

## Chemical Assessment and Antimicrobial Activity of Solvent Extracts from *Kalanchoe densiflora*

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### ABSTRACT

*Kalanchoe densiflora* leaves were sequentially extracted using hexane, ethyl acetate, chloroform, acetone and methanol. The solvents were evaporated through rotor evaporation under vacuum to yield five extracts. The extracts were then subjected to chemical and photochemical analyses to identify the components present and their functional groups. In addition the extracts were tested for antimicrobial activities against eight micro-organisms; *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Staphylococcus aureus*, *Proteus mirabilis* and *Pseudomonas aeruginosa*. The extracts were found to possess tannins, saponins, terpenoids, flavonoids and cardiac glycosides. It was also found that unsaturated bonds, carboxyl groups and aldehydes were present in the extracts. In addition, the extracts showed antimicrobial activity against *Bacillus spp.*, *E. coli* and *P. aeruginosa* as indicated by presence of zones of inhibition. Acetonic and methanolic extracts were sensitive to *P. aeruginosa*, *Bacillus spp.* and *S. aureus* while hexane extract was highly sensitive to *E.coli*. *K. densiflora* extracts was found to have broad antimicrobial effect since it was active against gram positive and gram negative bacteria. Crude extracts from *K. densiflora* can be used in control of diseases such as; acute enteritis, pneumonias and opportunistic diseases amongst other diseases caused by these microorganisms.

**Key words:** Antimicrobial, Antifungal, Extracts and *Kalanchoe densiflora*

### INTRODUCTION

World Health Organization (WHO) estimates that 4 billion people which constitute 80% of world population presently use herbal medicine as part of primary health care. In developing countries especially Africa and Asia, herbal medicine plays a key role in health care provision with up to 70% of population depending on it. The affordability and accessibility of herbal medicine entice the marginalized population to use it, for basic health provision (Luoga *et al.*, 2000). In Kenya an estimated 60% of the population uses herbal medicine as first option in management of disease or in combination with prescribed medication (Nyamwaya, 1992).

Although the prohibitive cost of modern medicine is one of the determinant factors, the socio-cultural traditions promote traditional medicine as superior (Kokwaro, 1993). Herbal medicines in most cases complement conventional medicine (Iwu *et al.*, 1999). The choice between the two

therapies depends on the socioeconomic status of a person. At the moment reexamining of traditional medicine and its practice through multidisciplinary studies for the efficacy and safety of these drugs is underway (Lewis, 1995).

*K. densiflora* is widely used in rural areas as a natural remedy for a variety of injuries and wounds (Verdicourt Trump, 1969, and Watt and Breyer-Brandwijk, 1962). The juice squeezed from its leaves is used topically as antiseptic for wounds. It has been shown through studies that plants from crassulaceae family are capable of inhibiting growth of parasites such as *Trypanosoma cruzi*, insects (Hostettmann *et al.*, 1982). In addition, steroidal alkaloids from *K. densiflora* are potential anti-cancer drugs. In the light of the variety of biological activities reported in the *Kalanchoe* genus (Hostettmann *et al.*, 1982) this plant was chosen for analysis for possible antimicrobial compounds against medically important microorganisms.

Moreover development of resistance to available drugs by microbes has been widely reported and has necessitated the search for new drugs. This therefore implies that there is need to find alternative drugs of which plant products is an option. Plant based antimicrobials have enormous therapeutic potential and are effective in the treatment of diseases while simultaneously mitigating many of the side-effects that are associated with synthetic antimicrobials. In addition plant based drugs are known not to easily develop resistance (Iwu *et al.*, 1999).

## METHODS AND MATERIALS

### Plant collection and identification

The plant materials were collected from Keiyo district and identified by a taxonomist in the Department of Botany, Egerton University.

### Chemical extraction and isolation

Mature leaves were washed with clean water to remove any dirt which could be a source of contamination. They were then chopped into small pieces and dried under shade until completely dry before being ground into fine powder. The powder was then sequentially extracted with hexane, ethyl acetate, chloroform, acetone and methanol. The solvents were removed by rotor evaporation under vacuum to yield five extracts. The solvents were stored in universal bottles.

