



Antibacterial Activity of *Ananas comosus* Fruit Extract against Clinically Isolated Bacteria from Urinary Tract Infected Patients

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Authors' contributions

This work was carried out in collaboration among all authors. Author AJSJS designed the study concept and carried out the pharmacognostic and phytochemical studies. Author AGKAV performed the isolation and identification of Bacteria and author DD drafted the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate the antibacterial activity of *Ananas comosus* (*A. comosus*) fruit extract against clinically isolated bacteria from urinary tract infected patients.

Study Design: Experimental study.

Place and Duration of Study: Research Lab, Department of Siddha Medicine, Tamil University, Thanjavur, India and Microbiology lab, School of Pharmacy, KPJ Healthcare University College, Malaysia, between February 2019 and January 2020.

Methodology: In the present study the ethanol and aqueous extracts of *A. comosus* were analysed for the phytoconstituents and the activity of the plant extract was compared with a standard antibiotic which is used for a wide range of Urinary Tract infection which is Ciprofloxacin (250 mg/mL) using cup plate method.

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Results: The ethanolic extract of *A. comosus* showed a great level of bacterial inhibition (27.3 mm) against *Bacillus cereus* as compared with standard (22.3 mm). Whereas the antimicrobial activity was moderate against *Klebsiella* organism and very less against *Staphylococcus*. Standard exhibits a huge difference in the zone of inhibition which is (34 mm) and ethanol extract (23 mm) against *Enterococcus*. While the aqueous extracts do not show any effect on the microorganism.

Conclusion: The ethanol extract *A. comosus* exhibited broad-spectrum activity against tested isolates compared to aqueous extract. *A. comosus* has broad inhibitory activities to pathogenic microorganisms and promising to act as potential antibacterial agents from natural plant sources.

Keywords: *Ananas comosus*; fruit extract; urinary tract infection; antibacterial activity.

1. INTRODUCTION

Urinary tract infection (UTI) is well-defined as manifestation of microbial pathogens in the urinary tract along with related symptoms. Urinary tract infections (UTIs) are a serious general medical issue and are caused by a wide range of pathogens, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus saprophyticus* [1]. They are a common reason for morbidity which can lead to increase in rate of mortality [2]. Numerous authors around the world have stated the Gram-negative bacteria *E. coli* and *Klebsiella sp.* being the most frequent organisms causing UTIs. *E. coli* causes 70-95% upper and lower UTIs [3]. In all cases of UTI effective treatment is very essential; hence exist the need of proper antibiotics for the treatment of effective management of UTIs. The outpatient setting has been hampered by the fact that many strains have developed resistance to several oral antimicrobial agents.

Natural medicine is widely assumed that it has the ability to increase the body's capacity on defending illnesses. Even in areas where modern medicine is obtainable, the attentiveness on herbal medicines and their use have been increasing rapidly. Herbal plants always play a vital source of bioactive constituents which include an integrative approach on investigation of plant as a treatment for progressing diseases. Analysis on its phytochemicals may grow the discoveries to invent new synthetic compound [4]. Utilisation of *A. comosus* tends to be more effective for long-standing UTI infections complaints that don't respond well to antibiotics. Natural antibiotics act in a unique mechanism, in addition to its bactericidal action. The success of this study can be useful for UTI infection formulation which will be conducted in future.

A. comosus is a fast-growing tree which usually grows up to 10 or 12 m in height. The roots of this plant are developed via seedlings which

have fast spreading property. It develops to a swollen, tuberous, white taproot which has a characteristic pungent odour, and very sparse lateral roots. The leaves grow up to 45 cm long that are alternately and spirally arranged (tripinnate) on the twigs. Leaflets are 1.2 to 2.0 cm long and 0.6 to 1.0 cm wide that are finely hairy. Individual flowers are approximately 0.7 to 1 cm long and 2 cm broad. The fruits are pendulous, linear, three-sided pods with nine longitudinal ridges, usually 20 to 50 cm long, but occasionally up to 1 m or longer, and 2.0 to 2.5 cm broad. Each pod usually contains up to 26 seeds which mature 3 months after flowering. They turn brown and split open longitudinally along the three angles, releasing the seeds upon maturing which are 1 cm in diameter.

