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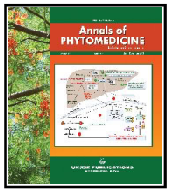

Original Article : Open Access

Antioxidant and antimicrobial potential of *Areca catechu* L. (Arecaceae) inflorescence extracts

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Abstract

Areca catechu L., a medium-sized palm tree comprising over 200 genera and approximately 2600 species, is commonly referred to as the areca nut palm and is extensively utilized in traditional Indian and Chinese medicines. This study primarily focused on the antioxidant and antibacterial activities of petroleum ether (ACPE), chloroform (ACCE), and methanol (ACME) extracts of *A. catechu* inflorescence. Quantitative assessment of the total phenolic, flavonoid, and alkaloid contents using the Folin-Ciocalteu reagent, aluminium chloride, and ammonium hydroxide methods revealed that both ACCE and ACME were rich in phenolics (27.97 and 21.525 µg GAE/g dry extract), flavonoids (12.823 and 11.939 µg QE/g dry extract), and alkaloids (36 and 32%). Both ACCE and ACME exhibited good antioxidant activity, as confirmed by different *in vitro* assays including total antioxidant capacity, DPPH radical scavenging, ABTS radical scavenging, hydrogen peroxide scavenging, and reducing power assays. Furthermore, all *A. catechu* inflorescence extracts (ACPE, ACCE, and ACME) showed marked antibacterial activity against both Gram-positive bacteria, including *Bacillus subtilis* (ACCE AI = 1.01), *Enterococcus faecalis* (ACPE and ACME AI = 0.74), and *Staphylococcus aureus* (ACPE AI = 0.79), and Gram-negative bacteria, including *Escherichia coli* (ACME AI = 0.93), *Pseudomonas aeruginosa* (ACME AI = 0.96), and *Salmonella typhi* (ACME AI = 1.23), and antifungal properties against both *Aspergillus niger* and *Penicillium chrysogenum* strains at the highest concentration tested (250 mg/ml). Finally, in this study, the defatted methanol extract of *A. catechu* inflorescence was tested for cytotoxicity using the MTT assay in L929 fibroblast cell lines, and the IC_{50} value was 267.40 µg/ml. These findings suggest that *A. catechu* inflorescences hold promise as a valuable natural source of compounds with potential therapeutic applications.

1. Introduction

Areca catechu L., also known as betel nut or areca nut palm, is a commercially significant crop that has traditionally been used for chewing and in various Ayurvedic and veterinary applications (Salehi *et al.,* 2020; Ijinu *et al.,* 2023). It is primarily grown in India, China, Myanmar, Indonesia, Thailand, and Bangladesh. The major states involved in cultivation in India include Kerala, Karnataka, Tamil Nadu, West Bengal, Assam, and Meghalaya (Mitra and Devi, 2018). Areca nuts also have cultural, social, and religious significance and are commonly used in Hindu religious rites, such as birth, marriage, and hospitality for guests (Strickland, 2002; Kumar *et al.,* 2021). The alkaloids found in areca nuts, which comprise around 0.2-1.7% of their composition, possess various medicinal properties, including astringent, anthelmintic, narcotic, and vermifuge qualities (Chavan and Singhal, 2012). Traditionally, areca nuts have been used to treat conditions such as leucoderma, cough, fit, anaemia, obesity, and specific skin ailments (Caius, 1934; Raghavan and Baruah, 1958; Amudhan *et al.,* 2012). Areca nuts exhibit various beneficial activities such as anthelminthic, antifungal, antibacterial, anti-inflammatory, antioxidant, insecticidal, and larvicidal effects. Its components, particularly areca tannin, have shown potential in regulating blood pressure by inhibiting responses to angiotensin I and II. Polyphenols found in areca nuts have anti-inflammatory, antiallergic, antiviral, and anticancer properties (Peng *et al.,* 2015; Osborne *et al.,* 2017; Salehi *et al.,* 2020). Ayurveda uses areca nuts to alleviate *pitta* and *kapha* doshas, treat mental confusion, swollen eyes, pus formation, and chronic urinary distress, and is used as a nervine tonic and aphrodisiac. Chewing areca nut strengthens the gums, checks perspiration, removes bad taste from the mouth, and sweetens the breath. It is also used as a carminative, diuretic, digestive, antiulcer, antidiarrheal, anthelmintic, anti-heartburn, and laxative agent (Ijinu *et al.,* 2023).

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Free radicals, which are molecules with unpaired electrons that are unstable and contribute to the development of various diseases when their production exceeds the body's antioxidant defences, can lead to oxidative stress and cellular damage (Pushpangadan *et al.,* 2015; Pizzino *et al.,* 2017). Diseases such as cancer, cardiovascular disease, neurological disorders such as Alzheimer's and Parkinson's disease, diabetes, and inflammatory conditions may result from this damage. A balanced diet rich in antioxidants and lifestyle choices that minimises exposure to harmful environmental factors can help manage free radicals (Poprac *et al.,* 2017). Phytochemicals, which are naturally occurring compounds found in plants, possess antioxidant properties that scavenge and neutralise free radicals. Compounds, such as flavonoids, carotenoids, and polyphenols, can reduce the harmful effects of free radicals by donating electrons to stabilise them (Zhang *et al.,* 2015). In addition to their antioxidant properties, phytochemicals have several therapeutic properties that are beneficial to human health and well-being (Patel *et al.,* 2019; Prakash *et al.,* 2020). Various studies have investigated the antimicrobial activities of phytochemicals (Wali *et al.,* 2019) and some have focused on the possible relationships between their chemical structures and activities (Xie *et al.,* 2014; Górniak *et al.,* 2019). According to a review by Yuan *et al.* (2021), plant flavonoids may target the cell membranes of Gram-positive bacteria, causing damage to phospholipid bilayers and inhibiting the respiratory chain, ATP synthesis, or other mechanisms. Therefore, a diet rich in plant-based foods containing phytochemicals can support antioxidant defense mechanisms and improve overall health (Pandey *et al.,* 2017). This study aimed to assess the levels of total phenolics, flavonoids, and alkaloids in the inflorescences of *A. catechu* and to evaluate their ability to neutralise free radicals and display antimicrobial and cytotoxic properties.

2. Materials and Methods

2.1 Drugs and chemicals

2,2-Diphenyl-1-picrylhydrazyl and 2,22 -azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) were purchased from Sigma-Aldrich (USA). Mueller Hinton broth, Mueller Hinton agar (MHA) for bacterial culturing, Sabouraud dextrose broth (SDB), and Sabouraud dextrose agar (SDA) for yeast culturing were obtained from HiMedia Laboratories, India. All other chemicals, reagents, and solvents were sourced from HiMedia and Nice Chemicals (India). Nunc (Thermo Fisher Scientific) supplied the tissue culture plates and flasks. Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM), foetal bovine serum (FBS), penicillin, and streptomycin were procured from Gibco (Thermo Fisher Scientific, USA).

2.2 Collection of plant material and preparation of extracts

Fresh inflorescences of *Areca catechu* L. were obtained from the Southern Western Ghats region of Thiruvananthapuram, India (latitude: 8.4321° N, longitude: 77.0503° E). The authenticity of the plant material was verified by Dr. P. Pushpangadan of the Amity Institute for Herbal and Biotech Products Development, Thiruvananthapuram. The collected plant material was shade-dried, powdered, and subsequently extracted based on polarity with petroleum ether (ACPE), chloroform (ACCE), and methanol (ACME) using a Soxhlet extractor for 24 h each, resulting in 0.8, 1.7, and 4.3% of the extract, respectively. The extracts were concentrated using a rotary evaporator and stored at 4°C until further use (Ijinu *et al.,* 2016).

