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Invitro Antibacterial Activity of *Muricata L.* Leaves Extract on Some Bacteria Isolates

Obiazi Helen Akinagwu Kuhodu[1, *], Dic-Ijiewere Ebenezer Oseremen[2], Amhanre Idi Napoleon[1], Aitufe Felix Okoro[3], Igwe Reginald Marvin Ndubuisi[4]

[1]Department of Microbiology, Ambrose Alli University, Ekpoma, Nigeria

[2]Department of Chemical Pathology, Ambrose Alli University, Ekpoma, Nigeria

[3]Department of Medical Laboratory Science, Ambrose Alli University, Ekpoma, Nigeria

[4]Edo State Hospital Management Board, Central Hospital, Benin City, Nigeria

Email address

ebenexar@gmail.com (O. H. A. Kuhodu)

[*]Corresponding author

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Abstract

Annona Muricata L. or infamously known as soursop is one of Malaysian exotic fruits from the family *Annonaceae* which has been shown to have medicinal properties. The study was carried out to evaluate the invitro antibacterial activity of *A. muricata L.* leaves extract on organisms isolated from Urine, Stool and wound of patients. Aqueous decoctions of the leaves were prepared by adding 10 grams of Soursop leaf in 100ml sterile distilled water and boiled over a low flame for 15 minutes. After cooling, the contents of the flasks were filtered with double filter paper and sterile filters to remove impurities and concentrations of 5% and 10%of extract were prepared using sterile distilled water. The minimum inhibitory concentration gave the lowest concentration of the aqueous extract that can inhibit the growth of the bacteria isolates. The percentage of bacterial isolate to various concentrations of soursop fruits and juice extracts in which 50% concentration/dilution of fruit extracts recorded the highest of 14(93.3%) with *E. coli, Salmonella* spp. *and shigella* was 10(100%), 2(50%) and 0(0%) respectively. However, the 10% of fruits extracts was second with 12(80%) and *Escherichia coli* recorded the highest of 10(100%) followed by *Salmonella spp* least of 2(50%) while absent in *shigella*. As for 50% Juice extracts, a total of 2(13.3%) was recorded with *Salmonella* spp and *shigella* with 1(100%) respectively. While for 10% juice extracts, only *Shigella* of 1(100%) was recorded. The present study demonstrated the in-vitro efficacy of Soursop fruits extracts which was highest against *E. coli* followed by *Salmonella spp*. Hence, this study proves to an extent that the Soursop fruits extract when used against microbiota has sufficient anti-microbial property.

Keywords

Annona Muricata L., Leaves, Extract, Bacteria, Soursop

1. Introduction

Studies have shown that medicinal plants are good materials for the formation of new drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy [1]. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity [2]. As a result, a number of medicinal plants used in indigenous medicine have been tested and found to possess bactericidal properties [3]. Phytochemical screening is very important methods of identifying bioactive compound that useful in creating new drugs. These simple, cheap, sensitive, selective and rapid chemical tests to determine the presence of certain groups of compounds is an initial step to select plants for further phytochemical studies [4]. Along with the increasing public

interests on medicinal plants, recently there are lots of researches done on various potential medicinal plants, One of them is *Annona Muricata L.* (*A. muricata L.*) commonly known as soursop. However, previous research on *A. muricata L*. has focused on the bark of the tree and roots for pharmaceutical purposes [5] and little attention has been paid to the leaves which is more commonly used in traditional medicine remedies. To date, there are only few research publications about phytochemical screening of *A. muricata L.* leaves and their antimicrobial activity against Gram-positive and Gram-negative bacteria.

