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Apple, Guava and Pineapple Fruit Extracts as Antimicrobial Agents against Pathogenic Bacteria

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Abstract This investigation was conducted to compare the antimicrobial properties of alcohol fruit extracts from Apple (*Malus pumila*), Guava (*Psidium guajava*), and Pineapple (*Ananas comosus)* against eight bacteria: S*taphylococcus aureus, Enteroaggregative E.coli (EAEC), Enterotoxigenic E.coli (ETEC), Enterobacter cloacae, Shigella flexineri, Enterococcus faecalis, Klebsiella,* and *Pseudomonas aeruginosa*. All the fruits were bought fresh from the market, cut into small cubes, and sun dried to a crisp over 4-5 days. After being blended to a fine powder, 75g of each powder was passed through a Soxhlet apparatus containing 250 ml of 99% ethanol to obtain a crude extract. This process was repeated with 250 ml of methanol to obtain a methanolic crude extract. The antimicrobial properties of the extracts were tested using agar well diffusion, Norfloxacin used as positive control and water as negative control. The inhibition zones from each extract were measured and an activity index was calculated from the mean zone sizes. All fruits showed some degree of antimicrobial properties with the highest activity index (2.6) being from Pineapple ethanolic and methanolic extracts against *EAEC*. Both Apple ethanolic and methanolic extracts showed activity only against *Staph. aureus* and *EAEC*. Guava extracts were effective against all the strains except for *ETEC*. Pineapple extracts were strongly active against all the bacteria. The methanolic extracts of Apple and Guava showed slightly larger zones compared to their ethanolic extracts. For Pineapple, inhibition zones from the ethanolic extracts were slightly larger. The results of this investigation show great promise for potential antimicrobial drugs.

Keywords: *Soxhlet, activity index, crude extract, well diffusion*

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1. Introduction

The first case of antibiotic resistance was perhaps recorded in the late 1930s, against sulfonamides, one of the earliest antimicrobials [1]. Since then, bacteria have rendered our strongest antibiotics useless and have turned the most easily curable infections into life threatening diseases. This problem is accompanied by huge economic costs. An estimated USD 500 million dollars will be needed to compensate for over 300 million treatment failures occurring yearly due to resistant gonorrhoea [2]. According to a WHO 2016 report, 480,000 cases of multi drug resistant TB are reported annually. Additionally, patients are now at a much higher risk of developing post-surgery infections [3]. A hospital in Bangladesh reportedly found 75% of *Salmonella typhi* to be resistant to Ciprofloxacin [4]. In the face of such adversity, it is important that we search for other alternatives. Thus research has circulated back to plants and their antimicrobial properties.

Plants have been used in folk medicine for over centuries as a panacea. The apple fruit is rich in flavonoids and polyphenols. Low doses of flavonoid reduce the chances of arterial plaque formation [5]. The barks and leaves of *Psidium guajava* have been used for centuries in many countries for its numerous salutary qualities. Leaves have been notably used to treat wounds, ulcers, skin diseases and toothaches [6]. The use of bark, leaf and root concoctions further extend to the treatment of diarrhea, dysentery, epilepsy and cholera [7,8]. The aqueous and alcoholic extracts of guava root and leaves have proven antimicrobial properties against *Staphylococcus aureus, Streptococcus mutans, Salmonella enteridis, Shigella, Bacillus cereus, Pseudomonas aeruginosa* and *E. coli* [9]. *Ananas comosus* has been used through the ages for various medicinal purposes. It has also been used to treat allergies, inflammation, burns and blocked sinuses [10]. Thanish et al. [10] tested the antimicrobial activity of a pineapple water extract and observed zones of 26 mm and 22 mm against *Streptococcus mutans* and *Enterococcus faecalis* respectively. Bromelain, extracted from pineapple, is a proven and promising antibacterial agent, being most efficacious at 25°C and 37°C against *E. coli* and *Proteus spp*. respectively [11]. Bromelain is also a strong antifungal agent, inhibiting the growth of *F. verticillioides* by 90%, at only 0.3 micromoles per litre [12]. Juice from Pineapple has also exhibited antibacterial action against *E. coli, Shigella sonnei* and *Salmonella para.B* [13].