### Pharmacological screening

The antimicrobial activity of the extracts was tested according to the National Committee of Clinical Laboratory Standards against the following microorganisms: *Staphylococcus aureus* KEMRI, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Candida albicans*, all sourced from KEMRI. Freshly grown microbial suspensions in Mueller Hinton Broth were standardized to a cell density of  $1.5 \times 10^8$  (McFarland No. 0.5). The positive antimicrobial and antifungal activities were established by the presence of clear measurable zones of inhibition after 48 hours at 4°C to allow for diffusion of the extracts into the media and 24 hours of incubation at 37°C. Chloramphenicol and nystatin were used as standards.

### Phytochemical tests

The solvent extracts from *K. densiflora* were subjected to phytochemical tests to test for general

classes of compounds. The results obtained are as shown in **Table 1**.

**Flavonoids:** 5ml of dilute aqueous ammonia solution were added to a portion of the plant extract followed by addition of concentrated sulphuric acid. Positive test was indicated by yellow colouration which disappeared on standing.

**Saponins:** 3 drops of olive oil were added to the plant extract and shaken vigorously in a tube. Positive test was indicated by formation of an emulsion.

**Tannins:** A few drops of 1% Ferric chloride were added to the plant extract in a tube. Positive test was indicated by formation of brownish green coloration.

**Phlobatannins:** A small portion of the extract was boiled with an equal volume of 1% aqueous hydrochloric acid. Positive test was indicated by depositions of a red precipitate.

**Terpenoids (Salkowski test):** 5ml of the extract was mixed with 2ml of chloroform followed by addition of 3ml of concentrated sulphuric acid to form a layer. Positive test was indicated by formation of a red coloration at the interface.

**Steroids:** 2ml of acetic anhydride and 2ml sulphuric acid were added to about 5ml of the extract. Positive test was indicated by change of colour from violet to blue or green.

**Cardiac glycosides (keller-killiani test):** 2ml of glacial acetic acid containing 1 drop of Ferric chloride solution was added to the extract. Then 1ml of concentrated sulphuric acid was slowly added to the solution. Positive test was indicated by formation of a brown ring at the interface. In most cases a violet ring may appear below the brown ring while in acetic acid layer a greenish ring may form gradually throughout the thin layer.

### **Chemical tests**

The extracts were tested for the following functional groups: permanganate test for unsaturated bonds, Lucas test for the presence of secondary and tertiary alcohol groups, sodium hydroxide test for the presence of phenolic groups, sodium hydrogen carbonate test for the presence of carboxyl groups and Tollens' test for the presence of aldehyde groups. The observations were noted and recorded. The results obtained were as shown in **Table 2**.

### **Antimicrobial sensitivity tests**

**Media preparation and sterilization:** 9.5g of Mueller- Hinton Agar and 16.25g of Sabouraud Dextrose Agar were reconstituted in 250ml of distilled water in 250ml flasks. They were thoroughly shaken to completely dissolve the nutrient and autoclaved at 121°C for 15mins. The sterilized media was allowed to cool in a water bath at 45-50°C. The freshly prepared and cooled medium was poured into plastic flat-bottomed petri dishes to give a uniform depth of about 4mm. The surface of the media was flamed to remove bubbles on the surface to prevent cracking of the media. The media was allowed to cool at room temperature and stored at 4°C in a refrigerator till used (within 7 days). The results obtained are as shown in **Table 3**.

### **Preparation and sterilization sensitivity disks**

Using a paper punching machine, discs of 6mm diameter were punched from whatman filter paper No 1. The disks were dispensed into a screw capped glass bottle and sterilized by dry heat in a hot air oven at 160°C for 60min. 10 discs were then immersed into each of the extracts and allowed to dry on a glass plate to rid them of any residual solvent. These were then stored separately in a sterile container at 4°C.

**Inoculation into liquid media:** The inoculums were prepared by making a direct broth of isolated colonies selected from an 18-24hours growth for each of the test micro-organisms. The suspension was standardized to a cell density of  $1.5 \times 10^8$  (McFarland No. 0.5).

**Inoculation into solid media:** 15 minutes after adjusting the pH of the inoculum suspension, a sterile cotton swab was dipped into the suspension, rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.

The dried surface of the Mueller-Hinton Agar prepared earlier were inoculated by stirring two or more times, while rotating the plate each to ensure an even distribution of the inoculum. The rim of the agar plate was then finally swabbed. This was left for about 5 minutes to allow for any excess surface moisture to evaporate before applying the impregnated discs.

