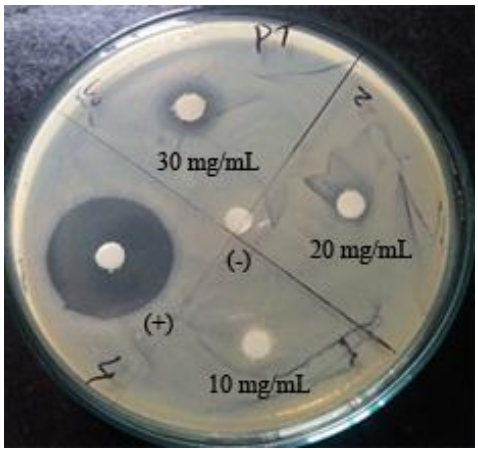
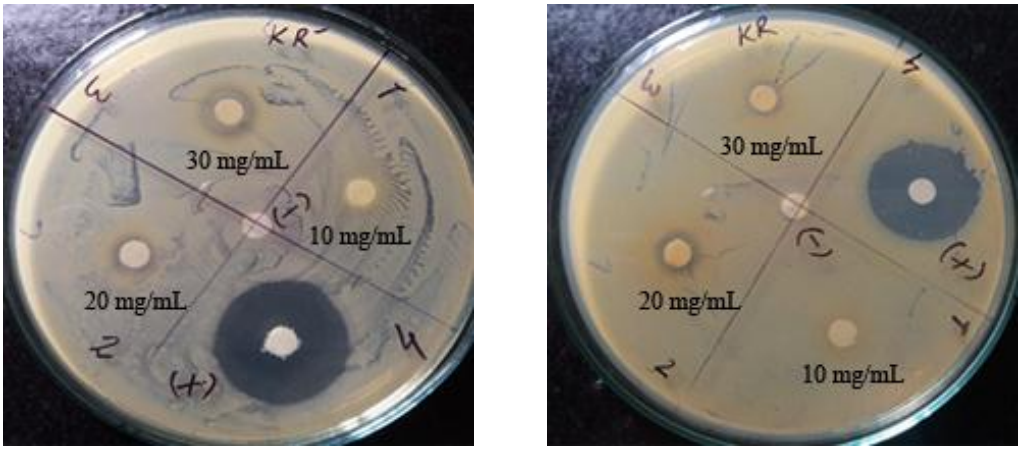
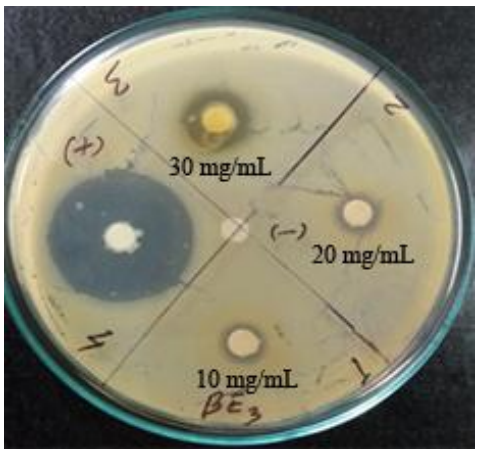


E

Fig. V.1: Antibacterial activity of methanol extracts of fruits against *B. cereus*. A = *G. sapida*; B = *A. bunius*; C = *E. operculata*; D = *A. dioica*; E = *O. alismoides*; (+) = Positive control; (-) = Negative control.