Pharmacologically, *A. comosus* leaves extract possesses good antioxidant and radical scavenging property which is due to the presence of kaempferol. *A. comosus* leaves, roots, bark and seeds has antimicrobial activities against bacteria, yeasts, dermatophytes and helminths. The leaves and flower extracts are capable of controlling parasitic worms. Anti-inflammatory activity is present in the flowers, leaves, roots, seeds and stalks or bark of *A. comosus*. Both the seeds and leaves have analgesic activity however the antipyretic effect is only present in the *A. comosus* seeds extracts. The leaves of *A. comosus* contain polyphenols including quercetin-3-glycoside, kaempferol glycosides, rutin and other polyphenols which results in antidiabetic activity. Furthermore, presence of mustard oil glycosides, thiocarbamate glycosides and nitrile are compounds leading to blood pressure lowering effect in leaves. *A. comosus* seeds have anticancer activity, act as cardiac stimulant and recede liver fibrosis. *A. comosus* seeds have plant alkaloid which has same activity as ephedrine in treatment of asthma. The seeds, leaves and pods are effective against eye problems and helpful in preventing night

blindness. Vitamin A presence will improve and delay cataract development.

Traditional medicines have been used since ancient times to treat urinary infections. Few studies have been published involving tests with medicinal plants. In view of these aspects, the present work is carried out to evaluate the antimicrobial activity of *A. comosus* against Urinary tract pathogens.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant

A. comosus was collected from organic farm which is located at Kanyakumari district in TamilNadu, India. Then it was authenticated by a Dr. N. Nagarajan, Botanist, Tamil University, Tamil Nadu, India. The fruit of *A. comosus* was cleaned with deionized water. The thorns excluded and the pith of the inner part of was separated from the foliage. The outer green part of the fruit was peeled off and its yellow inner portion cut into smaller portion (500gm). The small pieces were crushed mechanically and 600ml of pure juice was extracted [5] recommended being stored at 4°C.

2.2 Preparation of *A. comosus* Extract

A. comosus fruit juice consecutively extracted 600ml with Ethanol and Aqueous solvent using separating funnel and supernatant liquid was collected. Excessive solvent was evaporated and concentrated by using freezing technique [6]. The extracts were kept in refrigerator for 2 weeks then it was transferred to Microbiology lab, Department of Microbiology, KPJ healthcare university college, Malaysia for antibacterial study.

2.3 Phytochemical Analysis

Several phytochemical tests were done using the *A. comosus* fruit juice extract to study the presence of constituents. Preliminary phytochemical studies were done based on qualitative standard subjective techniques as portrayed by different authors. The *A. comosus* extract was screened for the existence of compounds like glycosides, phenolic, alkaloids, tannins, flavonoids, saponins and steroids [7,8].

2.4 Collection and Physical Examination of Urine Samples

A total of 8 UTI infected urine samples (Middle Adults, Age 36-55 years) were collected

aseptically in sterile plastic container from KPJ Specialist Hospital. All the specimens were brought to KPJUC microbiology lab for isolation and identification of UTI pathogen. Urine samples were stored at 4°C for up to 24 hours exposed to minimal light or in darkness, no specimen older than 24 h was used [9]. The physical characteristics of urine specimen were determined based on its volume, colour, pH, turbidity and odour [9].

2.5 Isolation and Identification of Bacterial Pathogens from Urine Samples

A total of 8 urine samples were collected in sterile containers from UTI infected patients. The samples in which bacterial count >10⁵ CFU/ml was taken for identification of uro-pathogen. For the isolation of UTI bacterial strains, loop full of urine samples were streaked in to the nutrient agar using streak plate method and incubated at 37 ± 2°C for 24 h [10,11]. All samples were plated in triplicate. Identification of bacterial pathogens was performed based on gram reactions, morphology and biochemical characteristics [12].

