

In Vitro Analysis of Antibacterial and Antifungal Potency of Tissue Cultured and Indigenous Aloe Secundiflora Plant Extracts

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Abstract

In vitro studies on the antimicrobial potency of extracts from tissue cultured and indigenous Aloe secundiflora plants were conducted. Mature tissue cultured and indigenous A. secundiflora plants were collected from Jomo Kenyatta University of Agriculture and Technology (JKUAT) and Kenya Forestry Research Institute (KEFRI), Gede forest respectively. Crude, chloroform (non polar solvent) and ethanol (polar solvent) extracts were prepared from both indigenous and tissue cultured A. secundiflora plants. The antimicrobial activities was evaluated using a standard strain of Pseudomonas aeruginosa (ATCC 27853), Rhizopus stolonifer isolate cultures obtained from bread, clinical isolates of Candida albicans, Staphylococcus aureus and Escherichia coli obtained from Mewa hospital in Mombasa district, Mombasa county. It was observed that the crude extracts from tissue cultured and indigenous A. secundiflora plants had antibacterial activities on both P. aeruginosa and S. aureus but was not active against R. stolonifer, E. coli and C. albicans. The antimicrobial potency between the same extract from tissue cultured and indigenous A. secundiflora plants had no significant difference ($P>0.05$). However, the antimicrobial potency of different extracts from either the tissue cultured or indigenous A. secundiflora plants varied significantly ($p<0.05$). The data suggests that both tissue cultured and indigenous A. secundiflora could be a rich source of antimicrobial agents. Therefore tissue culture is recommended as a means of propagating the plant to meet the demand in the production of antimicrobial agents other products.

Keywords: Antimicrobial potency; Aloe secundiflora tissue cultured; indigenous; antimicrobial agents

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1. Introduction

1.1 Problem Statement

The growing popularity of tissue cultured *A. secundiflora* seems to replace the indigenous *A. secundiflora*. The adoption of tissue culture method for faster propagation may have an impact on the phytochemical present in the plant. There may be alteration of active components during propagation leading to differences between tissue cultured and indigenous *A. secundiflora*.

1.2 Importance of the Problem

Naturally *Aloe secundiflora* propagates through the use of seeds. This natural method of propagation is too slow for commercial cultivation and hence the need for tissue culture techniques of propagation to produce large numbers of plants for establishing plantations. The method facilitates faster multiplication and growth of the plants by providing them with the right hormonal balance. This helps in production of infinite number of plants from one piece in a relatively short period of time. Plantations of *Aloe secundiflora* are being established to protect wild populations and increase production (Newton *et al.*, 2006).

1.3 Research Scholarship

The aloe plant is known to have several medicinal uses and has been used for the treatment of digestive disorders like heartburn and irritable bowel syndrome as well as in the treatment of wounds (Davies *et al.*, 1987). The plant's extracts have also been found to stimulate skin fibroblast (Danhoff *et al.*, 1983). In the cosmetic industry, the extracts are used in lotions as skin moisturizers for dry skins. The plant is also used as a live fence and in flower beds to add aesthetic value to the environment. Antimicrobial properties of *Aloe* have been reported (Waihenya *et al.*, 2002; Richard *et al.*, 2011).

The major components of the leaf exudates of *Aloe secundiflora* are the anthrones aloenin, aloenin B, aloin A (barbaloin) and other aloin-derivatives. The exudates also contain chromones and phenylpyrones, and the phenyl-ethylamine alkaloid N-methyltyramine (Newton *et al.*, 2006).

The tannins could be disrupting the cell membranes of the microorganisms, hence their inhibitory activities (Richard *et al.*, 2011). *Aloe secundiflora* contains phenol compounds like anthraquinones in their sap. These compounds are potent antimicrobial agents (Lorenzetti *et al.*, 1964, Sim *et al.*, 1971) and can be used to inhibit growth of both bacteria and fungi (Msoffe *et al.*., 2009) a property making them popular when used in herbal medicine. Provision of cheaper sources of preservative agents in baking industry cosmetics and medication for opportunistic diseases is a priority. The current study seeks to establish the difference in anti-microbial potency between the indigenous and tissue cultured *Aloe secundiflora*.