2.3 Qualitative phytochemical analysis

Preliminary phytochemical analysis was performed on the prepared plant extracts (ACPE, ACCE, and ACME) following protocols established by Harborne (1998) and Trease and Evans (2002).

2.4 Quantitative phytochemical analysis

2.4.1 Estimation of total phenolic content

The total phenolic content (TPC) of the extracts (ACPE, ACCE, and ACME) was determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Each extract (0.5 ml of each extract was mixed with Folin-Ciocalteu reagent (2.5 ml of Folin-Ciocalteu reagent and thoroughly blended. Subsequently, 2 ml of 7.5% sodium carbonate solution was added after 5 min, and the mixture was incubated at 45°C for 15 min, during which time a blue colour developed and was measured at 765 nm. The total phenolic content of the extracts was calculated using the equation $T = C \times (V/M)$, where 'T' represents the TPC (mg/g) of the extracts, 'C' is the concentration of gallic acid (mg/ml) obtained from the calibration graph, 'V' is the volume of the extract (ml), and 'M' is the weight of the extract (g). Gallic acid served as the reference standard, and the TPC was estimated from the calibration graph of gallic acid. The results were expressed as milligrams of gallic acid equivalents (mg GAE)/gram of sample dry weight.

2.4.2 Estimation of total flavonoid content

The total flavonoid content (TFC) in the extracts (ACPE, ACCE, and ACME) was determined using the aluminum chloride colorimetric method (Woisky and Salatino, 1998). To each extract, 0.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water were added sequentially. The mixture was then incubated at room temperature for 30 min and the absorbance was measured at 415 nm. Quercetin was used as the standard for calibration, and the TFC was calculated from the calibration graph plotted for quercetin using the formula $T = C \times (V/M)$, where 'T' represents the TFC (mg/g) of the extracts, 'C' denotes the concentration of quercetin (mg/ml) obtained from the calibration graph, 'V' is the volume of the extract (ml), and 'M' is the weight of the extract (g). The results were expressed as mg quercetin equivalent (mg QE)/g sample dry weight.

2.4.3 Estimation of total alkaloid content

Each 5 g extract (ACPE, ACCE, and ACME) was precisely measured and placed in a 250 ml beaker, followed by the addition of 200 ml of 10% acetic acid in ethanol. The mixture was then covered and allowed to stand for 4 h. Subsequently, the mixture was filtered and the resulting extract was concentrated in a water bath until it reached one-quarter of its original volume. Ammonium hydroxide was added dropwise to the concentrated extract until complete precipitation was achieved. The mixture was allowed to settle and the precipitate was collected, washed with dilute ammonium hydroxide, and filtered. The obtained residue represented the alkaloid, which was subsequently dried and weighed (Harborne, 1998). The percentage of alkaloids present in the sample was calculated using the following formula:

Percentage of alkaloid = (weight of alkaloid/weight of extract) ×100

2.5 *In vitro* antioxidant assays

2.5.1 Total antioxidant capacity

The total antioxidant capacity (TAC) of various extracts (ACPE, ACCE, and ACME) was evaluated using the phosphomolybdic acid method (Prieto *et al.,* 1999). For each sample, 0.1 ml of the extract, prepared at a concentration of 2 mg/ml in dimethyl sulfoxide (DMSO), was combined with 1 ml of a reagent mixture comprised of 0.6 M sulphuric acid, 20 mM sodium phosphate, and 4 mM ammonium molybdate. The tubes were covered and incubated in a water bath at 95°C for 90 min. Subsequently, the tubes were allowed to cool to room temperature, and the absorbance was measured at 695 nm against a blank. Gallic acid was used as the standard, and the TAC was calculated using the equation T = C × (V/M) where 'T' represents the total antioxidant content in the extract (mg/ml), 'C' denotes the concentration of gallic acid (mg/ml) obtained from a calibration graph, 'V' signifies the volume of the extract (ml), and 'M' is the weight of the extract (g). The resulting TAC is expressed as mg/g of the extract.

2.5.2 DPPH radical scavenging assay

A methanolic solution of DPPH (1 ml, 0.1 mM) was added to 1 ml of different concentrations of each extract at different concentrations (ACPE, ACCE, and ACME) and allowed to react at room temperature for 30 min in the dark. The absorbance was measured at 517 nm (Mensor *et al.,* 2001). Methanol served as the blank, DPPH in methanol without extract served as the control, and ascorbic acid was used as the reference standard. The lower the absorbance of the reaction mixture, the higher the free radical scavenging activity. The percentage of DPPH radical scavenging was calculated as follows:(absorbance of control-absorbance of sample)/absorbance of control × 100.

2.5.3 ABTS radical scavenging assay

The antioxidant activities of the extracts (ACPE, ACCE, and ACME) were evaluated by the ABTS radical scavenging method (Re *et al.,* 1999). ABTS radicals were generated by mixing ABTS (7 mmol/l) with potassium persulphate (2.4 mM) in the dark for 12-16 h to produce a solution with an absorbance of 0.700 ± 0.02 at 734 nm. ABTS solution was diluted with ethanol (1:89, v/v). ABTS radical solution (1 ml) was added to 3 ml of the plant extract solution in alcohol at varying concentrations. After six minutes, the percentage inhibition was determined by measuring the absorbance at 734 nm relative to the blank absorbance. Ascorbic acid was used as the reference standard. The percentage of ABTS radical scavenging was calculated as follows: (absorbance of control - absorbance of sample)/ absorbance of control×100.

2.5.4 Hydrogen peroxide scavenging assay

The ability of the extracts (ACPE, ACCE, and ACME) to scavenge hydrogen peroxide was estimated (Ruch *et al.,* 1989), with minor modifications (Pavithra and Vadivukkarasi, 2014). A solution of hydrogen peroxide (50 mM) was prepared in a phosphate buffer (1 M, pH 7.4). Different concentration of extracts was added to a hydrogen peroxide solution (0.6 ml, 2 mM). The absorbance of hydrogen peroxide at 230 nm was measured after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as the standard. Percentage of hydrogen peroxide radical scavenging = (absorbance of control-absorbance of sample)/absorbance of control × 100.

2.5.5 Reducing power assay

The reducing power assay was performed according to the procedures described by Gow-Chin and Pin-Deer (1994) and Uddin *et al.* (2014). Various concentrations of extracts (ACPE, ACCE, and ACME; 500 µl) were mixed with 1.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, followed by the addition of 5 ml of 10% trichloroacetic acid which was then centrifuged at 6000 rpm for 5 min at 4°C. The upper layer of the solution (1.5 ml) was mixed with 1.5 ml of distilled water and 300 µl of ferric chloride (0.1%), and the absorbance was read at 700 nm. Ascorbic acid was used as the standard. The increased absorbance of the reaction mixture indicated an increase in the reducing power. Concentration at 0.5 absorbance was used as the reference to determine the scavenging potential of each sample.

2.6 Antimicrobial assays

Bacterial and fungal cultures were acquired from the Department of Biotechnology, University of Kerala (MTCC and ATCC, respectively). Six reference microbial strains, comprising of *Bacillus subtilis* (MCC 2511; Gram-positive), *Enterococcus faecalis* (ATCC 29213; Gram-positive), *Staphylococcus aureus* (ATCC 25923; Gram-positive), *Escherichia coli* (MTCC 443; Gram-negative), *Pseudomonas aeruginosa* (ATCC 27853; Gram-negative), *Salmonella typhi* (MTCC 733; Gram-negative), and two filamentous fungal strains, namely *Aspergillus niger* (ATCC 9043) and *Penicillium chrysogenum* (ATCC 10108) were utilized. Bacterial strains were cultured in Mueller-Hinton broth (MHB) and grown on Mueller-Hinton agar (MHA) at 37°C. The yeast strains were cultured in Sabouraud dextrose broth (SDB) and grown on Sabouraud dextrose agar (SDA) at 28°C. The medium was sterilised by autoclaving at 121°C for 20 min.

