BACTERIAL EMPIRE
2020, VOL. 3, NO. 3, 20-24

PHYTOCHEMICAL AND ANTIMICROBIAL PROPERTIES OF LEAF EXTRACTS OF *Calliandra calothyrsus, Leucaena diversifolia* AND *Sesbania sesban*

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https://doi.org/10.36547/be.2020.3.3.20-24

ABSTRACT

Phytochemical compounds are secondary metabolites of plants useful as antimicrobial agents. Botanicals are being explored for bioactive compounds with antimicrobial properties against phytopathogens. Little information is available on the phytochemical and antimicrobial activity of *Calliandra calothyrsus, Leucaena diversifolia* and *Sesbania sesban* against *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *musacearum*. The aim of the study was to determine the phytochemical and antimicrobial properties of leaf extracts of *C. calothyrsus, L. diversifolia* and *S. sesban* against *C. zeae-maydis* and *Xc.* pv. *musacearum*. Dried leaves were extracted in methanol and aqueous solvents and screened for phytochemical and antimicrobial activity using Kirby-Bauer's disk diffusion and poisoned food technique methods. *Sesbania sesban* extracts contained all the phytochemical tested; tannins, terpenoids, steroids, saponins, flavonoids, and alkaloids, *Leucaena diversifolia* lacked alkaloids while *Calliandra calothyrsus* lacked steroids and alkaloids. The extracts were active against *Cercospora zeae-maydis* and *Xc.*pv. *musacearum* with *Sesbania sesban* having greater radial inhibition activity. There was no significant difference in the antimicrobial activity between the lowest concentrations (25% and 25mg/ml) and highest concentrations (75% and 75mg/ml) in all the three plant extracts. Growth inhibition observed could be as a result of the different chemical compound observed in the extracts. Presence of alkaloids in *Sesbania sesban* could explain the greater growth inhibition of the pathogens under study. The results form the basis for further research that could lead to isolation and development of antimicrobial agents. Therefore, these plants can be used as an alternative to synthetic chemicals to control *Cercospora zeae-maydis* and *Xanthomonas campestris* pv. *Musacearum.*

Keywords: Phytochemical, antimicrobial**,** *Cercospora zeae-maydis* and *Xanthomonas campestris*pv. *musacearum*

INTRODUCTION

Plants have been a source of novel metabolites useful in therapeutics and antimicrobial since invent of traditional medicine (**Araya-Contreras and Bittner, 2019; Alemu *et al.*, 2017**). They synthesize array of mixtures of secondary metabolites called phytochemicals that are used in treatment of some diseases and management of microbial related diseases (**Ugweko *et al.*, 2017; Banu and Cathrine, 2015**). The type, quality and concentration of phytochemical in a plant is a function of both agronomic and environmental factors of ecological zone in which the plant is growing **(Borges *et al.*, 2018; Kumar *et al.*, 2017; Liu *et al.*, 2016; Liu *et al.*, 2015**). Besides environmental factors, age of the plant, relative humidity of harvested materials and method of extraction have great influence on the variation of phytochemical concentration and toxicological activity of the plant extracts (**Borges *et al.*, 2018; Izah 2018**). It is therefore important to screen different plant species from various regions to reveal their chemical compounds distributions. Plants with useful secondary metabolites may be cultivated to be used as food supplements (**Borges *et al.*, 2018; Hossain *et al.*, 2013**) or may be harvested for medicinal purposes (**Ugweko *et al.*, 2017**). However, most of the plants used in folk medicine are understudied in relation to their phytochemical composition which are pillars of traditional medicines. Phytochemical compounds which have been studied in different plants include flavonoids, phenols and phenolic glycosides, saponins and cyanogenic glycosides, stilbenes, tannins, nitrogen compounds (alkaloids, amines, betalains), terpenoids and steroids (**Borges *et al.*, 2018; Igbal *et al.*, 2015; Vaghasiya *et al.*, 2011**).