2. Materials and Methods

2.1. Strains of Bacteria

The following strains of bacteria were used: *Staphylococcus aureus, Enteroaggregative E. coli (EAEC), Enterotoxigenic E. coli (ETEC), Enterobacter cloacae, Shigella flexineri, Enterococcus faecalis, Klebsiella,* and *Pseudomonas aeruginos*a. All strains of bacteria were maintained in laboratory fridge through regular subcultures.

2.2. Preparation of Extracts

Fruits were bought fresh from local market and cut into small cubes. Seeds were carefully removed. The fruit cubes were kept under direct sunlight for about 4-5 days until they lost all moisture and became dried to a crisp. After that, the cubes were put in a blender and turned to a fine powder. Seventy five grams of finely ground powder was measured, placed in a thimble and run through a Soxhlet apparatus. The flask in the Soxhlet was filled with 250 ml absolute ethanol and 250 ml methanol as solvents, individually, to obtain ethanolic and methanolic crude extracts respectively. The cycles were continued for about 4-5 hours until the cotton inside turned white.

2.3. Agar Well Diffusion

Fresh subculture plates, incubated for 24 hours, were placed under the laminar chamber and used to make standard bacterial suspensions in labeled test tubes. Next, an autoclaved cotton swab was used to perform lawn culture for the uniform growth of bacteria. After that, a cork borer was dipped into the agar to make three holes or wells in the media. Each well was labelled and accordingly filled with 30 microlitres of an ethanol fruit extract, methanol fruit extract and water for negative control. Norfloxacin, an antibiotic disc, is also placed in the dish in the form of positive control. The plates are incubated for 24 hours at 37°C, after which clear zones were formed around the control disc and the extracts which gave positive results. These inhibition zones were measured in millimetres using a ruler and recorded. All antimicrobial tests were repeated twice and the average of the inhibition zones is noted. An activity index was calculated from the results to measure the relative efficacy of the fruit extracts. The following formula was used:

$$\text{Activity Index}\left(\text{AI}\right)=\frac{\text{Zone of inhibition of fruit extract}}{\text{Zone of inhibition of Norfloxacin}}.$$

3. Results and Discussion

The problem of antibiotic resistance is not a new one, but it is progressively turning into an alarming issue. Colistin, one of the last-reserve antibiotics, had been saved for 'nightmare bacteria' that are resistant to other first and second line antibiotics. Since 2015, cases of colistin-resistant infections have been found in both animals and people in Europe, China, Canada and America [14]. Hence, in such dire circumstances, it has become an imperative need to turn our immediate attention to other alternatives.

3.1. Apple

The results of our investigation showed that apple has moderate antimicrobial activity. Both ethanol and methanol extracts showed positive results against *Staph. aureus* and *EAEC,* but no zones were seen against any other microorganism.

These results were partially concordant with a study conducted by Sunilson et al. (2016) [15]. According to their research, the ethanol extract showed inhibition zones of 17.2 mm diameter against *Staph. aureus.* Contrary to our results, the ethanol extract was also potent against *Pseudomonas* (16.3 mm) and *Klebsiella spp.* (9.2 mm). In a similar study, the antimicrobial properties of different apple cultivars from Kashan area were investigated. The apple fruit pulp was simply pulverized and concentrated in a rotary evaporator. One cultivar showed 11 mm against *P. aeruginosa* while another showed 12 mm and 16 mm against *K. pneumonia* and *E. coli* respectively [16].