2.6.1 Antibacterial activity assay

The agar well diffusion method, as detailed in the European Pharmacopoeia, with minor modifications, was used for antibacterial testing (Thankamani *et al.,* 2011). Sterile MHA plates were used for the assessment. Wells, 8 mm in diameter, were meticulously cut into plates. Freshly sub-cultured bacterial strains were suspended in 1 ml of nutrient broth and incubated at 37°C for 2 h to obtain a log-phase culture. The opacity of the culture was verified using McFarland turbidity standards (0.5), which correspond to approximately $1\text{-}2\times10^8$ colony forming units per ml. Pure cultures of the test strains (100 µl) were uniformly applied to the surface of the MHA plate using a sterile swab. The plates were allowed to dry for a period of 5 min, after which 100 µl of different concentrations of the extracts (ACPE, ACCE, and ACME), DMSO (0.05%; negative control), and the standard drug (0.125 mg/ml streptomycin; positive control) were precisely dispensed into each well. The plates were incubated for 24 h at 37°C. The zone of inhibition surrounding each well was measured in millimetres, and the antibacterial activity was observed. The activity index (AI) was calculated as the ratio of the zone of inhibition of the extract (mm) to the zone of inhibition of the standard (mm).

2.6.2 Antifungal activity assay

The antifungal activities of the extracts (ACPE, ACCE, and ACME) were tested against fungi using the test-tube method (Thankamani *et al.,* 2011). The extracts were added directly to the molten medium (SDA) to a volume of 1 µl in each tube. The extracts were mixed well

with the media and kept in slanted positions for solidification. After checking the sterility of the tubes, test fungi were inoculated into the respective test tubes. Tubes inoculated with *Aspergillus niger* and *Penicillium chrysogenum* were incubated at room temperature, and the results were recorded after 14-21 days. Imidazole (100 µg/ml) and 0.05% DMSO were used as the positive and negative controls, respectively.

2.7 Cytotoxicity assay

2.7.1 Cell culture

The L929 fibroblast cell line was obtained from the National Centre for Cell Sciences, India, and cultured in DMEM supplemented with 10 % FBS, L-glutamine, sodium bicarbonate, and an antibiotic solution containing penicillin (100 U/ml), streptomycin (100 µg/ml), and amphoteracin B (2.5 µg/ml). Cells were maintained at 37°C and 5% CO_2. After 24 h, the growth medium was removed, freshly prepared defatted *A. catechu* inflorescence methanol extract in DMEM was serially diluted two-fold (100, 50, 25, 12.5, and 6.25 µg in 500 µl DMEM), and each concentration of 100 µl each concentration was added in triplicates to the wells and incubated at 37°C in a humidified 5% CO_2 incubator. The untreated control cells were maintained (Chithra *et al.,* 2020).

2.7.2 Cell viability assay by microscopy

After 24 h of treatment, the entire plate was observed under an inverted phase-contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD Camera, Japan), and microscopic observations were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolisation in the cytoplasm, were considered indicators of cytotoxicity or apoptosis (Chithra *et al.,* 2020).

2.7.3 MTT assay

After a 24-hour incubation period, the contents of the wells were removed, and 30 µl of reconstituted MTT solution (0.5 mg/ml) was added to all test and control wells of each cell line. The plates were shaken gently and incubated at 37°C in a humidified incubator containing 5% CO_2 for 4 h. Following the incubation period, the supernatant was removed and 100 µl of MTT solubilisation solution (dimethyl sulfoxide) was added to the wells, which were then gently mixed by pipetting to solubilise the formazan crystals. The absorbance of the formazan solution was measured at 540 nm wavelength using an ELISA microplate reader (Erba LisaScan II, Germany). The optical density of the formazan produced in the untreated control cells was designated as 100% viability (Talarico *et al.,* 2004).

2.8 Statistical analysis

All data are presented as the mean ± SD. Linear regression analysis was performed to calculate the total phenolic, total flavonoid, and total antioxidant capacity, while the IC_{50} value was analysed using non-linear regression with GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Preliminary phytochemical analysis

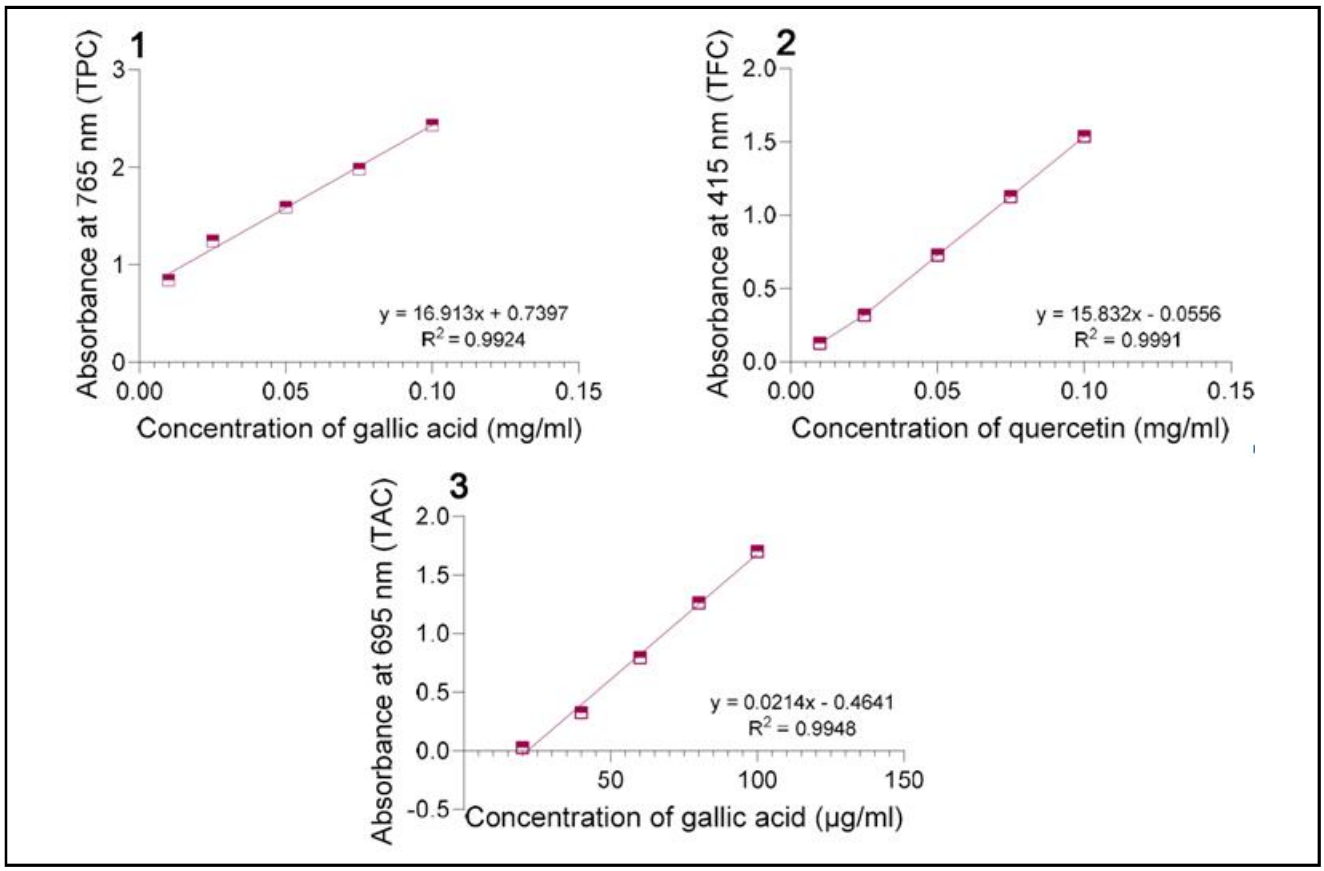
The preliminary phytochemical analysis of *A. catechu* inflorescence extracts indicated the presence of steroids, terpenoids, flavonoids, coumarins, alkaloids, and glycosides and the details are depicted in Table 1.