Most of the studies on the antimicrobial activity of plant crude extracts have focused majorly on human and animal pathogens (**Ugweko *et al.*, 2017; Zayed *et al.*, 2011**) while neglecting phytopathogens which have ravaged food crops hence compromising food security. Although little has been done on antimicrobial activity of plant extracts against phytopathogens, some plants have shown promising results in management of plant pathogens. For instance, some plant extracts have been reported to inhibit the growth of *Fusarium guttiforme* responsible for fusariosis in pineapples (**Sales *et al.*, 2016**). Extracts from *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana* are active against *Aspergillus niger* and *Aspergillus parasiticus* pathogens of fruits (**Mahlo *et al.*, 2016**). Similarly extracts from *Bidens pilosa* and *Euphorbia hirta* demonstrated antimicrobial activity against *Xanthomonas campestris* pv. *vescatoria* (**Emitaro *et al.*, 2018**). Considering the fact that most plants if not all have biologically active compounds against pathogens (**Salhi *et al.*, 2017; Mahlo *et al.*, 2016**), it is therefore necessary to screen *Calliandra calothyrsus*, *Leucaena diversifolia* and *Sesbania sesban* for phytochemical and antimicrobial properties.

Calliandra calothyrsus Meisn. is a small leguminous shrub that is predominantly cultivated as fodder for ruminant livestock (**Setyawati *et al.*, 2019**) but other uses are also found within different farming systems and include the provision of green manure, fuel wood, shade for coffee and tea, land rehabilitation, erosion control, and honey production (**Setyawati *et al.*, 2019; Abia *et al.*, 2006**). *Sesbania sesban* (L.) Merrill is a multipurpose tree that is widely distributed in tropics and subtropics of Africa and Asia and usually planted by smallholder farmers mostly for its fodder and soil improvement values **(Nigussie and Alemayehu, 2014; Mythili and Ravindhran, 2012**). It is used also as a source of green manure, anti-inflammatory activities, reproduction and milk production enhancement, nitrogen fixation, bioenergy source, antibacterial and anti-parasitic effect, antioxidant and mosquito repellant effects (**Nigussie and Alemayehu, 2013; Degefu *et al.*, 2011**). *Leucaena diversifolia* is an erect tree shrub that grows well in cool and seasonally wet locations and provides crude protein for livestock, control soil erosion and fix nitrogen in soils (**Walker, 2012; Orwa *et al.*, 2009**).

Even though there are no reports on the antimicrobial activity of *Calliandra calothyrsus* and *Leucaena diversifolia*, extracts from related species *Calliandra haematocephala* and *Leucaena leucocephala* have been reported to be active against Gram positive and Gram negative bacteria (**Josephine *et al.*, 2017; Chew *et al.*, 2011**). *Sesbania sesban* has been shown to possess phytochemical compounds with antimicrobial activity (**Gomase *et al.*, 2012; Kathiresh *et al.*, 2012**). While plants are being considered reliable sources of antimicrobial compounds, the antimicrobial activity of *C. calothyrsus*, *L. diversifolia* and *S. sesban* against *Cercospora zeae-maydis* and *Xanthomona scampestris* pv. *musacearum* has not been documented. Similarly, because of the effect of environment on phytochemical constituents, it is necessary to study the phytochemical composition and distribution among the three plant species. This will go along with identifying alternative measures of disease control using botanicals and understanding the variation in phytochemical components of plant in varying ecological zones. This study therefore aimed at identifying the phytochemical constituents and evaluating the antimicrobial properties of *C. calothyrsus*, *L. diversifolia* and *S. sesban* leaf extracts.

MATERIALS AND METHODS

Collection and processing of plant materials

Leaves of *C. calothyrsus*, *L. diversifolia* and *S. sesban* were collected in nonwoven bags from demonstration plots of Maseno University located 0° 10' 0" South, 34° 36' 0" East along Kisumu Busia road in western Kenya. They were transported to the botany laboratory for extraction. They were washed in tap water, air dried under shade for 14 days with periodic turning of two days then crushed into fine powder using electrical motor. Fine powder was used for extraction with both methanol and aqueous solvents according to Dent *et al.* (2013) where 50 grams of each powdered plant leaf materials were separately kept in 500 ml conical flask and 500 ml methanol and aqueous added and thoroughly mixed respectively. The mixtures were left to stand overnight on a shaker for complete extraction, filtered using muslin cloth followed by Whatman no 1 filter paper. Methanol was evaporated using rotary vacuum evaporator with the water bath temperature of 45ºC. The filtrate was used to test for the presence of phytochemicals and antimicrobial activity of the extracts.

