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Antioxidant, urobactericidal and antibiotic modulating activity of the methanolic extract of the stem and resin of *Acacia catechu* (L.f.) Willd

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Abstract

Background Emergence of multidrug resistant pathogens has opened new vistas for novel drug discovery or combinatorial drug surveillance, often in form of some natural products, which is considered to be cheap and safe. In this study, the urobactericidal activity of the methanolic extract of the stem and resin of *Acacia catechu* (L.f.) Willd (Fabaceae) was explored against five uropathogenic bacterial strains i.e. *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Staphylococcus aureus*.

Methods Varieties of antibacterial (disc diffusion, agar well diffusion, modified agar well diffusion) and antioxidant assays (DPPH and OH free radical scavenging assay) were tried to prove the efficacy of stem and resin extracts of *A. catechu* and to compare their urobactericidal and free radical scavenging properties.

Results Phytochemical analysis envisaged that the stem and resin contained phytoconstituents like alkaloids, phenols, tannins, proteins, glycosides, flavonoids, steroids and terpenoids, which were reported to have excellent antioxidant and antibacterial activities. The total phenolic contents of the methanolic extract of *A. catechu* stem (ACs) and *A. catechu* resin (ACr) were calculated as 37.74 ± 0.023 and 51.98 ± 0.011 mg/g Gallic Acid equivalents. The total flavonoid contents of methanolic extract of ACs and ACr were calculated to be 71.33 ± 0.004 and119.6 ± 0.010 mg/g Rutin equivalent. ACs had IC_{50} value of 93.68 ± 0.71; 90.92 ± 0.54 µg/mL and ACr had 79.21 ± 0.54; 85.74 ± 0.61 µg/mL in comparison to an IC_{50} value of 72.33 ± 1.20; 66.96 ± 0.61 µg/mL for standard Ascorbic acid in the DPPH and hydroxyl free radical scavenging assay. Phytocompounds present in both ACs and ACr were proved to have improved the urobactericidal efficacies of conventional antibiotics especially against the *E. faecalis* and *E. coli*, the prime etiological agents of uropathogenesis.

Conclusion Our results indicated the excellent urobactericidal effects of the stem and resin extracts of a least explored natural remedy against uropathogens, which will be beneficial for treating urinary tract infections and augmenting the quest for novel therapies in future for uropathogenesis.

Keywords *Acacia catechu* (L.f.) Willd, Antioxidants, Urobactericidal activity, Phytochemicals, Uropathogens

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Introduction

Since antiquity, human and animals mostly rely on plant based natural therapeutics for different common ailments and more than 30% of the plant species are used for remedial purposes [1]. India being one of the epicenters of mega biodiversity, contribute a lot to drug discovery and production of pharmaceuticals that are of herbal origin. Medicinal plants rich in secondary metabolites, have been explored extensively for their bioactive potential. Herbal medicines are always accepted and considered safe as compared to synthetic medicines. Over 80% of the total world population depends mostly on plants and their products for their major health concerns, especially in developing and underdeveloped countries [2].

Acacia catechu (L.f.) Willd belonging to family Fabaceae (subfamily Mimosoideae) known as Khair or Cutch tree, is native to south-Asian countries. The hot water extract of its heartwood is known as catechu or '*kattha*' and its resin part is a crucial ingredient for '*pan*' which is a betel leaf preparation chewed in India and Pakistan [3]. In Ayurvedic medicines, it has been used in the treatment of cough, dysentery, throat infection, chronic ulcers and wounds. *A. catechu* has been reported for its antimicrobial, anti-inflammatory, antipyretic, antiproliferative, immunomodulatory, and hypoglycemic activity [4]. This plant is known for its influential astringent and antioxidant activities. Ethanolic stem extract of *A. catechu* exhibited significant antimicrobial activity [3]. The plant based antimicrobial compounds belong to different categories like alkaloids, flavonoids, essential oils, sesqui-terpene lactones and naphtoquinones, etc [5, 6]. A. *catechu* is an important medicinal plant with immense medicinal potential for a variety of health issues, which is extensively used in ayurveda and other systems of medicine for treatment of different diseases. The heartwood is inhaled to check bleeding from nose. Stem is bitter and known for its astringent, acrid, cooling, depurative, anthelmintic, antiseptic, antidysenteric, antipyretic, appetizer, hyperglycaemic, anti-inflammatory, hypotensive, haemoptysis, haematemesis, haemostatic properties. It is also used for the treatment of catarrh, cough, pruritus, leprosy, leucoderma, skin diseases, helminthiasis, anorexia, diarrhoea, dysentery, vitiligo, foul ulcers and wounds, haemorrhages, fever, anaemia, diabetes and pharyngodynia [7].

The stem and resin are reported to have many therapeutic phytocomponents. Important compounds like catechins (66.9%) and epicatechins or acacatechin (23.1%), 4-hydroxybenzoic acid, quercetin, baicalin, baicalein, afzelechin, mesquitol, ophioglonin, epiafzelechin, aromadendrin, and kaempferol, were isolated from *A. catechu.* Similarly, phenolic compounds such as 5-hydroxy-2-[2-(4-hydroxyphenyl) acetyl]-3-methoxylbenzoic acid, (2 S,3 S)-3,7,8,3′,4′-pentahydroxyflavane, rhamnetin, 4-hydroxyphenyl ethanol, 3,3′,5,5′,7-pentahydroxyflavane, and fisetinidol along with ellagic acid, rutin, quercetin, gallic acid, chlorogenic acid, umbelliferone, kaempferol, coumaric acid, and caffeic acid were isolated from aqueous extract of *A. catechu* and they are majorly responsible for the tested pharmacological applications [8].

In common scenario, urinary tract infection (UTI) occurs when bacteria enter the urinary tract through the urethra (urethritis) and begin to multiply in the bladder (cystitis). About 80–90% of UTI are caused by *Escherichia coli* found in the digestive tract of human and located around the rectal area. About 10–20% of UTI is caused by *Staphylococcus saprophyticus*, which has a higher occurrence in summer. About 5% or less of UTI is caused by other bacteria like *Proteus sp.*, *Klebsiella sp.*, *Citrobactor sp.*, *Enterobacter sp.*, *Pseudomonas sp.* and *Enterococcus faecalis* [9]. Uncomplicated UTI can be treated with antibiotic or alternative therapy except in few instances where the infection reaches and infects the kidneys (pyelonephritis), where the patient requires emergency care and prolonged antibiotic therapy. Often these therapy leads to dysbiosis and post therapeutic antibiotic side effects. To address such problems, herbal therapy with minimum side effects is often sought. Although there are many prescribed plant based natural remedies for urological issues, reports on the curative roles of *Acacia catechu* or phytocompounds derived from its different parts against uropathogenic bacteria are limited. Hence the present research is aimed to investigate the antioxidant, antibacterial and antibiotic combinatorial effects of the stem and resin extract of *A. catechu* against clinically isolated and standard strains of uropathogens.