A. muricata L. or infamously known as soursop is one of Malaysian exotic fruits from family Annonaceae. The tree is a low branching and bushy but slender plant. It can reach a height of 25 to 30 ft. It is a typical tropical tree with heart shaped edible fruits with which the flesh is white and juicy. The leaves are lanceolate with glossy and dark green in color. This species are widely distributed in most of tropical countries [6-7]. *A. muricata L.* has a long, rich history of use in herbal medicine as well as a lengthy recorded indigenous use. All parts of the soursop plant are used in natural medicine in the tropics, including the bark, leaves, roots, fruit, and fruit seeds. Different properties and uses are attributed to the different parts of the tree. Generally, the fruit and fruit juice are taken for worms and parasites, to cool fevers, to increase mother's milk after childbirth, and as an astringent for diarrhea and dysentery. The crushed seeds are used against internal and external parasites, head lice, and worms. The barks, leaves, and roots are considered sedative, antispasmodic, hypotensive, and nervine, and a tea is made for various disorders toward those effects [8]. *A. muricata L.* has quite high nutritional value. The white juicy pulp of the fruit is high in carbohydrates and sugars and fair amount of vitamin C, vitamin B1, vitamin B2, potassium and dietary fibre. However, it is poor in vitamin A. 6 Recent studies have supported many of *A. muricatas* traditional medicinal uses and also showed that various parts of the tree contain acetogenins, which have been shown to be responsible for its myriad array of its medicinal attributes. Annonaceous acetogenins are only found in the Annonaceae family (to which *Annonamuricata L.* belongs). These chemicals in general have been documented with antitumor and antimicrobial activities [9].

Preliminary phytochemical analysis revealed the presence of secondary metabolites like tannins, steroid, cardiac glycosides, etc. were present in trace amounts in the leaves of *A. muricata* [2]. Other phytochemical analysis of the n-butanolic leaf extract of *A. muricata* revealed the presence of flavonoids, terpenoids, tannins, cardiac glycosides and reducing sugars. Whereas, the extract showed the absence of saponins, steroids, phlobatannins, oil and anthraquinones tested [10]. The phytochemical screening of the *A. muricata* different plant parts also showed the presence of flavonoids, terpenoids, reducing sugar, anthraquinone, tannins and cardiac glycosides. Phytoconstituents in the leaves of A. muricata L. contain an alkaloidal principle named 6-Hydroxyundulatine and other alkaloids [11]. In other study of Annona squamosa, the results of phytochemical screening of ethanolic extract, chloroform and water fractions of the plant revealed the presence of alkaloids, flavonoids, reducing sugars, saponins, steroids, 7 tannins and glycosides. These metabolites have been reported to possess antimicrobial activity [12]. In particular the flavonoids were reported to be responsible for antimicrobial activity associated with some ethnomedicinal plants [12-13]. In each studies there were different findings on the phytochemical constituents obtained, thus it is not surprising that there are differences in the antimicrobial effects of plant species, due to the phytochemical properties and differences among species [2]. Phytochemical analysis helps detect the chemical constituents of plants extract in search of bioactive agents as basis for drug synthesis [14]. The presence of saponins, condensed tannins and glycosides as the major constituents and trace amounts of flavonoids contribute immensely to the bioactivity of *A. muricata* and also to its usage in treating various diseases. These have included antioxidant activity [15] as well as hepatoprotective effect and antibacterial agent [16].

Along with the phytochemical screening of *Annona sp.*, their antimicrobial properties also had been evaluated through screening process. In one of the study, it was revealed that the aqueous extracts of *A. muricata L* showed an antibacterial effect against *S. aureus* and *V. cholera,* but the antibacterial activity by the ethanol extracts of this plant was not demonstrated [17]. A study also had been conducted in which *A. muricata* extract was screened against Herpes simplex virus-1 (HSV-1) and clinical isolate (obtained from the human keratitis lesion) In order to check whether they inhibit the cytopathic effect of HSV-l on vera cells which is the indicative of anti-HSV-l potential. The minimum inhibitory concentration of ethanolic extract of *A. muricata* was found to be 1 mg/ml which shows that the A. muricata could be used as the potential antiherptic drugs [18]. In one study of Annonasquamosa antimicrobial properties, it was observed that water fraction were active against *S. pneumoniae* and a-haemolytic streptococci but inactive against the other test isolates while chloroform fraction was active against *S. aureus* and *S. pneumonia* respectively but inactive against all other test isolates. In contrast, ethanolic extract was inactive against the other test isolates at the same disc concentration of 50l-lg [12].

2. Materials and Methods

2.1. Study Area

This study was carried out in Ekpoma, Esan West Local Government Area of Edo State. Edo state lies between longitude 06° 04^{l}E and 06° 43^{l}E and latitude 05° 44^{l}N and 07°34^{l}N with a land mass of 17,450 sq.km located in the South-South geopolitical zone of Nigeria. This study area is a semi- urban town with civil servants, students, farming and trading as the major occupation [19].