A B



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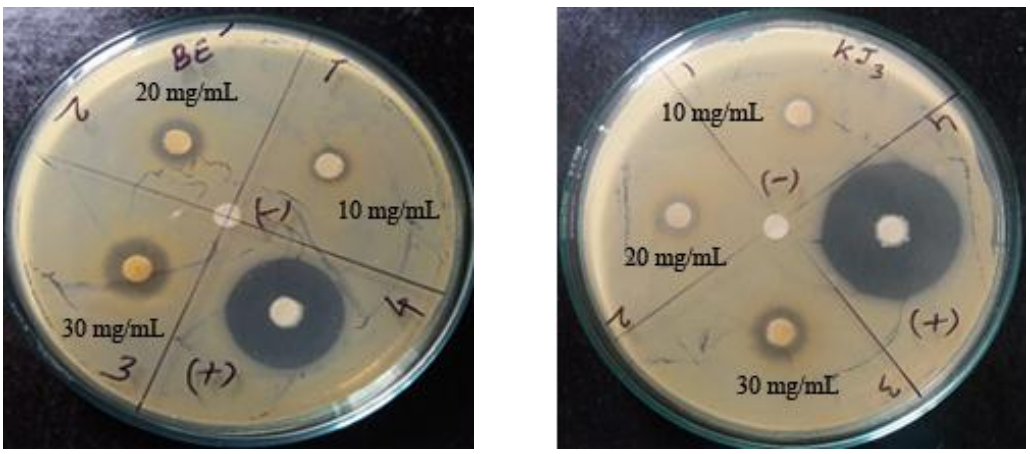
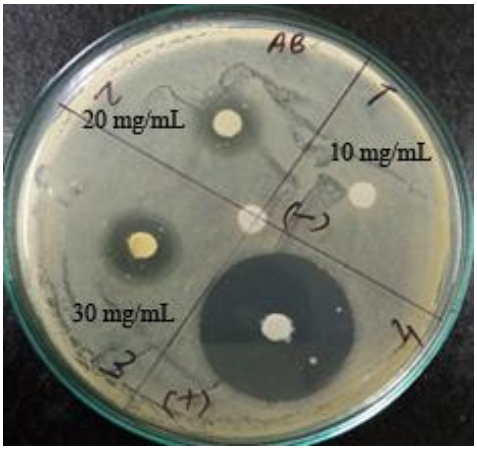


D E

Fig. V.2: Antibacterial activity of methanol extracts of fruits against *S. aureus*. A = *G. sapida*; B = *A. bunius*; C = *E. operculata*; D = *A. dioica*; E = *O. alismoides*; (+) = Positive control; (-) = Negative control.



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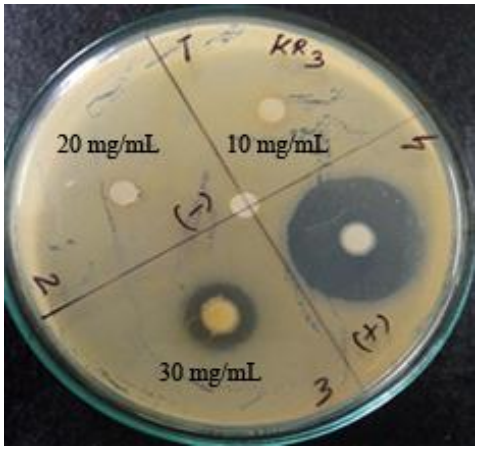

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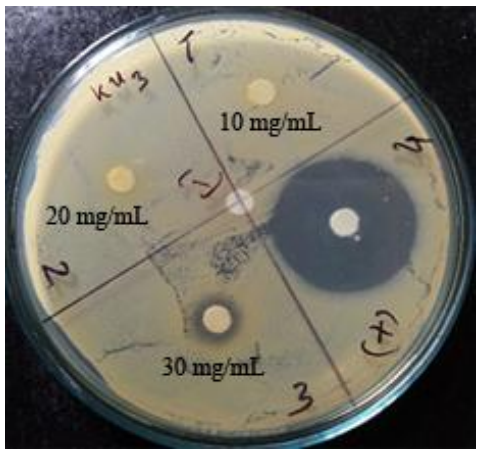

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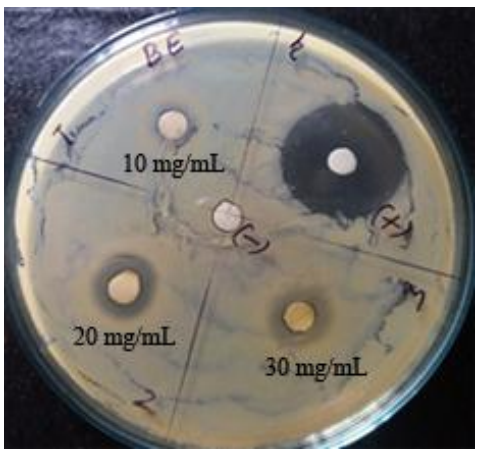

D

E

Fig. V.3: Antibacterial activity of methanol extracts of fruits against *E. coli*. A = *G. sapida*; B = *A. bunius*; C = *E. operculata*; D = *A. dioica*; E = *O. alismoides*; (+) = Positive control; (-) = Negative control.