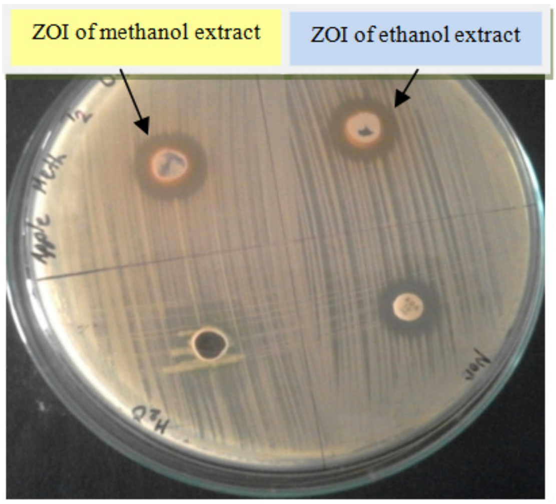

Figure 1. Antimicrobial effect of apple extracts against EAEC

Table 1. Antimicrobial test results for Apple extracts

Organism	Mean ZOI* for Nor* (mm)	Distilled Water (mm)	Mean ZOI for Ethanol Extracts (mm)	AI* for Ethanol Extract	Mean ZOI for Methanol (mm)	AI for Methanol Extracts
S. aureus	24.3	0.0	13.0	0.5	14.3	0.6
EAEC	14.3	0.0	14.7	1.0	16.3	1.1

*ZOI = Zone Of Inhibition *AI = Activity Index *Nor = Norfloxacin.

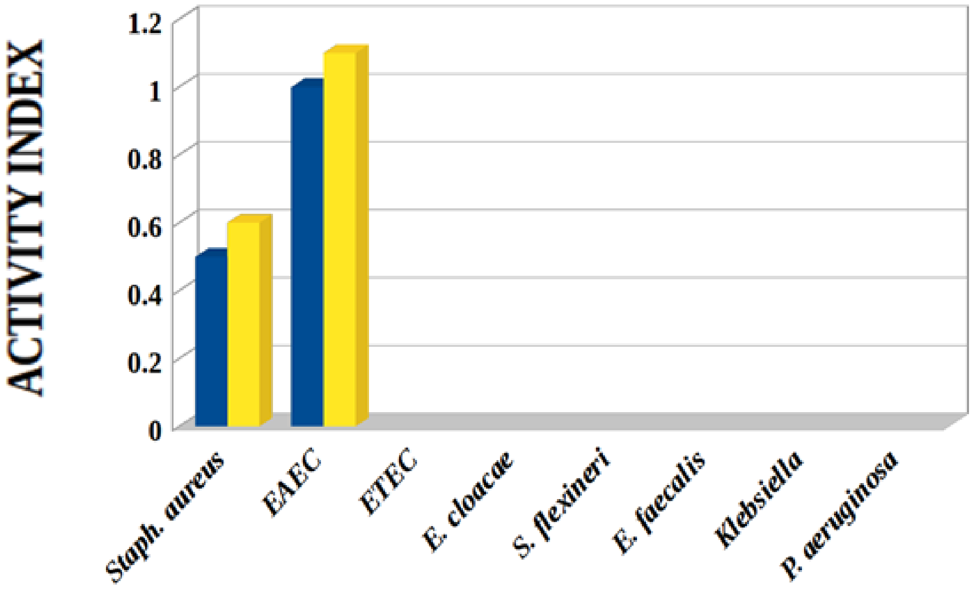

Figure 2. Activity Indices of Apple ethanol and methanol extracts

The antimicrobial properties of apple could be contributed to the presence of flavonoids, terpenoids, phenolic compounds and tannins [15]. Studies have shown that *Staph. aureus* is particularly susceptible to phenolic compounds as they can destabilize the cell membrane [17,18]. Furocumarins, a type of flavonoid, can interfere with the process of DNA replication in microbes, while tannins can inactivate enzymes and other important proteins [19,20]. According to our study, the methanol extract seemed relatively more potent compared to the ethanol extract. The highest activity index (1.1) of apple was of the methanol extract against *EAEC,* proving to be more potent than the control antibiotic disc, Norfloxacin. This suggests that the bioactive compounds in the extract were more polar.

3.2. Guava

Guava shows great promise as an antimicrobial agent according to the results of our investigation. Guava extracts were active against all the pathogens except *ETEC.* Also the methanol extract did not show activity against the *Klebsiella spp.* With the exception of *Klebsiella spp.* and *Staph. aureus,* all the organisms were more susceptible towards the methanol extract. This observation is supported by several studies conducted previously. Thus the exceptions could be just an anomaly. The largest ethanol (16.3 mm) and methanol (19.0 mm) zones were seen against *EAEC,* both proving to be more effective than the control antibiotic, Norfloxacin.