Phytochemical screening of the extracts

The presence of steroids, alkaloids, flavonoids, saponins, tannis and terpenoids in leaves of *C. calothyrsus*, *L. diversifolia* and *S. sesban* were determined as indicated below.

Steroids: Fifty (50) mg of sample was dissolved in chloroform, filtered, then heated plus anhydrous acetic acid and cooled. Concentrated sulfuric acid was added through the walls of the tube drop wise and formation of a brown ring indicated the presence of steroids (**Setyawati *et al.*, 2019**).

Terpenoids: The crude extract was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulfuric acid was added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. Formation of deep red color in the lower layer indicated a positive test for terpenoids (**Bhandary *et al*., 2012**).

Saponins: Test solution was mixed with water, shaken and observed for the formation of froth, which is stable for 15 minutes for a positive result (**Gul *et al.*,2017**).

Alkaloids: The plant extract was warmed with 2% H_2SO_4 for two minutes, filtered and few drops Mayer's reagent added. Appearance of a creamy- white color precipitate indicated a positive result (**Sheel *et al.*,2014**).

Flavonoids: 2 ml of 2.0% NaOH mixture was mixed with aqueous plant crude extract. A concentrated yellow color was produced, which became colorless when 2 drops of dilute H_2SO_4 acid was added. Colorless appearance indicated presence of flavonoids (**Gul *et al.*, 2017**).

Tannins: A small quantity of the extract was boiled with 5 ml of 45% solution of ethanol for 5 minutes, cooled and filtered. 1ml of filtrate was diluted with distilled water and two drops of ferric chloride added. A transient greenish to black color indicated the presence of Tannins (**Sheel *et al.*, 2014**).

Isolation of fungal pathogen *Cercospora zeae-maydis.*

Maize leaves showing characteristic symptoms were collected from the fields, cut into pieces of approximately 5cm, placed on sterile moist blotter in a sterile petridish and incubated at 25ºC for 5 days for the pathogen to sporulate. Conidia were picked with an isolation needle under dissecting microscope and plated on PDA. Plates were incubated at 25ºC for 5-7 days and hyphal tips from the advancing colony margins with typical morphological characteristics were transferred onto PDA with isolating needle as pure culture and kept at 5ºC (**Nega *et al.*, 2016**).

Isolation of bacterial pathogen *Xanthomonas campestris* pv. *musacearum*

The bacterial pathogen was isolated from diseased banana leaves according the procedure of **Adriko *et al.* (2016)**. Approximately 1g sample of infected leaves was crushed in 1 ml of sterile distilled water in a petridish. The suspension was spread on semi-selective YPGA (Yeast extract-5 g l−1, Peptone-5 g /l, Glucose-4 g /1, Agar-12 g/1) medium containing antibiotics cephalexin (40 mg/1), 5-fluorouracil (10 mg/1) and cycloheximide (120 mg/1). The inoculated plates were incubated at 28ºC for 48–72 hr and mucoid yellow-pigmented colonies were picked and purified on nutrient agar (NA) medium.

Determination of antimicrobial activity

Methanol extracts were reconstituted by Dimethyl sulfoxide (DMSO) to make concentrations of 12.5mg/ml, 25mg/ml/ 50mg/ml and 75mg/ml while aqueous extracts were reconstituted into concentrations 12.5%, 25%, 50% and 75% and used for antimicrobial studies. Disc diffusion method was used to assess the sensitivity of the bacterial pathogen to plant extracts (Bauer *et al*., 1966). Colonies from pure culture were lawn spread on MHA plates and discs impregnated with 10µl of each test extract placed on the surface aseptically while discs impregnated with pure water and DMSO served as negative control. Every treatment was replicated thrice, plates arranged in completely randomized design and incubated at 30ºC for 48 hours and zone of inhibition measured in millimeters. Antifungal activity of the extracts was determined using poisoned food technique according to **Durgeshlal *et al.* (2019)**, by dispensing 4 ml of each extract in petri plates and adding 16 ml of PDA then mixing and allowing them to set. A 5mm mycelia plug from 7 day old mycelia was inoculated at the center of the plates and plates without extracts served as control in triplicates. Plates were incubated for 7 days at 28ºC where fungi mycelia radial growth was measured and inhibition percentage determined using the formula of **Durgeshlal *et al.* (2019)**;