2.5.1 Gram staining method

Staining method was carried out by using Crystal Violet along with a heat fixed smear of bacterial culture. Addition of Gram's iodine along with decolourization with 95% ethyl alcohol. Finally, its counterstained with safranin. Then slides were rinsed with water and blot dried continued by observing under microscope with oil. Biochemical tests were carried out for the identification of bacteria species based on the enzymatic activity of the bacteria.

2.5.2 Biochemical tests

2.5.2.1 Starch hydrolysis

A medium containing starch was used. After inoculation and overnight incubation, iodine reagent is added to identify the presence of starch. Clear halos surrounding colonies is revealing their ability to digest the starch in the medium due to the occurrence of alpha-amylase.

2.5.2.2 Triple sugar iron (TSI) agar test

The well isolated colony was inoculated by using inoculation needle into Triple sugar iron agar media by first stabbing through the centre of the

medium to the bottom of the tube and then streaked on the surface of the agar slant. The tubes were incubated at 37°C for 18 to 24 hrs. After incubation the following change were observed [13].

- i) Alkaline slant (red) and acid butt (yellow) with (or) without gas production; Indicating glucose fermentation.
- ii) Acid slant (yellow) and acid butt (yellow) with (or) without gas production; Indicating lactose and (or) sucrose fermentation.
- iii) Alkaline slant (red) and alkaline butt (red) or no change (orange-red) butt; There by showing no carbohydrate fermentation.

2.5.2.3 Indole production test

An inoculum from a pure culture transferred to Sulphide Indole Motility medium and Tryptone broth. An inoculating wire used for inoculating SIM and a loop for Tryptone broth. SIM stabbed all the way to the butt followed by incubation at 35-37°C for 24 hours. Five to ten drops of Kovac's reagent were added to the tube to identify the reaction of Indole. The reagent will react with indole to produce a ring that is cherry red in colour. The production of H₂S and motility was observed.

2.5.2.4 Methyl red

Inoculated MR-VP Broth with a pure culture of the microorganisms and incubate it at 35 °C. Added about 5 drops of the methyl red indicator solution. A positive reaction shows the changes in the medium to red colour.

2.5.2.5 Voges Proskauer test

Inoculated MR-VP Broth with a pure culture of the microorganisms and incubate it at 35°C.

2 drops of 5% alpha-naphthol were be added. Then 1 drop of 40% potassium hydroxide was added. The tube was shaken and left undisturbed for about 10-15 minutes. A positive reaction shows pink-red colour on the surface of the medium.

2.5.2.6 Citrate utilization test

Inoculated Simmons citrate agar with a pure culture of the microorganisms on the slant by using a colony that is 18-24 hrs old with an inoculating loop. Followed by incubation 35°C-37°C for up to 7 days for formation of blue colour [14].

2.6 Stock Culture Maintenance of Clinical Isolates

Stock cultures were maintained on Nutrient Agar Slants and nutrient broth medium at 37° C under aerobic condition for 24 hours. After incubation the bacterial inoculum stored in refrigerator at 4°C.

