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Antimicrobial, cytotoxicity and preliminary phytochemical determination of commonly used medicinal plants to treat oral cavity, urinary tract and gut infections by inhabitants of Borabu sub-county, Nyamira County, Kenya

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ABSTRACT

Aims: The study aimed at determining the antimicrobial activities and cytotoxicity properties of medicinal plants collected from southwestern Kenya.
Methods and results: A total of 23 ethanol extracts of selected medicinal plants were bio-assayed against Gram-negative bacterial strains (*Escherichia coli* NU14, *Helicobacter pylori* ATCC 700824, and *Porphyromonas gingivalis* ATCC 33277). Cytotoxicity tests were also carried out on mammalian cell lines (AGS, KB, and TR146). Preliminary type of phytochemical compounds present in the extracts was determined by thin-layer chromatography. *Cassia didymobotrya* plant extract (1 mg/mL) had strong antimicrobial activity against *P. gingivalis* (average zone of inhibition of 21.70 ± 0.88 mm, MIC 0.13 ± 0.00 mg/mL and MBC 0.50 ± 0.00 mg/mL). *Escherichia coli* was resistant to all the extracts bioassayed. *Leonotis nepetifolia* (15.80 ± 0.20 mm) and *Clerodendrum myriacoides* (14.20 ± 0.44 mm) showed only moderate activity against *H. pylori*. Cell cytotoxicity results indicated a dose-dependent response against KB, TR146 and AGS cell lines with *C. didymobotrya* having IC_{50} values of 47.64 and 704.00 µg/mL on KB and TR146 cell lines, respectively. *L. nepetifolia* and *C. myriacoides* did produce IC_{50} of 0.1883 mg/mL and 0.1061 mg/mL against the AGS cell line respectively.
Conclusion, significance and impact of the study: Most of the extracts had no or weak activity against test isolates, but *C. didymobotrya* leaves extracts showed strong activity against *P. gingivalis*. *C. didymobotrya* can offer alternative medicare to *P. gingivalis* conditions.

Keywords: Antimicrobial, medicinal plants, resistance, cytotoxicity

INTRODUCTION

With the extensive use and abuse of antibiotics, a serious problem of bacterial resistance has developed globally (Luo *et al.*, 2016). This has worsened in the last few decades due to a series of aggravating factors, such as overuse of antibiotics in patients and animals, lack of surveillance, lack of vaccination, shortage of investment in research and development by the pharmaceutical industry and governmental agencies. Most microorganisms have evolved several mechanisms for antimicrobial resistance like biofilm formations, which has worsened their management with antibiotics (Mu *et al.*, 2016).

This situation of multidrug resistances is coupled with the undesirable side effects of certain antibiotics and the emergence of previously uncommon infections. This global healthcare crisis is amplified by the current state of our antibiotic pipeline; since the late 1960s, there has been little success in bringing new antimicrobial agents to the clinic (Abouelhassan *et al.*, 2015). Therefore, our ability to bring new antibacterial entities to market is outpaced by the drug and multidrug resistance and there is a need to discovery new antibacterial agents that operate via new modes of action and hit new bacterial targets when compared with current antibiotic arsenal (Abouelhassan *et al.*, 2015). The search for antimicrobials from higher plants is not in vain because it is estimated

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that over 75% of the antibacterial drugs in clinical used today are natural origin (Omori and Okemo, 2012). Bérdy (2012) further indicates that 60,000-80,000 microbial compounds have been characterized from natural products. Thus, a clear indication that natural products research could be promising.

Medicinal plants have been used by societies across the world for centuries to manage various ailments with about 80% of this population globally relying on traditional plant-derived medicines (Taid *et al.*, 2014). With estimates of about 250,000 to 500,000 species of plants on the Earth (Borris, 1996), plants could be an important source of medicines, especially in many developing countries, where the plant-based traditional medicines are ingrained in the culture and still used to meet the health-care needs. Despite the recent interest in drug discovery using molecular modelling, combinatorial chemistry, machine learning, and other synthetic chemistry high-throughput methods, natural product-derived compounds are still proving to be an invaluable source of medicines for humans with an only small portion of them been evaluated for their activity.