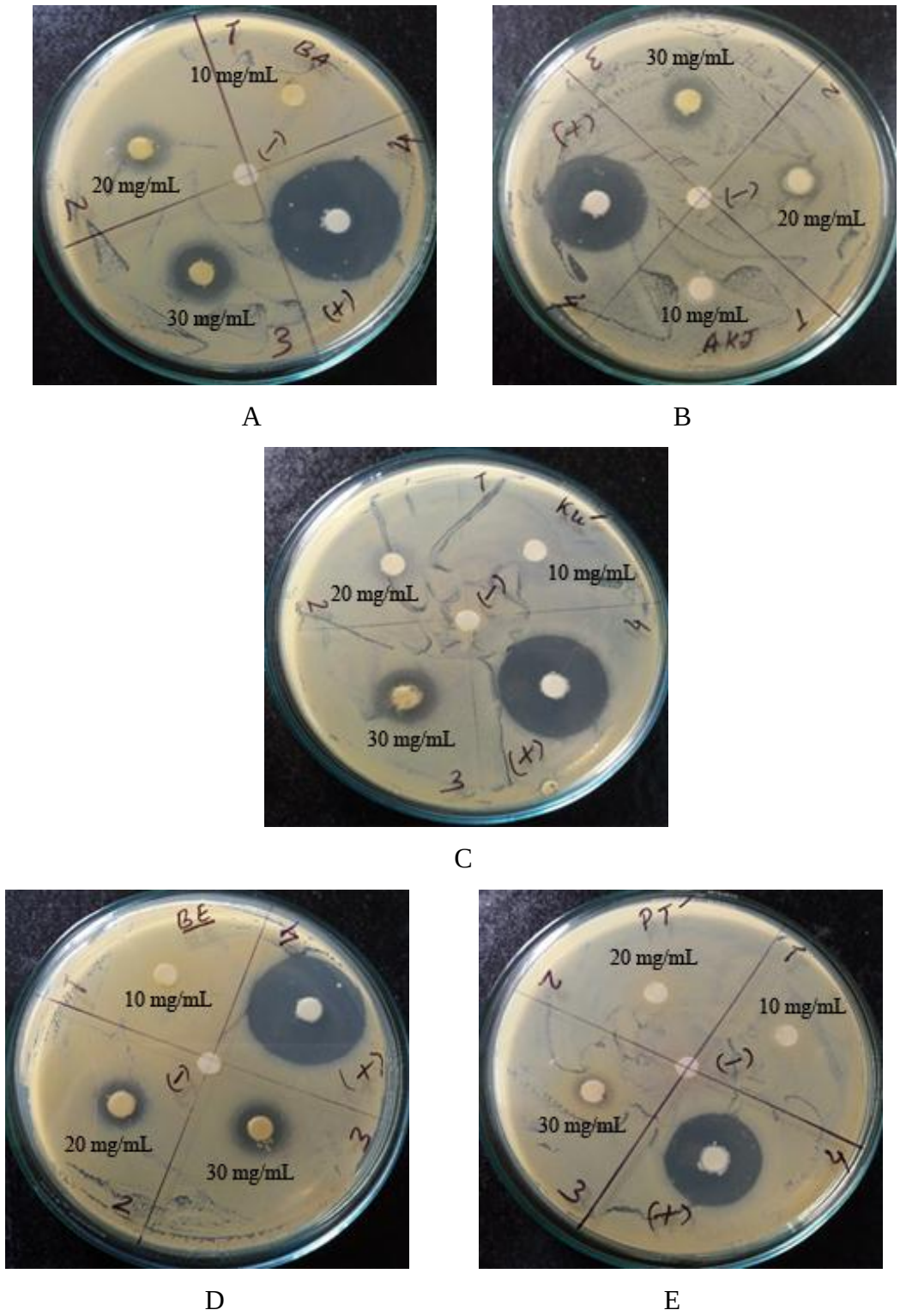

Fig. V.4: Antibacterial activity of methanol extracts of fruits against *P. vulgaris*. A = *G. sapida*; B = *A. bunius*; C = *E. operculata*; D = *A. dioica*; E = *O. alismoides*; (+) = Positive control; (-) = Negative control.