1.4 Hypotheses

HO: Indigenous and tissue cultured *Aloe secundiflora* has no antibacterial inhibitory effects

HO: Indigenous and tissue cultured *Aloe secundiflora* has no antifungal inhibitory effects

HO: Methanol and chloroform extracts of both tissue cultured and indigenous *Aloe secundiflora* do not show antimicrobial effects.

2. Materials and Methods

2.1 Plant Material Collection, Identification and Extract Preparation

Mature tissue cultured and indigenous *Aloe secundiflora* were collected from Jomo Kenyatta University of Agriculture and Technology (JKUAT) and Kenya Forestry Research Institute (KEFRI) Gede forest (Malindi) respectively between January and March 2011. The plants were authenticated by a plants taxonomist from the JKUAT and KEFRI, in whose herbarium the voucher specimens are deposited.

The fresh leaves of both the tissue cultured and indigenous plants were chopped into small pieces, blended in to fine particle using a blender. The blended material was transferred into a 250 mL beaker and extracted using chloroform, a non polar solvent for 24 hours. The mixture was separated using separating funnel. The liquid part composed of chloroform and non polar active components were recovered after which the solvent was evaporated in a fume chamber.

The remaining layer of plant materials in the separating funnel was transferred into a 250 mL beaker and extracted using ethanol, a polar solvent for 24 hours. The mixture was separated using separating funnel. The liquid part composed of ethanol and polar active components were recovered then the solvent evaporated in a fume chamber.

2.2 Test Microorganisms

The three bacterial strains and two fungal species were used for the antimicrobial assays. *Escherichia coli* and *Pseudomonas aeruginosa*, clinical isolate of *Staphylococcus aureus*, clinical isolate *Candida albicans* and laboratory isolate of *Rhizopus stolonifer* commonly known as black bread mold (BBM) were used. *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 25853) were obtained from the Department of Pure and Applied Science, Mombasa Polytechnic University College (MPUC) *S. aureus* (ATCC 20591) and *C. albicans* (ATCC EK138), were obtained from Mewa Hospital in Mombasa, Kenya.

2.3 Evaluation of Antibacterial and Antifungal Activity

The antibacterial and antifungal activities of the extracts were assayed *in vitro* using agar disc diffusion (DD) method (Parekh and Chanda, 2007). Mueller Hinton agar (MHA) and Potato Dextrose Agar (PDA) were prepared using manufacturer's instructions for purposes of culturing the bacteria and fungi respectively. Normal saline solution was used to dilute a 24-hours culture of the bacterial type culture or clinical isolate to attain a 0.5 McFarland standard.

1 mL of the bacterial suspensions cultured using spread plate method (Meite et al., 2009) was introduced into separate Petri dishes. Dry sterile filter paper disk (6 mm in diameter) were prepared and impregnated with the plant extracts made by dissolving 300 mg of the extracts in 10 mL of dimethylsulfoxide (DMSO), air-dried and placed on the spread plates at reasonable distances using forceps. Filter discs impregnated with DMSO were used as negative control and various standard conventional antibiotics streptomycin, tetracycline and gentamycin as positive controls. The plates were then incubated at 35°C for 24 h. This was replicated three times for each pathogen.

1 mL of cultured suspension of *C. albicans* and BBM in a broth were spread on PDA plates separately.

A prepared inoculum of young mycelium was placed at the centre of the PDA plates. Dry sterile filter paper disk prepared and impregnated with 1mL of the plant extracts placed at a distance around the mycelium inoculums. The inoculums were incubated at 25°C for 24 hours. Fluconazole (Pfizer, UK) and dry discs treated with DMSO were also used as positive and negative controls respectively. All tests were performed in triplicate. Microbial growth inhibition was determined by measuring the zones of inhibition using a transparent ruler.