Inhibition (%) = $[(D_c – D_T)/D_c] \times 100$

D_C and D_T are the colony diameters of the control and treated sets respectively

RESULTS

Phytochemical screening

Phytochemical screening revealed that terpenoids, flavonoids and saponins were present in the leaf extracts of the three plant species (Table 1). Tannins were present in *C. calothyrsus* and *S. sesban* but absent in *L. diversifolia*. Steroids were present in *L. diversifolia* and *S. sesban* but absent in *C. calothyrsus*. Alkaloids were present only in the leaf extracts of *S. sesban*. *Calliandra calothyrsus* had higher concentrations of tannins, terpenoids, saponins and flavonoids while *S. sesban* contained higher amounts of steroids and alkaloids.

Table 1 Leaf phytochemical compounds of *C. calothyrsus, L. diversifolia* and *S. sesban*

Plant species	Tannins	Terpenoids	Steroids	Saponins	Flavonoids	Alkaloids
C. calothyrsus	++	++	-	++	++	-
L. diversifolia	-	+	+	+	++	-
S. sesban	+	+	++	++	+	++

Key. - = Absent, += Present in low concentration, ++= Present in high concentration.

Antimicrobial activity of the extracts

Antimicrobial activity of leaf extract of *S. sesban, C. calothyrsus* and *L. diversifolia* against *Xc*.pv. *musacearum* and *Cercospora zeae-maydis*

The antimicrobial activity of leaf methanol and aqueous extracts of *S. sesban* , *C. calothyrsus* and *L. diversifolia* against *Xc*. pv *musacearum* and *Cercospora zeae-maydis* were significantly different with the P value of (P=0.0014 and P=0.0001) in methanol extract and (P=0.0016 and <.0001) in aqueous extract for *Xc*. pv *musacearum* and *Cercospora zeae-maydis* respectively. *Sesbania sesban* produced largest mean zone of inhibition of 13.9 mm for bacteria and inhibition percentage of 72.2% for fungi in methanol extracts and 13mm for bacteria and 78.3 % for fungi in aqueous extracts compared to *C. calothyrsus* and *L. diversifolia* (Tables 2 and 3). There was no significant ($p \leq 0.05$) difference in the mean zone of inhibition between different concentrations for both methanol and aqueous extracts against *Xc*. pv *musacearum* except for *L. diversifolia* whose concentrations exhibited significant difference against *Cercospora zeae-maydis* with concentration 75% having greatest mean inhibition percentage in both methanol and aqueous extracts (Tables 2 and 3).

Table 2 Antimicrobial activity of *S. sesban*, *C. calothyrsus* and *L. diversifolia* leaf methanol extract against *Xc*. pv*musacearum* and *Cercosporazeae-maydis*

Xc*. pv *musacearum				***Cercospora zeae-maydis***		
Plant species	Mean radial growth inhibition (mm)			% growth inhibition		
S. sesban	13.9[a]			72.2[a]		
C. calothyrsus	11.0[b]			68[a]		
L. diversifolia	10.0[b]			55.2[b]		
P value	0.0014			0.0001		
LSD	1.98			7.0789		
Xc*. pv *musacearum				***Cercospora zeae-maydis***		
Mean growth inhibition (mm)				**% growth inhibition**		
Treatments (mg/ml)	***S. sesban***	***C. calothyrsus***	***L. diversifolia***	***S. sesban***	***C. calothyrsus***	***L. diversifolia***
12.5	14.3[a]	10.3[a]	8.3[b]	74[a]	57[a]	64[a]
25.0	13.3[a]	11[a]	8.3[b]	75[a]	78.3[a]	61.6[a]
50.0	13.6[a]	10.3[a]	12.6[ab]	64.6[a]	62.6[a]	68.6[a]
75.0	14.3[a]	12.3[a]	11[ab]	75[a]	74[a]	23.3[b]
P value	0.95	0.65	0.09	0.2	0.17	<.0001
LSD	5.09	4.1	3.99	11.73	21.95	11.45

Means followed by the same letters down the column are not significantly different at P = 0.05.