2.2. Preparation and Extraction of the Plant Leaves

Collection of materials: The Soursop leaf (*Annonamuricata*) was obtained from the trees from the surroundings of Ekpoma in the month of July. After washing the leaf they were separated from the twigs and dried in shade for further catharsis. The leaves were then grounded to dry coarse powder using a homogenizer and about 500 grams of dry course powder was obtained. Specimens were identified by the botany Department of Ambrose Alli University, Ekpoma, Nigeria.

Preparation of extracts: Aqueous extracts of the Soursop leaf were prepared in the Department of microbiology Laboratory. The procedure of aqueous decoction followed to prepare the extracts of Soursop leaf and juice was based on a previous study by Pai *et al.*[20]. The aqueous decoctions were prepared by adding 10 grams of Soursop leaf in 100ml sterile distilled water and boiled over a low flame for 15 minutes. The flasks were then plugged, removed from heat and allowed to cool for 45 minutes. After cooling, the contents of the flasks were filtered with double filter paper and sterile filters to remove any impurities while for the juice it was squeezed [21].

Concentrations of 5% and 10% of extract were prepared using sterile distilled water. The extracts were stored separately in sterile air tight containers and labeled accordingly. These containers were stored in refrigerator and transported for microbiological assays. A positive control Chlorhexidine Gluconate (0.2%) was used, with benchmark controls of standard anti-microbials in disc diffusion for comparison. All the samples were stored in a refrigerator at 4°C until the analyses were accomplished.

Source of Bacterial Isolates

The different test organisms were isolated from stool, urine and wound swab from patient visiting Specialist Teaching Hospital (ISTH) located in Irrua, Edo State and were analyzed in the Microbiology Laboratory of Ambrose Alli University, Ekpoma, Nigeria for a period of Four months (May 2017 - August 2017). The test organisms isolated for the study included; *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Streptococcus species*, *Pseudomonas aureginosa, Proteus mirabilis* and *Enterobacter species.*

2.3. Identification of Test Organisms

All isolates for this study were identified by their colonial appearance on the media which include Size, Shape, Elevation, Opacity, Edge, Colour, haemolysis and fermentation.

2.4. Gram Staining

Gram staining was carried out on culture that yielded growth using standard procedures;

The growth on the culture plate was carefully placed on a sterile, grease-free microscope slide and allowed to air-dry.

It was fixed by passing it over the pilot flame of the Bunsen burner three times.

The fixed smear was flooded with Crystal violet for 30 seconds before washing off with tap water.

Lugol's iodine was added and washed up after about 30 seconds and subsequently decolourised rapidly using acetone and washed off immediately.

Neutral red (Counter stain) was then added and washed off after about 60 seconds.

The slides were placed in a draining rack for the smear to air dry.

After drying, a drop of immersion oil was applied on the smear and viewed microscopically using oil immersion objectives [22].

2.5. Biochemical Characterization and Identification

Catalase test was done on Gram positive cocci. Catalase negative Gram positive Cocci in chains were identified as *Streptococcus species* while the catalase positive cocci in clusters was identified as *Staphylococcus* species. Coagulase test was carried out on all the catalase positive cocci. The coagulase positive organism was identified as *Staphylococcus aureus* while the coagulase negative organism was identified as *Staphylococcus albus.*

For the Gram negative bacilli, overnight broth cultures was made for each by adding the colonies to sterilized peptone water and incubated for 24 hours at 37°C and motility test was done to ascertain their motility.

Among the Gram negative bacilli isolated they were non-lactose fermenting but motile, oxidase test was done by placing the filter paper on the petri dish and adding two drops of oxidase reagent (which contain tetramethyl paraphenylene diamine dihydrochloride). A glass edge was used to pick the colony from the agar plate and smeared on the moist filter paper with oxidase reagent and was observed for less than 30 seconds for the production of deep blue colouration, which indicates oxidase positive and this biochemical test confirmed *Pseudomonas species.* The lactose fermenting Gram negative bacilli and motile, Indole test was done by adding some drops of Kovacs reagent (Para-dimethyl amino benzldehyde) to the overnight broth cultures. Development of red ring at the surface after ten seconds indicated a positive reaction and such bacteria was regarded as *Escherichia coli*. For those colonies that were Lactose Fermenters and Oxidase negative, Urease test was performed on them. Having been positive to Urease and negative to Indole confirmed *Klebsiella pneumonia.*

2.6. Biochemical Tests

2.6.1. Motility Test

This test was performed to distinguish the motile organisms (*E. coli*) from the non-motile ones.