2.4 Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC)

The Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of the chloroform and ethanol extract against the bacterial strains were determined by tube-dilution method. The MIC test was done in test-tubes in Nutrient broth with chloroform and ethanol extracts with serial dilutions ranging from 9.2×10^0 mg/ml to 9.2×10^{-9} mg/ml. Approximately 1×10^7 cfu/ml of inoculums of test- bacterial strains were prepared. 1 mL of the chloroform and ethanol extracts for each concentration was then added to 1 mL of the test-bacterial suspension. Test tubes containing 1 mL of the extracts and 1 mL of DMSO were used as positive and negative control respectively. The test-tubes were incubated at 37°C for 24 hours. The tube with lowest concentration yielding no visible growth but giving colonies on media was considered as MIC. For MLC, the test-tube without growth from the MIC procedure were streaked onto Muller Hinton agar plates. The plates were then incubated at 37°C for 24 hours. The lowest concentration that killed 100% of the test-bacteria (no growth on plate) was recorded as MLC. All tests were performed in triplicate.

3. Results

3.1 Data Analysis

Data was analyzed using the Minitab Statistical Software 13.20, © 2000 Minitab Inc. PA 16801-9928, USA. Among the groups, significance test was performed using student's t-test and one-way ANOVA at 95% significance levels and $P < 0.05$ was considered to be statistically significant.

3.2 Inhibitory Effects

The antibacterial and antifungal activities of the chloroform and ethanol extracts of both tissue cultured and indigenous *A. secundiflora* were evaluated. The extracts tested showed varying degree of antibacterial activities against the test microorganisms [Table 1].

Table 1: Antibacterial and Antifungal Activities of Tissue Cultured and Indigenous *a. Secundiflora* Chloroform and Ethanol Extracts

Isolates	Diameters of zones of inhibition(mm)±SE					
	Chloroform extract			Ethanol extract		
	Tissue culture	Indigenous	Standard	Tissue culture	Indigenous	Standard
<i>P. aeruginosa</i>	11.33±1.43	12.00±0.00	24.00±0.00	8.66±3.79	8.33±1.43	24.00±0.00
<i>E. coli</i>	7.67±3.79	8.00±3.48	12.00±0.00	7.33±1.43	6.33±1.43	12.00±0.00
<i>S. aureus</i>	16.00±0.00	16.33±1.43	29.00±0.00	17.00±2.48	17.33±1.43	29.00±0.00
<i>R. stolonifer</i>	-	-	-	-	-	-
<i>C. albicans</i>	-	-	-	-	-	-

The plant extracts generally had antimicrobial activity [Table 1]. The activity of the different plant extracts was not significantly different ($p \geq 0.05$).

The extracts were not as active as the positive controls, streptomycin (24.00 mm) for *P. aeruginosa*, tetracycline (12.00 mm) for *E. coli* and gentamycin (29.00 mm) for *S. aureus* [Table 1]. The chloroform extract of indigenous plant was higher than the chloroform extract of tissue cultured plant while the ethanol extract of tissue cultured plant was higher than the ethanol extract of indigenous plant against the evaluated microorganism.

The antibacterial activities of both chloroform and ethanol extracts of tissue cultured and indigenous plants followed the *S. aureus*, *P. aeruginosa* and *E. coli*. *C. albicans* and *R. stolonifer* were not sensitive to all extracts tested [Table 1]. The antibacterial activities of the chloroform and ethanolic extracts from tissue cultured and indigenous plants compared favorably and had no significant difference ($p \geq 0.05$).