There were more than 10 research articles published in the last five years (2018–2023) on the various pharmacological activities of *A. catechu*. *A. catechu* ethanolic extract improved anxiety symptoms at dose-dependent levels in mice and at 400 mg/kg showed comparable antianxiety activity to that of standard drug diazepam (2.5 mg/kg) because of its antioxidant properties [10]. The *A. catechu* ethanolic seed extract induced cytotoxicity and oxidative stress by increasing apoptotic marker gene expressions such as Bax, Cyt-c, caspase-9 and 3, and decreasing anti-apoptotic marker Bcl-2 in HepG2 cells, a human hepatocellular carcinoma cell line [11]. The butanol fraction of ethanol extract of *A. catechu* heartwood that was rich in catechin, had immunomodulatory effects on non-specific, humoral, and cell-mediated immune functions. At a dose of 400 mg/kg b. w., it enhanced the number of antibody producing cells in the spleen and at 100 mg/mL, it increased phagocytic response in peritoneal macrophages. It inhibited the production of NO and the release of TNF-a and increased interleukin-10 production [12]. *A. catechu* and *Scutellaria baicalensis* played a potent anti-inflammatory role in LPS-induced

acute lung injury in rats and significantly decreased lung wet-to-dry weight ratio and reduced the release of inflammatory mediators such as TNF-α and IL-1β in bronchoalveolar lavage fluid (BALF) and blocked NF-κB activation [13]. *A. catechu* is reported to have significant cytotoxicity that was accompanied by increased apoptotic cells and ROS generation, caspase-9 and 3 activities in human colorectal adenocarcinoma HT-29 cells without affecting the healthy rat ileum and colon [14]. Heartwood extract of *A. catechu* was investigated for its neuroprotection activity against neurodegenerative diseases in both human neuroblastoma SH-SY5Y cells and rat brain slices treated with hydrogen peroxide. *A. catechu* increased cell viability, sub-diploid, DAPI positive cells, reduced ROS formation, and recovered the mitochondrial potential and caspase-3 activation [15]. *A. catechu* is reported to have potentiality against COVID-19 as evident from the virtual docking results that baicalein, (+)-catechin and fisetin (1-) exhibited high affinity to SARS-CoV-2 3CL pro that was validated by the FRET-based enzymatic inhibitory assays with the IC_{50} of 11.3, 23.8, and 44.1 μM, respectively. And also, a concentration-dependent inhibition of baicalein, quercetin and (+)-catechin against SARS-CoV-2 ACE2 was observed with the IC_{50} of 138.2, 141.3, and 348.4 μM, respectively [16]. A randomized, triple-blind, placebo-controlled, parallel study was conducted to verify the role of an *A. catechu* and *S. baicalensis* formulation, UP446, in supporting immune function in response to influenza vaccination. In the post-vaccination period, total IgA and IgG levels increased in participants supplemented with UP446 vs. those on Placebo ($p \leq 0.026$), besides, influenza B-specific IgG increased 19.4% from Day 28 to 56 and 11.6% from baseline at Day 56 ($p \leq 0.0075$) [17]. The role of (-) epicatechin derived from *A. catechu* in diabetic wound healing was best observed in mice treated with a combination of both topical (10% gel) and oral (extract at 200 mg/kg) followed by only topically and orally treated groups after 14 days of treatment [18]. As per the available literature, the role of *A. catechu* in different urological conditions or its effective antiurobacterial or antibiotic combinatorial activities are not yet explored, which has been investigated and presented in this paper.

Materials and methods

Plant material collection, storage and crude extract preparation

The dried stem of *Acacia catechu* and the dried, processed *A. catechu* resin was collected from the local market of Bhapur bazar, Berhampur, Odisha and since this plant samples are publicly available, it did not need any permission before using. However, the samples were authenticated by Prof. M. K. Misra, Taxonomist, Berhampur University and relevant guidelines for study on plant materials were followed. A voucher specimen was submitted to the departmental herbarium with a voucher number BOTBU231. The dried stems were powdered and subjected to exhaustive solvent extraction in methanol (300mL) for 72 h at 60–70˚C via Soxhlet apparatus. The filtered extract was concentrated in a rotary evaporator and kept in incubator at 37˚C for complete solvent evaporation. The crude extract was kept in sealed petri plate and stored at 4˚C for future use [19]. For different assays, the working concentration was prepared by dissolving 0.2 g of the crude extract in 1mL of lukewarm water and stored in labeled Eppendorf tubes for further use. The dried and processed *Acacia catechu* resin were powdered and directly used for phytochemical test after dissolving it in the solvent for phytochemical and antibacterial assays.

Phytochemical screening and qualitative test

The methanolic extract of *A. catechu* stem (ACs) and processed *A. catechu* resin (ACr) was tested for the presence or absence of different phytocompounds using standard lab procedures to ascertain the availability of the primary as well as secondary metabolites like alkaloids (Mayer's test, Wagner's test), anthocyanin, anthraquinones, carbohydrates (Benedict's test, Fehling's Test, Molisch Test), coumarin, emodin, flavonoids, glycosides (Liebermann's test, Acetic acid Test), leucoanthocyanin, phenol compounds ($FeCl_3$ test, Lead acetate test, Potassium dichromate test), protein (Biuret test, Conc. HNO_3 test, Ninhydrin solution test), Saponin (Foam test with Water and $NaHCO_3$), steroid, tannin, terpenoid [20–22].

Quantitative test

Standard procedures were followed to plot calibration curves for total phenolic content (TPC) and total flavonoid content (TFC). The Folin-Ciocalteu method [23] was used to determine the TPC, which was then quantified as mg/g Gallic Acid equivalent. For the analysis of TFC, aluminium chloride assay [24] was used, which was then quantified as mg/g Rutin equivalent.

Antioxidant tests

1, 1 diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity

The free radical scavenging capacity of methanolic extracts of ACs and ACr was assessed using the DPPH assay [25]. Variable concentrations of plant extracts (20, 40, 60, 80, 100, 200, 500 μl) were taken in a series of test tubes and the volume was made up to 3 mL by addition of methanol, which were then combined with 1 mL of methanolic solution of 0.4 mM DPPH. After 30 min of vigorous shaking and standing, the mixture was tested for absorbance at 517 nm. Ascorbic acid was used as

standard. The following formula was used to determine the sample's percentage of DPPH decolourization:

$$\%\ \text{decolorization} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

Hydroxyl radical scavenging activity

The reaction mixture (3 mL) contained 1 mL $FeSO_4$ (1.5 mM), 0.7 mL hydrogen peroxide (6 mM), 0.3 mL sodium salicylate (20 mM), and extract concentrations ranging from 10 to 500 µg/mL. The absorbance of the hydroxylated salicylated complex was measured at 562 nm after 1 h of incubation at 37 °C [26]. Ascorbic acid was employed as the standard. The percentage scavenging effect was calculated as

$$\%\ \text{scavenging activity} = 1 - \frac{\text{A1} - \text{A2}}{\text{A0}} \times 100$$

Where A0 represents the absorbance of the control (without extract), A1 represents the absorbance when sodium salicylate was present in the extract, and A2 represents the absorbance when sodium salicylate was absent.

Antibacterial activities tests

The antibacterial effect of ACs and ACr were investigated against *E. faecalis*, *E. coli*, *P. aeruginosa*, *P. vulgaris* and *S. aureus* through different methods i.e. disc diffusion, agar well diffusion (pour plate method), modified agar well diffusion and modified antibiotics susceptibility test (AST) in which the conventional antibiotics were supplemented with a low dose of ACs or ACr to check its combinatorial effect.

Minimum inhibitory concentration (MIC) determination

Before antibacterial testing, MIC for each test organism was determined using 96-well microtiter plate with some modifications [27, 28]. Stock extract solution was prepared by dissolving 250 mg/mL of ACs/ACr in Luria Bertani Broth. Around 5 mL of bacterial suspension of 10^6 cfu/mL in sterile broth was prepared. Ciprofloxacin was taken as the positive control. In the first column wells of a microtiter plate, media was taken, in second 200 µL bacterial culture, then positive control 0.5 mg of CIP and 5 mg of extract (ACs/ACr) in 200 µL of broth was taken in the first row in duplicates. These are subjected to serial half fold dilution and then 100 µL of bacterial suspension was added to each well. ACs/ACr without bacteria served as blank. Each plate was wrapped loosely with para-film to prevent dehydration, and incubated at 37^0C for 20–24 h. Following the incubation, 40 µL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide) was added at a concentration of 0.2 mg/mL to each of the well and incubated at room temperature for 30 min and the optical densities of microplates were taken in microplate reader at 595 nm. Bacterial growth was observed as purple coloration of the wells. The well of lowest coloration was observed and the corresponding concentration was referred as the MIC value for that bacteria.

Disc diffusion method

Discs of size 5 mm diameter were taken on petri plate and different doses (0.5 mg/disc, 1 mg/disc, 1.5 mg/disc, 2 mg/disc) of ACs and ACr extract were added on it and left for some time in laminar hood for complete drying. Nutrient agar (NA) plates were prepared and swabbed with sterilized cotton bud containing bacterial culture. The drug treated discs were aseptically placed on them. The plates were incubated overnight at 37˚C. The clear zones formed around the discs were measured.