2.7 Antibacterial Assay Method

The antibacterial assay was carried out using agar well diffusion technique or called as Cup Plate method. The Ethanol and Aqueous extract of *A. comosus* with each concentration of 500mg/ml were diluted with Dimethylsulfate (DMSO₄). Ciprofloxacin (250mg) used as a standard drug to study the effective antimicrobial activity of *A. comosus* fruit juice extract. The drug was prepared in the concentration of 250mg/ml. Thus, the standard drug was weighed accurately and dissolved in 2ml of distilled water to make a stock solution. Antimicrobial activity of *A. comosus* extract was compared with standard antibiotic Ciprofloxacin 250mg/ml. Mueller-Hinton agar plates was used for this study. Whereas 20 ml of Mueller-Hinton agar was dispensed into sterile universal bottles. These were then inoculated with 0.2 ml of the cultures, mixed gently and poured into sterile petri dishes. After setting, a number 4 cup borer (8 mm diameter) was properly sterilised by flaming and used to make three uniform cups in each petri dish. A drop of molten agar was used to seal the base of each cup. The organism was inoculated vertically and horizontally on the Muller Hinton agar using cotton swab. Ethanol and aqueous extracts of *A. comosus* at concentration of 500mg/ml allowed to diffuse for 45 minutes. The solvents used for reconstituting the extracts were similarly analysed. The plates were incubated at 37 °C for 24 hr. The tests were repeated about three times to check on its reliability. The diameter of inhibition zone either around the treat discs or around the control discs will be measured as mean diameter in mm for the antimicrobial evaluation [15].

2.8 Determination of Minimum Inhibitory Concentration (MIC) of *A. comosus* Extract against Isolated UTI Pathogen

The broth dilution method was used to determine the minimum inhibitory concentration (MIC) of the ethanolic and aqueous extracts against isolated UTI pathogens. Ciprofloxacin was prepared at a

concentration of 250 µg/mL and both aqueous and ethanolic extracts were tested beginning from 1000 µg/mL concentration. Twenty test tubes were sterilized by autoclaving at 121°C for 15 minutes and arranged as two rows in a test tube rack. The first test-tube in each row is kept as negative control (no inoculum) and the second test tube is kept as a positive control (with antibiotic). Using a micropipette, 1mL of sterile nutrient broth was added to all twenty test tubes. For the first row, take 1mL of aqueous extract of 1000µg/mL and add to the first (negative control), second (positive control) and third test tubes (aqueous extract). The third test tube now contains 1000 µg/mL and is diluted into consequent concentrations by taking 1mL from the third test tube and added into the fourth test tube, taking 1 mL from the fourth test tube and added into the fifth test tube and so on to obtain concentrations of 500, 250, 125, 62.5, 31.25 and 15.63 µg/ml. This procedure was repeated for the second row by replacing the aqueous extract with ethanolic extract. All the test tubes were incubated for 24 hours at 37°C. The test tubes were observed following incubation to confirm absence or growth of bacteria. The test tubes with absence of growth or turbidity during MIC determination were selected.

2.9 Statistical Analysis

The MIC data was collected and analysed using one sample T test, as it shows the mean comparison of a distinct group to the mean of another group from the same sample. The one sample T-test was run whereby the distinct group was ciprofloxacin 250mg/ml zone of inhibition values and the other group was ethanol extract 1000µg/mL zone of inhibition values. The mean values obtained were recorded. The one sample T-test was allowed to run again by replacing ethanol extract values to that of aqueous extract values and the results were recorded. The statistical significance is determined by a P value less than 0.05.

3. RESULTS

3.1 Morphological Characteristics of *A. comosus* Fruit Juice

Collected fruit juice were kept in schott bottle. The characteristics were classified based on colour odour texture and taste. The morphological characters were listed in Table 1.

3.2 Nature and Yield of Extract

The nature of *A. comosus* juice extract is classified based on its colour, and consistency. The percentage yield was calculated using the following formula:

$$\text{Yield} = \frac{\text{Weight of the extract}}{\text{Weight of mass used for extraction}}$$

The Ethanolic extract of *A. comosus* were found to be dark brown in colour, semi solid in consistency. The %yield of the exact obtained was 6.38. The aqueous extract of *A. comosus* was found to be light brown in colour, semi solid in consistency. The %yield of the extract obtained was 7.28.