**Application of discs to inoculated plates:** The pre-determined battery of discs for each particular isolate were taken out of the refrigerator and brought to room temperature. These discs were individually placed using fine sterile forceps on the inoculated agar plate with each plate taking 8 discs. This was done in duplicate for each of the eight microbes.

**Incubation:** The plates were placed in refrigerator at  $4^\circ\text{C}$  to allow the extracts to diffuse throughout the media. The petri plates were then incubated at  $37^\circ\text{C}$  in an inverted position for 24hours. The results were then read and recorded after 24hours.

### Minimum Inhibitory Concentration (MIC) Determination

Kirby- Bauer test was performed to determine the MIC of the extracts. This method is also called disc diffusion method. This was done as follows;

Serial tenfold dilutions (i.e.  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  etc) of the plant active fraction was carried out. Each serial dilution of the extract was impregnated with blank sterile sensitivity discs. The discs with respective dilutions were placed on a Mueller-Hinton agar plate to which 1ml bacterial broth culture had been inoculated. Eight discs with plant extracts of different dilutions were placed in the petri plate with inoculated micro-organisms. The plates were kept at  $4^\circ\text{C}$  for 48 hours to allow the extract to diffuse into the media. After which the plates were removed and incubated at  $37^\circ\text{C}$  for 24 hours. Zones of inhibition caused by the different dilutions of the extracts were measured in mm and were tabulated as shown in **Table 4**. The lowest concentrations of the different extracts at which inhibition occurred were deduced as minimum inhibitory concentrations.

## RESULTS

### Phytochemical tests and Chemical analysis

**Table 1:** Results of Phytochemical test for different extracts of *K. densiflora*

Test	Phytochemical test of compounds using various solvents					Conclusion
	Hexane	Ethyl Acetate	Chloroform	Acetone	Methanol	
Tannins	+	+	+	+	+	All extracts positive
Phlobotannins	-	-	+	-	-	Chloroform extract, only positive
Saponins	-	-	+	-	+	Chloroform extract, only

						positive
<b>Flavonoids</b>	-	-	+	-	+	Chloroform and Methanol extracts positive
<b>Steroids</b>	+	+	+	+	+	All extracts positive
<b>Terpenoids</b>	+	+	+	+	+	All extracts positive
<b>Cardiac glycosides</b>	+	+	+	+	+	All extracts positive

All the five extracts of *K. densiflora* tested positive for tannins, steroids, terpenoids and cardiac glycosides. Phlobotannins were present only in chloroform extract. In addition saponins were present in both chloroform and methanolic extracts (Table 1).

Analysis for the functional groups revealed that all the extracts contained unsaturated bonds, carboxyl groups and aldehydes as evidenced by the decolorization of  $\text{KMnO}_4$ , formation of effervescence and silver mirror respectively (Table 2).

**Table 2:** Results of *K. densiflora* extract functional group test using various solvents

Functional group	solvents				
	Hexane	Ethyl acetate	Chloroform	Acetone	Methanol
<b>Sodium bicarbonate</b>	++	++	++	+	+
<b>Sodium hydroxide</b>	-	-	-	-	-
<b>Lucas (alcohol group)</b>	-	-	-	-	-
<b><math>\text{KMnO}_4</math></b>	+	+	+	+	+
<b>Tollen's reagent</b>	+	+	+	+	+

### Antimicrobial sensitivity tests

**Table 3:** Results of antimicrobial sensitivity tests of different solvent extracts from *K. densiflora*

Microorganism	Source	Standard	Inhibition zone in millimeters (mm)				
			Hexane	Ethyl acetate	Chloroform	Acetone	Methanol
<i>E. coli</i>	<sup>b</sup> ATCC 25922	40	20	0	0	10	12
<i>S. aureus</i>	<sup>a</sup> KEMRI	30	0	0	0	18	17
<i>K. pneumoniae</i>	<sup>a</sup> KEMRI	20	0	0	0	10	18
<i>S. typhi</i>	<sup>a</sup> KEMRI	30	0	0	0	16	22
<i>P. mirabilis</i>	<sup>a</sup> KEMRI	25	0	0	0	12	20
<i>P. aeruginosa</i>	<sup>a</sup> KEMRI	20	0	0	0	14	16
<i>B. subtilis</i>	<sup>a</sup> KEMRI	40	0	0	0	18	25

