ANTIMICROBIAL ACTIVITY OF THE DIFFERENT EXTRACT PART OF *KALANCHOE PINNATA* AGAINST ESKAPE PATHOGEN

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Abstract

Antibiotic resistance has emerged as a threat to global health, food security, and development today. Antibiotic resistance can occur naturally but mainly due to misuse or overuse of antibiotics, which results in recalcitrant infections and Antimicrobial Resistance (AMR) among bacterial pathogens. These mainly include the MDR strains (multi-drug resistant) of ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter Baumann, Pseudomonas aeruginosa, and Enterobacter species). These bacterial pathogens have the potential to "escape" antibiotics and other traditional therapies. ESKAPE Pathogens have been placed in the list of 12 bacteria by World Health Organization (WHO), against which development of new antibiotics is vital. In the present study, physico-chemical and phytochemical analysis of various extracts from flower, stem and roots was done. Further, methanolic and ethanolic extracts of Kalanchoe pinnata (Crassulaceae),was screened for antimicrobial activity against ESKAPE Pathogens. The results of the antimicrobial activity showed that the methanol stem extract significantly ($P< 0.01$) demonstrated antibacterial activities against S. aureus, while the aqueous stem extract significantly ($P< 0.01$) showed antibacterial activities against P. aeruginosa and E. faecium, both at a concentration of 25 mg/ml and above. Also, the methanol and the aqueous extracts showed the least antibacterial activities at a concentration of 12.5 mg/ml for S. aureus and E. faecium, respectively.For MIC, the antibacterial activities of the methanol extract were significantly ($P< 0.01$) different from those of the aqueous extract and Gentamicin.

Keywords: *Kalanchoe pinnata***,** ESKAPE pathogens, antimicrobial activity, minimum inhibitory concentration, guaianolides.

Introduction

In recent times, there have been increases in antibiotic resistant strains of clinically important pathogens, which have led to the emergence and spread of antibiotic resistance, as well as the evolution of new strains of disease-causing agents, are of great concern to the global health community[1–3]. Effective treatment of a disease entails the development of new pharmaceuticals or some potential source of novel drugs. Commonly used medicinal plants of

our community could be an excellent source of drugs to fight off this problem[4].Humans benefited partially from plants and metals to treat microbial infections. Later these infections were cured with antibiotics but further suffered from resistance issues[5]. Antimicrobial resistance has become a cosmopolitan problem and it has been a challenge in the medical and pharmaceutical fields from 20th century. With major advances in medicine, huge surgical procedures, such as heart surgery and kidney transplantation, are being victorious; but the infection after the surgeries is a major issue due to microbial resistance. Thus, our competitive medical world is deadlocked in the evolutionary arms of microbes[6].The prime class of opportunistic pathogens that are a universal threat to humankind are entitled as 'ESKAPE' (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.) as they are known to "escape" antibiotics and other traditional treatments [7]. This imminent health threat has activated the development of novel antimicrobial therapies, where better care of the patient and improved governance happens to be the requirement of the hour[8]. Antimicrobial activity can be described as any natural, semi-synthetic or synthetic origin used to kill or inhibit the growth of microorganisms[9]. Many different types of medicinal herbs and their formulation have been used since time immemorial. Practical experience and several modern research works have clearly shown that therapy using medicinal plants is more esteemed than using synthetic chemicals. The world is rich in medicinal plants growing wild or cultivated, forming a huge natural and economical health which must be protected, increased for the development of economy, wealth of nation and health of people[10].Plants in the genus Kalanchoe (Family: Crassulaceae)comprises 125 species, most of them native to Madagascar[11].These plants are used in traditional medicine throughout the tropics for treating a variety of conditions[12]. Crassulacean acid metabolism (CAM) and succulent leaves allow its acclimation to environmental factors such as periodic drought and hot [13]. In addition, the leaves of this species are rich in flavonoids [11], substances that are considered protective against UV-B radiation [14], and that also play a role in many of the proven biological activities of this species [11].This aromatic plant is of extreme therapeutic potential and high medicinal importance due to the presence of distinctive chemical constituents such as essential oils having alkaloids, lipids, triterpenes, bufadienolides, glycosides, steroids, flavonoids and cardienolides[15]. Leaves of Kalanchoe pinnata contain a biologically active group of chemicals called "bufadienolides" including bryotoxin-A, bryotoxin-B, bryotoxin-C, cardiac glycoside, digitoxin and digoxin possessing the insecticidal, chemo-preventive, anti-tumor and anti-bacterial potentials[16]. The plant is rich in both macro and micro elements, vitamins, calcium, phosphorus, ascorbic acid, inulin [17] and other compounds like saponin, flavonoids, anthraquinones, xanthones, bryophyllin A and B [18]. Anti-inflammatory, hypoglycemic, anti-diabetic and anticancer properties have been also reported[19].A lot of work on antimicrobial activity of leaf extract against ESKAPEpathogen has been reported by different researchers. There are a very few data reported on the antimicrobial activity of different parts of kalanchoe pinnata against

ESKAPEpathogen. This study is focused on antimicrobial activityof the different extract part of kalanchoe pinnataagainst ESKAPEpathogen.