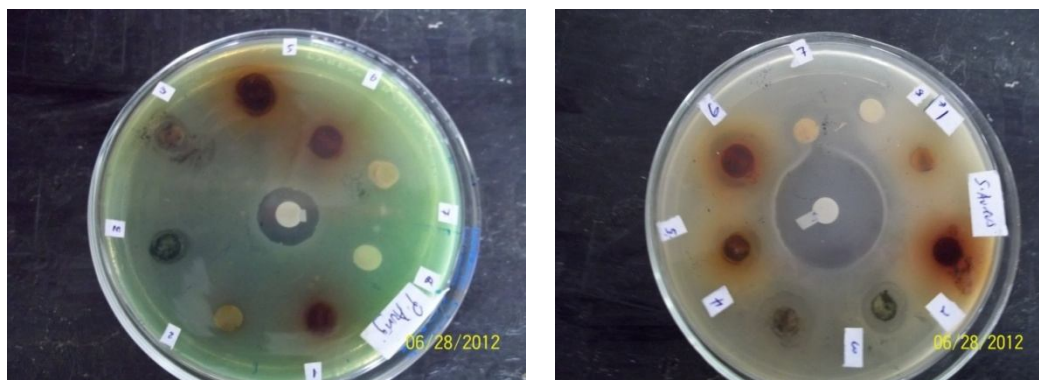


Plate I

Plate II

Fig 1: Shows Plates I and II with Inhibition Zones

Plate I; Zones of inhibitions of *P. aeruginosa*, at 3 and 4, is chloroform tissue culture extract and chloroform indigenous extract respectively. Plate II; Zones of inhibition of *S. aureus* at 3,4,5 and 6 is chloroform tissue culture extract, chloroform indigenous extract, ethanol tissue culture extract and ethanol indigenous extract respectively.

Ethanol and chloroform extracts of both tissues cultured and indigenous *A. secundiflora* was only effective for *P. aeruginosa* at MIC value $9.2 \times 100 \text{ mg mL}^{-1}$. The extracts did not show any MLC properties, as the subculture of the tubes with the MIC of the plant extracts resulted in to growth of *P. aeruginosa* colonies on Muller Hinton agar.

Table 2: Minimum Inhibitory/ Bactericidal Concentrations (Mg MI⁻¹) Of Extracts of Aloe Secundiflora

Plant Extract	Microorganism	Concentration (9.2 x10 ^x mg mL ⁻¹)									
		10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹
Chloroform extract from Tissue cultured plant	<i>P. aereginosa</i>	+	-	-	-	-	-	-	-	-	-
	<i>E. coli</i>	-	-	-	-	-	-	-	-	-	-
	<i>S. aureus</i>	-	-	-	-	-	-	-	-	-	-
	<i>R. stolonifer</i>	-	-	-	-	-	-	-	-	-	-
Chloroform from Indigenous plant	<i>P. aereginosa</i>	+	-	-	-	-	-	-	-	-	-
	<i>E. coli</i>	-	-	-	-	-	-	-	-	-	-
	<i>S. aureus</i>	-	-	-	-	-	-	-	-	-	-
	<i>R. stolonifer</i>	-	-	-	-	-	-	-	-	-	-
Ethanol extract from Tissue cultured plant	<i>P. aereginosa</i>	+	-	-	-	-	-	-	-	-	-
	<i>E. coli</i>	-	-	-	-	-	-	-	-	-	-
	<i>S. aureus</i>	-	-	-	-	-	-	-	-	-	-
	<i>R. stolonifer</i>	-	-	-	-	-	-	-	-	-	-
Ethanol extract from Indigenous plant	<i>P. aereginosa</i>	+	-	-	-	-	-	-	-	-	-
	<i>E. coli</i>	-	-	-	-	-	-	-	-	-	-
	<i>S. aureus</i>	-	-	-	-	-	-	-	-	-	-
	<i>R. stolonifer</i>	-	-	-	-	-	-	-	-	-	-

Key: (+) Indicates growth, (-) No growth

4. Discussion

In this study, the activity of crude, chloroform and ethanol extracts of the tissue cultured and indigenous *A. secundiflora* (9.2 mg/ml) against tested microbial strains (Table 1) showed that the plants contain pharmacologically active substances. Bacterial strains, *S. aureus* was sensitive to both chloroform and ethanol extracts while *P. aeruginosa* was only sensitive to chloroform extract from both tissue cultured and indigenous *A. secundiflora* with zones of inhibition being ≥ 9.00 mm. The antimicrobial potency could be attributed to the presence of active antimicrobial compounds. These results agree with those of Ejoba, (2012), who showed that *Aloe Species* plant leaves contain alkaloids, tannins, flavonoids, carbohydrates, and terpenoids which have antimicrobial activity.

