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Research Article

Antimycobacterial Activity, Cytotoxicity and Phytochemical Screening of Organic Extracts of *Commiphora Africana* Stem Bark from Kenya

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Abstract: Tuberculosis (TB) is a communicable disease that kills approximately three million people annually. Efforts to treat the disease have been made difficult due to the development of drug-resistant strains and co-infection with HIV/AIDS. There is a need to develop new, inexpensive, safe, and effective anti-TB drugs. *Commiphora africana* (*C. africana*, Burseraceae) is a very useful plant and has been known to treat several ailments. The plant contains various secondary metabolites and has been found to possess many pharmacological activities such as antifungal, antimicrobial, antioxidant, hepatoprotective, anti-inflammatory and anti-ulcer effects. The study aimed at screening the extracts for their antimycobacterial activity against *Mycobacteria smegmatis*; cytotoxicity in Vero cells; qualitative phytochemical analysis and Thin Layer Chromatography (TLC) profiling. Phytochemical screening revealed the presence of alkaloids, flavonoids, terpenoids, coumarins, phenols, saponins, sapogenins, and tannins. The dichloromethane and ethyl acetate extracts of *C. africana* stem bark were the most active extracts against the *M. smegmatis* strain used with a minimum inhibitory concentration (MIC_{99}) of 1.30 and 2.60 mg/mL, respectively. Cytotoxicity studies revealed that most of the extracts had CC_{50}>20 µg/mL thus considered safe. However, hexane extract of *C. africana* stem bark showed CC_{50}<5 µg/mL and thus considered toxic. The study confirms the antimycobacterial activity of *C. africana*. Further studies on the isolation of specific phytochemicals are ongoing and elucidating mechanisms of action are needed with the aim of developing a novel anti-TB regimen.

Keywords: *Commiphora africana*, *Mycobacteria smegmatis* ATCC607, Synergy, Additive, Minimum Inhibitory Concentration, and Cytotoxicity.

INTRODUCTION

Tuberculosis (TB) is a major public health concern with over 2 billion people currently infected, 8.6 million new cases per year, and more than 1.3 million deaths annually (Aryee *et al*., 2018; Kumar *et al* ., 2014). Kenya is among the 22 countries with the highest burden of TB. The estimated prevalence of all forms of TB was 233 per 100,000 population while the mortality from all forms of TB was 20 per 100,000 population ((WHO, 2018). The high incidence rate is due to resistance of TB to the two main first-line anti-tuberculosis drugs (isoniazid and rifampicin) which has increased over the last few years, from 0.04% in 2005 to 0.16% in 2016—a four-fold increase in eleven years, and partly because of poor adherence (Kipruto *et al.*, 2015).

The current drug regimen combination for TB consists of isoniazid, rifampicin, ethambutol, and pyrazinamide, administered over six months (Zhang *et al.*, 2013). Although this treatment has a high success rate, the utility of this regimen is limited by compliance issues, which has resulted in the rise of strains that are resistant to some or all of the first- and second-line antibiotics (Loddenkemper *et al.*, 2016). These strains, called multidrug-resistant (MDR), extensively drug-resistant (XDR) and totally drug-resistant (TDR) strains

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of *Mycobacterium tuberculosis* (*Mtb*), have worse disease outcomes (Sloan *et al.*, 2013).

It has been estimated by the World Health Organization (WHO, 2016) that about 80% of the inhabitants of the world, especially in developing countries, rely mainly on medicinal plants for their primary health care. Plant products also play an important role in the health care systems of the remaining 20% (developed countries) where they use an active pure plant-derived compound in the development of conventional medicines (Sofowora *et al.*, 2013). Widely used agents like morphine, digoxin, digitoxin, reserpine, codeine, atropine, hyoscyamine, scopolamine, quinine, quinidine, pilocarpine, artemisinin, and physostigmine are examples of plant-derived drugs.

Natural products or their semi-synthetic derivatives have in recent years provided novel drug leads in TB chemotherapy (Shu, 1998). Examples of such compounds include Streptomycin and Kanamycin from *Streptomyces griseus* (Copp, 2003) and Capreomycin isolated from *S. capreolus* (Shu, 1998). Rifampicin is a semi-synthetic drug that has been derived from rifamycin a product of *Amycolatopsis mediterranei* that has been in natural environments for a long time (Tribuddharat & Fennewald, 1999).