<i>C. albicans</i> (fungus)	<sup>a</sup> KEMRI	20	0	0	10	15	18
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**Key:**

- <sup>a</sup> - American type culture collection
- KEMRI - Kenya Medical Research Institute

**Table 4:** Minimum inhibition concentrations (MIC) of solvent extracts from *K. densiflora*

Microorganism	Source	Standard	Inhibition zone in millimeters (mm)				
			Hexane	Ethyl acetate	Chloroform	Acetone	Methanol
<i>E. coli</i>	<sup>b</sup> ATCC 25922	25	93.3	0	0	82	0
<i>S. typhi</i>	<sup>a</sup> KEMRI	25	0	0	0	0	0
<i>K. pneumoniae</i>	<sup>a</sup> KEMRI	22.5	0	0	0	0	0
<i>P. aeruginosa</i>	<sup>a</sup> KEMRI	30	0	0	0	68.3	92
<i>S. aureus</i>	<sup>a</sup> KEMRI	31.3	0	0	0	0	0
<i>B. subtilis</i>	<sup>a</sup> KEMRI	26.3	0	0	0	0	92

**Antimicrobial activity**

**Table 3** shows the results of antimicrobial sensitivity of the different extracts against seven bacteria and a fungus. Not all the extracts exhibited antimicrobial activity. Hexane and methanol solvent extracts showed antimicrobial activity against *E. coli*. Acetone solvent extract showed antimicrobial activity on *K. pneumoniae* and *P. aeruginosa*. Methanolic extract showed antimicrobial activity against *K. pneumoniae*, *P. aeruginosa* and *B. subtilis*. However ethyl acetate as well as chloroform extracts did not show any antimicrobial activity. *C. albicans* showed resistance to all extracts except chloroform. The observed activity by the different extracts were however lower than those of the standard. The best activity was shown by hexane extract against *E. coli* with a zone of inhibition of 20mm and methanolic extract against *P. aeruginosa* also with 20 mm inhibition. This was equivalent to that of standard. However that of *E. coli* was below that of standard (40mm). The observed antimicrobial activity was suspected to be due presence of flavonoids, steroids, terpenoids and cardiac glycosides in the extracts. These compounds are known to possess antimicrobial activities (Cohen, 1999).

On the other hand resistance was observed against the extracts by *S. typhi*, *C. albicans*, *P. mirabilis*, *P. aeruginosa* and *S. aureus*. This resistance was suspected to be due to the ability of the microorganisms to modify the original antimicrobial target in such a way that the drug no longer binds to it. Other microorganisms such as *S. aureus* are capable of synthesizing enzymes that inactivate the antimicrobial agent. Most of the gram negative bacteria exhibited resistance. This could have been due to the fact that gram negative bacteria possess an additional outer membrane that confers resistance factors. Production of biological targets having a reduced activity for antibiotic is a very common source of resistance mechanism used by various organisms (Spratt, 1994).

### Minimum Inhibitory Concentration

Minimum inhibitory concentration was deduced as the lowest concentration of the extract that inhibited visible microbial growth. Generally the extracts showed minimum inhibitory concentration (MIC) ranging between 82-127.5mg/ml and 92-93.3mg/ml for gram positive and gram negative bacteria respectively, **Table 4**. It was therefore evident that the extracts had better activity against gram positive bacteria. The MICs values were lower than those of the standard.

### Conclusion

*K. densiflora* leave extracts had antimicrobial activity against gram positive bacteria (*S. Aureas* and *B. subtilis*) and gram negative bacteria (*E. coli*, *P. aeruginosa* and *K. pneumoniae*). Thus the extracts can be used in the treatment of diseases caused by the micro-organisms inhibited by the extracts.

Extracts of *K. Otensiflora* using acetone and methanol solvents have antimicrobial activity against *S. Aureus* (13mm), *P. aeruginosa* (20mm) and *B. subtilis* (14mm). *E. coli* are highly sensitive to *K. densiflora* extracts using hexane as solvent (20mm). Extracts of *K. densiflora* have antimicrobial activity against both gram positive (*S. Aureus* and *B. subtilis*) and Gram negative organisms (*P. aeruginosa* and *E. coli*). *P. aeruginosa* and *K. pneumoniae* show least sensitivity.



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