The activity on *S. aureus* of the chloroform and ethanol extract could also be traced to the plant possessing acemanan, which has been scientifically proved to be able to stimulate the defenses of the body against staphylococcal infections (George and Pamplona, 1998).

Escherichia coli, fungi and yeast were not sensitive to either chloroform or ethanol extracts from both tissue cultured and indigenous *A. secundiflora*. This could have been due to the absence or low concentration of active antimicrobial component in both chloroform and ethanol extract of tissue cultured and indigenous *A. secundiflora*. The ineffectiveness of these extracts on the bacteria and all the fungi tested may also be attributed to the fact that the solvents could not extract active components of the plant. It could also be that the main active components of the *A. secundiflora* plant are only antibacterial. Except for *R. stolonifer* and *C. albicans*, general antibacterial results were also noted (table 3). The activity of extracts against various test microorganisms was statistically significant at $P \leq 0.05$.

There was no significant difference in the potency of extracts from the tissue cultured and indigenous *A. secundiflora* plants. A zone of inhibition ≥ 9.00 mm is an indication of strong antimicrobial activity. The zones of inhibition from the three treatments were statistically different. These results were in agreement with the previous studies concerning the *in vitro* antimicrobial activity of *Aloe spp.* which showed that *Aloe* Extracts had antimicrobial activities against both Gram positive and Gram negative bacteria including *S. aureus*, and *Klebsiella pneumonia* and that organic extracts of *Aloe*, including ethanolic or petroleum ether extracts, were more effective than aqueous extracts (Ndhala *et al.*, 2009; Pandey *et al.*, 2010).

Comparative study of the activities of ethanol extract correlated to chloroform extract irrespective of whether tissue cultured or indigenous *A. secundiflora* plants did not show statistical significant difference at $P \leq 0.05$. These results were in agreement with the previous studies concerning "*in vitro* Antioxidant activities of *Aloe spp.* leaf skin extracts", by Sonia Miladi and Mohamed Damak (2008) where the chloroform-ethanol fractions showed the highest amount of phenolic compounds followed by the ethyl acetate extract, the butanol extract and the hexane extract.

Minimum inhibitory concentration of both chloroform and ethanol extract of tissue cultured and indigenous *A. secundiflora* against *S. aureus* and *P.*

aeruginosa were determined. Potency effectiveness was only shown against *P. aeruginosa* at MIC of 9.2×10^0 mg mL⁻¹. These results support those by Mariita *et.al* (2009) which showed antimicrobial activity against *P. aeruginosa* at MIC of 9.375 mg mL⁻¹. On the other hand, there was no MIC recorded for both extracts on *Staphylococcus aureus*. In the same study there were no MLC recorded for both extracts on the two microorganisms tested. This was due to low concentration of both extract as a study by Mariita *et.al* (2009) shows ethanolic extract was effective against various microorganisms as in *P. aeruginosa* (MBC/MLC of 18.75 mg mL⁻¹) and *S. aureus* (with MICs and MBCs of 37.5 mg mL⁻¹).

5. Conclusion

Comparing the antimicrobial activity of the crude chloroform and ethanol extracts of the tissue cultured and indigenous *A. secundiflora* there was no significant difference noted on the potency of tissue cultured extracts compared to indigenous *A. secundiflora* extract. The crude extracts from either tissue cultured or indigenous *A. secundiflora* plants could thus be useful in the development of new, alternative and cheap antimicrobial drugs, especially against infections caused by the *S. aureus* and *P. aeruginosa*. Therefore tissue culture is recommended as a means of propagating the plant to meet the demand in the production of antimicrobial agents other products.

6. Recommendation

Further work is needed to speed up the growth and maturity of the *A. secundiflora* plants through genetic engineering. Research is also needed to increase the amount of active components in *A. secundiflora* plants as the current amount is slow. There is also the need for continued research on the presence of antimicrobial active components in the roots of both tissue cultured and indigenous *A. secundiflora* plants. Otherwise this study lays the basis and open the way for enhanced phytochemical and pharmacological research.

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7.0 References

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