Table 3 Antimicrobial activity of *S. sesban, C. calothyrsus* and *L. diversifolia* leaf aqueous extract against *Xc*. pv *musacearum* and *Cercospora zeae-maydis*

Xc*. pv *musacearum				***Cercospora zeae-maydis***		
Plant species	Mean radial growth inhibition (mm)			% growth inhibition		
S. sesban	13[a]			78.3[a]		
C. calothyrsus	9.4[b]			59.1[c]		
L. diversifolia	12[a]			71[b]		
P value	0.0016			<.0001		
LSD	1.87			2.7		
Xc*. pv *musacearum				***Cercospora zeae-maydis***		
Mean growth inhibition (mm)				% growth inhibition		
Treatments (%)	***S. sesban***	***C. calothyrsus***	***L. diversifolia***	***S. sesban***	***C. calothyrsus***	***L. diversifolia***
12.5	9.7[b]	8.7[ab]	11.6[a]	80[a]	55.3[b]	68.6[b]
25.0	15[a]	10.3[a]	11.3[a]	78[a]	53.3[b]	72a[b]
50.0	14.3[ab]	8.3[b]	12.3[a]	79[a]	55.6[b]	69.3[b]
75.0	13.3[ab]	10.3[a]	12.6[a]	80[a]	72[a]	74[a]
P value	0.16	0.08	0.91	0.71	0.0028	0.05
LSD	5.24	1.95	4.64	4.61	8.31	4.06

Means followed by the same letter down the column are not significantly different at P = 0.05.

DISCUSSION

Plant species used in traditional medicines continue to be reliable sources for discovery of useful compounds (**Musyimi *et al.*, 2008; Emitaro *et al.*, 2018**). Plants have become the subject of human curiosity in search for novel natural products relevant to pest and disease management in food crops (**Izah *et al.*, 2018; Sales *et al.*, 2016**). Medicinal plant extracts contain secondary metabolites such as alkaloids, quinones, flavonoids, glycosides, saponins, tannins and terpenoids with antimicrobial properties (**Izah *et al.*, 2018; Salhi *et al.*, 2017; Sales *et al.*, 2016; Musyimi *et al.*, 2008**). The variation in the concentration of the of phytochemicals in the leaf extracts of *C. calothyrsus* and *S. sesban* than *L. diversifolia* could be attributed to the type of solvent used and response of individual plant to biotic and abiotic factors as the plants were obtained in the same ecological zone. The concentration of bioactive compounds in each plant species depends on the environmental conditions, age of the plant, relative humidity of harvested materials and method of extraction (**Borges *et al.*, 2018;**

Izah 2018; Musyimi *et al*., 2008). The presence of all the phytochemical tested in *S. sesban* is in agreement with the results of **Nirosha *et al.* (2019)** and this explains its significance in antimicrobial activity (tables 2 and 3). *Callindra calothyrsus* extracts lacked steroids and alkaloids contrary to the results by **Setyawati *et al.* (2019).** This may be due to the fact that these plant occupy different ecological zones (**Kumar *et al*., 2017**). Phytochemicals differences can also occur depending on the type of solvents used in extraction. Phytochemical compounds take into account different parameters and factors such as species, ecological factors and environmental conditions (**Musyimi *et al*., 2008**).