Table 1: The preliminary phytochemical analysis of *A. catechu* inflorescence extracts

Tests	ACPE	ACCE	ACME
Steroids and terpenoids			
Liebermann Burchard test	+++	++	+
Salkowski's test	++	+	+
Phenolics and tannins			
Ferric chloride test	++	+++	++
Gelatin test	-	+++	++
Shinoda test (for flavonoids)	+	++	+++
Lead acetate test (for flavonoids)	-	+	++
Alkaline reagent test (for flavonoids)	-	-	+
Coumarins	-	+	-
Alkaloids			
Mayer's test	-	++	++
Wagner's test	+	+++	++
Dragendorff's test	+	+++	++
Saponins			
Froth test	-	-	-
Carbohydrates			
Molisch's test	-	-	++
Benedict's test	-	-	+
Fehling's test	-	-	-
Proteins and amino acids			
Millon's test	-	-	+
Ninhydrin test	--	--	+

3.2 Quantitative phytochemical analysis

The TPC in ACPE, ACCE, and ACME was found to be 7.865, 27.97, and 21.525 µg GAE/g dry extract, respectively. The TFC in ACPE, ACCE, and ACME were 6.759, 12.823, and 11.939 µg QE/g dry extract, respectively. TPC and TFC were calculated from gallic acid (Figure 1) and quercetin (Figure 2) standard curves, and the highest TPC and TFC values were observed for the chloroform extract. The percentages of alkaloids in ACPE, ACCE, and ACME were 2.1, 36, and 32%, respectively. The chloroform extract contained the highest percentage of alkaloids.



Figures 1-3: Standard curves for 1. gallic acid for the total phenolic content assay, 2. quercetin for the total flavonoid content assay, and 3. gallic acid for the total antioxidant assay.

3.3 Antioxidant activity

3.3.1 Total antioxidant capacity

The total antioxidant capacity was expressed as gallic acid equivalent (GAE) per gram of extract and was calculated from the gallic acid standard curve (Figure 3). All extracts possessed a significant total antioxidant capacity. The TAC values for ACPE, ACCE, and ACME were 23.950, 31.340, and 27.050 mg GAE/g, respectively.

3.3.2 DPPH radical scavenging activity

Both extracts and ascorbic acid demonstrated an increase in DPPH radical scavenging activity in a dose-dependent manner (Figures 4a and b). At a concentration of 1000 µg/ml, ACCE exhibited radical scavenging activity of 96.12%. ACPE and ACME, at their highest tested concentrations, showed radical scavenging activities of 71.75% and 92.51%, respectively. Ascorbic acid exhibited a significant radical-scavenging activity (92.38%) at a concentration of 100 µg/ml.

3.3.3 ABTS radical scavenging activity

All extracts displayed concentration-dependent enhancement in their ability to scavenge ABTS radicals (Figures 5a and b). At a concentration of 1000 µg/ml, ACCE exhibited radical scavenging activity of 90.77%. The same concentrations of ACPE and ACME displayed radical scavenging activities of 71.08% and 86.84%, respectively. Furthermore, ascorbic acid exhibited radical scavenging activity of 69.12% at a concentration of 100 µg/ml.

3.3.4 Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of ACCE at 1000 µg/ml was 89.12%, which was the highest among all the tested samples. ACME showed a hydrogen peroxide-scavenging activity of 88.70%. Compared to the other samples, ACPE exhibited the lowest hydrogen peroxide scavenging activity of 65.03% (Figures 6a and b).

3.3.5 Reducing power activity

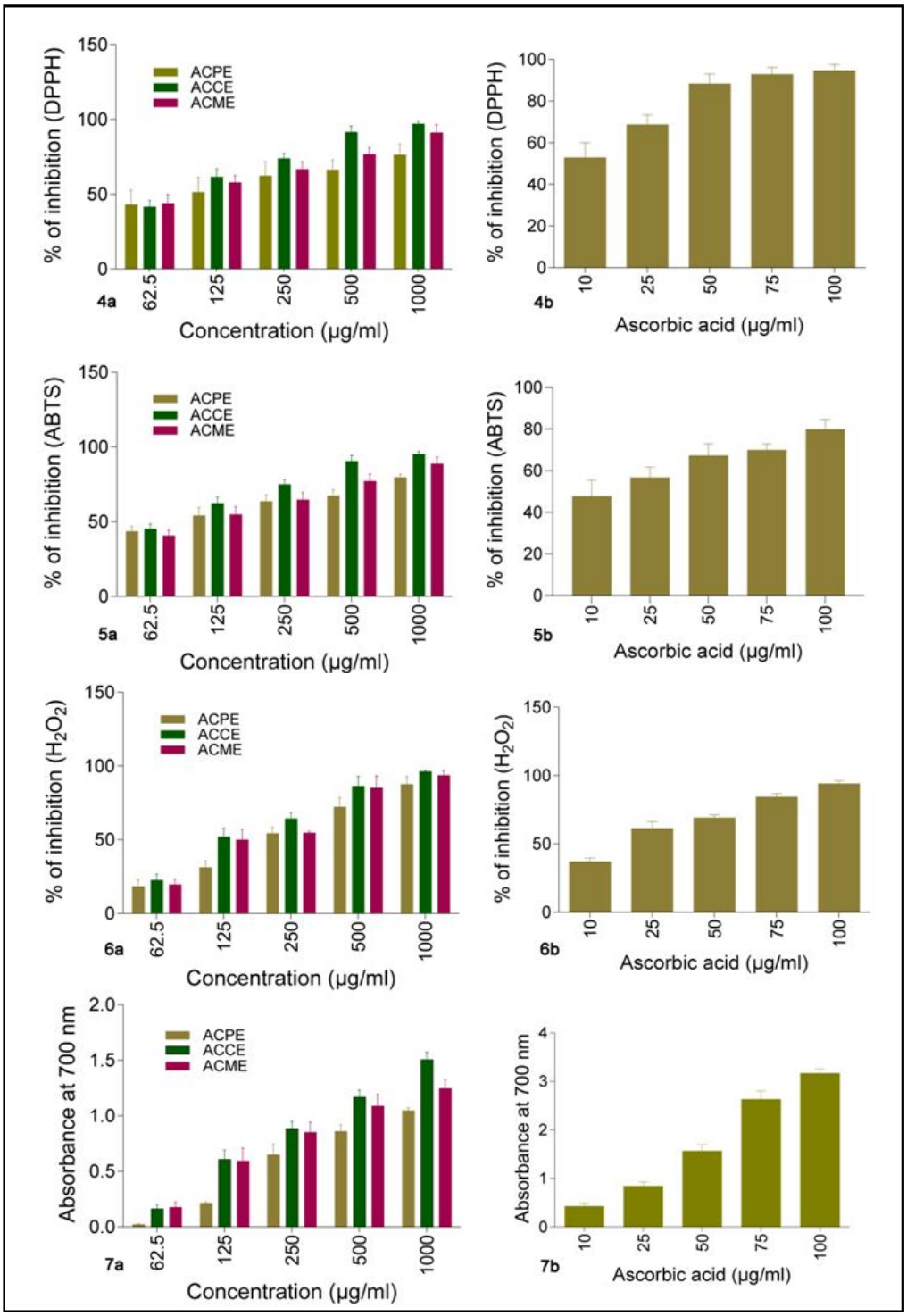
The ability of various extracts to decrease ferric ions increased as the concentration of the extract increased. ACPE displayed a lower potential for reducing ferric ions than ACCE and ACME. The sequence of reducing ability of the extracts, from highest to lowest, was ACCE > ACME > ACPE (Figures 7a and b). The reducing power of ascorbic acid was superior to that of all the examined extracts.