Commiphora africana (Burseraceae) and commonly known as African Myrrh is widely used in sub-Saharan Africa including Kenya (Shen *et al* ., 2012). African myrrh is a spiny, low-branching deciduous shrub with a short bole and a dense, rounded crown. It usually grows 3 - 5 m tall. The tree is often bare of leaves for several months in the dry period (Orwa *et al*., 2009). *Commiphora africana* has been reported in traditional medicine as a remedy for ailments such as stomach pains and dysentery, heartburn, malaria, healing wounds, male sterility, leprosy, and snake-bites ((Nuhu *et al*., 2016. The leaves are pounded with bulrush millet and taken as a stomachic. The root is cooked with millet or sorghum to treat heartburns. The stem bark is used to treat diabetic patients, heartburns and as a remedy for stomach pains and dysentery (Johnson *et al.*, 2012).

Phytochemical studies have shown that the pharmacological activities of *Commiphora africana* are due to the presence of secondary metabolites such as flavonoids, coumarins, triterpenoids, saponins, and alkaloids (Ezekiel *et al.*, 2010). Resins contain the highest mixtures of the terpenoids (Ahmed *et al*., 2016). Ethanol leaf extract was found to have lipid profile activity in laboratory rats and antimicrobial activity (Adebayo *et al*., 2006). Sesquiterpene present has been reported to have antibacterial and antifungal activity against pathogenic strains of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* (Mohammed *et al*., 2013). The stem bark has also been reported to have anti-ulcer activity (Nuhu e*t al*., 2016). Therefore, current research is focused on the discovery of natural antimycobacterial compounds from the plants for new and safer treatment options with fewer side effects and also able to inhibit the efflux pumps which is one of the drug resistance mechanisms by the mycobacteria. Thus, the objective of the present investigation was to evaluate *in vitro* cytotoxicity and antimycobacterial activity of organic extracts of *C. africana* stem bark.

MATERIALS AND METHODS

Plant Collection

The stem bark of *Commiphora africana* was collected from the North Rift region (Marakwet) in Kenya in February 2019. The plant was taxonomically authenticated at the Herbarium Unit, Department of Biological Sciences, University Eldoret, Kenya with a Voucher specimen number CFW/31/1/19/004.

Chemicals and Reagents.

All the drugs, solvents and chemicals used in the study were of analytical grade. Silica gel 60 F254 (Merck, Germany) and HEPES (Goldbio.com technology, USA). All other reagents, drugs, and culture growth media such as methanol, hexane, ethyl acetate, dichloromethane, Dimethyl sulphate, PBS, Middlebrook 7H9 broth base, Middlebrook OADC, penicillin-streptomycin, MEM Eagles media, MTT dye, Resazurin Dye, Rifampicin, and the 96 microtitre well plates were purchased from Sigma Aldrich Germany.

Sources Of Micro-Organisms

The *M. smegmatis* strain ATCC607 used in the bioassay analysis was seeded from a glycerol frozen stock obtained at Centre of Respiratory Disease Research (CRDR) in Kenya Medical Research Institute (KEMRI) which was maintained at a nutrient broth at -20^0C while the Vero cells for cytotoxicity were seeded from a passaged stock at Centre of Traditional Medicine and Drug Research (CTMDR) -KEMRI.

The Sequential Maceration Extraction Process

Commiphora africana stem bark was air-dried at room temperature (28 -32 ^{0}C) for a period of one month and mechanically pulverized. The sequential method of extraction was carried out using four solvents in the order of increasing polarity (hexane, dichloromethane, ethyl acetate, and methanol). A 100 g of the powdered material was extracted with the four organic solvents using the maceration process with each solvent left to stand for 48 h with constant shaking. The extracts were filtered, concentrated by rotary evaporator (Buchi Rotavapor® R-114) at 40 ^{0}C and stored in a clean sterile vial. Finally, percentage yields of the dried extracts were calculated.