Humankind to treat diseases for millennia has used plant-derived compounds and they represent a large reservoir of chemical species, which work together, exhibiting a wide range of biological activities (Sadlon and Lamson, 2010; Omori and Okemo, 2012). Unlike most antibiotics, which function by influencing directly the cell viability, plant-derived compounds at sub-lethal concentrations, can inhibit virulent traits and hence, disarm the bacterial pathogenic capacity, thus generating less pressure for the evolution of resistance (Zhao *et al.*, 2013). Such alternative approaches for treating of microbial infections that rely on plant natural compounds are finding their way into pharmaceuticals, nutraceuticals and food supplements (Sen and Batra, 2012).

This study aimed at validating the traditional use of selected medicinal plants that used by the inhabitants of Borabu sub-county in Nyamira County, Kenya to treat gastrointestinal tract, oral cavity, and the urinary tract infections by evaluating their *in vitro* antibacterial activity based on a survey that was carried earlier (Omwenga *et al.*, 2015).

MATERIALS AND METHODS

Preparation of plant extracts

A set of 23 samples of the plant material (Omwenga *et al.*, 2015) obtained from different parts (root, leave, fruit, and bark) were harvested and air-dried at room temperature (25 °C) under a shade. The plant roots and hard barks were chopped into small pieces before they were ground into a fine powder using the laboratory grinding mill (IKA M20-Staufen, Germany). Other dry plant parts like the leaves and some soft barks were ground into powder by the same mill (Omwenga *et al.*, 2017).

Extraction of the plant extracts for antibacterial activity bioassays

The extraction and preparation of the 23 plant samples were done as documented in our preceding study (Omwenga *et al.*, 2017). Briefly, 5 g of each of the plant powder was weighed and mixed with 20 mL of 50% ethanol (w/v) and was shaken for 10 min at 80 rpm. Then the mixture was placed in an ultrasonic machine (Sonorex Bandelin RK-100) for 30 min before it was centrifuged at 5,000 rpm for 10 min. The supernatant was sucked carefully by use of a micropipette into the round-bottomed volumetric flask. The same procedure was repeated on the pellet 2 times. Then, the solvent in the supernatant was evaporated by using rotary evaporator (Buchi Rotavapor R-210) until its volume decreased to around 5-10 mL. Then, it was deep-frozen with liquid nitrogen and placed into the freeze drier (Christ Alpha 1-4 LD plus) overnight to completely get rid of the solvent and remain with the powder. The powder was weighed and then packed in vials for bio assaying purposes (Supplementary data S1). One milligram of each extract was then mixed with 1 mL of 50% ethanol and this was used as a treatment for the bioassays below. For minimum inhibitory concentration (MIC) determination, 4 mg of the plant extract was diluted with 1 mL of 50% (v/v) ethanol and then serially diluted to concentrations ranging between 2.0 and 0.016 mg/mL.

Test organisms

The bacterial strains were selected based on the various uses of the medicinal plants by the inhabitants of Borabu sub-county in Nyamira County, Kenya, as documented in our preceding study (Omwenga *et al.*, 2015). *Escherichia coli* NU14 bacteria was used as a surrogate of the urinary tract pathogens, particularly, uropathogenic *E. coli* (UPEC); *P. gingivalis* ATCC 33277 was chosen to represent a widespread pathogen of the oral cavity bacteria, and *H. pylori* ATCC 700824 bacteria represented a gastric pathogenic bacterium.

Antibacterial assays

Agar well diffusion assay

The antimicrobial activity of the 50% (v/v) ethanol extracts of the 23 medicinal plants was determined by agar well diffusion method (Alsaraf *et al.*, 2016). Briefly, a few colonies were taken from the sub-cultured plate of the bacterial strains and mixed with 1 mL of the corresponding broth (*E. coli*: LB medium; *P. gingivalis*: a special medium-Supplementary data S1; *H. pylori*: Fetal Calf Serum (FCS) 10% (w/v), tryptic soy broth (TSB) and trypticase-supplemented soy agar medium). All media were made according with the manufacturer's (Sigma-Aldrich GmbH, Steinheim, Germany) instructions.