Procedure: A drop of uncentrifuged, uniformly mixed, mid-stream urine (MSU) sample was place on a cover slip and inverted over a hollow ground slide (cavity slide), avoiding air bubbles. The preparation was examined microscopically for motile organisms, using the X10 and

X40 objectives [23].

2.6.2. Catalase Test

This test helps to differentiate staphylococci from streptococci.

Principle: The enzyme catalase, acts as a catalyst in the breakdown of hydrogen peroxide to oxygen water.

$$2H_2O_2 \xrightarrow{Catalase} 2H_20 + O_2$$

When the organism containing catalase comes in contact with hydrogen peroxide, bubbles of oxygen are given off [23].

Procedure: Few colonies of the organism was emulsified in distilled water on a clean grease free slide placed in a petri dish. 2 drops of H_2O_2 was added to the colony and the petri dish covered. Gas bubbles were observed for some and were not observed in others [23].

2.6.3. Citrate Test

This test is one of the important tests used in the identification of enterobacteria.

Principle: It is based on the ability of an organism to utilize citrate as its only source of carbon and ammonium as its only source of nitrogen. The citrate is metabolized to acetoin and CO_2 [23].

Procedure: A light suspension of the organism was made in saline and inoculated into Koser's citrate medium with a straight wire. Growth (of blue colour) indicated by turbidity in Koser's medium in Simmons agar indicates positive result meaning that citrate has been utilized [23].

2.6.4. Coagulase Test

This test is used to differentiate *Staphylococcus aureus* from other staphylococci.

Principle: The enzyme, coagulase causes plasma to clot by converting fibrinogen to fibrin. Coagulase is produced by *Staphylococcus aureus.* It is considered a pathogenicity test for *Staphylococcus aureus.* Free coagulase – this converts fibrinogen to fibrin by first activating the coagulase reacting factor (CRF) in plasma. This coagulase is detected by the formation of a clot in the tube test in which bacterial broth culture is mixed with plasma. The free coagulase is released in the surrounding medium [23].

Procedure: Human plasma was diluted 1 in 10 in saline. Into each of the three test tubes for each sample, 0.5ml of the diluted sample was added. Five (5) drops of 18-24 hour broth culture of the test organisms was added to the 1st test tube. Five (5) drops of 18-24 hour broth culture of *Staphylococcus aureus* was added to test tube 2 and five (5) drops of sterile broth was added to test tube 3. They were all mixed gently and incubated in a water bath and examined for clot after one hour, two hours and at 30 minutes interval for up to 6 hours. The tubes were observed for clot. The positive control produced clot within 1 hour and the negative control did not produce any fibrin clot [23].

2.6.5. Oxidase Test

This test is employed to aid in the identification of *Pseudomonas*, *Neisseria*, *Vibrio* and other groups.

Principle: The enzyme oxidase will oxidize a redox dye such as tetramethyl paraphenylene diamine dihydrochloride (TMPPDH) to deep purple colour. This enzyme is produced by some aerobic bacteria as part of their respiratory oxidation mechanism (Ochei and Kolhatkar, 2000).

Procedure: A few drops of oxidase reagent was added to a few colonies on the culture plate. Colour change of blue to deep purple was looked out for within 5-10 seconds [23].

2.6.6. Urease Test

This is an important test for differentiating enterobacteria.

Principle: The enzyme urease is possessed by some bacteria. The urease is able to decompose urea by hydrolysis to give ammonia and carbon dioxide. This reaction turns the medium alkaline which is shown by a change in colour of the indicator to red-pink [23].

$$\mathrm{CO}\begin{matrix}NH_2\\NH_2\end{matrix} + H_2O \xrightarrow{urease} 2NH_3 + CO_2$$

Procedure: The entire surface of Christensen's urea slope was inoculated and incubated at 37°C in water bath. It was examined four (4) hours later. Colour change was observed [23]; *Proteus vulgaris* – Positive and *E. coli*– Negative.

2.6.7. Indole Test

This test for Indole production is used as an aid in differentiation of Gram negative bacilli.

Principle: Some bacteria are able to break down the amino acid tryptophan present in peptone water to release indole [23].

Procedure: The organism was grown in peptone water overnight. A few drops of Kovac's reagent was added to the overnight peptone water culture. Colour change was looked out for. Red colouration indicated positive indole production [23].