Phytochemical Screening And TLC Profiling

Organic extracts of *C. africana* stem bark were subjected to qualitative phytochemical screening and TLC fingerprinting techniques for the identification of the different secondary metabolites using standard tests

and procedures (Cooper-Driver & Harborne, 2007). Thin layer chromatographic analysis was performed on pre-coated silica gel plates with silica gel 60 F254 using the one-way ascending technique. Hexane: ethyl acetate 7:3 and 8:2 were used as mobile phases. The developed TLC plates were viewed under Ultra-Violet light and then sprayed with appropriate reagents (Cooper-Driver & Harborne, 2007).

Inoculum Preparation and Antimycobacterial Assay (Resazurin Microtiter Assay -REMA)

The frozen stock of the inoculum was thawed and sub-cultured in solid Lowenstein Jensen Media for three days. A loop of the freshly grown mycobacteria was drawn and inoculated in 20 mL of the broth (MADC-Tw) and grown to mid-log phase at 37^0C for 16 h at an OD_{600} (0.6-0.8) in a filter-sterilized 7H9 media supplemented with 10% OADC, 0.2% glycerol and 0.25% Tween80. After 16 h the sub-culture was diluted ×1000 and used for the assay. The susceptibility test was done in 96 microtiter plates using the resazurin dye as an indicator of cell viability as described by (Palomino *et al*., 2002). Working solutions of the tested extracts were serially diluted in the enriched Middlebrook 7H9 broth to obtain the final sample concentrations that ranged from 25 mg/mL to 0.0976 mg/mL. Rifampicin (10 mg/mL) was dissolved in distilled water and used as a positive control drug at a concentration of 1 to 0.00097 mg/mL. Medium with strain suspensions was used as a negative control. Fifty microliters (50µL) of 7H9 broth were added into all 96 wells of the plate, and 50 µL of the extracts were introduced to the wells in the first row (1) and mixed thoroughly. The sample mixture (50 µL) was removed from wells of row (1) to perform a two-fold serial dilution across the rows (2-12). The last 50 µL was discarded. Later, 50 µL of the inoculum was introduced into the corresponding wells. The final volume in each well was 100 µL. Each extract concentration was assayed in duplicate. The microplate was sealed with parafilm and stored in a tight plastic container and incubated for 2 days at 37 ^{0}C in a normal atmosphere. After the incubation period, 20 µL of 0.1% resazurin dye was added to each well. The plates were then re-incubated for 24 h at 37 ^{0}C in the dark. The minimum inhibitory concentration (MIC) results were presented as a mean value of the three experimental tests. The lowest concentration that resulted in 99% inhibition was defined as the MIC.

Cytotoxicity Assay

Cytotoxicity of the crude extracts was evaluated on Vero E6 African Green monkey kidney epithelial cells. Cells were grown in MEM (Eagles Media) culture medium with L-glutamine and 25 mM HEPES. The medium was supplemented with 2 mg/mL $NaHCO_3$, 10 µg/mL hypoxanthine, 11.1 mM glucose, 10% FBS and 5µg/mL Penicillin-Streptomycin. The cells were incubated at 5% O_2, 5% CO_2, and 90% N_2 in a humidified incubator (SHEL LAB™, Sheldon MfgInc, OR, USA) at 37°C until confluent before they were used for cytotoxicity assay. Trypsinated cells were distributed in 96 well plates at 20,000 cells in 100 µL per well and incubated for 24 h to allow them to attach before adding the extract. After 48 h the medium was removed completely from each well, and 100 µL of fresh culture medium was then added. Thereafter, 100 µL of crude extracts (1000 µg/mL) was added in row H and a 3-fold serially diluted to row B to give concentrations ranging from 1000 – 0.457 µg/mL. Cells in row A served as controls without the drug (100% growth). The cells with or without extracts were incubated at 37°C for 72 h before determining their viability. Each concentration level was tested in duplicate. Cell viability was determined using Thiazolyl Blue Tetrazolium Bromide (MTT) assay (Bahuguna *et al*., 2017). Ten µL of 5 mg/mL MTT was added into each well and incubated for 3 h at 37°C. After 3 h, 100 µL dimethyl-sulfoxide was added to dissolve formazan crystals and then incubated for 1-2 h before recording the optical density (SkanIt Software 4.1 for Microplate Readers RE, ver. 4.1.0.43) at 570 and 720 nm. Percentage cell viability was calculated as shown below

$$\%\text{viable cells} = abs_{sample} - abs_{blank} / abs_{control} - abs_{blank} \times 100$$

The values obtained were plotted against concentration and the equation used to calculate the Inhibitory concentration CC_{50}.