Material and Method

Material

Collection and Authenticationof Plantmaterial

The plant partutilised in the study were gathered in the months of March and April from a local market in Gurgaon, Haryana, India.The Department of Botany, Govt. Agriculture College, Indore, recognised and authenticated the obtained plant material (the entire plant) based on macroscopic and microscopic features. To serve as supplementary references, the voucher specimens have been deposited at our institute's department of pharmacognosy museum.

Method

Physicochemical study

Determination of moisture content/ Loss on drying

10gm of leaf powder was dried at 105^0C in a hot air oven using a pre-weighed porcelain dish to measure the moisture content. The percentage was determined using the initial leaf powder weight.

Ash Value: Total ash. acid insoluble ash, water soluble ash values

Determination of Ash values

The following techniques were used to determine the ash values of Kalanchoe pinnata leaf, including total ash, acid insoluble ash, and water-soluble ash:

Determination of Total ash

A muffle furnace was used to burn 2 gramme of flower, stem, and root powder at temperatures between 5000°C and 6000°C until carbon-free ash was produced. Calculating the percentage of ash used the dry powder's initial weight as a reference.

Determination of acid insoluble ash

To remove insoluble material, the ash made from complete ash was boiled for 5 minutes with 25 ml of 1 N HCl. The ashless filter paper was then used to collect the residue. The filter paper was put into a silica crucible that had been pre-weighed before being burned at 650 ^{o}C in a muffle furnace until it was carbon-free. Calculating the percentage of acid-insoluble ash required dried powder.

Determination of water-soluble ash value

With 25 ml of water, the ash from the total ash was boiled for 5 minutes. On ashless filter paper, soluble material was gathered. The filter paper was put into a silica crucible that had been pre-weighed before being burned at 450°C in a muffle furnace. The percentage of water-soluble ash was calculated using dried powder as the standard.

Preparation of Extracts

Different types of extracts were prepared from the flower, leaves, stem and roots of *Kalanchoe pinnata.*The flowers (100.0 g), the stem (200.0 g) and roots (200.0 g) of *Kalanchoepinnata* (Lam.) were separated, washed with tap water and air dried. The separated components were

macerated in 70 percent methanol at room temperature for 7 days with intermittent shaking after drying. Pet ether, ethyl acetate, benzene, chloroform, alcohol, and water were also used in the extraction process. Following that, Whatmann's filter paper No. 1 was used to filter each extract. In a rotary evaporator, the solvent was evaporated at a controlled temperature and reduced pressure (BUCHI Rotavapour R-200, Switzerland) [20], [22].

Extractive Values

The following techniques were used to determine the extractive values of Kalanchoe pinnata's flower, stem, and roots:

Determination of water-soluble extractive value:

100 ml of water was added to 3gm of powdered dried leaf, flower, stem, and root, which was then thoroughly combined. The mixture was heated to a boil on a water bath (100 0C), and then filtered. In a pre-weighed porcelain dish, the filtrate was evaporated and dried at 1050C. The extractive value of water was computed.

Determination of alcohol soluble extractive value:

100 ml of alcohol was mixed with 4 gm of powdered material while it was being shaken, and the mixture was allowed to stand for 16 hours before being filtered.The filtrate was then evaporated in a porcelain plate that had been previously weighed and dried at 105^0C. The extractive value of alcohol was determined[23].

Qualitative phytochemical screening of plant extracts

The presence of different phytochemicals in the plant's extracts, such as alkaloids, flavonoids, phenolic compounds, steroids, terpenoids, glycosides, tannins, balsams, saponins, and anthraquinones, etc.

Test for carbohydrates

Molisch's test: Two drops of freshly made, 20 percent alcoholic (α-napthol solution were put to a test tube containing drug extract, along with two drops of concentrated sulphuric acid. If carbohydrates are present, a purple or reddish violet tint will form where two liquids converge.

Benedict's test: In a test tube containing the drug extract, add Benedict's solution, stir well, and then quickly boil the mixture for two minutes. The mixture should then be cooled. The presence of carbohydrates causes the formation of a red precipitate.

Barfoed's test: To the 0.5 ml of the solution under inspection, add the barfoed solution and heat until it boils. It was discovered that carbohydrates were present when a red copper oxide precipitate formed.

Anthrone test: Add the drug extract to the two ml of anthrone test solution. The presence of carbohydrates was indicated by a green or blue colour.

Test for Alkaloids

Dragendorff's Test:A few mg of the drug's extract was dissolved in 5 ml of water, 2 M hydrochloric acid was added, and an acid reaction ensued. 1 ml of the dragendorff's reagent (potassium bismuth iodide solution) was then added, and an orange-red precipitate appeared, indicating the presence of alkaloids.

Wagner's test: Add a few drops of Wagner's reagent after acidifying the drug extract with hydrochloric acid at a ratio of 1.5 percent by volume (iodine potassium iodide solution). Alkaloids were detected by reddish-brown precipitate formations.

Mayer's Test:A dull white precipitate that formed when two ml of extract solution was treated with two to three drops of Mayer's reagent (potassium mercuric iodide solution) indicated the presence of alkaloid.

Hager's Test:A yellow precipitate that formed after treating an extract of the drug solution with 3 ml of Hager's reagent (a saturated solution of picric acid) indicated the presence of alkaloids.

Test for glycosides

Legal's test: Extract solution was dissolved in pyridine before being made alkaline by the addition of sodium nitroprusside solution. The presence of glycosides was identified by the pink-red colour.

Baljet's test: Sodium picrate solution was added to the drug extract; its yellow to orange colour showed the presence of glycosides.