3.3 Phytoconstituent Present in Aqueous and Ethanolic Extract

The preliminary phytochemical screening was done qualitatively and the below table shows the phytochemicals present in both aqueous and ethanolic extracts. Based on the preliminary phytochemical screening, ethanol extract of *A. comosus* contains alkaloids, carbohydrates, flavonoids, tannins and terpenoids whereas aqueous extract contains carbohydrates and flavonoids. Phytoconstituent present in Aqueous and Ethanolic Extracts of *A. comosus* were mentioned in Table 2.

3.4 Isolation and Identification of Microorganisms from the Patients with UTI

The collected urine samples were inoculated to Nutrient Agar Media incubated for 37 ± 2°C for 24 h at room temperature respectively. The isolated colonies and the colony characteristics of bacteria observed.

The isolated bacterial samples were identified using by gram staining technique and biochemical technique. The observations were recorded in Fig. 1 and Table 3.

3.5 The Antimicrobial Activity of *A. comosus* Extract against *Klebsiella*, *Enterococcus Faecalis*, *Bacillus cereus* and *Staphylococcus aureus*.

The antimicrobial activity of aqueous and ethanolic extracts of *A. comosus* Extract juice against isolated UTI pathogen was done using

cup plate method. The results show great antimicrobial activity when treated with Ethanol extracts of *A. comosus* whereas no activity shows against Aqueous Extract of *A. comosus* juice. The P value obtained from the One Sample

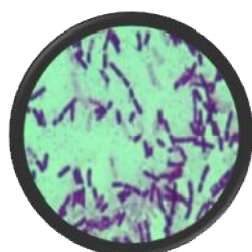
T Test shows that fruit extract shows significant antibacterial activity against *Klebsiella*, *Enterococcus Faecalis*, *Bacillus cereus* and *Staphylococcus aureus*. The results were recorded in Table 4.

Table 1. Morphological characteristics of *A. comosus* fruit juice

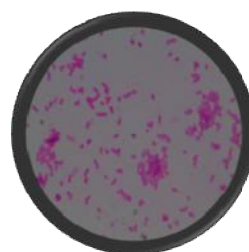
Odour	Rancid
Color	Amber
Texture	Watery
Taste	Sweet and tart with a slightly bitter after taste

Table 2. Phytoconstituent present in Aqueous and Ethanolic Extract of *A. comosus*

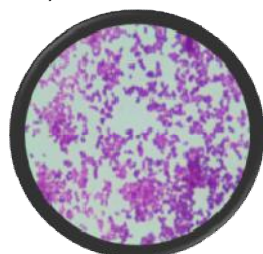
Test	Ethanol	Aqueous
Alkaloids	+	-
Carbohydrates	+	+
Phenols	-	-
Tannins	+	-
Flavonoids	+	+
Proteins	-	-
Terpenoids	+	-