2.7. Preparation of Test Organisms

The different organism isolated, Staphylococcus aureus, Klebsiella pneumonia, Streptococcus species, Proteus species, Pseudomonas species, and Escherichia coli and Enterobacter were sub-cultured into peptone water overnight before antibiogram extract testing.

2.8. Minimum Inhibitory Concentration (MIC)

Using the Tube dilution method as described by Cowan and Steel (1985), the minimum inhibitory concentration was the lowest concentration of the aqueous extract that inhibited the growth of the bacteria isolates. 0.5ml prepared nutrient broth was dropped into the test tube 2 to 10, 0.5ml of the extract was added to tube 1 and 2. Serial dilutions were made resulting to decreasing concentration of the aqueous extract. The extract in tube 3 was diluted until tube 9 from which 0.5ml was discarded. 0.5ml of the test bacteria isolates was added to tube 2 to tube 10. Tube1 which contained nutrient broth and organism served as control tube.

The entire procedure was done for the bacteria isolates that

was susceptible to the extracts with zone of inhibition above 10. The tubes was thoroughly mixed and incubated at 37°Cfor 24hours after which they were examined for visible growth which is seen as turbidity. The MICs reported as the lowest concentration of the orange peel extracts that prevent visible growth [25].

The minimum inhibitory concentration gave the lowest concentration of the aqueous extract that can inhibit the growth of the bacteria isolates. Freshly prepared sterile nutrient broth was used. Ten test tubes were used. 0.5ml of nutrient broth was dropped in the test tube 2 to 10, 0.5ml of the extract was added to tube 1 and 2. Serial dilutions were made resulting to decreasing concentration of the extract. The extract in tube 3 was diluted until tube 9 from which 0.5ml were discarded. 0.5ml of the test bacteria isolates were added to tube 2 to tube 10. Tube 10 which contain nutrient broth and organism serves as control tube [24].

The entire procedure was done for the bacteria isolates that were susceptible to the extracts with zone of inhibition above. The tubes were thoroughly mixed and incubated at 37°C for 24 hours after which they were examined for visible growth which is seen as turbidity. The MICs reported as the lowest concentration of the plants extracts that prevented visible growth [25].

2.9. Statistical Analysis

SPSS software was used for statistical analysis (version 20). The disc diffusion values of all the different concentrations were entered in the SPSS software for statistical analysis. Descriptive statistics were retrieved and data was analysed.

3. Results

Table 1 shows the percentage of bacterial isolate to various concentrations of soursop fruit juice extracts in which 50% concentration/dilution of fruit extracts recorded the highest of 14(93.3%) while *E. coli, Salmonella* spp. *and shigella* was 10(100%), 3(75%) and 1(100%) respectively. However, the 10% of fruits extracts was second with 12(80%) and *Escherichia coli* recorded the highest of 10(100%) followed by *Salmonella* spp lest of 2(50%) while absent in *shigella*.

As for 50% Juice extracts, a total of 2(13.3%) was recorded with *Salmonella* spp and *shigella* with 1(100%) respectively. While for 10% juice extracts, only *Shigella* of 1(100%) was recorded. The susceptibility rate for *Escherichia coli* was 7.07±0.60 and 5.56±0.62 for fruits and juice respectively. While for *Salmonella spp.* was 5.56±0.84 and 6.73±0.64 for fruits and juice respectively

Table 1. *The Percentage of Bacterial Isolate to Various Concentrations of Soursop Extract.*

Bacteria isolates	Number	Leave extracts concentration/dilution		Juice extracts concentration/dilution	
		10%	50%	10%	50%
Escherichia coli	10	10(100%)	10(100%)	0(0%)	0(0%)
Salmonella spp.	4	2(50%)	3(75%)	0(0%)	1(100%)
Shigella	1	0(0%)	1(100%)	1(100%)	1(100%)
Total	15	12(80%)	14(93.3%)	1(6.6%)	2(13.3%)

Table 2. *The Zone of Inhibition in Millimeter (Mm) of the Bacterial Isolates to various Concentration of Soursop Extract.*