3.4 Antimicrobial assays

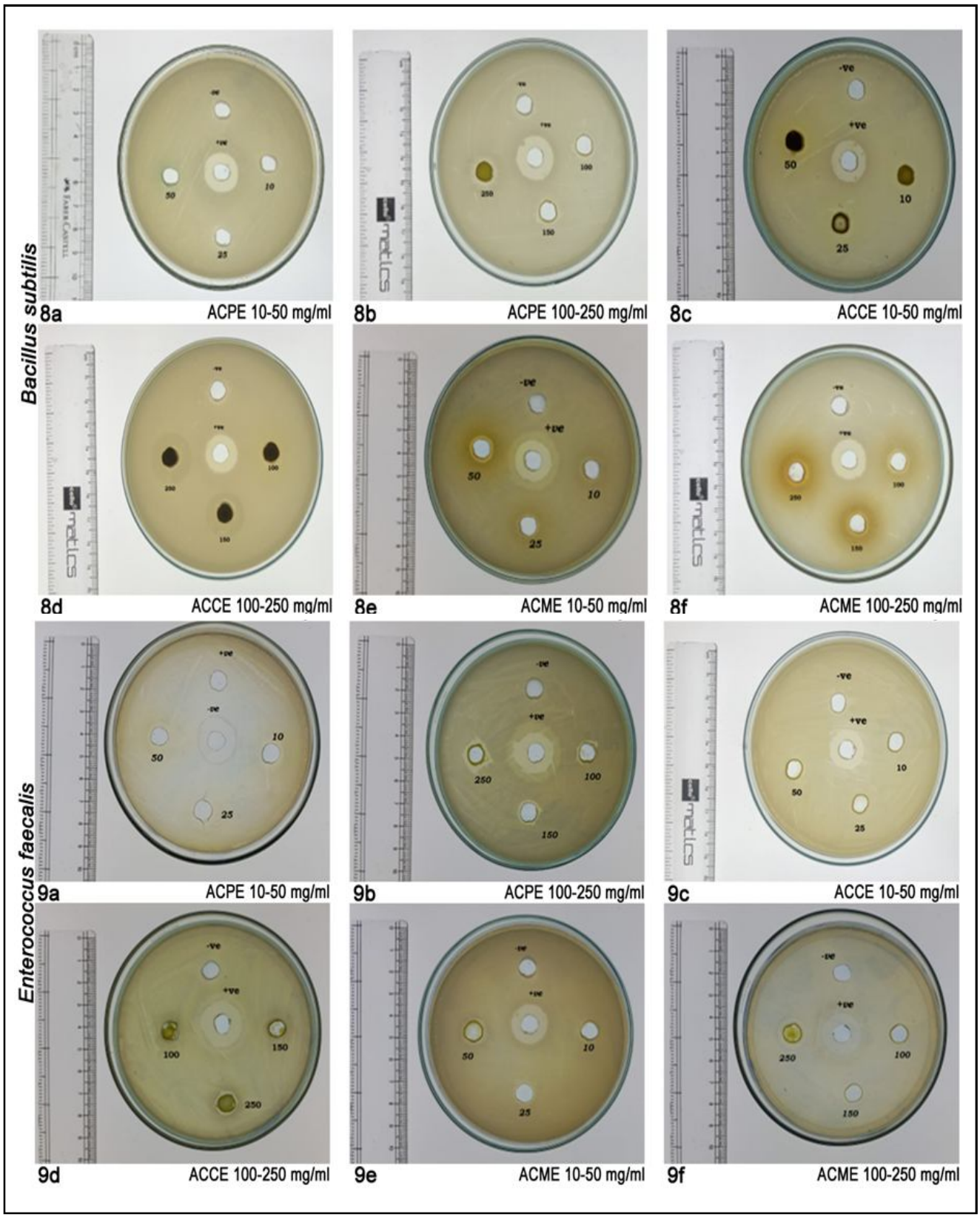
3.4.1 Antibacterial activity

The inhibitory effects of ACPE, ACCE, and ACME were evaluated against six bacterial strains at different concentrations (10, 25, 50, 100, 150, and 250 mg/ml) using the well diffusion method. The activity was quantitatively assessed based on the inhibition zone (Figures 8-13a-f), and the activity index was calculated using the corresponding inhibition zone value of the drug standard (Table 2). All the microorganisms tested were effectively inhibited by all the