Agar well diffusion (pour plate method)

Activated culture of all the strains were added to different sterile petri plates, then lukewarm nutrient agar media was added and left for solidification. After that three wells were dug and different concentrations (2 mg/well, 4 mg/well, 6 mg/well) of ACs/ACr were added and left for 1 h at room temperature for extract diffusion and then incubated overnight at 37˚C. The clear zones formed around the wells were measured.

Modified agar well diffusion method

To find sensitivity of different bacteria (*E. coli*, *P. aeruginosa*, *E. faecalis*, *S. aureus*, *P. vulgaris*) on a single plate, this method was used. Nutrient agar plates were prepared for both ACs and ACr. After solidification, a well was dug in the center. Bacterial strains were streaked on the plate from periphery towards center in a zigzag manner. Then particular dose of drug was loaded into the well. Then the plates were left for 1 h at room temperature for extract diffusion and then incubated overnight at 37˚C. The bacterial growth inhibition zone was then measured.

Modified antibiotic sensitivity test for ACs and ACr

A sterile cotton swab was dipped into the dilute culture medium and swabbed on agar plate. Different antibiotic disc (AMC 30-Amoxycillin and clavulanic Acid 30, S 10-Streptomycin 10, NIT 300-Nitrofurantoin 300, CIP 5-Ciprofloxacin 5, CFM 5-Cefixime 5) were aseptically placed on bacteria swabbed agar plates leaving appreciable gap between two discs. Plates were incubated at 37˚C for 18 h. The clear zone found around the discs was a measure of the susceptibility of the organism to an antibiotic at a particular concentration. This antibiotic susceptibility test was considered as standard and used for comparison with other plates processed similarly with

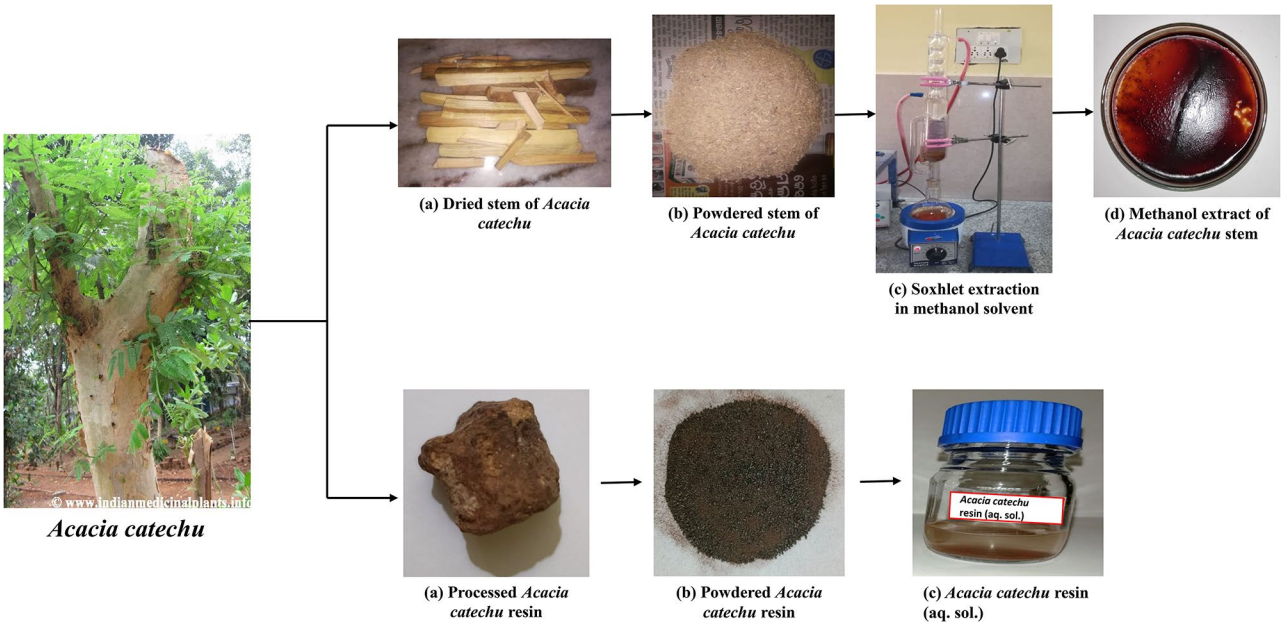

Fig. 1 The procedure of *Acacia catechu* stem and resin extract preparation

ACs/ACr (2 mg/disc) supplemented to conventional antibiotics. The difference in the ZOIs present the supplementary or complementary effects of ACs and ACr on inhibitory potential of the antibiotic discs.

Statistical analysis MS Excel 2019 and GraphPad Prism 8 software were used to conduct the statistical analysis, and findings were expressed as mean of three replicates ($n = 3$) ± standard error of mean (SEM). $p < 0.05$ was considered significant.

Results

Preparation of plant extracts

Dark reddish-brown colored methanolic crude extract of *A. catechu* stem (ACs) was obtained which was soluble in lukewarm water. In case of the *A. catechu* resin (ACr), the powdered resin was mixed with lukewarm water for phytochemical and antibacterial assays. The procedure of extract preparation is presented in Fig. 1. The percentage yield of the plant extract was calculated using the below mentioned formula. The average yield percentage of ACs extract was calculated to be 5.78%.

$$\% \text{ Yield} = \frac{W_1}{W_2} \times 100$$

Where,

W_1 = The weight of the methanolic extract in grams (7.75 g),

W_2 = The weight of the initial sample (134.01 g).

Table 1 Phytochemical screening of bioactive compounds in *Acacia catechu* stem and resin

Phytochemical Constituents	Tests	Acacia catechu stem	Acacia catechu resin
Alkaloid	Mayer's test	+	+
Terpenoid	Liebermann-Burchard test	+	
Phenol and Tannin	Ferric chloride test	+	+
Reducing Sugar	Fehling's test		+
Saponin	Foam test		+
Protein	Xanthoproteic test	+	+
Steroid	Salkowski test	+	+
Anthocyanin	Pigment-dependent test		
Coumarin	NaOH test		
Leucoanthocyanin	Bate-Smith test		
Flavonoid	Shinoda's test	+	+
Phoblatannins	Hydrochloric acid test		
Glycosides	Salkowski's test	+	+

Note- '+' indicates the presence and '-' indicates the absence of phytochemicals

Qualitative phytochemical analysis

The presence of different phytochemicals like alkaloids, flavonoids, phenols, tannins, proteins, steroids, and glycosides were observed in both the stem and resin extracts, whereas terpenoids were detected in ACs and saponins and reducing sugars were found in ACr. The results are presented in Table 1.

Quantitative phytochemical analysis of *A. catechu* stem and resin

Total phenolic content (TPC) determination

The major antioxidants and antimicrobials belong to this group. Using Gallic acid standard, total amount of phenolic compounds of ACs and ACr were calculated from the calibration curve ($R^2 = 0.9691$) were 37.74 ± 0.023 and 51.98 ± 0.011 mg/g of Gallic acid equivalent, respectively.

Total flavonoid content (TFC) determination

Using Rutin standard, total amount of flavonoids of ACs and ACr were calculated from the calibration curve ($R^2 = 0.9847$) were 71.33 ± 0.004 and 119.6 ± 0.010 mg/g of Rutin equivalent, respectively.

Antioxidant activities

DPPH scavenging assay

Using Ascorbic acid, which is a potent antioxidant agent as standard, antioxidant potential of both ACs and ACr was evaluated. The results revealed that ACs had IC_{50} value of 93.68 ± 0.71 µg/mL and ACr had 79.21 ± 0.54 µg/mL in comparison to an IC_{50} value of 72.33 ± 1.20 µg/mL for standard Ascorbic acid in the DPPH scavenging assay. The methanolic extract of ACr exhibited better scavenging activity than the ACs extract as evident from the results that are presented in Table 2; Fig. 2.