Borntrager's test: The test solution of the extract was added along with a few ml of diluted sulphuric acid solution. After filtering, ether or chloroform was boiled with the filtrate. After separating the organic layer and adding ammonia, the orange layer formed a pink, red, or violet colour, which indicated the presence of glycosides.

Keller Kiliani test: One ml of concentrated sulfuric acid was carefully added by the side of the test tube after the methanolic extract had been thoroughly dissolved in glacial acetic acid that had traces of ferric chloride. The presence of glycosides was shown by the colours blue in the acetic acid layer and red at the intersection of the two liquids.

Test of saponins:20 ml of distilled water were used to dilute 1 ml of alcohol extract, which was then agitated in a graduated cylinder for 15 minutes. The presence of saponins was indicated by a 1 cm layer of foam.

Test for flavonoids

Shinoda test: Add 5–10 drops of dilute hydrochloric acid to the test tube containing the drug's alcoholic extract, then add the little piece of magnesium. Flavonoids caused a pink, reddish pink, or brown tint to be created.

Test for tannins

The presence of tannins was detected when ferric chloride solution was added to the extract sample, giving off a dark blue or greenish-black colour. When potassium cyanide was added to the extract sample, the presence of tannins was confirmed by the deep red colour. When potassium dichromate solution was applied to the extract sample, yellow precipitate was created.

Test for protein and amino acid

Biuret's test: When 1 ml of 40 percent sodium hydroxide solution and 2 drops of 1 percent copper sulphate solution are added to 2-3 ml of drug extract and thoroughly mixed, a purplish-violet or pinkish-violet colour result, indicating the presence of proteins.

Ninhydrin's test: The extract was mixed with two drops of freshly made 0.2 percent ninhydrin reagent, which was then heated for one to two minutes till boiling point and then allowed to cool. Proteins, peptides, and amino acids were detected by the development of a blue colour.

Xanthoprotein test: Add concentrated nitric acid to the extract in the test tube. The solution was gently cooled after obtaining a white precipitate that, when heated, becomes yellow. An overabundance of orange colour in the sodium hydroxide solution after 20 percent addition showed the existence of aromatic amino acids.

Millon's test: There were 5–6 drops of Millon's reagent added to the little amount of drug extract dissolved in distilled water. In the form of a white precipitate that turned red when heated, proteins were found.

Lead acetate test: The extract was prepared by adding two millilitres of a 40 percent sodium hydroxide solution, boiling it, adding glacial acetic acid, cooling it, and then adding one millilitre of lead acetate solution. A grey, black precipitate resulted, indicating the presence of an amino acid containing sulphur. Test of fixed oils or fats Using sodium hydroxide: The extract was combined with 1 ml of 1% copper sulphate solution, 10% sodium hydroxide solution, and a clear blue solution was obtained, indicating the presence of glycerine in the sample. Using sodium hydrogen sulphate: After adding a small amount of sodium hydrogen sulphate to the extract in the test tube, a pungent odour was produced, indicating the presence of glycerine in the sample.

Saponification: A 2 percent concentration of the extract was diluted into four millilitres of sodium carbonate solution. shaken and heated quickly. After cooling a clean soapy solution, a few drops of concentrated HCl were added, and it was noticed that the fatty particles separated out and floated to the top [24], [25].

Quantitative phytochemical screening of plant extract:

The resulting plant extracts were additionally analysed for the quantitative presence of phytochemicals. Each sample's total phenolic and flavonoid content was measured.

Determination of Total Flavanoids

The aluminium chloride method was used to quantify flavonoids in triplicates. In a test tube, 0.5 ml of each quercetin standard (Merck, Germany) (100, 200, 400, 600, 800, and 1000 µg/ml) and plant extract were diluted in 4.5 ml of 70% ethanol before being mixed with 0.3 ml of NaNO2.0.3 ml of 10% $AlCl_3$ was added and incubated for an additional 5 minutes after the initial 5 min. This was followed by the addition of 2 ml of 1M NaOH and the addition of distilled water to bring the volume up to 10 ml. A spectrophotometer was used to measure the absorbance at 510 nm after 15 minutes of incubation. The standard calibration curve was drawn using the standards' absorbance. In terms of mg of quercetin equivalent (QE)/100 g of dry mass, the total flavonoid content was calculated.

Determination of Total Phenolics

Using the Folin-Ciocalteu reagent (Merck, Germany) method, the total phenolic compounds were quantified in triplicates. As a standard, gallic acid (Merck, Germany) was used to prepare various concentrations, including 10, 20, 40, 60, 80, and 100 µg/ml. After adding 5 ml of

distilled water and 0.5 ml of Folin-reagent, Ciocalteu's a test tube was filled with one millilitre of each plant extract and Gallic acid standard. This was combined and left to stand for 5 minutes. Then, 1.5 ml of 20 percent sodium carbonate was added, and the volume was brought up to 10 ml using distilled water.The absorbance of the test and standard was measured at 750 nm in comparison to a reagent blank after two hours of incubation. The standard calibration curve was created using the standards' optical densities. Gallic acid equivalent (GAE)/100 g of dry mass was used to express the total phenolic components in the plant extracts [24], [26], [27]. The methanolic and aqueous extracts of Kalanchoe pinnata showed most satisfactory results after qualitative and quantitative phytochemical screening of different extracts, hence selected for anti-microbial activity.