No	Organisms	Leave extracts concentration/dilution		Juice extracts concentration/dilution	
		10%	50%	10%	50%
1	*Escherichia coli*	7.0	6.0	-	-
2	*Escherichia coli*	7.5	5.0	-	-
3	*Escherichia coli*	6.2	5.0	-	-
4	*Escherichia coli*	7.2	6.3	-	-
5	*Escherichia coli*	6.0	4.5	-	-
6	*Escherichia coli*	7.5	5.5	-	-
7	*Escherichia coli*	7.9	5.2	-	-
8	*Escherichia coli*	6.8	6.0	-	-
9	*Escherichia coli*	7.5	6.4	-	-
10	*Escherichia coli*	7.1	5.7	-	-
11	*Salmonella spp.*	6.2	7.0	-	5.2
12	*Salmonella spp.*	5.0	7.2	-	-
13	*Salmonella spp.*	-	-	-	-
14	*Salmonella spp.*	-	6	-	-
15	*Shegella*	-	7.2	6.0	6.0

Table 3. *The Susceptibility of the Bacterial Isolates (Mean±SD) to the Various Soursop Extract Concentrations (in mm).*

Bacterial Strains	Inhibitions diameters			
	Leave extracts concentration/dilution		Juice extracts concentration/dilution	
	10%	50%	10%	50%
Escherichia coli	7.07±0.60	5.56±0.62	-	-
Salmonella spp.	5.56±0.84	6.73±0.64	-	5.2
Shigella	-	7.2	6.0	6.0

4. Discussion

Plants have been used as medicines from time immemorial. The main advantages of using plants as alternative medicine include its diversity and flexibility of use, their availability and affordability in the region and mainly to reduce adverse reactions. The widespread acceptance of plants in low- and middle-income countries, its comparatively low cost and the relatively low level of technological input required, make them the ideal alternative to costly therapies. Hence, plant extracts may prove to be better and safer alternatives if they are supported by scientifically based evidence [26]. The use of Soursop extract on microorganisms has a strong traditional foundation; many countries in the world use this extract for treatment of various diseases. In countries like Peru, Brazil and Togo the extracts have been used for various treatments such as liver disorders, diarrhoea, dysentery, fevers, hypertension, sores, internal ulcers and diabetes [17]. These observed curative properties that have been published in many studies and its effect against cancer prompted this present study to investigate the possible effect of Soursop leave extract on some Bacteria.

Preliminary susceptibility tests (Table 3) showed effective inhibition of microorganisms growth. At 50% concentration, the inhibition diameters obtained from leaf extract were ranged between 5.60 and 7.07 mm while 10% concentration was varied from 5.56 and 6.73mm. Those obtained from 10% and 50% concentration juice extract varied from 00 to 6.0 mm and 5.2 to 6.0mm respectively. This difference could be due to the fact that, the sensitivity of a microorganism to an extract depends not only on the extract but on the microorganism itself [26]. The results of these susceptibility tests are in accordance with those obtained by Vijayameena *et al.*[27] who had proven the susceptibility of bacteria such as *Staphylococcus aureus, Bacillus, Pseudomonas aeruginosa, Klebsiella pneumonia* to barks and leaves extracts of *Annonamuricata*. This justifies the traditional uses of this plant for the treatment of bacterial infections.

The mode of action of Soursop extract against microorganisms is presently unknown but the common mechanism as to how they act against microbes, insects, and herbivores in their natural environment might prevail. Biologically as to what makes Soursop potent against micro-organisms is the presence of acetogenins. These are bioactive compounds found in the annonacea family, these acetogenins, are known to have tumoricidal, anti-malarial, anti-helmintic, anti-viral, and anti-microbial effects, suggesting many potentially useful application. Of the annonaceous-acetogenins, bullatacin, an acetogenin is a powerful tumoricidal and antibacterial agent [26].

5. Conclusion

In conclusion, the Soursop fruits extracts of 50% and 10% concentration were efficient for all test organisms except *shigella* in 10% concentration. Whereas for soursop juice 50% and 10% concentration were less efficient for all test organism. The present study demonstrated the in-vitro efficacy of Soursop fruits extracts which was highest against *E. coli* followed by *Salmonella spp*. Hence, this study proves to an extent that the Soursop fruits extract when used against microorganisms, has sufficient anti-microbial property. Thus, it is recommended that going to nature for finding cure now has become an essential part of medicine, natural products that are also available at homes of individuals might be an adjuvant to empirical therapy, as best use of resources that are already available might be the solutions going forward therefore, it should be encouraged even at policy making levels.

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