Hydroxyl radical scavenging assay

The IC_{50} value was determined to be 90.92 ± 0.54 µg/mL for ACs and 85.74 ± 0.61 µg/mL for ACr in comparison to an IC_{50} value of 66.96 ± 0.61 µg/mL for Ascorbic acid standard in hydroxyl radical scavenging. From the figure, we conclude that the scavenging effect of the extracts increase as the concentration increases. The methanolic extract of ACr showed better scavenging activity than the ACs, which is presented in Table 2; Fig. 3.

Antibacterial tests

Minimum inhibitory concentration

A minimum inhibitory concentration (MIC) of 2.5 mg/mL of ACs was noted each against *E. col* and, *E. faecalis* and 1.25 mg/mL against *P. aeruginosa*, *P. vulgaris* and *S. aureus*, respectively. MIC of 1.25 mg/mL of ACr was noted each against *E. coli* and *E. faecalis* and 0.62 mg/mL against *P. aeruginosa*, *P. vulgaris* and *S. aureus*, respectively At the same time the MIC of the positive control CIP was recorded as 0.03 mg/mL against *E. faecalis*, *E. coli* and 0.015 mg/mL against *P. aeruginosa*, *P. vulgaris* and *S. aureus*.

Growth Inhibition of uropathogens caused by A. catechu stem and resin by using disc diffusion method

The zone of inhibition (in mm) was measured for different concentration of ACs and ACr extracts. ACs and ACr were added at conc.of 0.5, 1, 1.5, 2 mg per disc. ACs was found to be more effective against *P. aeruginosa* with Zone of Inhibition (ZOI) of 15.45 ± 1.73 to 20.45 ± 1.00 mm followed by *P. vulgaris* with 10.56 ± 1.73 mm to 20.12 ± 1.00 mm, *E. coli* from 9.25 ± 1.73 mm to 15.07 ± 1.00 mm and then *S. aureus* from 6.26 ± 1.00 mm to 16.14 ± 1.73 mm and at last *E. faecalis* from 12.00 ± 1.73 mm to 12.65 ± 1.70 mm (Table 3; Fig. 4). ACr was found to be more effective against *P. vulgaris* at higher dose (2 mg/disc) with ZOI of 18.25 ± 1.73 mm then followed by other strains (Table 4; Fig. 5). ACr extract exhibited better inhibitory effects than ACs against all the tested urobacteria and it was also

Table 2 Determination of 50% inhibitory concentration (IC_{50}) value of both *Acacia catechu* stem and resin

Extract/ positive control	50% Inhibitory concentration (IC_{50}) in µg/mL	
	DPPH radical scavenging activity	Hydroxyl radical scavenging activity
Ascorbic acid	72.33 ± 1.20	66.96 ± 0.61
A. catechu stem	93.68 ± 0.71	90.92 ± 0.54
A. catechu resin	79.21 ± 0.54	85.74 ± 0.61

Values represent the mean ± standard error of mean (SEM) of triplicate sets of experiments

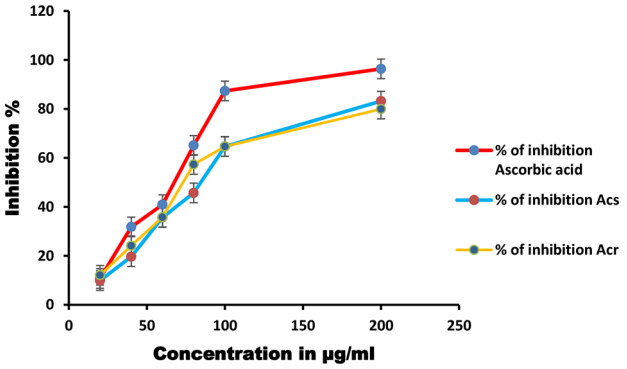

Fig. 2 Evaluation of antioxidant activities of *Acacia catechu* stem and resin by DPPH scavenging assay

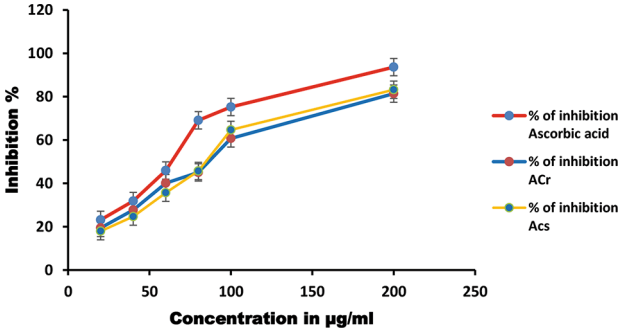

Fig. 3 Evaluation of antioxidant activities of *Acacia catechu* stem and resin by hydroxyl free radical scavenging assay

Table 3 Measurement of ZOI in disc diffusion method for *A. catechu* stem

Zone of inhibition (in mm)/conc of extract in mg				
Bacterial strains	**0.5 mg**	**1 mg**	**1.5 mg**	**2 mg**
E.coli	0.0 ± 0.0	9.25 ± 1.73	10.02 ± 1.73	15.07 ± 1.00
E.faecalis	0.0 ± 0.0	0.0 ± 0.0	12.00 ± 1.73	12.65 ± 1.70
P.aeruginosa	0.0 ± 0.0	15.45 ± 1.73	18.25 ± 1.00	20.45 ± 1.00
P.vulgaris	0.0 ± 0.0	0.0 ± 0.0	10.56 ± 1.73	20.12 ± 1.00
S.aureus	0.0 ± 0.0	0.0 ± 0.0	6.26 ± 1.00	16.14 ± 1.73

Values represent the mean ± standard error of mean (SEM) of triplicate sets of experiments

dose dependent, as evident from the results of disc diffusion assay.

Agar well diffusion (pour-plate method)

From the results, it was evident that ACs was more effective against *E. coli* giving ZOI from 10.17 ± 2.64 to 14.23 ± 1.73 mm followed by other bacterial strains. ACr was more effective for *P. aeruginosa* as ZOI is 15.01 ± 1.73 to 33.05 ± 1.73 mm followed by *P. vulgaris*, *E. coli*, *S. aureus* and least effective in case of *E. faecalis* having ZOI from 15.65 ± 2.64 to 21.06 ± 1.00 mm. The resin extract showed significant bactericidal activity against all the tested organisms in comparison to the ACs extract. The results of agar well diffusion by pour plate method were presented in Tables 5 and 6 and graphically depicted in Figs. 6 and 7.

Modified agar well diffusion

The efficiency of ACs and ACr against all five bacterial strains were studied by modified agar well diffusion method. ACs was more effective against *E. faecalis* with a ZOI up to 16.00 ± 1.73 followed by *P. aeruginosa* (13.33 ± 5.29) and *E. coli* (13.00 ± 1.73) followed by *S. aureus* (11.33 ± 2.64 mm) and *P. vulgaris* giving ZOI of

Table 4 Measurement of ZOI in disc diffusion method for *A. Catechu* resin

Zone of inhibition (in mm)/conc of extract in mg				
Bacterial strains	**0.5 mg**	**1 mg**	**1.5 mg**	**2 mg**
E. coli	0.0 ± 0.0	12.45 ± 1.73	15.25.±2.64	16.50 ± 1.00
E. faecalis	0.0 ± 0.0	10.05 ± 1.73	13.42 ± 2.64	17.50 ± 1.00
P. aeruginosa	0.0 ± 0.0	13.27 ± 3.60	14.59 ± 2.00	15.02 ± 1.73
P. vulgaris	0.0 ± 0.0	12.42 ± 1.73	14.05 ± 1.73	18.25 ± 1.73
S. aureus	0.0 ± 0.0	11.56 ± 2.64	14.98 ± 4.35	17.11 ± 1.73