Antimicrobial activity of Kalanchoe pinnata extracts

Antimicrobial tests were carried out on the methanol and aqueous extracts using the agar diffusion method [28]. The selected extracts were tested against strains of ESKAPEpathogens. Two species were Gram-positive,Enterococcus faecium (EU-44) and S. aureus (UAMS-1); therest were Gram-negative: Klebsiella pneumoniae (CDC-16),Acinetobacter baumannii (CDC-33), P. aeruginosa (AH-71),and Enterobacter cloacae (CDC-08). Strains were streaked fromfreezer stock onto tryptic soy agar (TSA) plates and incubated at37◦C overnight. About 200 mg of each extract was dissolved in 2 ml of dimethyl sulphoxide (DMSO) to obtain 100 mg/ml concentration. Further dilutions of the stock solution were made using a two-fold serial dilution technique, to give 50, 25, 12.5 and 6.25 mg/ml concentrations. This same dilution was carried out on gentamicin (as a reference drug) to obtain 100, 50, 25, 12.5 and 6.25 µg/ml, concentrations. About 20 ml of sterile solidified Mueller-Hinton nutrient agar was poured into a sterile Petri dish and seeded with 0.1 ml of standardized broth culture of the test micro-organism (1.0 × 107 cfu/ml). This was carried out for all the test micro-organisms. Five equidistant wells were made in each of the plates with a sterile 6.0 mm diameter cork borer. Using a sterile dropper, 0.3 ml of each of 100, 50, 25, 12.5 and 6.25 mg/ml concentration of the extracts were dispensed into each corresponding well, made in the plates. A well containing gentamicin was made in each of the plates seeded with the bacteria, while the plates seeded with fungi had ketoconazole in the well, as a reference drug. The plates were allowed to stand for 1 h for the diffusion of the extract to occur. The plates with bacteria were incubated at 37°C for 24 h while those with fungi were incubated at 25°C for 48 h. They were observed and the presence of zones of inhibition around the wells were measured and taken as an indication of antimicrobial activity [29]. The experiment was carried out in two replicates and the mean for each organism was determined. For the determination of MIC, methanol and aqueous extracts, as well as gentamicin (as a reference drug) were used on the susceptible micro-organisms. The agar diffusion method [28] was adopted. About 200 mg of each extract was dissolved in 2 ml of DMSO to obtain 100 mg/ml concentration. Two-fold serial dilution was made to obtain 50, 25, 12.5 and 6.25 mg/ml concentrations. The same two-fold dilutions of 100 µg/ml gentamicin were made to obtain 50, 25, 12.5 and 6.25 µg/ml concentrations. These concentrations were put into wells bored in the

seeded agar plates containing the susceptible micro-organisms, as above. The experiment was carried out in two replicates. The agar plates were placed in an incubator at 37°C for 24 h, after which the mean diameter of the zone of inhibition was measured. The graph of the square of the inhibition zone diameter was plotted against log concentration for each micro-organism. A regression line was drawn through the points. From these graphs, the representative MIC values were determined as the antilogarithm of the intercept on the logarithm of concentration axis. Analysis of variance (ANOVA) was determined on the data obtained, while the multiple comparisons were carried out between treatment means using Duncan's multiple range tests at $P< 0.05$ confidence level [30], [31].

Results

In the present study, the Kalanchoe pinnata plant was collected and authenticated. Many researchers have reported various pharmacological activity of different extracts obtained from leaves of kalanchoe pinnata, whereas, a very few data reported on the evaluation of different pharmacological activity of other parts of this plant. It is already reported that Kalanchoe pinnata species has many wonderful effects and used traditionally for many purposes. It is also reported that this plant has profound anti-microbial activity against various pathogens, however, most of the studies were concentrated on leaves, thus, the present study focused on evaluation of antimicrobial activity of other parts of plant. Initially, the plant material was run through physicochemical screening.

Physicochemical screening

The plant material was initially evaluated by physicochemical parameters viz., moisture content, ash value, total ash, acid insoluble ash and water-soluble ash. For the proper identification of plant, physicochemical parameters (moisture content (LOD), extractive values and ash values) provide useful information. Moisture content of drugs could be at minimal level to discourage the growth of bacteria, yeast or fungi during storage. These can serve as a valuable basis of information and provide suitable standards to establish the quality of this plant material as future prospects. In case of stem, moisture content was found to be 1.25% in *Kalanchoe pinnata* which was the lowest one. An ash values are used to decide quality and purity of crude drug, it indicates presence of various impurities like, silicate, oxalate and carbonate. The water-soluble ash is used to determine the quantity of inorganic compounds present in drugs. The acid insoluble ash helps to estimate the amount of silica present in the material. The total water-soluble portion of the ash is considered as water soluble ash. The results of physicochemical study were depicted in table 1.

Table 1: Physical Parameters of *Kalanchoe pinnata*

Parameters	**Flower**	**Stem**	**Root**
LOD	5.6%	1.25%	11%
Total ash	10.3%	8.5%	11.8%
Acid insoluble ash	1.5%	0.3%	2.5%
Water soluble ash	3.7%	2.15%	4.2%

Total ash obtained with stem sample was 8.5% in which 0.3% of ash was acid insoluble whereas 2.15% of ash was water soluble. The physical parameters of flower and root sample showed LOD-5.6% & 11%, Total ash-10.3%&11.8%, Acid insoluble ash-1.5%&2.5%, Water soluble ash-3.7%&4.2%, respectively. Less amount of these parameters in stem indicated that the inorganic matter and silica were less in Kalanchoe pinnata stem.