The toxicological activity of plant extracts on the pathogens depends on the presence of bioactive compounds (**Pizzi, 2019; Nirosha *et al*., 2019**). Saponins and tannins have antibacterial and antifungal activity as well as anti-insect activity (**Hossein *et al*., 2013; Nirosha *et al*., 2019; Pizzi, 2019**). Flavonoids have been used as scavengers of superoxide anions, anti-inflammatory and antimicrobial agent (**Nirosha *et al*., 2019; Hossein *et al*., 2013**) while alkaloids act as anti-inflammatory, antimalarial, antimicrobial and cytotoxicity (**Iqbal *et al*., 2015; Matsuura and Fett-Neto, 2015; Hussain *et al*., 2018**). Similarly, steroids and terpenoids have been reported to possess cardiotonic effect, antibacterial, insecticidal properties (**Iqbal *et al*., 2015; Tholl, 2015; Bergman *et al*., 2019**).

There was a significant inhibition of radial growth of *Xc.* pv *musacearum* and *Cercospora zeae-maydis* by the leaf extracts from *S. sesban*, *C. calothyrsus* and *L. diversifolia*. *Sesbania sesban* extract was more effective against *Xc.* pv *musacearum* and *Cercospora zeae-maydis* pathogen as it produced large zones of inhibition compared to *C. calothyrsus* and *L. diversifolia.* The difference in performance could be attributed to high concentration of saponins, steroids and alkaloids in the leaf extract of *S. sesban* (Table 1). These compounds are known to have antibacterial and antifungal activity when they work synergistically with flavonoids. It may also be because active compounds were polar which dissolved in methanol and aqueous solvents readily than those in *C. calothyrsus* and *L. diversifolia.* The results are in agreement with the results reported by **Ahmed *et al.* (2013)** that *S. sesban* extracts are active against plant bacterial pathogen *Erwinia amylovora*. *Sesbania sesban* and plant pathogenic fungi *Curvularia lunata* and *Fusarium oxysporum* (**Mythili and Ravindhran 2012**).

The antimicrobial activity of the three plant leaf methanol and aqueous extracts have a wide range of activity because different concentrations of the extracts inhibited the growth of *Xc.* pv *musacearum* and *Cercospora zeae-maydis.* This could be probably due to the active ingredients in the extract which interfered with pathogen's cell functioning hence arresting growth. The ability of *S. sesban*, *C. calothyrsus* and *L. diversifolia* extracts to inhibit the growth of *Xc.* pv *musacearum* is attributed to the presence of secondary metabolites with antibacterial and antifungal properties. Leaves of all the three plants were found to have terpenoids, flavonoids and saponins which have antibacterial and antifungal properties (**Deivasigamani, 2018; Ahmed *et al*., 2013**). The antimicrobial activity of the plant secondary metabolites is due to their ability to denature protein, interfere with pathogen's cell signaling, DNA alkylation and altering the reproductive system of the pathogen (**Ramírez-Gómez *et al*., 2019**).

CONCLUSION

This study aimed at isolating, identifying and evaluating the antimicrobial properties of the compounds from the leaf extracts of *S. sesban*, *C. calothyrsus* and *L. diversifolia*. Phytochemical screening revealed that terpenoids, flavonoids and saponins were present in the leaf extracts of the three plant species. Tannins were only present in *C. calothyrsus* and *S. sesban*. Steroids were present in *L. diversifolia* and *S. sesban* while alkaloids were present only in the leaf extracts of *S. sesban*. The study found a variation in the concentration of phytochemical compounds in the leaf extracts of three plant species even though the plants were from the same ecological zone. Antimicrobial activity of plant extracts is depended on the secondary metabolites that the plant synthesise. *Sesbania sesban*, *Callindra calothyrsus* and *Leucaena diversifolia* extracts showed antimicrobial activities against *Xc.* pv *musacearum* and *Cercospora zeae-maydis* which could form a basis of developing botanical pesticides to avoid the adverse effects of synthetic chemicals. The results in this study supports the use of plant extracts in controlling plant pathogens as they are readily available, cheap and ecofriendly. Future studies should focus on identifying the active ingredients in the extracts of *S. sesban*, *C. calothyrsus* and *L. diversifolia* for development of chemicals to optimize their use by smallholder farmers in disease control to reduce dependence on synthetic pesticides.

Acknowledgements: Authors acknowledge the National Research Fund, Kenya for supporting this research through their funding. We also appreciate Hisdon Owiti and Ambrose Ndiao of Jaramogi Oginga Odinga University of Science and Technology for their technical support during this research.

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