Values represent the mean ± SEM of triplicate sets of experiments

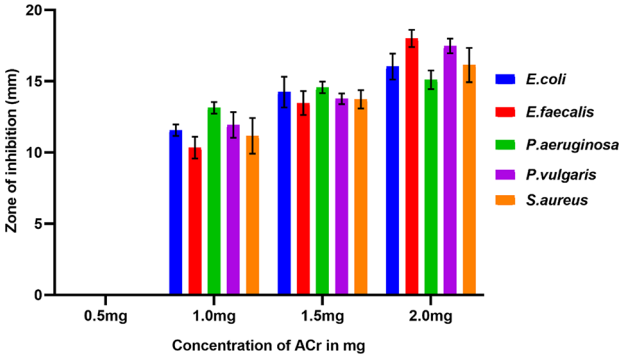

Fig. 5 Inhibition of bacterial growth by *A. catechu* resin extract by disc diffusion method

Table 5 Growth inhibitory effects of *Acacia catechu* stem in Agar well diffusion method

Zone of inhibition(in mm)/ conc of extract per well in pour plate method			
Bactrial strains	**2 mg**	**4 mg**	**6 mg**
E. coli	10.17 ± 2.64	12.5 ± 1.73	14.23 ± 1.73
E. faecalis	10.42 ± 1.73	11.25 ± 1.73	13.09 ± 1.00
P. aeruginosa	10.01 ± 2.64	11.42 ± 2.64	12.96 ± 1.73
P. vulgaris	9.45 ± 1.73	10.87 ± 1.73	13.25 ± 1.73
S. aureus	9.75 ± 1.73	12.75 ± 1.73	13.12 ± 1.73

Values represent the mean ± SEM of triplicate sets of experiments

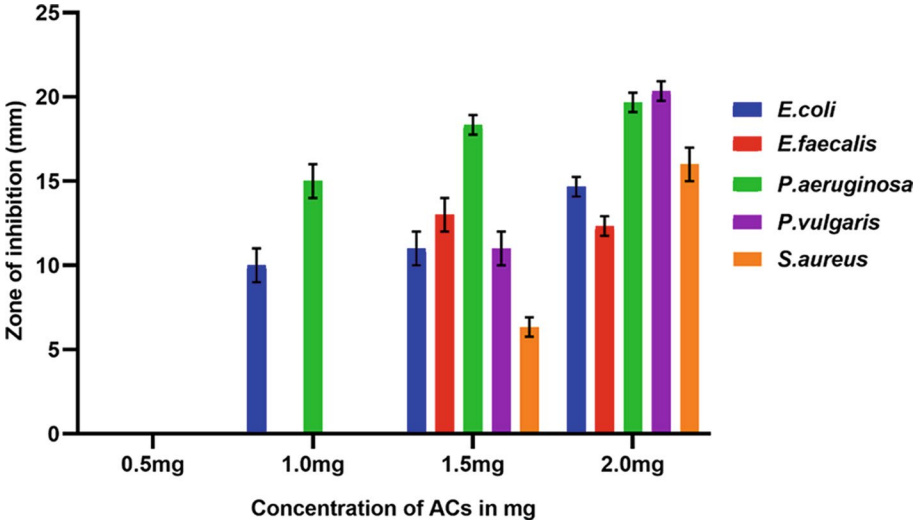

Fig. 4 Inhibition of bacterial growth by *A. catechu* stem extract by disc diffusion method

Table 6 Growth inhibitory effects of *Acacia catechu* resin in Agar well diffusion method

Zone of inhibition(in mm)/ conc of extract per well in pour plate method			
Bactrial strains	**2 mg**	**4 mg**	**6 mg**
E. coli	20.15 ± 1.73	25.22 ± 3.60	27.64 ± 2.64
E. faecalis	15.65 ± 2.64	18.87 ± 2.64	21.06 ± 1.00
P. aeruginosa	15.01 ± 1.73	25.14 ± 1.73	33.05 ± 1.73
P. vulgaris	20.87 ± 1.73	25.56 ± 2.64	28.45 ± 1.73
S. aureus	21.24 ± 2.64	22.54 ± 3.60	25.51 ± 1.73

Values represent the mean ± SEM of triplicate sets of experiments

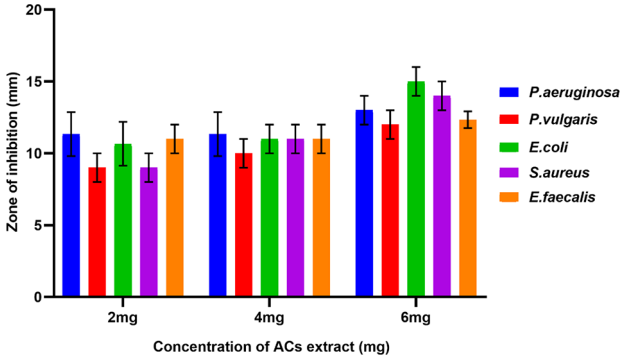

Fig. 6 Urobactericidal activity of *A. catechu* stem extract in agar-well diffusion method

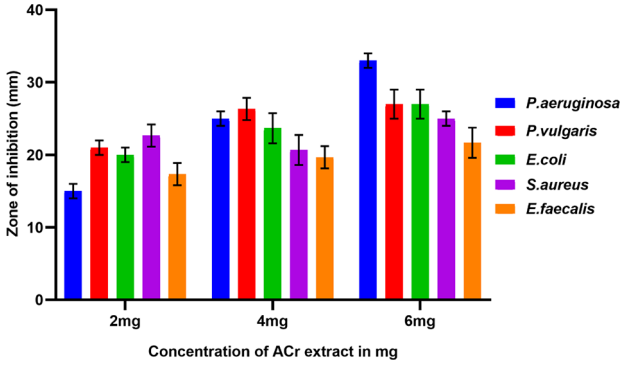

Fig. 7 Urobactericidal activity of *A. catechu* resin extract in agar-well diffusion method

Table 7 Growth inhibitory effects of *Acacia catechu* stem and resin extract in modified Agar well diffusion method

Bacterial strains	A. catechu stem (6 mg/well) ZOI in mm	A. catechu resin (6 mg/well) ZOI in mm
E. coli	13.00 ± 1.73	16.00 ± 0.173
E. faecalis	16.00 ± 1.73	13.66 ± 0.100
P. aeruginosa	13.33 ± 5.29	15.66 ± 0.100
P. vulgaris	9.66 ± 2.64	15.00 ± 0.173
S. aureus	11.33 ± 2.64	14.66 ± 0.100

Values represent the mean ± SEM of triplicate sets of experiments

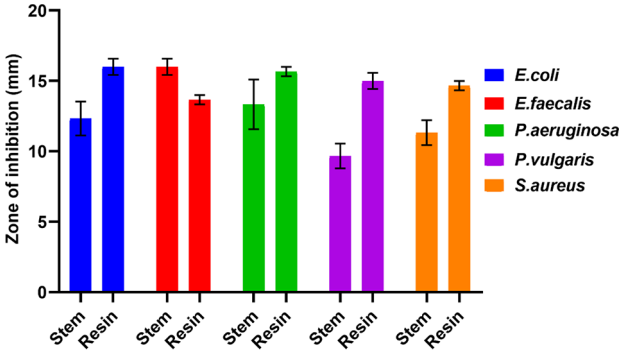

Fig. 8 Antibacterial activity of *A. catechu* stem and resin extract in modified agar well diffusion method

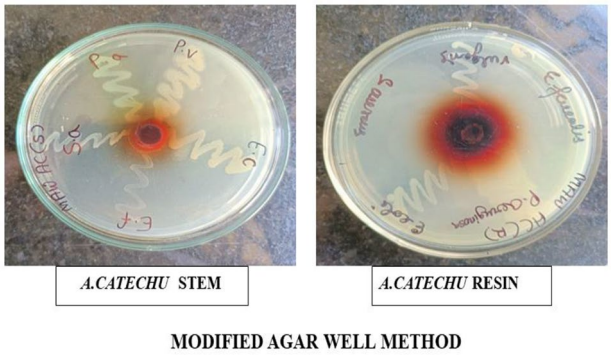

Fig. 9 Urobactericidal activity of *A. catechu* stem and resin extract in modified agar well diffusion method

9.87 ± 0.264 mm. ACr was more effective against *E. coli* giving ZOI of 16.00 ± 0.173 mm followed by *P. aerugenosa* (15.66 ± 0.10 mm) and *P. vulgaris* (15.00 ± 0.173 mm). The results were represented in Table 7; Figs. 8 and 9.