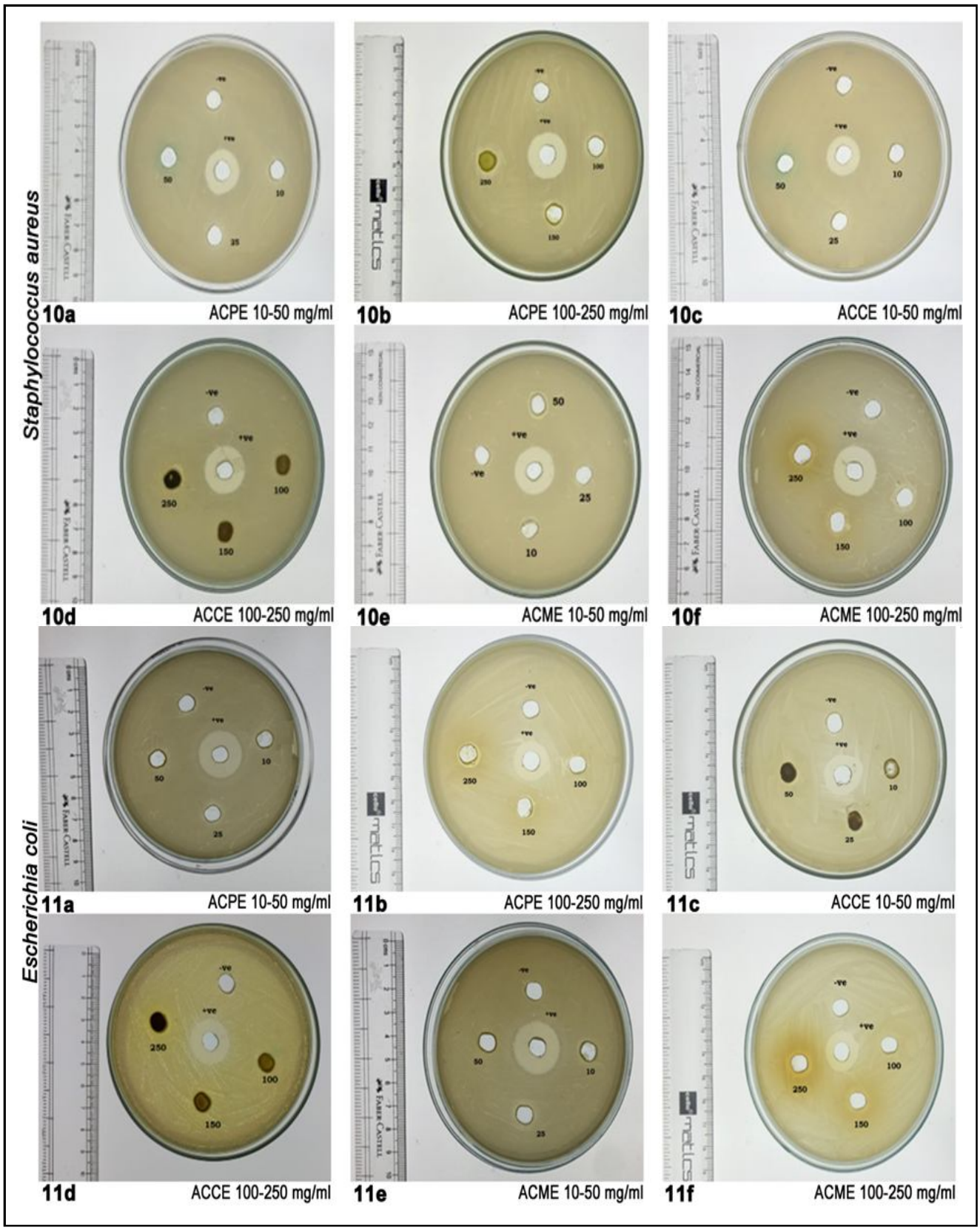
extracts at the highest concentration tested (250 mg/ml). All extracts demonstrated antibacterial properties against *Pseudomonas aeruginosa* at all concentrations tested (10-250 mg/ml), with the zone of inhibition ranging from to 9-18 mm. The maximum antibacterial activity was recorded for *Bacillus subtilis* (ACCE AI = 1.01), *Enterococcus faecalis* (ACPE and ACME AI = 0.74), and *Staphylococcus aureus* (ACPE AI = 0.79) and for the gram-negative bacteria *Escherichia coli* (ACME AI = 0.93), *Pseudomonas aeruginosa* (ACME AI = 0.96), and *Salmonella typhi* (ACME AI = 1.23) at the highest concentrations tested.



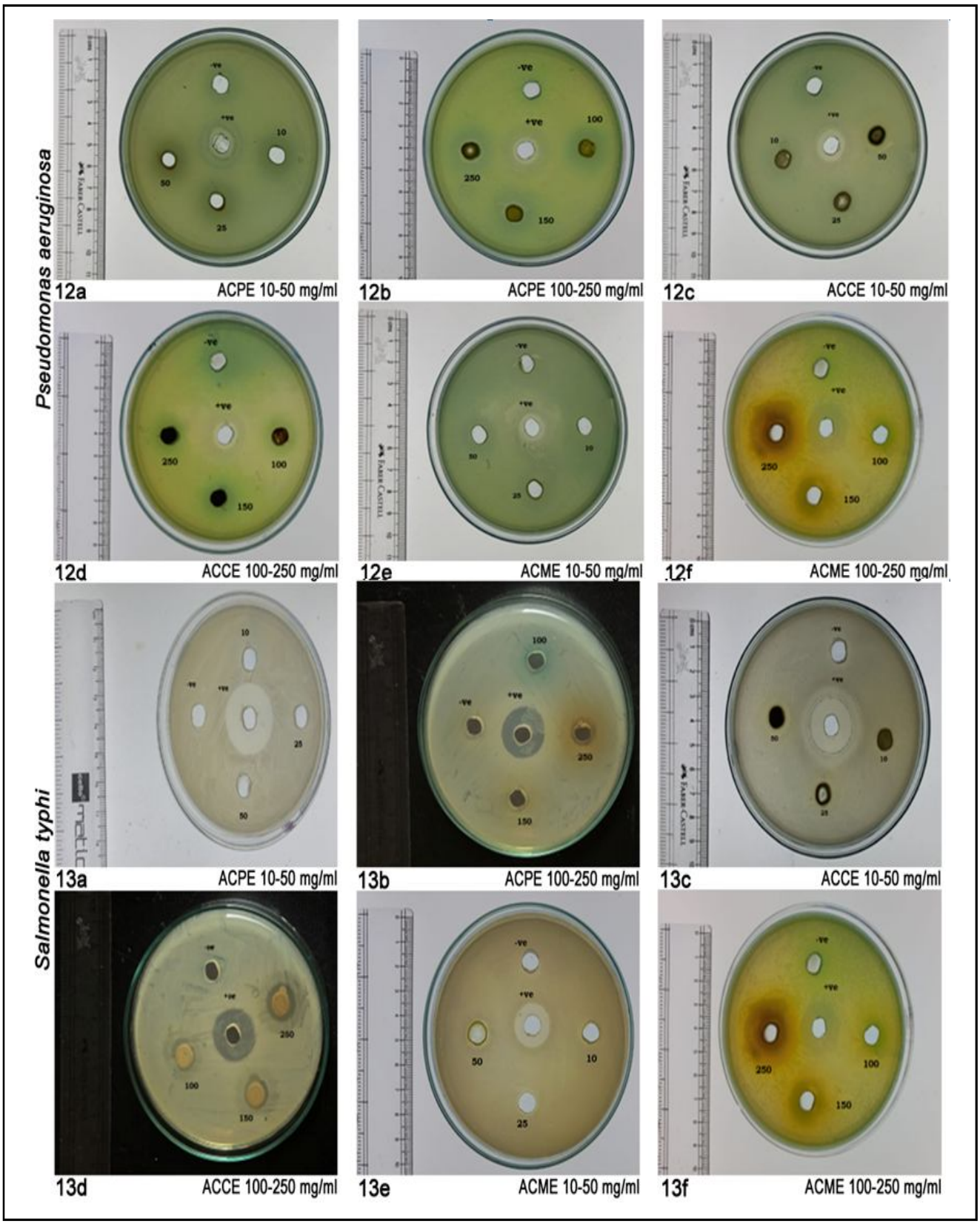
Figures 4-7: Antioxidant assays of different extracts of *A. catechu* and comparative standard ascorbic acid graphs 4. DPPH assay, 5. ABTS assay, 6. hydrogen peroxide assay, and 7. reducing power assay.



Figures 8-9a-f: Antibacterial assays of different extracts of *A. catechu* using the agar well diffusion method, 8a-f *Bacillus subtilis*, and 9a-f *Enterococcus faecalis*.



Figures 10-11a-f: Antibacterial assays of different extracts of *A. catechu* using the agar well diffusion method, 10a-f *Staphylococcus aureus*, and 11a-f *Escherichia coli*.