Preparation of Extracts

Different types of extracts were prepared from the flower, stem and roots of *Kalanchoe pinnata*. Reddish brown (flowers), greenish black (stem) and dark brown (roots) extracts were obtained.

Extractive Values

Extractive values of flower, stem and roots of Kalanchoe pinnata were determined. Alcohol soluble extractive value of stem was found greater (31.8%) than alcohol soluble extractive value (25.5%) which means alcohol soluble extract contains more components. Water soluble extractives obtained from flower & roots were 7.6%& 9.5% while alcohol soluble extractive value was about 10.7%& 12.5% from flower & roots, respectively. The results were summarized in table 2.

Table 2: Extractive values of different parts of *Kalanchoe pinnata*

Parameters	**Flower**	**Stem**	**Root**
Water soluble extractive value	7.6%	25.5%	9.5%
Alcohol soluble extractive value	10.7%	31.8%	12.5%

The different plant parts were further treated with different solvents and their extractive values were determined. The successive solvent extractive values were shown in table 3a-3c.

Table 3a: Successive solvent extractive values and nature of Kalanchoe pinnata Flower extracts

Solvents	**Colour**	**Consistency**	**Extractive value (%w/w)**
Petroleum ether	Reddish brown	Sticky mass	0.48%
Ethyl acetate	Reddish brown	Sticky mass	0.28%
n-hexane	Brown	Semisolid mass	1.05%
Chloroform	Brown	Sticky mass	0.99%
Methanol	Brown	Sticky mass	4.13%
Aqueous	Reddish brown	Sticky mass	3.81%

Table 3b: Successive solvent extractive values and nature of Kalanchoe pinnata Stem extracts

Solvents	**Colour**	**Consistency**	**Extractive value (%w/w)**
Petroleum ether	Greenish black	Sticky mass	0.59%

Ethyl acetate	Greenish	Sticky mass	0.31%
n-hexane	Greenish	Sticky mass	1.12%
Chloroform	Greenish black	Sticky mass	1.08%
Methanol	Greenish black	Sticky mass	4.27%
Aqueous	Brown	Sticky mass	3.57%

Table 3c: Successive solvent extractive values and nature of Kalanchoe pinnata Root extracts

Solvents	**Colour**	**Consistency**	**Extractive value (%w/w)**
Petroleum ether	Blackish brown	Semisolid sticky	0.68%
Ethyl acetate	Brown	Sticky mass	0.35%
Benzene	Brown	Sticky mass	1.41%
Chloroform	Chocolate brown	Sticky mass	1.18%
Methanol	Dark brown	Sticky mass	4.62%
Aqueous	Yellowish brown	Sticky mass	3.91%

The highest extractive value was obtained with stem sample (methanolic extract) of Kalanchoe pinnata among the all three parts used for the study.

Qualitative phytochemical screening of plant extracts

The presence of various phytochemicals viz., alkaloids, flavonoids, phenolic compounds, steroids, terpenoids, glycosides, tannins, saponins, proteins, gum & mucilage and volatile oil etc. in the extracts from the plant. The results of preliminary phytochemical analysis were shown in table 4a-4c.

Table 4a: Preliminary phytochemical analysis of Kalanchoe pinnata flower extracts

Parameters	**Extracts**					
	Pet. ether	**Benzene**	**Chloroform**	**Acetone**	**Methanol**	**Aqueous**
Alkaloids	+	-	-	-	+	+
Carbohydrates and glycosides	-	-	+	-	+	+
Phytosterols	-	+	+	+	+	-
Fixed oils and fats	+	+	+	-	+	-
Phenolic compounds and tannins	-	-	+	+	+	+

Saponins	-	-	-	-	+	+
Flavonoids	+	-	-	-	+	+
Gums and mucilage	+	-	+	+	+	+
Proteins	-	-	+	+	+	+
Volatile oils	-	-	-	+	+	+

+, present, -, absent

Table 4b: Preliminary phytochemical analysis of Kalanchoe pinnata stem extracts

Parameters	**Extracts**					
	Pet. ether	**Benzene**	**Chloroform**	**Acetone**	**Methanol**	**Aqueous**
Alkaloids	-	-	-	-	+++	++
Carbohydrates and glycosides	-	+	+	-	+++	+++
Phytosterols	-	+	+	+	+	-
Fixed oils and fats	+	+	+	-	-	-
Phenolic compounds and tannins	-	-	+	+	+	+
Saponins	-	+	-	-	+	+++
Flavonoids	+	-	-	-	+++	++
Gums and mucilage	+	-	+	+	-	+
Proteins	-	-	+	+	+++	+++
Volatile oils	-	-	-	-	+	-

+, present, -, absent

Table 4c: Preliminary phytochemical analysis of Kalanchoe pinnata root extracts

Parameters	**Extracts**					
	Pet. ether	**Benzene**	**Chloroform**	**Acetone**	**Methanol**	**Aqueous**
Alkaloids	-	-	-	-	+	+
Carbohydrates and glycosides	-	+	+	-	+++	++
Phytosterols	+	+	+	+	++	-

Fixed oils and fats	-	+	+	-	++	++
Phenolic compounds and tannins	-	-	-	+	++	+
Saponins	-	-	-	-	-	+
Flavonoids	-	-	-	-	+++	++
Gums and mucilage	-	-	-	-	-	+
Proteins	-	-	+	+	+	+
Volatile oils	-	-	-	-	+	-

+, present, -, absent

From the phytochemical screening of the different extracts obtained from different plant parts, it was observed that the methanolic and aqueous extracts obtained from stem sample showed presence of phytoconstituents in appreciable quantity. Hence, these two extracts were evaluated for quantitative phytochemical parameters.