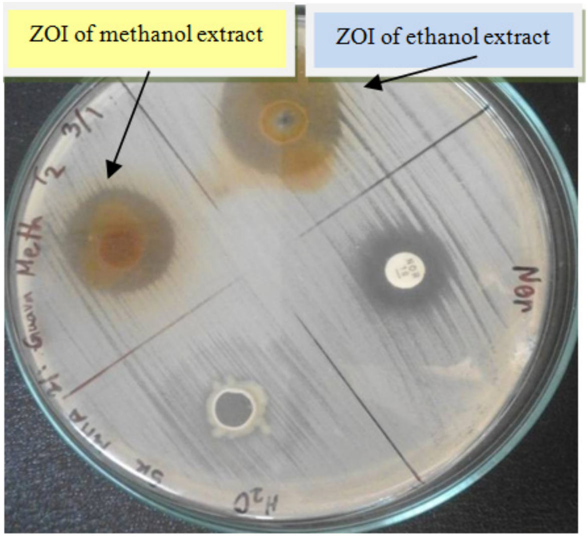

Figure 3. Antimicrobial effect of guava extracts against EAEC

Table 2. Antimicrobial test results for Guava extracts

Organism	Mean ZOI* for Nor* (mm)	Distilled Water (mm)	Mean ZOI for Ethanol Extracts (mm)	AI* for Ethanol Extract	Mean ZOI for Methanol (mm)	AI for Methanol Extracts
S. aureus	23.3	0.0	16.0	0.7	14.7	0.6
EAEC	12.3	0.0	16.3	1.3	19.0	1.5
ETEC	24.7	0.0	0.0	0.0	0.0	0.0
E. cloacae	25.3	0.0	10.7	0.4	13.7	0.5
S. flexineri	24.7	0.0	9.0	0.4	12.3	0.5
E. faecalis	19.3	0.0	13.0	0.7	15.3	0.8
Klebsiella	23.7	0.0	9.0	0.4	0.0	0.0
P. aeruginosa	27.3	0.0	10.0	0.4	12.3	0.5

*ZOI = Zone Of Inhibition *Nor = Norfloxacin *AI = Activity.