RESULTS AND DISCUSSION

Four organic extracts were obtained after the maceration process as represented in Table1. The methanolic extract had the highest yields.

Table 1: A Table Showing Extraction Yields.

Plant code	Solvent used	Yields (g)	% yields	Physical appearance
CA2a	Hexane	1.36	0.68	Dark yellow slurry
CA2b	Ethyl acetate	1.06	0.58	Dark green slurry
CA2c	Methanol	3.00	1.50	Brownish shinny powder
CA2d	DCM	1.55	0.78	Dark green slurry

Key

CA- Commiphora Africana; 2- Stem bark; a-hexane solvent ; b-ethyl acetate solvent ; c- methanol solvent; d- dichloromethane solvent

Phytochemical screening and the TLC profiles of the extracts identified the various class of phytochemical compounds such as alkaloids, saponins, sapogenins, flavonoids, coumarins, glycosides and terpenoids as shown in Table 2 and figure 1.

Table 2: Phytochemical Screening Of The Extracts

Phytocompound	**CA2a**	**CA2b**	**CA2c**	**CA2d**
Alkaloids	+	+	-	+
Saponins	-	-	+	-
Phenols	-	+	-	+
Flavonoids	-	+	-	+
Tannis	-	+	+	-
Terpenoids	+	+	+	+
Glycosides	-	+	-	+
Coumarins	+	+	+	+
Sapogenins	+	+	+	+

Key
-Absent
+Present
CA- Commiphora Africana; 2- Stem bark; a-hexane solvent
b-ethyl acetate solvent ; c- methanol solvent; d- dichloromethane solvent

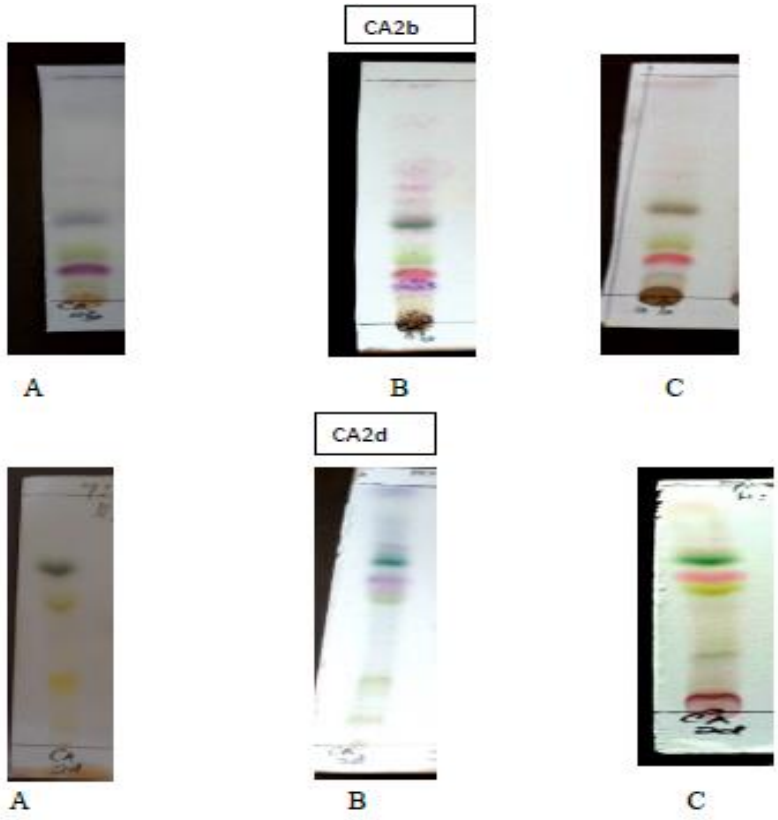

Figure 1: TLC Profile of the most active extracts

Key
A- Naked eye visualization
B- Spraying with vanillin- ethanol sulphuric acid
C- Spraying with saturated antimony trichloride
CA2b solvent system hexane: ethyl acetate (8:2)
CA2d solvent system hexane: ethyl acetate (7:3)

Phytochemical screening of *C. africana* has been reported in several studies showing they have a wide range of secondary metabolites. Nuhu *et al*., 2016 reported the presence of secondary metabolites in the *C. africana* stem bark while Ezekiel *et al* .,2010 also reported the presence of flavonoids, coumarins, triterpenoids, saponins, and alkaloids in the resins. This agrees with my findings since alkaloids, phenols, tannins, anthraquinones, coumarins, flavonoids, terpenoids, and glycosides were present.