The bacterial cell density was adjusted to match the turbidity of 0.5 McFarland standards. One hundred microliters of the adjusted bacteria in the broth was then

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cultivated unto the agar via spread plate technique. After inoculation, plates were allowed to dry up under the sterile working bench for a few minutes before 6 mm wells were punched carefully onto the agar using sterile corn borer. They were then each filled with 50 µL of the 1 mg/mL plant extracts, 50 µL of gentamicin or amoxicillin (1 mg/mL) as a positive control, or broth as a negative control. Plates were incubated for 24 h at 37 °C and specific condition depending on the bacteria of interest to allow extracts to diffuse through the agar media.

The diameters of the zone of inhibition of the treatments exhibiting antibacterial activity were measured along with the diameter of the well for further analysis. Agar wells that did not show an inhibition zone were considered as deprived of antibacterial activity. All experiments were done in triplicate and the average values were calculated.

Determination of the Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC values of the 50% (v/v) ethanol plant extracts were determined by use of the micro titre-serial dilution technique in 96-well micro-plates (Omwenga *et al.*, 2009). Briefly, the plant ethanol extracts were serially diluted directly in the wells to obtain solutions of concentrations ranging between 2.0 and 0.016 mg/mL. Similar serial dilutions were performed for gentamicin, which used as a positive control, and 50% (v/v) ethanol was used as the negative control. A 100 µL fresh bacterial cultures that adjusted to match the turbidity of 0.5 McFarland standards were added to each of the wells and incubated at 37 °C for 24 h under the favourable conditions of the bacteria of interest (e.g., *H. pylori* was incubated under microaerophilic conditions, while *P. gingivalis* was incubated under anaerobic conditions).

The MIC values were determined as the lowest concentrations of the extract that suppressed the bacterial growth. All the wells that did not exhibit growth (i.e., lack of turbidity) were sub-cultured in agar medium. The lowest concentration of the examined plant extracts that showed no colony formation on the solid medium after sub-culturing and incubating for 24 h, was regarded as the MBC. All tests were performed in triplicate and the average values were calculated. FCS 10% (w/v), TSB and trypticase soy-agar medium were used for *H. pylori*, LB medium and its broth were used for the *E. coli* NU14, while a special medium was used for the *P. gingivalis* (Supplementary data S1).

Mammalian cell cytotoxicity tests

Three mammalian cell lines, namely AGS–stomach lining cell line, KB and TR146 mouth cavity (Sigma-Aldrich GmbH, Steinheim, Germany), were used to examine the effect of the plant extracts on the metabolic competence using the MTT assay (Omwenga *et al.*, 2018). Aliquots of 100 µL each of the three cell lines were seeded in a 96 well polystyrene tissue culture plates at a cell density of 5×10^5 cells/mL using RPMI 1640 containing 1% (w/v) penicillin/streptomycin, supplemented with 10% (w/v) fetal bovine serum (FBS) for AGS cell line. For KB cell line, minimum essential medium (MEM) supplemented with 1% gentamycin and 10% FBS was used. As for TR146 cell line, DMEM plus Hams F12 mixture (1:1), containing 1% penicillin/streptomycin (100 times), 1% L-glycine, supplemented with 10% (w/v) FBS was used to culture the cells. All three cells lines were incubated in humidified atmosphere of 5% CO_2 at 37 °C for 24 h. After 24 h, the wells are washed twice with 100 µL/well of minimum medium to get rid of the unattached cells. Subsequently, 100 µL/well of the plant extract solutions which dissolved in minimum essential medium were added to the wells at the various concentrations and further incubated for 24 h in similar condition. Four percent Triton X-100 was used as a positive control, while the culture medium was used as a negative control. After incubation, the plant extracts are removed, and the wells were washed again twice with 100 µL/well of minimum medium. Then, 100 µL/well of minimum medium and 25 µL/well of MTT solution consisting of 5 mg/mL MTT in phosphate-buffered saline solution (PBS) were added and incubated for 4 h at 37 °C. This was followed by removal of minimum medium and the MTT mixture and 100 µL aliquots of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals, followed by incubation for 10 min to dissolve any air bubbles while shaking at 100 rpm. The culture plate was placed on a Safire-Tecan microplate reader and the absorbance was measured at λ=590 nm. The absorbance intensity is directly proportional to the number of metabolically competent cells. All assays were performed in three replicates for each concentration. Cell viability (%) was calculated using the equation below, as suggested by Rezk *et al.* (2015).

$$\%\ \text{Cell survival} = \frac{mean\ absorbance\ in\ test\ wells}{mean\ absorbance\ in\ control\ wells} \times 100\%$$

Preliminary phytochemical screening

The preliminary phytochemical determination was done by using the analytical thin layer chromatography (TLC plate/aluminium sheet, silica gel $60F_{254}$–absorbent thickness: 0.25 mm, Merck, Darmstadt, Germany) on the active extracts (Wagner and Bladt 1996). Table 1 summarizes the solvent and the spray used to deduce for the presence of the various phytochemicals screened.