Quantitative phytochemical screening of plant extract:

The resulting plant extracts were additionally analyzed for the quantitative presence of phytochemicals. Each sample's total phenolic and flavonoid content was measured. The determination of the total phenolic content, expressed as mg gallic acid equivalents and per 100 mg dry weight of sample. TPC of methanolic and aqueous extract of Kalanchoe pinnata stem showed the content values of 4.5473 and 2.5292, respectively. The total flavonoids content of the extracts was expressed as percentage of quercetin equivalent per 100 mg dry weight of sample. The total flavonoids estimation of methanolic and aqueous extracts of Kalanchoe pinnata stem showed the content values of 3.519 and 1.2863, respectively (Table 5).

Table 5: Estimation of total phenolics and total flavonoids content in Kalanchoe pinnata stem

S. No.	Kalanchoe pinnata Extracts	Total phenolic content (mg/100mg of dried extract)	Total flavonoids content (mg/ 100 mg of dried extract)
1.	**Methanol**	4.5473	3.519
2.	**Aqueous**	2.5292	1.2863

The above results showed that aqueous extract contain less phenolic and flavonoids content than the methanolic extract. It may due to the solubility of principle contents presence be higher in case of alcoholic solvent, thus it has been accepted that it is a universal solvent for the extraction of plant constituents. These extracts were further evaluated for anti-microbial activity.

Anti-microbial activity of Kalanchoe pinnata extracts

The methanolic and aqueous extracts obtained from stem part were selected for anti-microbial activity as both these extracts showed presence of phytoconstituents in appreciable quantity. The methanol stem extract revealed the presence of high concentrations of alkaloids, carbohydrates, glycosides, proteins, steroids, terpenoids and reducing sugar, while the aqueous extract showed carbohydrates, proteins and saponins in high concentration (Table 4a-4c). Fats and oils were not detected in both extracts. The results of the antimicrobial activity showed that the methanol stem extract significantly ($P< 0.01$) demonstrated antibacterial activities against S. aureus, while the aqueous stem extract significantly ($P< 0.01$) showed antibacterial activities against P. aeruginosa and E. faecium, both at a concentration of 25 mg/ml and above (Table 6). Also, the methanol and the aqueous extracts showed the least antibacterial activities at a concentration of 12.5 mg/ml for S. aureus and E. faecium, respectively. However, both extracts did not show any antimicrobial activity against Enterobacter spp. For both extracts, 100 mg/ml was significantly ($P< 0.01$) the most effective against all the bacteria used. However, for the methanol extract, 100 mg/ml was not significantly different from 50 mg/ml, as regards S. aureus. The lower the concentration, the lower the effectiveness to the extent that 6.25 mg/ml concentration exhibited no activity against any microorganism. Comparatively, gentamicin (the standard drug) showed significantly ($P< 0.01$) higher antibacterial activities against S. aureus,K. pneumoniae, P. aeruginosaand Enterobacter spp. at a concentration of 12.5 µg/ml and above.

Table 6: Mean inhibitory zone diameter (mm) of different concentrations of methanol and aqueous extracts of Kalanchoe pinnata stem and Gentamicin on the test microorganisms

Microorganism	Methanolic extract					Aqueous extract					Gentamicin				
	Concentration (mg/ml)					Concentration (mg/ml)					Concentration (mg/ml)				
	100	50	25	12.5	6.25	100	50	25	12.5	6.25	100	50	25	12.5	6.25
S. aureus	25.5	22.0	19.0	12.0	-	-	-	-	-	-	36.5	32.5	25.0	22.5	-
P. aeruginosa	-	-	-	-	-	24.0	20.5	14.5	-	-	29.0	27.5	22.0	19.5	-
E. faecium	-	-	-	-	-	19.5	15.0	10.5	7.5	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-	-	-	-	-	33.5	30.0	29.5	22.0	-
A. baumanni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter spp	-	-	-	-	-	-	-	-	-	-	27.5	24.0	20.5	17.5	-

-, no activity

For MIC, the antibacterial activities of the methanol extract were significantly ($P< 0.01$) different from those of the aqueous extract and Gentamicin (Table 7). S. aureus showed the lowest MIC in the methanol extract, while P. aeruginosa demonstrated the highest MIC in the aqueous extract.

However, for Gentamicin, the reverse was the case for MIC. The methanolic extract inhibited S. aureus more than the aqueous extract. These effects were significant at $P<0.01$.