Modified antibiotic sensitivity test of *A. catechu* stem and resin extract

Antibiotics susceptibility test was conducted for the bacterial strains in vitro against antibiotics S10, NIT300, AMC11, CIP5, CFM5. The ZOI was measured in millimetre and taken as standard. These results were compared with the ZOIs for antibiotic discs supplemented with ACs and ACr (2 mg/disc). Bacterial species differ in their susceptibility to different conventional antibiotics. As Streptomycin and Nitrofurantoin were found to be more effective against the tested bacterial strains in comparison to Amoxycilin, Ciprofloxacin, Cefixime. The effect of AST and modified AST with ACs and ACr was studied for five bacterial strains. The increase in ZOI was measured. ACs and ACr increased the inhibitory effect of antibiotic discs specifically AMC30, CIP5 and CFM5 for the tested bacterial strains especially against *E. coli* and *E. faecalis*, the major uropathogens. S10 was also supplemented in presence of both ACs and ACr against *E. faecalis.* The results of the combinatorial effects of ACs

were presented in Table 8; Fig. 10 and for ACr were represented in Table 9; Fig. 11.

Both the stem and resin exhibited excellent antiurobacterial activity against all the five test bacterial strains, which was evident from the various test results conducted using different antibacterial assays and in each assay, resin extract was found to be more effective than the stem extract as it had better antioxidant potentiality and probable presence of higher amount of antimicrobial compounds. Therefore, the results were compiled and presented as a summary sheet. The results of ACs were presented in Fig. 12 and the results for ACr were depicted in Fig. 13.

Discussion

Acacia catechu plant branches are being used as chewing sticks in various parts of the world, due to its antimicrobial effect, and hence it is considered as a valuable ingredient for dental care preparations. It is useful in dental, oral and throat infections, and as an astringent for reducing oozing from chronic ulcers and wounds. *A. catechu* is valuable for its influential astringent and antioxidant activities. Catechins have significant antioxidant and antimicrobial effects [3]. The *A. catechu* plant has been used traditionally to treat asthma, bronchitis, cancer, chest pain, diarrhea, mouth sores, sore throats, ulceration, vitiligo, and aids wound healing. It also possesses antifungal, antiviral, spasmolytic, and hypoglycemic properties [29]. Katha (resin) is used as the best substitute for gum arabic, and is also used as oral contraceptive, chemopreventive. It has algicidal, digestive, appetiser, aphrodisiac, vulnerary, anthelmintic, depurative and antibacterial properties. It is acrid, bitter, thermogenic, tonic and used in laryngopathy, flatulence, ulcers, wounds, leprosy, skin diseases, urine incontinence, colporrhagia, toothache, loss of voice. It is also used in cases of mercurial salivation, hoarseness, relaxed sore throat, bleeding, ulcerations and sponginess of gums, bed sores, gonorrhoea, otitis, otorrhoea [30, 31].

Ethanolic and aqueous extract of *A. catechu* were found to be effective against *E. coli*, *S. aureus*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Shigella sonnei* [32]. Aqueous extracts of *A. catechu* exhibited an antimicrobial effect against *S. aureus*, *P. aeruginosa*, *Proteus mirabilis*, *E. coli*, and *K. pneumonia* with a diameter of zone of inhibition (ZOI) of 17.66 ± 1.52, 16.66 ± 1.15, 14.0 ± 2.0, 8.33 ± 0.57, and 8.0 ± 0.0 mm, respectively [33]. Aqueous extract of

Table 8 Increase in antibiotic sensitivity of uropathogens by adding *Areca catechu* stem extract

Difference in Zone of inhibition(in mm) by addition of ACs extract (2 mg/disc)					
Antibiotics	***E. coli***	***E. faecalis***	***P. aeruginosa***	***P. vulgaris***	***S. aureus***
S10	2 ± 1.732	16 ± 2.642	0.0 ± 0.0	2.12 ± 0.132	2.25 ± 1.73
NIT300	2 ± 1.732	3 ± 2.645	0.0 ± 0.0	2.45 ± 1.104	2.08 ± 2.64
AMC30	14 ± 2.64	18 ± 1.732	9 ± 1.732	2.02 ± 1.732	2.30 ± 0
CIP5	10 ± 0.79	22 ± 1.000	0.0 ± 0.0	3.70 ± 1.126	1.17 ± 0.64
CFM5	12 ± 1.231	13 ± 1.732	0.0 ± 0.0	2.14 ± 1.732	2 ± 1.732

Values represent the mean ± SEM of triplicate sets of experiments

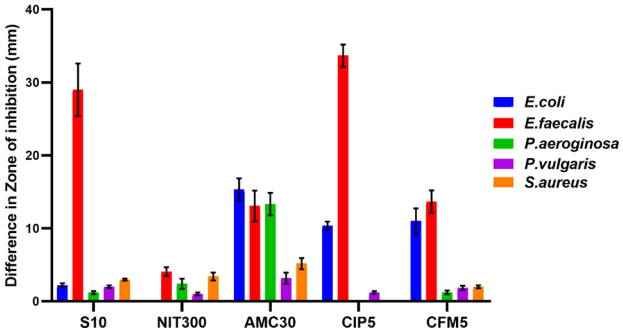

Fig. 10 Antibiotic complementary/supplementary effect of *A. catechu* stem extract effect against uropathogenic bacteria

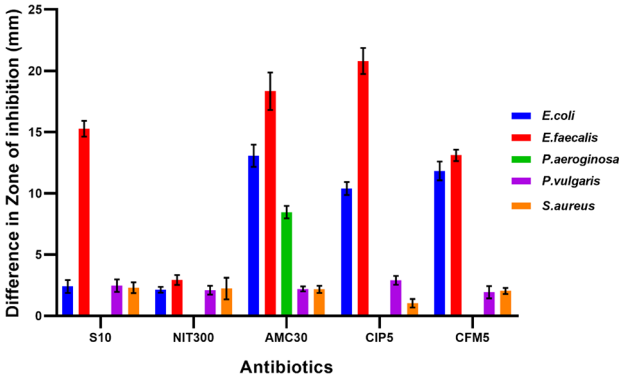

Fig. 11 Antibiotic complementary/supplementary effect of *A. catechu* resin extract against uropathogenic bacteria

Table 9 Increase in antibiotic sensitivity of uropathogens by adding *Areca catechu* resin extract

Difference in Zone of inhibition(in mm) by addition of ACr extract (2 mg/disc)					
Antibiotics	***E. coli***	***E. faecalis***	***P. aeruginosa***	***P. vulgaris***	***S. aureus***
S10	2 0.1 ± 1.732	30.55 ± 2.64	1.45 ± 0.58	2.25 ± 1.23	3 0.02 ± 0.173
NIT300	0.0 ± 0.0	4 0.67 ± 2.64	2.67 ± 2.64	1 0.26 ± 0.27	2.35 ± 0.264
AMC30	14.9 ± 2.64	13.4 ± 3.46	13.45 ± 2.64	4.14 ± 1.73	5.75 ± 1.4
CIP5	10 ± 2.64	34.78 ± 2.9	0.0 ± 0.0	1.0 5 ± 0.78	0.0 ± 0.0
CFM5	12 ± 1.732	15.56 ± 1.1	1.45 ± 0.73	2.00 ± 0.25	2 ± 0.173