V.2 Results and Discussion

The antibacterial activities in methanol extracts of five wild edible fruits were studied using different concentrations (10, 20 and 30 mg/mL) against four bacterial strains *viz*. two gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and two gram-negative bacteria (*Escherichia coli* and *Proteus vulgaris*) using disc diffusion method. All the fruit extracts showed antibacterial activities against both gram-positive and gram-negative bacterial strains which are shown in **Fig. V.1–Fig. V.4**. The antibacterial activities of fruit extracts were assessed in terms of zone of inhibition of bacterial growth and the results are presented in **Tables V.1–V.4**.

The zone of inhibition measured was ranged from 7.3 ± 1.52 mm to 16.6 ± 4.04 mm for all the sensitive bacteria. The largest zone of inhibition has been recorded against *E. coli* (16.6 ± 4.04 mm) and *P. vulgaris* (16.6 ± 1.15 mm) at 30 mg/mL concentration with the methanolic extracts of *O. alismoides* and *G. sapida*, respectively. *E. operculata* indicated inhibition zones between 7.3 ± 1.52 mm (10 mg/mL) and 13.3 ± 4.04 mm (30 mg/mL) and showed minimum activity of 7.3 ± 1.52 mm (10 mg/mL) against *B. cereus*. But in the other four fruits studied, no activity was observed at the lower concentration (10 mg/mL) against *B. cereus*. In all the tested bacteria, *G. sapida* fruits showed growth inhibition zones between 9.5 ± 2.78 mm (20 mg/mL) and 16.6 ± 1.15 mm (30 mg/mL) but no activity was observed at 10 mg/mL. Growth inhibition zones observed in *O. alismoides* fruit against *B. cereus* and *P. vulgaris* at 30 mg/mL were 10.6 ± 2.3 mm and 9.3 ± 2.51 mm, respectively. However, no activities were noted at 10 mg/mL and 20 mg/mL of *O. alismoides* fruit against *B. cereus* and *P. vulgaris*. At the concentration of 10 mg/mL, no antimicrobial activity was observed in *A. bunius* against all the tested bacterial strains as the potency to exhibit antimicrobial activity may be less at this concentration. But at the concentration of 20 mg/mL, *A. bunius* showed an inhibition zone of 8.3 ± 1.52 mm against *P. vulgaris* and 9 ± 2.64 mm against *S. aureus*, whereas no activity was observed against *B. cereus* and *E. coli* at the same concentration. Growth inhibition zones observed in *A. dioica* was 8.3 ± 3.21 mm (20 mg/mL) against *E. coli* and 14.6 ± 3.21 mm (30 mg/mL) against *S. aureus. A. dioica* showed inhibitory zones of 9.5 ± 3.04 mm at 10 mg/mL against *S. aureus* but no activity was observed at 10 mg/mL with other tested bacteria. These results were in accordance with the results reported by Pandey and Singh [12] and Hoque *et al*. [13]. Growth inhibition zone was studied by Saklani *et al*. [14] and reported as 15 ± 1 mm in ethanolic fruit extract of *Rubus ellipticus* against *E. coli* which was comparable to the results of the current study. Rauha *et al*. [15] also reported that

Rubus idaeus and *Rubus chamaemorus* displayed only slight antibacterial effects against *S. aureus* and *E. coli*, whereas the inhibition towards *Bacillus subtilis* was found to be moderate.

Table V.1: Antibacterial activity of methanol extracts of fruits against *B. cereus*

Sample	Zone of inhibition at different concentration (mm)				MIC (mg/mL)	MBC (mg/mL)
	10 mg/mL	20 mg/mL	30 mg/mL	Amoxicillin (30 µg/mL)		
G. sapida	0	9.5±2.78^{a}	11±2.64^{a}	23±2.64^{a}	15	<30
A. bunius	0	0	13.6±2.08^{b}	28.3±2.51^{b}	<30	<30
E. operculata	7.3±1.52^{a}	9.6±2.08^{a}	13.3±4.04^{b}	23.6±1.52^{c}	7.5	15
A. dioica	0	10.3±3.21^{b}	13.6±1.52^{b}	27.3±2.08^{d}	15	<30
O. alismoides	0	0	10.6±2.3^{a}	26.6±3.05^{e}	<30	<30

<30 = Concentration is below 30 mg/mL; Values were expressed as mean of three replicates ± standard deviation; The data with different letters in a column are significantly different from each other at p < 0.05.