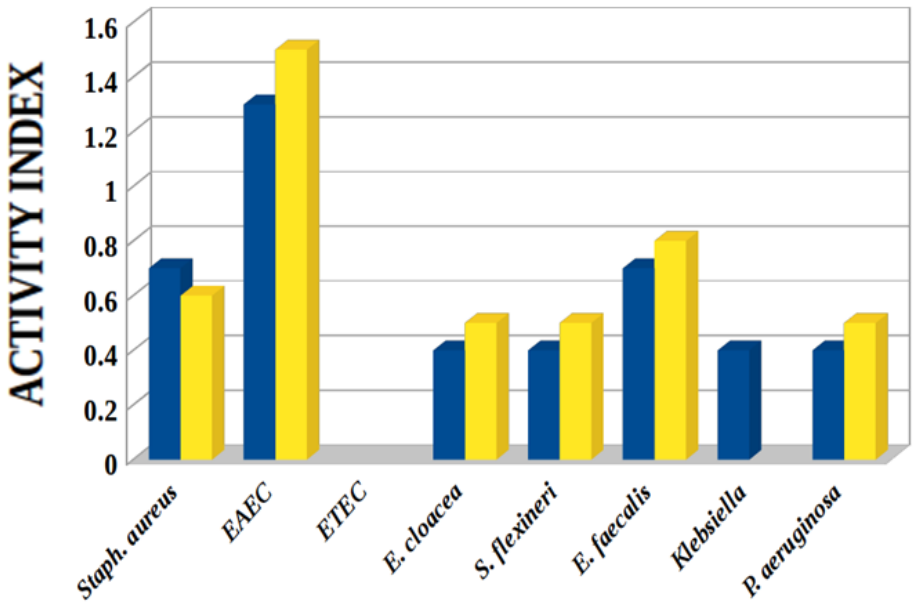

Figure 4. Activity Indices of Guava ethanol and methanol extracts

Farhana et al. (2016) investigated the activity of guava fruit extract at different concentrations [21]. The fruit pulp was blended and filtered, then diluted to form various concentrations. At 100%, guava extract showed 12.67 mm and 13.5 mm zones of inhibition against *Staph. aureus* and *E. coli* respectively. Tahera et al. (2014) carried out a similar study to compare the activities of guava peel and pulp and combined [22]. The research showed the pulp to be a strong antimicrobial agent with significant zones formed by the ethanol extracts against *Pseudomonas, Klebsiella, E.coli, Vibrio spp, Staphylococcus spp.* and *Bacillus spp.* But contrary to other studies, Tahera et al.'s investigation proved the ethanol extract to be more potent. With that exception, their results were also consistent with ours.

The phytochemical analysis of guava fruit has revealed several secondary metabolites, many of which have established antimicrobial properties. Some of these are tannins, saponins, terpenoids, guajavarin, quercetin and flavonoids. Tannins and flavonoids are polyphenolic compounds which inhibit the growth of bacteria by disrupting the synthesis of proteins or the integrity of cell walls [23].

3.3. Pineapple

Pineapple proved to be the strongest antimicrobial agent in our investigation. In fact, the highest inhibition zones in our entire research, of both ethanol (34.3 mm) and methanol (33.7 mm) extracts, were that of pineapple, against *EAEC,* appearing 2.6 times stronger than the control antibiotic Norfloxacin. Besides that, both pineapple ethanol and methanol extracts showed significant activity against all the microorganisms tested. The ethanol extract produced zones slightly larger than that of the methanol extract.

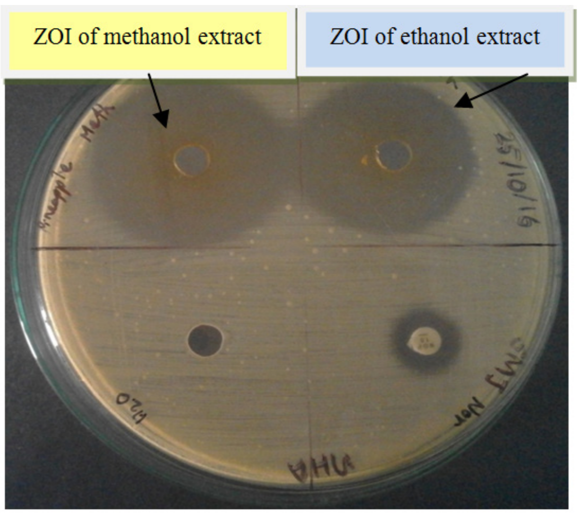

Figure 5. Antimicrobial effect of pineapple extracts against EAEC

Table 3.Antimicrobial test results for Pineapple extracts

Organism	Mean ZOI* for Nor* (mm)	Distilled Water (mm)	Mean ZOI for Ethanol Extracts (mm)	AI* for Ethanol Extract	Mean ZOI for Methanol (mm)	AI for Methanol Extracts
S. aureus	21.3	0.0	22.3	1.0	21.3	1.0
EAEC	13.0	0.0	34.3	2.6	33.7	2.6
ETEC	24.7	0.0	15.3	0.6	15.0	0.6
E. cloacae	25.3	0.0	16.3	0.6	15.0	0.6
S. flexineri	21.3	0.0	21.3	1.0	20.3	1.0
E. faecalis	22.0	0.0	19.0	0.9	16.3	0.7
Klebsiella	24.0	0.0	14.0	0.6	13.7	0.6
P. aeruginosa	27.7	0.0	17.7	0.6	19.3	0.7

*ZOI = Zone Of Inhibition*Nor = Norfloxacin*AI = Activity Index.