Values represent the mean ± SEM of triplicate sets of experiments

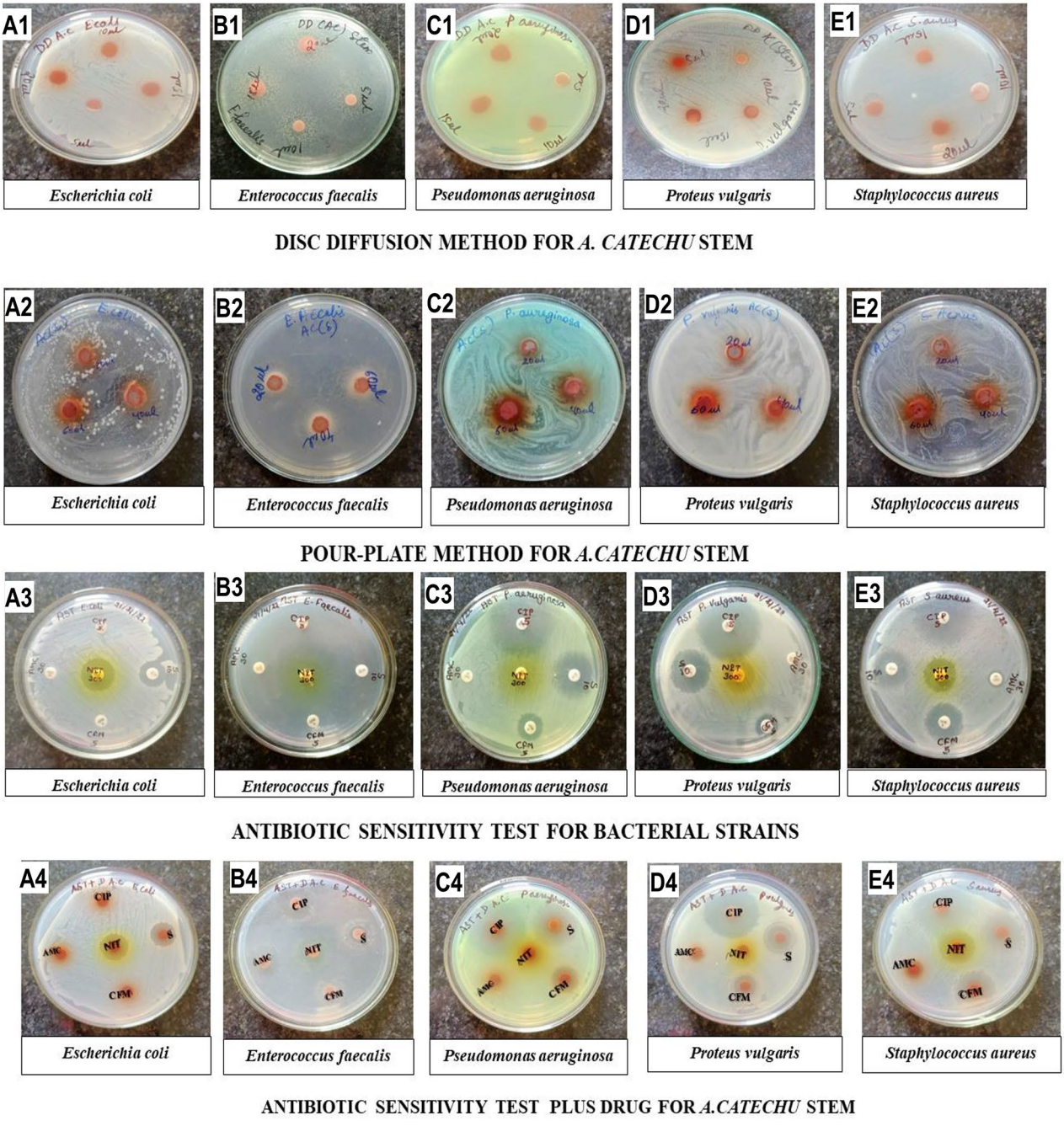

Fig. 12 Compiled summary of urobactericidal effects of *Acacia catechu* stem (ACs) extract in disc diffusion, agar well, modified agar well diffusion, modified antibiotic sensitivity assay

A. catechu resin exhibited an inhibitory effect against *Bacillus subtilis* (MIC: 20 µg/mL), *S. aureus* (MIC: 40 µg/mL), *P. aeruginosa* (MIC: 220 µg/mL), and *E. coli* (MIC: 330 µg/mL) possibly due to the presence of high contents of catechin and epicatechin [34]. Antimicrobial activities of methanol extract of *A. catechu* leaves was reported with an MIC of 1,000 µg/mL against Gram-positive bacteria *S. aureus* and *B. subtilis*, while that for Gram-negative *Salmonella typhimurium*, *E. coli*, and *P. aeruginosa* were 700, 1,500, and ≤2,000 µg/mL, respectively [35]. A study on the heartwood of plants from Nepal showed significant antibacterial activity of its ethyl acetate extract with an MBC of 50 mg/mL against *B. subtilis* and *Shigella sp.* and that was 100 mg/mL against *K. pneumonia* and *S. aureus* [36]. Methanol extract of the plant showed antibacterial activity with a diameter of ZOI of 18, 15, 14, and 12 mm against *E. coli*, *S. aureus*, methicillin-resistant *S. aureus*, and *Acinetobacter baumannii*, respectively [37].

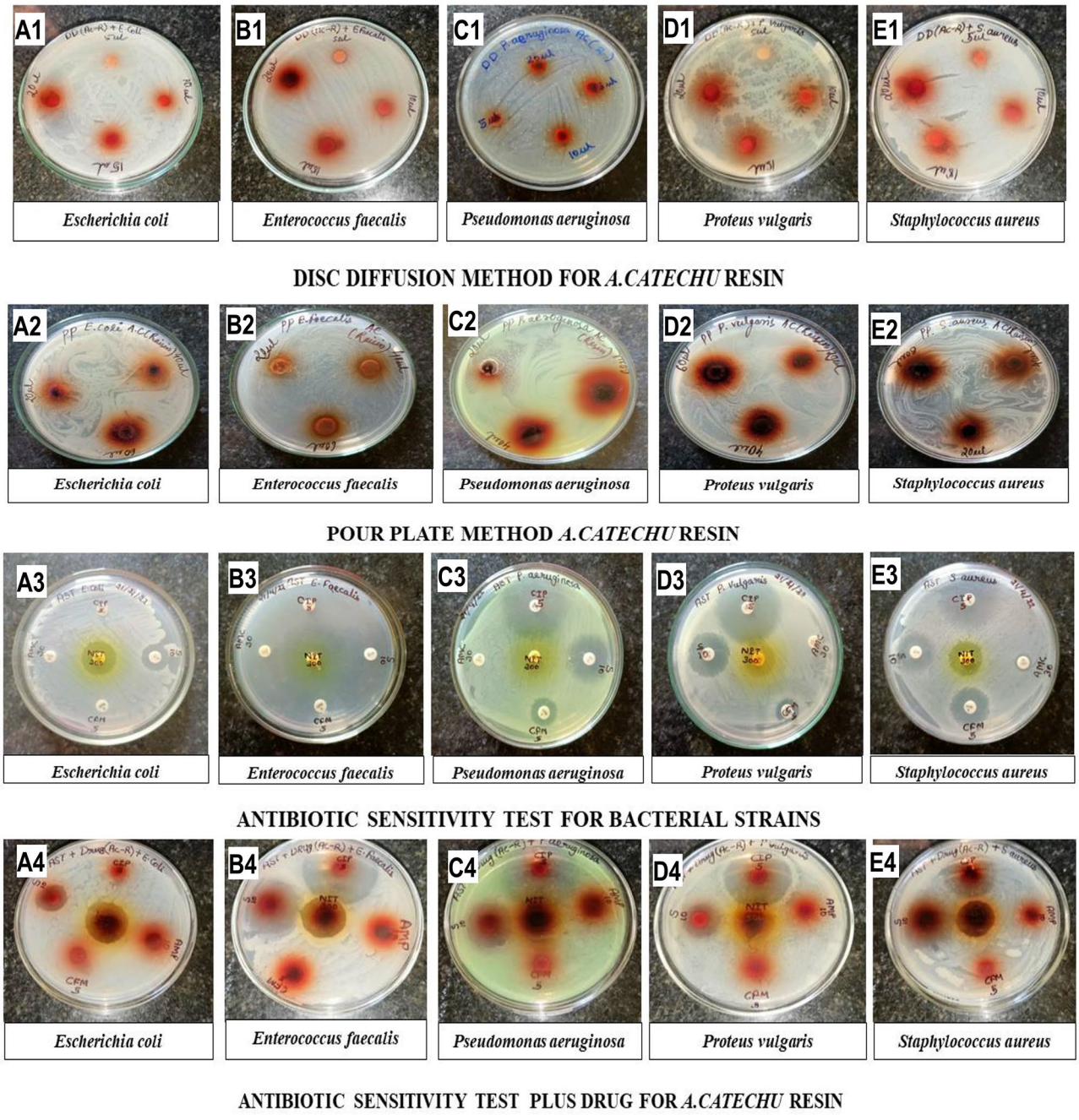

Fig. 13 Compiled summary of urobactericidal effects of *Acacia catechu* resin (ACr) extract in disc diffusion, agar well, modified agar well diffusion, modified antibiotic sensitivity assay

Aqueous fraction of bark showed antibacterial activity against *S. aureus* with an MIC and MBC of 6.25 and 12.5 mg/mL [32].