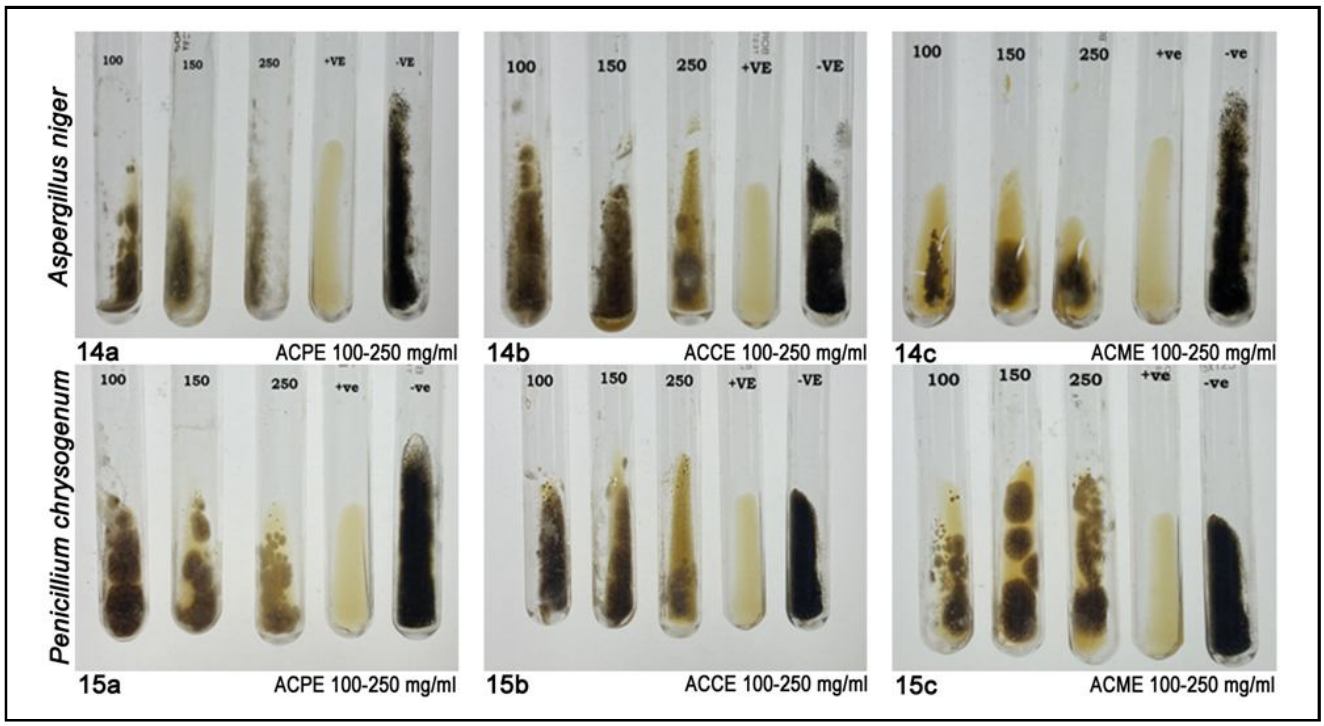
Figures 12-13a-f: Antibacterial assays of different extracts of *A. catechu* using the agar well diffusion method, 12a-f *Pseudomonas aeruginosa*, and 13a-f *Salmonella typhi*.

Table 2: Activity index (AI) of extracts of *A. catechu* inflorescence against various bacterial strains

S.No.	Bacterial strains	Concentrations (mg/ml)	AI		
			ACPE	ACCE	ACME
1.	*Bacillus subtilis*	10	-	0.56	0.68
		25	-	0.62	0.68
		50	-	0.69	0.81
		100	0.54	0.75	0.75
		150	0.66	0.96	0.87
		250	0.74	1.01	0.99
2.	*Enterococcus faecalis*	10	-	-	-
		25	-	-	-
		50	0.55	-	0.64
		100	0.56	-	0.61
		150	0.61	0.52	0.65
		250	0.74	0.71	0.74
3.	*Staphylococcus aureus*	10	-	-	-
		25	-	-	-
		50	-	-	0.60
		100	0.50	0.48	0.59
		150	0.68	0.60	0.64
		250	0.79	0.63	0.75
4.	*Escherichia coli*	10	-	-	-
		25	-	-	-
		50	-	0.53	-
		100	-	0.52	0.59
		150	0.55	0.73	0.79
		250	0.63	0.83	0.93
5.	*Pseudomonas aeruginosa*	10	0.69	0.55	0.75
		25	0.64	0.68	0.81
		50	0.76	0.76	0.94
		100	0.70	0.79	0.80
		150	0.75	0.82	0.88
		250	0.89	0.95	0.96
6.	*Salmonella typhi*	10	-	0.46	-
		25	-	0.49	-
		50	-	0.60	0.65
		100	-	0.76	1.00
		150	-	0.81	1.15
		250	0.59	0.89	1.23

3.4.2 Antifungal activity

ACCE and ACME showed antifungal activity against *Aspergillus niger* at the highest concentration tested (250 mg/ml), and all three extracts showed activity against *Penicillium chrysogenum* at 250 mg/ml. Maximum antifungal activity was recorded for ACME against *Aspergillus niger* (250 mg/ml), ACPE (250 mg/ml), and ACCE (250 mg/ml) was observed against *Penicillium chrysogenum* (Figures 14 and 15a-c and Table 3).



Figures 14 and 15a-c: Antifungal assays of different extracts of *A. catechu* using the test-tube method, 14a-c *Aspergillus niger*, and 15a-c *Penicillium chrysogenum*.

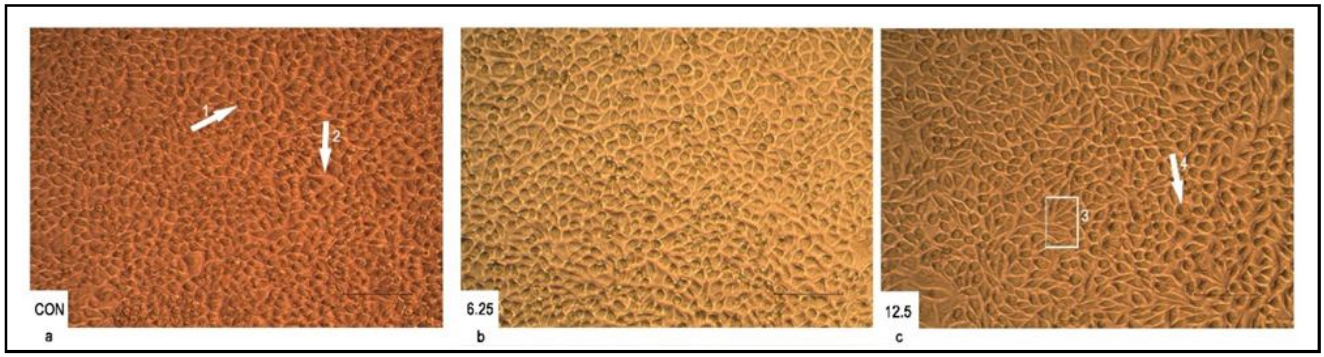
Table 3: Antifungal activity of *A. catechu* inflorescence extracts

S.No.	Fungal strains	Samples	Concentrations (mg/ml)			+ve controlImidazole (100 µg/ml)	-ve control (0.05% DMSO)
			100	150	250		
1.	*Aspergillus niger*	ACPE	+++	+++	+++	NA	+++
		ACCE	++	++	+	NA	+++
		ACME	++	++	++	NA	+++
2.	*Penicillium chrysogenum*	ACPE	+++	++	+	NA	+++
		ACCE	+++	++	+	NA	+++
		ACME	++	++	++	NA	+++

NA no growth, + mild growth, ++ moderate growth, +++ intense growth

3.5 Cell viability

The MTT assay was used to assess the cytotoxicity of the defatted methanolic extract of *A. catechu* inflorescence in L929 fibroblast cell lines. After a 24 h incubation with the defatted methanolic extract, the morphology of L929 (Figures 16a-f) was observed under an inverted phase-contrast microscope, revealing no significant changes in L929 fibroblasts. The cells showed >77.69% viability even at the higher concentration tested (100 µg/ml) compared with the control group ($p \leq 0.0001$) (Figure 17). Thus, the extract was less toxic to L929 fibroblasts. The IC_{50} value was 267.40 µg/ml.