Table V.2: Antibacterial activity of methanol extracts of fruits against *S. aureus*

Sample	Zone of inhibition at different concentration (mm)				MIC (mg/mL)	MBC (mg/mL)
	10 mg/mL	20 mg/mL	30 mg/mL	Amoxicillin (30 µg/mL)		
G. sapida	0	9.6±3.46^{a}	11.3±0.57^{a}	25.6±2.08^{a}	15	<30
A. bunius	0	9±2.64^{b}	14±2.64^{b}	26±2.64^{a}	15	<30
E. operculata	9.3±2.51^{a}	10.6±1.15^{c}	14.3±2.51^{b}	28.3±3.21^{b}	7.5	15
A. dioica	9.5±3.04^{a}	12.3±2.51^{d}	14.6±3.21^{b}	23.3±2.51^{c}	7.5	15
O. alismoides	9.6±2.08^{a}	10±1.73^{a}	12.6±1.15^{c}	29.6±2.08^{d}	7.5	15

<30 = Concentration is below 30 mg/mL; Values were expressed as mean of three replicates ± standard deviation; The data with different letters in a column are significantly different from each other at p < 0.05.

Table V.3: Antibacterial activity of methanol extracts of fruits against *E. coli*

Sample	Zone of inhibition at different concentration (mm)				MIC (mg/mL)	MBC (mg/mL)
	10 mg/mL	20 mg/mL	30 mg/mL	Amoxicillin (30 µg/mL)		
G. sapida	0	12.3±1.15^{a}	14.3±2.51^{a}	30.3±2.51^{a}	15	15
A. bunius	0	0	15.6±2.08^{b}	27.6±2.88^{b}	<30	<30
E. operculata	0	0	12.3±3.51^{c}	28.3±0.57^{c}	<30	<30
A. dioica	0	8.3±3.21^{b}	13±2.64^{d}	26.6±2.08^{d}	15	<30
O. alismoides	8.3±1.52^{a}	12.6±4.04^{a}	16.6±4.04^{e}	29±3.60^{e}	7.5	15

<30 = Concentration is below 30 mg/mL; Values were expressed as mean of three replicates ± standard deviation; The data with different letters in a column are significantly different from each other at p < 0.05.

Table V.4: Antibacterial activity of methanol extracts of fruits against *P. vulgaris*

Sample	Zone of inhibition at different concentration (mm)				MIC (mg/mL)	MBC (mg/mL)
	10 mg/mL	20 mg/mL	30 mg/mL	Amoxicillin (30 µg/mL)		
G. sapida	0	14±1.73^{a}	16.6±1.15^{a}	28±4.58^{a}	15	15
A. bunius	0	8.3±1.52^{b}	13.3±0.57^{b}	24±1.73^{b}	15	<30
E. operculata	0	0	13±3.60^{b}	25.3±3.21^{c}	<30	<30
A. dioica	0	12.3±2.08^{c}	14±1.73^{c}	28.6±0.57^{d}	15	15
O. alismoides	0	0	9.3±2.51^{d}	22±2.64^{e}	<30	<30

<30 = Concentration is below 30 mg/mL; Values were expressed as mean of three replicates ± standard deviation; The data with different letters in a column are significantly different from each other at p < 0.05.