Statistical analysis

All experiments were conducted at least in triplicate to validate reproducibility with results reported as mean ± standard error (SE). The graphs were constructed using Graph Pad Prism software (version 6.01; Graph Pad Software, Inc., La Jolla, CA, USA).

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Table 1: Summary of TLC analysis of families of compounds.

Phytochemical	Solvent used (ratio)	Spray used to detect the phytochemical	Expected outcome
Flavonoids	Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26)	Natural products reagent-polyethylene glycol solution	Intense yellow–orange sometimes green fluorescence when illuminated with UV light (λ=365 nm)
Alkaloids	Toluene: ethyl acetate: diethylamine (70:20:10)	Dragendorff's reagent and then with 10% sodium nitrite solution or 10% ethanolic sulphuric acid	Brown or orange-brown
Tannins	Ethyl acetate: forric acid: water (90:5:5)	Spray 1% ethanolic vanillin followed by concentrated HCl	Pinkish bands observed
Anthracenes	Ethyl acetate: methanol: water (100:13.5:10)	Natural products reagent-polyethylene glycol solution	Intense yellow fluorescence at when illuminated with UV light (λ=365 nm)
Saponins	Chloroform: glacial acetic acid: methanol: water (64:32:12:8)	1% vanillin-sulphuric acid	Blue, blue-violet and sometimes red or yellow-brown zones
Triterpenes	Ethyl acetate: glacial acetic acid: formic acid: water (100:11:11:26)	Anisaldehyde–sulphuric acid reagent. After spraying, the TLC plate was heated for 6 min at 100 °C and observations were made.	Blue-violet colour when illuminated with UV light (λ=366 nm)
Cardiac glycosides	Ethyl acetate: methanol: water (75:10:3)	Anisaldehyde–sulphuric acid reagent	Blue colour when illuminated with UV light (λ=366 nm)

RESULTS

Medicinal plants harvested and the amounts extracted

A total of 23 samples of the plant material (Omwenga *et al.*, 2015) obtained from different parts (namely, root, leave, fruit, and bark) were harvested, ground and extracted using 50% ethanol (w/v) as presented in Table 2.

Antimicrobial assays

Agar well diffusion test

Most extracts did not show antimicrobial activity against the test isolates at 1 mg/mL concentration. *Cassia didymobotrya* was the best extract against *P. gingivalis* (21.70 mm) (Table 3). Against *H. pylori*, extracts of *L. nepetifolia* (15.80 mm) and *C. myricoides* (14.20 mm), produced mild inhibitory activity (Table 4). Gentamicin and amoxicillin were used as positive controls while the respective broth media for culturing each isolate was used as negative control. Against uropathogenic *E. coli* NU14, all of the plant extracts tested against this isolate showed no activity as the zones of inhibition were 6.00 ± 00 mm, as shown in the Table 5 and Figure 1 below. Only the positive control (gentamicin) had a slight activity with a zone of inhibition of 19.30 ± 0.78 mm (n=3).

MIC and MBC assay

The plant extracts that were active against the pathogens of interest were selected for determination of the bactericidal and bacteriostatic activities. Extracts from *L. nepetifolia* and *C. myriacoides* did not show the bactericidal or bacteriostatic effects against *H. pylori* as shown in Table 6. However, *C. didymobotrya* produced a bacteriostatic activity at 0.13 ± 0.00 mg/mL and bactericidal activity at 0.50 ± 0.00 mg/mL against *P. gingivalis*.