Table 7: Minimum inhibitory concentrations (MIC) of Gentamicin, methanol and aqueous extracts of Kalanchoe pinnata stem on the test micro-organisms

Micro-organism	Methanol extract (mg/ml)	Aqueous extract (mg/ml)	Gentamicin (µg/ml)
Staphylococcus aureus	**9.45±0.01**	**-**	**5.98±0.05**
P. aeruginosa	**-**	**8.95±0.15**	**5.65±0.15**
E. faecium	**-**	**8.52±0.02**	**-**

Discussion

In this investigation, the methanol and the aqueous extracts of *Kalanchoe pinnata* stem demonstrated antibacterial activity against three out of the six micro-organisms used for the test. This antibacterial activity may be attributed to the alkaloids, glycosides, steroids, terpenoids, saponins, flavonoids and perhaps resins, since these secondary metabolites were detected in the extracts. This is similar to the work of Marriage and Wilson (1971), who reported that a number of active compounds, including flavonoids, glycosides, steroids, guaianolides and organic acids have been identified in *Kalanchoe pinnata*. These constituents are known to demonstrate activity against micro-organisms. The extracts used in this work exhibited antibacterial activity at a concentration of 12.5/25 mg/ml and above. The results are similar to the work of Ugochukwu A (2011) who found that the methanol and aqueous extracts of the stem of B. pinetum inhibited the growth of three out of six bacteria used, at a concentration of 25 mg/ml or above. Taylor (2010) explained that the traditional use of B. pinetum for the treatment of internal and external infections is supported by the fact that the leaves have antibacterial, antiviral and antifungal activities.In this investigation, 100 mg/ml was the most effective against the susceptible bacteria used and the lower the concentration, the lower the effectiveness, to the extent that 6.25 mg/ml showed no activity against any micro-organism.

Conclusion

From the present investigation, it was found that the physicochemical and preliminary phytochemical investigation study of Kalanchoe pinnata leaves yielded a set of standards that can serve as an essential basis of evidence to determine the identity and to determine the quality and purity of the plant material as per its future perspectives. The phytochemical investigation gave valuable information about the different phytoconstituents present in the plant, which helps the future investigators concerning the selection of the particular extract for further investigation of isolating the active principle and also gave idea about different phytochemical have been found to possess a wide range of activities. The total phenolic and flavonoid content in methanolic extract was found to be higher than aqueous extract. The selected extracts were further evaluated for anti-microbial potential. In conclusion, *Kalanchoe pinnata* stem has the

potential to be used as an antimicrobial agent, just like the leaves. However, further laboratory and clinical studies are required to determine its potency and safety.

References

[1] I. Aibinu, E. Aednipekun, and T. Odugbemi, "Emergence of Quinolone Resistance amongst *Escherichia coli* strains isolated from Clinical infections in some Lagos State Hospitals, in Nigeria.," *Nigerian Journal of Health and Biomedical Sciences*, vol. 3, no. 2, Sep. 2004, doi: 10.4314/njhbs.v3i2.11513.

[2] I. E. Aibinu, V. C. Ohaegbulam, E. A. Adenipekun, F. T. Ogunsola, T. O. Odugbemi, and B. J. Mee, "Extended-spectrum β-lactamase enzymes in clinical isolates of Enterobacter species from Lagos, Nigeria," *J Clin Microbiol*, vol. 41, no. 5, pp. 2197–2200, May 2003, doi: 10.1128/JCM.41.5.2197-2200.2003.

[3] *The promotion and development of traditional medicine.* World Health Organization, 1978.

[4] S. Manandhar, S. Luitel, and R. K. Dahal, "In Vitro Antimicrobial Activity of Some Medicinal Plants against Human Pathogenic Bacteria," *J Trop Med*, vol. 2019, 2019, doi: 10.1155/2019/1895340.

[5] L. K. Ruddaraju, S. V. N. Pammi, G. sankarGuntuku, V. S. Padavala, and V. R. M. Kolapalli, "A review on anti-bacterials to combat resistance: From ancient era of plants and metals to present and future perspectives of green nano technological combinations," *Asian Journal of Pharmaceutical Sciences*, vol. 15, no. 1. Shenyang Pharmaceutical University, pp. 42–59, Jan. 01, 2020. doi: 10.1016/j.ajps.2019.03.002.

[6] H. Nikaido, "Multidrug resistance in bacteria," *Annual Review of Biochemistry*, vol. 78. pp. 119–146, 2009. doi: 10.1146/annurev.biochem.78.082907.145923.

[7] Y. X. Ma *et al.*, "Considerations and Caveats in Combating ESKAPE Pathogens against Nosocomial Infections," *Advanced Science*, vol. 7, no. 1. John Wiley and Sons Inc., Jan. 01, 2020. doi: 10.1002/advs.201901872.

[8] P. Bhatia *et al.*, "Antibacterial activity of medicinal plants against ESKAPE: An update," *Heliyon*, vol. 7, no. 2. Elsevier Ltd, Feb. 01, 2021. doi: 10.1016/j.heliyon.2021.e06310.

[9] S. C. Burnett-Boothroyd and B. J. McCarthy, "Antimicrobial treatments of textiles for hygiene and infection control applications: an industrial perspective," in *Textiles for Hygiene and Infection Control*, Elsevier, 2011, pp. 196–209. doi: 10.1533/9780857093707.3.196.

[10] F. A. Tamboli, P. Tambare, and H. N. More, "STANDARDIZATION OF HERBAL DRUGS: An Overview Development and Validation of RP-HPLC Method for Determination of Doxorubicin Hydrochloride from Vacuum Foam Dried Formulation View project STANDARDIZATION OF HERBAL DRUGS View project Standardization of herbal drugs: An overview," 2021, [Online]. Available: https://www.researchgate.net/publication/351489626

[11] S. S. Costa, M. F. Muzitano, M. M. Camargo, and M. A. S. Coutinho, "Therapeutic Potential of Kalanchoe Species: Flavonoids and other Secondary Metabolites."