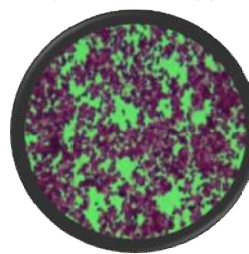
a) *Bacillus cereus*



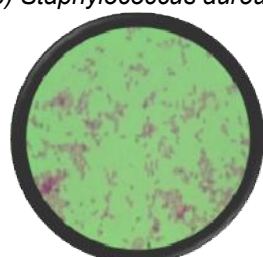
e) *Klebsiella spp*



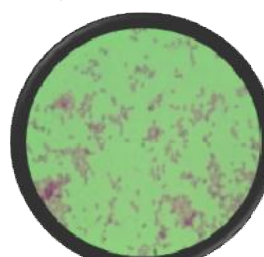
b) *Staphylococcus aureus*



f) *Enterococcus*



c) *Escherichia coli*



g) *Escherichia coli*

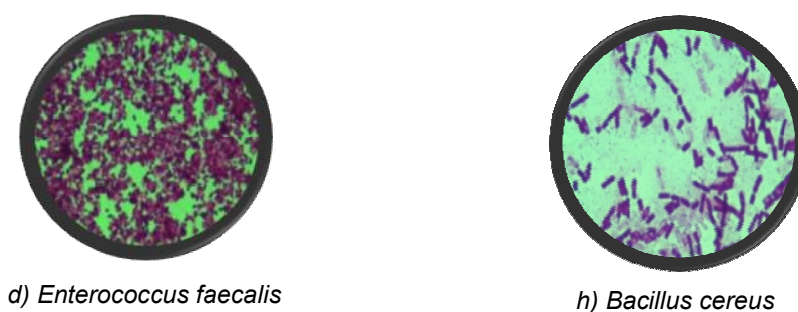


Fig. 1. Identification of isolated bacteria by gram staining technique

3.6 The Minimum Inhibitory Concentration (MIC) of *A. comosus*

The Minimum Inhibitory Concentration (MIC) of ethanolic and extract of *A. comosus* juice extract was determined by broth dilution method starting from 1000 µg/ml. The results obtained were tabulated in Table 5.

The Minimum Inhibitory Concentration (MIC) of the ethanolic extract of *A. comosus* juice showed visible growth at concentrations of 15.63, 31.25 and 62.5µg/mL for all organisms. The lowest concentration of ethanolic showing no visible growth or turbidity for *Klebsiella* and *Enterococcus faecalis* was found to be 250µg/ml. Whereas the lowest concentration exhibited for *Staphylococcus aureus* and *Bacillus Cereus* was found to be 500 µg/ml and it was recorded as the MIC value.

4. DISCUSSION

A huge number of individuals around the world experience urinary tract infection (UTI) which eventually worsens their health and wellbeing. Generally, Antibiotics treat individual infections but the recurrent ones are not looked through. Useful treatment is needed to overcome this issue. The significant of the current study is to utilise plant as antimicrobial source for the on-going antibiotic resistance issue. Antimicrobial activity of the stem juice extract undoubtedly could provide an effective treatment against UTI infections. Since herbal plants are being the most favoured remedies due to their medicinal functional traits with minimal side effects. *A. comosus* juice contains ascorbic acid and is a good source of vitamin c. ascorbic acid fights bacterial and viral infections which is an effective antioxidant and helps the body absorb iron [16]. In this study the cup plate method is used to identify the antibacterial activity of the *A. comosus* plant extract with a standard drug ciprofloxacin (500mg/ml) which is used for the

treatment of UTI. The various extracts of the *A. comosus* were compared with the standard in which the ethanolic extract of the plant exhibited a greater bacterial inhibition of 23.58mm against *Bacillus cereus*. Whereas the aqueous extract does not exhibit any result against the microorganism. The ethanol extracts show better antibacterial activity compared to aqueous extracts as the active components of the crude drug has better solubility in organic solvents. This observation can be rationalised in terms of polarity of the compounds being extracted by each solvent and in addition to their intrinsic bioactivity by their ability dissolve or diffuse in the different media used in the assay.

In the present study negligible inhibitory activity with aqueous extract may be due to loss of active compound during extraction process of the plant [17]. Preliminary Phyto-chemical screening of plant is very useful for determination of the active constituents in different solvents and their yields. Many primary metabolites lie in their impact as precursors or pharmacologically active metabolites in pharmaceutical compounds [18]. The antibacterial activity exhibited by *A. comosus* Juice extract could be due to the presence of phytochemical presents such as alkaloids, tannins, flavonoids and sugars present in the plant extracts [19]. Flavonoids and tannins present in the ethanol extract may be responsible for the antibacterial activity. Tannin is known to show the antibacterial activity by precipitation the microbial proteins [20]. Flavonoids are produced by the plants for the defence against the infection. So, use of the crude ethanol extract of this plant as an agent to control microbial pathogens needs further extensive research for their better economic and therapeutic utilization. Further Phyto-chemical studies are required to determine the purified fractions or bioactive compounds responsible for the antibacterial activities of these species, which could serve as useful sources for new antimicrobial agents.