Figures 16a-f: Photomicrographs of the MTT assay of L929 fibroblasts treated with various concentrations (6.25-100 µg/ml) of defatted methanolic extract of *A. catechu* inflorescence examined using an inverted phase-contrast microscope (arrows: 1 normal cells, 2 fibroblast-shaped cells, 3 cell clones, 4 oocyte-like cells, 5 cell aggregates, 6 damaged cells, and 7 altered morphology).

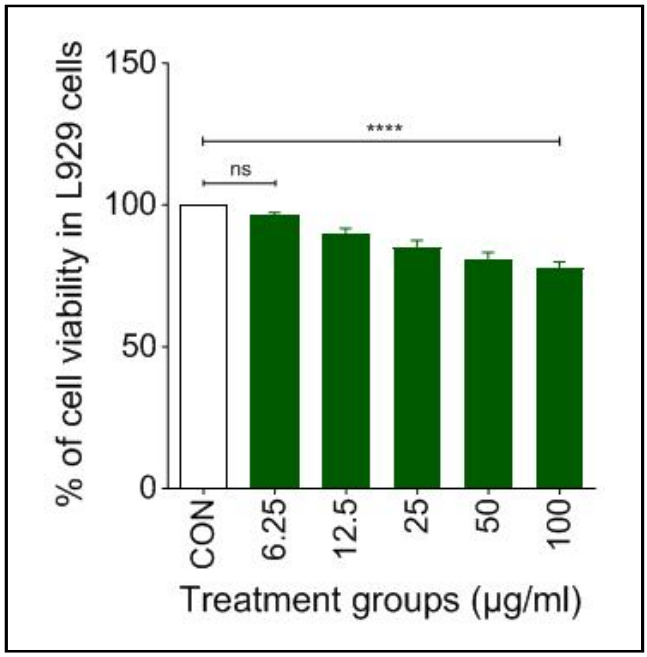


Figure 17: Viability of L929 fibroblasts treated with different concentrations (6.25-100 µg/ml) of defatted methanolic extract of *A. catechu* inflorescence.

4. Discussion

A. catechu inflorescences are rich in polyphenols, polysaccharides, alkaloids, diverse physiologically active compounds, and microelements. In Chinese culture, it has a reputation as a source of miniature nourishment and longevity (Chen *et al.,* 2012). However, the full potential of areca inflorescence has not been completely explored. The current study found that chloroform (ACCE) and methanol (ACME) extracts of *A. catechu* had higher antioxidant activities, including free radical scavenging and reducing properties, than the nonpolar petroleum ether extract (ACPE). Previous research has also demonstrated that *A. catechu* inflorescence extracts can delay the formation of malondialdehyde during low-density lipoprotein peroxidation induced by Cu^{2+} (Chen *et al.,* 2012). Although areca nut/fruit is rich in polyphenols, its health benefits are limited because of the high content of carcinogenic alkaloids, such as arecoline, arecaidine, and guvacine (Yi *et al.,* 2022). However, areca nut extracts have been reported to exhibit antioxidant (Chavan and Singhal, 2013) and antibacterial activities (Jam *et al.,* 2021, 2022). Wetwitayaklung *et al.* (2006) found that the methanol extract of *A. catechu* showed higher antioxidant activity than other extracts. Additionally, some compounds isolated from areca nuts have demonstrated notable DPPH radical-scavenging activity (Zhang *et al.,* 2010). Based on these findings, the current study supports earlier reports, and the radical scavenging and reducing activities were observed to be concentration-dependent. In the present investigation, the chloroform extract exhibited the highest scavenging activity compared to the other extracts. This could be attributed to the fact that the chloroform extract possessed higher levels of antioxidant molecules, such as phenolic and flavonoid compounds, as evidenced by phytochemical analysis. Previous reports have shown that *A. catechu* contains polyphenols, primarily flavanols, accounting for approximately 12% (+)-leucocyanidin, 2.5% epicatechin, and 10% (+)-catechin (Mathew and Govindarajan, 1963). A diverse range of tetrameric, trimeric, and dimeric procyanidins have been isolated from the seeds of *A. catechu* (Nonaka *et al.,* 1981). Flavonoids identified in *A. catechu* include isorhamnetin, chrysoeriol, luteolin, quercetin, 4' , 5' -dihydroxy-3', 5', 7' -trimethoxyflavone, 5',7',4' -trihydroxy-3', 5' -dimethoxyflavanone, liquiritigenin, and jacareubin (Yang *et al.,* 2012; Zhang *et al.,* 2009). Areca nut extract phenolic compounds show high antioxidant activity *in vivo* (Li *et al.,* 2017). In a mouse model of ovariectomy-induced osteoporosis, the extract protected against bone loss, as demonstrated by its effects on the trabecular bone microarchitecture and bone mineral density. Extract supplementation for 6 months at a dose of 300 mg/kg body weight reduced serum hydrogen peroxide and malondialdehyde levels while increasing glutathione and catalase levels.

The excessive and improper use of antibiotics and antifungals has engendered multidrug resistance in microbes, presenting a significant peril and obstacle in the medical field. Consequently, the discovery of novel compounds that can combat antimicrobial resistance is imperative. This study further evaluated the antimicrobial properties of three extracts (ACPE, ACCE, and ACME) derived from *A. catechu*. All tested microorganisms were effectively inhibited by all solvent extracts at the highest concentration tested (250 mg/ml). The chloroform and methanol extracts showed the highest activity and a broad spectrum of activity against all Gram-positive and Gram-negative pathogenic bacterial strains tested, as well as antifungal

activity against both *Aspergillus niger* and *Penicillium chrysogenum* at the highest tested concentration (250 mg/ml). The findings showed that the chloroform and methanol extracts were rich in flavonoids, which may be responsible for their antimicrobial activity. This is supported by previous studies, such as that by Jam *et al.* (2021), who found that the methanolic fruit extract of *A. catechu* showed antibacterial activity against both Gram-positive and Gram-negative bacteria, with the most significant activity against *E. coli* (MIC 1.56 mg/ml). Additionally, Boniface *et al.* (2014) found significant antimicrobial activity of the methanol extract, ethyl acetate, butanol, and water fractions against four bacterial strains, *Staphylococcus aureus* 96, *S. aureus* 2940, *Streptococcus mutans*, and *Mycobacterium smegmatis*, with MIC values of 125, 250, 62.5, and 250 µg/ml, respectively, for the methanol extract, ethyl acetate, butanol, and water fractions towards *S. aureus* 96. According to a study conducted by Chin *et al.* (2013), the ethanolic extract of *A. catechu* nut displayed antimicrobial activity against *E. coli*, *Klebsiella pneumoniae*, *P. vulgaris*, *P. aeruginosa*, non-typhoidal *Salmonella*, *S. typhi*, *S. flexneri*, and *V. cholerae*. The extract inhibited the growth of all microorganisms, with inhibition zones ranging from 7 to 18 mm. The highest activity was observed against *P. vulgaris* and *V. cholerae*, with mean inhibition zones of 16-18 mm.

5. Conclusion

The current study revealed that the inflorescence extracts of *A. catechu* contain a significant concentration of phenolics and flavonoids, which have been found to exhibit beneficial effects, such as antioxidant and antimicrobial activities. As a result, this plant can be considered a potential natural source for obtaining therapeutically valuable metabolites that can be used to combat various diseases. However, further research, particularly extensive and comprehensive *in vivo* and clinical trials, are necessary to assess the safety and efficacy of this plant.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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