[12] N. Richwagen, J. T. Lyles, B. L. F. Dale, and C. L. Quave, "Antibacterial Activity of Kalanchoe mortagei and K. Fedtschenkoi Against ESKAPE Pathogens," *Front Pharmacol*, vol. 10, no. FEB, 2019, doi: 10.3389/fphar.2019.00067.

[13] K. Winter and lAC Smith, "An Introduction to Crassulacean Acid Metabolism. Biochemical Principles and Ecological Diversity," Springer-Verlag, 1978.

[14] G. Agati, C. Brunetti, M. Di Ferdinando, F. Ferrini, S. Pollastri, and M. Tattini, "Functional roles of flavonoids in photoprotection: New evidence, lessons from the past," *Plant Physiology and Biochemistry*, vol. 72. pp. 35–45, Nov. 2013. doi: 10.1016/j.plaphy.2013.03.014.

[15] R. Rahman, J. N. Al-Sabahi, A. Ghaffar, F. Nadeem, and A. Umar, "Phytochemical, morphological, botanical, and pharmacological aspects of a medicinal plant: Kalanchoe pinnata-A review article," 2019. [Online]. Available: www.iscientific.org/Journal.html

[16] A. Kamboj and A. K. Saluja, "Bryophyllumpinnatum (Lam.) Kurz.: Phytochemical and Pharmacological Profile : A Review," 2009. [Online]. Available: www.phcog.net

[17] D. E. Okwu and C. Josiah, "Evaluation of the chemical composition of two Nigerian medicinal plants," *Afr J Biotechnol*, vol. 5, no. 4, pp. 357–361, 2006, [Online]. Available: http://www.academicjournals.org/AJB

[18] M. M. Iwu, *Handbook of African medicinal plants*. 2014.

[19] P. K. Sahu, V. Masih, S. Gupta, D. L. Sen, and A. Tiwari, "Ethnomedicinal Plants Used in the Healthcare Systems of Tribes of Dantewada, Chhattisgarh India," *Am J Plant Sci*, vol. 05, no. 11, pp. 1632–1643, 2014, doi: 10.4236/ajps.2014.511177.

[20] B. Shruti, D. Bhavita, Z. Maitreyi, C. Divya, and C. Chandel Divya, "A comparative pharmacognostical and phytochemical analysis of Kalanchoe pinnata (Lam.) Pers. leaf extracts," ~ *1519 ~ Journal of Pharmacognosy and Phytochemistry*, vol. 7, no. 5, 2018.

[21] Q. Majaz, "PHARMACOGNOSTIC EVALUATION OF KALANCHOE PINNATA ROOTS," 2011. [Online]. Available: http://www.irjponline.com

[24] K. Waititu*et al.*, "Associated with Management of Diabetes," *Biomedicine and Biotechnology*, vol. 6, no. 1, pp. 15–20, 2018, doi: 10.12691/bb-6-1-3.

[25] R. Mishra, Y. Pounikar, and M. Gangwar, "Extraction, Phytochemical Screening and Quantitative Determination of Phenols and Flavonoids in Extract of Kalanchoe pinnata and Pongamia pinnata," *Journal of Drug Delivery and Therapeutics*, vol. 9, no. 4, pp. 192–196, 2019, doi: 10.22270/jddt.v9i4.3024.

[26] S. Kamtekar, V. Keer, and V. Patil, "Estimation of phenolic content, flavonoid content, antioxidant and alpha amylase inhibitory activity of marketed polyherbal formulation," *J Appl Pharm Sci*, vol. 4, no. 9, pp. 61–65, 2014, doi: 10.7324/JAPS.2014.40911.

[27] S. A. Baba and S. A. Malik, "Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of Arisaema jacquemontii Blume," *Journal of Taibah University for Science*, vol. 9, no. 4, pp. 449–454, 2015, doi: 10.1016/j.jtusci.2014.11.001.

[28] B. T. Pelczar, H. G. Weed, D. E. Schuller, D. C. Young, T. E. Reilley, and A. G. James, "Identifying High-Risk Patients Before Head and Neck Oncologic Surgery a total of 119 patients who underwent head and neck oncologie surgery lasting more than 2 hours at the Comprehensive Cancer Center at the," 1993. [Online]. Available: http://archotol.jamanetwork.com/

[29] P. I. Alade and O. N. Hobib, "Antimicrobial activities of crude leaf extracts of Acalypha wilkesiana," 1993.

[30] I. O. Edafiogho, K. V. V. Ananthalakshmi, and S. B. Kombian, "Anticonvulsant evaluation and mechanism of action of benzylamino enaminones," *Bioorg Med Chem*, vol. 14, no. 15, pp. 5266–5272, Aug. 2006, doi: 10.1016/j.bmc.2006.03.049.

[31] A. O. Nwadinigwe, "Antimicrobial activities of methanol and aqueous extracts of the stem of Bryophyllumpinnatum Kurz (Crassulaceae)," *Afr J Biotechnol*, vol. 10, no. 72, pp. 16342–16346, Nov. 2011, doi: 10.5897/AJB11.1000.