Antimycobacterial Activity And Cytotoxicity

Minimum inhibitory concentration (MIC) was used to determine the active extract by colourimetry. The 'blue' colour in the wells was termed as no growth occurrence of the mycobacteria (Palomino *et al*.,2002). Antimycobacterial activity of *Commiphora africana* stem bark was evaluated against *Mycobacteria smegmatis* ATCC 607 strain. Among the four extracts, two extracts showed moderate activity against the strain used with dichloromethane extract showing the lowest MIC of 1.3 mg/mL. Detailed results are shown in Table 3.

Cytotoxicity studies with normal cell culture have not been studied extensively on plant extracts and this is vital for safety. In this study, Vero cells were used to determine the cytotoxic effects of the extracts and they were considered safe when CC_{50}>20 µg/mL ((Kigondu *et al*., 2009; Zirihi *et al*., 2005). Only the hexane extract showed cytotoxic effects with an Inhibitory concentration of 4.23 µg/mL. Detailed results are shown in Table 3.

Table 3: Antimycobacterial Activity And Cytotoxicity Of The Extracts

Plant code	**Antimycobacterial activity mg/mL**	**Cytotoxicity activity µg/mL**
CA2a	>25	4.23±2.12
CA2b	2.60± 0.90	30.16±3.99
CA2c	>25	371.97±5.02
CA2d	1.30± 0.45	344.64±5.20
Rifampicin	0.013±0.005	>1000

Key
CA- *Commiphora africana*
2- Stem bark
a-hexane solvent
b-ethyl acetate solvent
c- methanol solvent
d- dichloromethane solvent

The DCM and ethyl acetate crude extracts of *C. africana* stem bark were active at a MIC concentration of 1.30 and 2.60 mg/mL respectively. This was an indication that the compounds in the extracts may be acting synergistically hence leading to a higher antimycobacterial activity of the crude extracts. These findings agree with Adebayo *et al*., 2006; Idris *et al*., 2019 who reported anti-microbial and anti-fungal activity of *C. africana* resins against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* strains although it's activity against *M. smegmatis* has not been reported and this finding is being reported for the first time.

The antimycobacterial activity could be attributed due to the presence of terpenoids and flavonoids which were most abundant in the DCM and ethyl acetate extracts. Terpenoids have been reported to influence cell membrane structures by increasing membrane fluidity and permeability, changing the topology of membrane proteins and inducing disturbances in the respiration chain of a microbial pathogen (Sieniawska *et al*., 2017). Flavonoids have been associated with inhibition of cytoplasmic membrane functions and DNA gyrase enzyme (Cao *et al*., 2019). These properties may explain why the two *C. africana* crude extracts had high activity against *M. smegmatis* strain tested.

Commiphora africana (stem bark) hexane extract was the only cytotoxic extract as it showed CC_{50}<5 µg/mL. These results corroborate with a study done by Paraskeva, 2008 in South Africa on Commiphora species, namely *C. schimperi* (stem), *C. neglecta* (stem), *C. tenuipetiolata* (stem and leaf), and *C. edulis* (stem), who reported the LC_{50} of extracts of these species to be below 10 µg/mL and Mkangara *et*

al., 2014 who also reported chloroform root extract of *C. swynnertonii* to have LC_{50} of 4.5642 µg/mL.

CONCLUSION

Commiphora africana stem bark has antimycobacterial activity against Mycobacterial smegmatis strain ATCC507 at 1.30mg/mL which support the traditional use of the plant for management of bacterial and fungal infections. Encouragingly the extracts are not cytotoxic thus considered safe for use. However, hexane extract exhibited high cytotoxic effects on Vero cells suggesting the presence of secondary metabolites that can be evaluated for the development of anticancer agents. Isolation and characterization of the active compounds are in progress. However, further studies on the mechanism of action of the active extracts are highly needed and also In Vivo studies of the active non-toxic extracts.

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