In this study, the antiuropathogenic effect of ACs and ACr was explored against UTI causing bacteria *E. coli*, *E. faecalis*, *P. aeruginosa*, *P. vulgaris* and *S. aureus* using different methods i.e. disc diffusion method, pour plate method, modified agar well method, modified antibiotic susceptibility test (combinatorial effect of the extracts of *A. catechu* stem and resin with the conventional antibiotics).

Earlier reports showed that the chief phytoconstituents are catechutannic acid, acacatechin, catechin, epicatechin, phlobatannins, tannic acid in ACr [34]. In this present study, the presence of alkaloid, flavonoid, reducing sugar, saponin, phenol and tannin, protien, steroid, and glycoside in the ACs and ACr extract was confirmed. The polyphenolic compounds Epicatechin and catechin

in ACs showed high antioxidant activities [38]. *A. catechu* showed the antioxidant potential through oxygen radical absorbance capacity (41589 ± 151.30 µMTE/g), DPPH scavenging assay (IC_{50} = 7.40 ± 1.16 µg/mL), ABTS radical scavenging assay (IC_{50} = 2.28 ± 0.14 µg/mL), and cellular antioxidant activity (EC_{50} = 230.50 ± 6.40 µg/mL) [39]. Methanol extract of *A. catechu* showed antioxidant activity with an IC_{50} value of 1.3 µg/mL [37].

ACs is reported to have TPC value of 175.48 ± 4.67 mg/g gallic acid equivalents and TFC of 7.66 ± 1.0 mg/g quercetin equivalent [32]. The TPC of the methanolic extract of ACs and ACr, calculated from the calibration curve (R^2 = 0.9691), were 37.74 ± 0.023 and 51.98 ± 0.011 mg/g Gallic Acid equivalents, respectively. TFC of methanolic extract of *A. catechu* stem and resin, calculated from the calibration curve (R^2 = 0.9847), were 71.33 ± 0.004 and 119.6 ± 0.010 mg/g Rutin equivalent/g, respectively. Both TPC and TFC were found to be significantly higher in the methanolic extract of resin in comparison to *A. catechu* stem, which might be responsible for the better antiuropathogenic activity of resin in comparison to stem extract. However, both the extracts had significant bioactivities as evident from the various results of different antibacterial studies.

In disc diffusion method, ACs was more effective against *P. aeruginosa* with a ZOI of 15 ± 1.73 mm to 20 ± 1.00 mm and ACr was more effective against *P. vulgaris* with a ZOI from 12 ± 1.73 mm to 18 ± 1.73 mm followed by other strains. ACr extract was found to be more effective against all bacterial strains as compared to ACs. In agar well diffusion method (Pour plate method), ACs was more effective against *E. coli* with ZOI of 11 ± 2.64 mm to 14 ± 1.73 mm followed by other bacterial strains. ACr was more effective against *P. aeruginosa* showing ZOI of 15 ± 1.73 mm to 33 ± 1.00 mm followed by *P. vulgaris*, *E. coli*, *S. aureus* and least effective in case of *E. faecalis* having ZOI from 16 ± 2.64 mm to 21 ± 3.64 mm. The efficiency of ACs and ACr were studied against all five bacterial strains by Modified Agar well diffusion method on a single plate. ACs was more effective against *E. faecalis*, *E. coli* and *P. aeruginosa* followed by *S. aureus* and *P. vulgaris*. ACr was most effective against *E. coli* followed by *P.aerugenosa*, *P. vulgaris*, *S. aureus* and *E. faecalis*. Moreover, ACr was found to be very effective against all five test bacteria in comparison to ACs at a dose of 6 mg/well concentration.

Though there are multiple reports on the antibacterial activity of *Acacia* plant, we found only one report on the antibacterial efficacy of the *A. catechu* seed extract against selected urinary tract pathogens i.e. *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae* [40]. However, antimicrobial property of *A. catechu* bark extract against periodontal pathogens like *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in subgingival plaque samples of generalized periodontitis patients was reported earlier [41] and potent in vitro antibacterial activity of ethanolic bark extract of *A. catechu* was also reported against selected oral microbes like *Streptococcus mitis*, *S. sanguis* [42].

Emergence of MDR strains is the major threat and challenge for the biomedical fraternity who are always in need of exploring novel alternative and combinatorial therapy as antibiotic use is often combined with serious side effects and herbal remedies are often slow in action. And to resolve this, combination of both antibiotic and phytoextracts are suggested for better efficacy and minimum side effects. In this present study, the combination of ACs or ACr had significantly increased the capacity of all the tested antibiotics against all the urobacterial strains specifically against *E coli* and *E. faecalis*, the major causative agents of urinary tract infections. In all the antibacterial assays, ACr was found to have better efficacy in comparison to the stem extract, which might be due to the presence of better bactericidal phytocomponents in the resin of *A. catechu*. The most habituated adaptability of these uropathogens is the antibiotic resistance which is manifested mainly due to their high mutation rate and horizontal gene transfer ability. The combinatorial effect of the desired antibiotics and biocompounds of *A. catechu* plant parts may increase the efficiency of antibiotics to treat and eradicate the UTIs.

Conclusion

From the results of the above study, it can be concluded that the extracts of *Acacia catechu* stem and resin can be used for treatment of various urinary infections. Since *A. catechu* stem and resin exhibited excellent antibacterial activity due to the presence of their bactericidal phytochemicals. Further study may be conducted to decipher the molecular mechanism lying behind these activities. Additionally, the mechanism behind the antibiotic synergistic effects of resin and stem components can be identified, which will be beneficial for the society and mankind as UTI is considered to be the most frequently occurring and most discomforting ailment, which can be treated or prevented by using such natural therapies.

Abbreviations

AC	Acacia catechu
ACs	Acacia catechu stem
ACr	Acacia catechu resin
AK	Amicncin
AMC10	Ampicillin
ºC	Degree Celsius
AZM	Azithromycinn
NIT300	Nitrofurantoin
CFM5	Cefixime
CIP5	Ciprofloxacine
S10	Streptomycin
UTI	Urinary tract infection UPEC-Uropathogenic Escherichia coli
MIC	Minimum inhibitory concentration FC-Folin-Ciocalteu
$FeCl_3$	Ferric chloride

g	gram
L	litre
TPC	Total phenolic content
TFC	Total flavonoid content
MDR	Multi Drug Resistant
mg	milligram
Min	Minute
mL	Millilitre
µl	Microlitre
MS excel	Microsoft excel
gdw	gram dry weight
LB	Luria-bertani broth
OD	Optical density
Sp.	species
Temp.	Temperature
ZOI	Zone of inhibition
cfu	Colony forming unit
SEM	Standard Error of Mean

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Author contributions
LP and SRP conducted research work, drafted manuscript; SA and NSP helped in artwork; SD designed content, discussion and edited the MS. All authors read and approved the final manuscript.

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Data availability
All data generated or analysed during this study are included in this published article and they will be available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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