Table 3. Biochemical tests for bacterial species identification

Sample	Indole	MR	VP	CIT	Strach hydrolysis	TSI	Identified bacterial pathogen
1	-	+	+	+	+	A	<i>Staphylococcus aureus</i>
2	-	-	+	-	-	A/ALK	<i>Enterococcus faecalis</i>
3	-	-	+	-	-	AG	<i>Klebsiella</i>
4	-	-	+	-	-	A/ALK	<i>Enterococcus faecalis</i>
5	+	+	-	-	-	AG, A	<i>E-coli</i>
6	-	-	+	-	-	A/ALK	<i>Enterococcus faecalis</i>
7	-	-	+	-	-	AG	<i>Klebsiella spp</i>
8	-	-	+	+	+	A	<i>Enterococcus faecalis</i>

+ indicates Positive; - indicates Negative A indicates production of acid
AG indicates presence of acid and gas ALK indicates alkaline

Table 4. Zone of inhibition of aqueous and ethanolic extracts of *A. comosus*

Organism	Ethanol 500 mg/ml	Standard ciprofloxacin 250 mg/ml	P value
<i>Klebsiella</i>	27.333 ±0.333	22.333±1.45	0.028
<i>Enterococcus</i>	23.00±0.57	34.000±0.57	0.000
<i>Bacillus cereus</i>	21.00±0.57	25.66±0.881	0.011
<i>Staphylococcus</i>	17.667±0.33	29.66±0.33	0.000

Note: zone of inhibition in mean ± S.E.M (n=9) Values * p<0.05 are considered significant compared to standard.

Table 5. Minimum inhibitory concentration of the Ethanolic extract of *A. comosus*

Concentration of Ethanol extract (µg/ml)	1000	500	250	125	62.5	31.25	15.63
<i>Klebsiella</i>	-	-	-	+	+	+	+
<i>Enterococcus faecalis</i>	-	-	-	+	+	+	+
<i>Staphylococcus aureus</i>	-	-	+	+	+	+	+
<i>Bacillus Cereus</i>	-	-	+	+	+	+	+

The growth medium used also plays a crucial role to evaluate the antimicrobial activity. Muller-Hinton is the most suitable medium to determine the antibacterial activity and the same was used in the present study. This indicates that the plant extracts could possess more antimicrobial property if the plant has some purification. Some researchers report that there is a relationship between the chemical structures of the most abundant compounds in the tested extracts or essential oils and the antimicrobial activity [21].

Based on the results obtained from MIC of the ethanolic and aqueous extracts of *A. comosus* ethanolic extract shows bacteriostatic at 250 µg when compared with other concentrations. The lowest concentration of the plant extract required for inhibiting the growth was considered as the MIC of the extracts against bacterial and fungal strains. The MIC values of each extract against the tested microorganisms were presented. It was found from the data obtained that extract of *A. comosus*, required relatively lesser quantity for arresting the growth of tested organisms. The results obtained in this study prove the efficacy of *A. comosus* juice in reducing the bacterial growth of urinary tract infection with its antimicrobial property to a considerable extent, thus justifying in the indigenous system of medicine. *A. comosus* has broad inhibitory activities to pathogenic microorganisms and promising to act as potential antibacterial agents from natural plant sources.

5. CONCLUSION

The results of the present study indicate presence of secondary metabolites such as alkaloids, carbohydrates, flavonoids and tannins, in the ethanolic extract of *A. comosus* fruit juice. The ethanolic extract actively inhibits the bacterial growth of the organism while aqueous extract has no antibacterial activity against the microorganism. Based on the results obtained, it can be concluded that the *A. comosus* ethanolic fruit juice extracts contain potential antimicrobial compounds acting against UTI inducing uropatogens and they can be used in the treatment UTI infection. Thus, from the findings, it is concluded that the studied plant can be a potential source of useful antibacterial drug. Further studies are however recommended on the plant in order to isolate, identify, characterize and elucidate the structure of the bioactive compounds from the medicinal plant *A. comosus*.

CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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