The antimicrobial activities in methanol extracts of five wild edible fruits against four species of bacteria were assessed by evaluating the minimum inhibitory concentration (MIC) values. It is observed from **Tables V.1–V.4** that all the fruit extracts showed varying degrees of antibacterial activity against all tested strains. The MIC and MBC values of microbial strains were in the range from 7.5 mg/mL to <30 mg/mL and 15 mg/mL to <30 mg/mL,

respectively depending on the nature of the bacterial strains. *E. operculata* showed the lowest MIC values (7.5 mg/mL) with respect to the two strains such as *B. cereus* and *S. aureus*. *O. alismoides* also showed the lowest MIC values (7.5 mg/mL) against *E. coli* and *S. aureus* whereas *A. dioica* exhibited the lowest MIC value (7.5 mg/mL) only against *S. aureus*. On the other hand, *G. sapida* showed the lowest MIC value of 15 mg/mL against *E. coli* and *P. vulgaris.* The MIC values of the fruit extracts studied were in accordance with the results reported by Tshikalange *et al*. [8]. The methanol extract of *Psidium sartorianum* reported by Pio-Leon *et al*. [16] had MIC value of 2 mg/mL and MBC value of 4 mg/mL against *Staphylococcus aureus* which are lower than the MIC and MBC values of the present study. The lower MIC and MBC values indicate higher effectiveness [17]. The MIC values of wild berry fruit species studied by Radovanovic *et al.* [18] against several bacterial species ranged from 15.6 to 500 µg/mL and MBC values ranged from 31.2 to 500 µg/mL which are higher in comparison to the values of the fruits studied herein. Dua *et al*. [19] reported MIC value of cumin extract effective against *E. coli, S. aureus, P. aeruginosa* and *B. pumilus* that ranged from 6.25 to 25 mg/mL and these values are comparable to the results of the present study. Akinpelu *et al*. [20] also reported crude and butanolic extracts of *Persea americana* against *Bacillus cereus* and both the extracts exhibited antibacterial activity at concentrations of 25 and 10 mg/mL with MBC values of 3.12 and 12.5 mg/mL respectively. *G. sapida* fruit extract showed potential bactericidal activity against the tested pathogenic bacteria (*E. coli* and *P. vulgaris*) with MBC value of 15 mg/mL. Likewise, *E. operculata* also showed bactericidal activity against *B. cereus* and *S. aureus*. *A. dioica* fruit also showed bactericidal activity against *S. aureus* and *P. vulgaris* with MBC value of 15 mg/mL while MBC value of *A. bunius* fruit reached to <30 mg/mL against all the four tested strains which was less sensitive.

The results revealed that all plant extracts were potentially effective in suppressing microbial growth of food poisoning bacteria with variable potency. *E. operculata* was the most effective extract retarding the microbial growth of *S. aureus* and *B. cereus* at all concentrations (10, 20 and 30 mg/mL) while the extract of *O. alismoides* was effective against *S. aureus* and *E. coli*. *A. dioica* fruit was found to be effective only against *S. aureus*. Other fruit extracts showed variable antimicrobial activity against food poisoning bacterial strains. *G. sapida* exhibited inhibitory effect against all bacterial strains (*B. cereus, S. aureus, E. coli* and *P. vulgaris*) at two concentrations (20 and 30 mg/mL), whereas *A. bunius* was effective against two of them (*S. aureus* and *P. vulgaris*). Results of antimicrobial activity of the five wild fruit extracts could suggest that *P. vulgaris* was the most resistant strain to fruit extracts followed by *E. coli* and *B. cereus*, whereas *S. aureus* was the most susceptible strains

to the extracted fruits. Moreover, *E. operculata* and *O. alismoides* extracts were the most effective extracts and showed a strong antibacterial activity against food poisoning bacteria.

V.3 Conclusion

From the study, it was found that all the five wild fruit extracts exhibited different degrees of antibacterial activities. *O. alismoides* and *G. sapida* fruit extracts are exhibiting the highest inhibition zone (16.6 mm) at 30 mg/mL against *E. coli* and *P. vulgaris*, respectively. When compared to other fruit extracts, *G. sapida* fruit extract was found potentially effective against all bacterial strains (*B. cereus, S. aureus, E. coli* and *P. vulgaris*) at two concentrations (20 and 30 mg/mL). *O. alismoides* and *A. dioica* fruits exhibited the lowest MIC value (7.5 mg/mL) against *S. aureus*, whereas *G. sapida* fruit showed MBC value of 15 mg/mL against *E. coli* and *P. vulgaris*. The present study clearly indicates that the fruit extracts possess antibacterial properties. Therefore, these fruits can be used as natural food preservatives to prevent foodborne pathogens in order to avoid human health hazards of chemically antimicrobial agent applications.

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