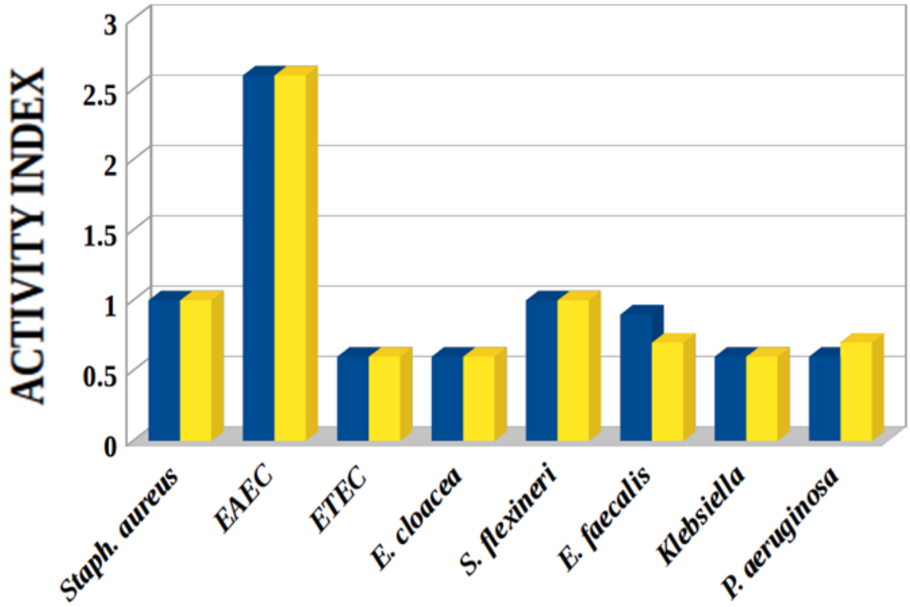

Figure 6. Activity Indices of Pineapple ethanol and methanol extracts

Although pineapple has long been used in traditional medicine, significant research has not been done on it. There have been several studies on pineapple's antioxidant properties, but remarkably few on the fruit's antimicrobial activity. Bansode and Chavan (2013) extracted pineapple juice using a syringe and conducted agar well diffusion tests. They found that the juice formed inhibition zones of 6 mm against *E. coli* and 4 mm against *Shigella sonnei* and *Salmonella para B* [13]. Another study by Rodriguez (2011) found that commercial pineapple juice has no antibacterial activity [24]. Ajibade (2015), in his investigation found the highest zone of inhibition to be 13 mm at 1.0 g/ml against *Streptococcus pneumoniae,* followed by 12 mm against *P. aeruginosa* and *Staphylococcus aureus* [25].

The phytochemical analysis of pineapple has revealed several compounds such as flavonoids and steroids, which are known antimicrobial agents. Also, pineapple contains a protease called bromelain. Bromelain has shown antimicrobial properties as well against *P. aeruginosa, K. pneumonia* and *Staphylococcus aureus* [26].

3.4. Limitations

There were certain limitations of the investigation that need to be addressed. Firstly, the method of extraction, using Soxhlet apparatus, had certain disadvantages. For efficient Soxhlet extraction, the sample needs to be ground into a finely divided powder. This was not the case for apple. Even after extensive drying, the sticky nature of the fruit caused it to clump together. Hence efficient extraction could not be achieved. Secondly, Soxhlet does not allow for agitation during the extraction process. Also since extraction is carried out at the solvent's boiling point, it could disintegrate heat labile compounds that could have been important to the study.

3.5. Further Scope

As the antimicrobial nature of the tested fruits have been established, further research is required with the limitations in mind. Several other extraction techniques can be tried such as supercritical fluid extraction and accelerated solvent extraction.

In this investigation, only the fruits were considered. Further research can be done to explore the antimicrobial properties of other parts of the plants, using a variety of solvents.

Analysis by HPLC could help isolate and identify the major components contributing to the antimicrobial properties of the fruits. Once that is done, fruits can be genetically enhanced to produce GM fruits, with increased concentrations of the bioactive compounds, thus making them much more potent antimicrobial agents.

4. Conclusion

Though the use of fruits in medicine has been employed over thousands of years, it is only recently that scientific research has taken an interest in them. Antibiotic resistance is a growing global issue. Especially in our country, where more than half the population is not educated, this is an even bigger problem. Antibiotics are bought and sold without prescriptions. Often they are prescribed unnecessarily and the courses are not completed. Consequently, many resistant strains of bacteria have also been reported in Bangladesh. Using fruits to treat diseases could greatly decrease the pressure on antibiotics. Also, in the context of Bangladesh, health care is inaccessible to many. Eating fruits that are readily available and relatively cheaper could greatly help the people.

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