Mammalian cell cytotoxicity assays

The cytotoxic of the plant extracts that exhibit antimicrobial activity was examined on three different mammalian cell lines. *Porphyromonas gingivalis* affects the buccal cavity, hence, cell lines KB and TR146 of the buccal cavity were assayed against *C. didymobotrya* that inhibited its growth (Figure 2). Plant extracts (*L. nepetifolia* and *C. myriacoides*) that exhibited activity against *H. pylori*, which mainly invades the gut, were assessed against the AGS cell line (Figure 3). The various concentrations of the examined plant extract showed dose-response behaviour. From such a concentration-dependent response, it was possible to calculate the IC_{50} values. The IC_{50} of *C. didymobotrya* against KB and TR146 cell lines were 47.64 and 704.00 µg/mL respectively. These values are indicative of the plant extracts dosage that can kill 50% of the mammalian cells tested. Also, *L. nepetifolia* and *C. myricoides* did produce IC_{50} of 0.1883 mg/mL and 0.1061 mg/mL against the AGS cell line respectively.

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Table 2: The various medicinal plant samples harvested (Omwenga *et al.*, 2015) and the weight (grams) per medicinal plant extracted with 50% ethanol as solvent.

Plant No.	Botanical name	Plant family	Plant part used	Native plant use	Flask weight (g)	Flask + extract weight (g)	Extract weight (g)
P2	*Urtica dioica* L.	Urticaceae	Leaves	Skin and stomach infections	77.84	78.22	0.36
P3	*Erlangea marginata* S. Moore	Asteraceae	Leaves	Skin, stomach, oral cavity infections	74.43	74.65	0.22
P6	*Spilanthes mauritiana* DC.	Asteraceae	Leaves	Oral cavity infections	71.46	71.70	0.24
P7	*Orthosiphon hildebrandtii* Vatke	Lamiaceae	Leaves	Stomach and oral cavity infections	79.70	80.03	0.33
P8	*Dichrocephala integrifolia* Kuntze	Asteraceae	Leaves	Oral cavity infections	79.39	79.76	0.37
P9	*Leonotis nepetifolia* (L) R. Br.	Lamiaceae	Leaves	Stomach infections	72.66	77.08	0.42
P10	*Rhoicissus tridentata* (L. f) Wild and Drumm	Vitaceae	Leaves	Stomach, oral cavity infections	75.95	76.40	0.45
P11	*Toddalia asiatica* (L.) Lam	Rutaceae	Roots	UTIs	78.54	79.22	0.58
P12	*Asparagus africanus* Lam.	Asparagaceae	Roots	Skin infections, UTIs	80.59	80.93	0.34
P13	*Balanites orbicularis* Sprague	Balanitaceae	Roots	UTIs	78.85	79.15	0.30
P14	*Clerodendrum myricoides* (Hochst) R.Br. and Vatke	Lamiaceae	Roots	Stomach infections, UTIs	77.87	78.97	1.10
P15	*Caesalpinia decapetala* (Roth) Alston	Caesalpiniaceae	Roots	Skin infections, UTIs	78.97	79.55	0.58
P16	*Solanum renschii* Vatke	Solanaceae	Roots	Stomach infections	61.34	61.55	0.21
P17	*Croton macrostachyus* Hochst. ex Delile	Euphorbiaceae	Bark	Oral cavity infections	74.45	74.72	0.27
P18	*Erythrina abyssinica* Lam.	Fabaceae	Bark	UTIs	79.88	80.86	0.98
P19	*Rhus natalensis* Bernh. Ex Krauss	Anacardiaceae	Bark	UTIs	80.39	80.85	0.46
P20	*Warburgia ugandensis* Sprague	Canellaceae	Bark	Stomach and tooth infections	79.96	80.18	0.22
P21	*Elaeodendron buchananii* Loes	Celastraceae	Bark	UTIs	78.38	79.08	0.70
P22	*Acacia gerrardii* Benth.	Mimosaceae	Bark	Stomach infections	66.97	67.71	0.74
P23	*Carissa edulis* Vahl	Apocynaceae	roots	UTIs	78.37	78.76	0.39
P24	*Cassia didymobotrya* Fresen	Fabaceae	Leaves	Stomach, skin, oral cavity infections	74.40	74.67	0.27
P25	*Acacia nilotica* (L.) Delile.	Mimosaceae	Bark	Stomach infections, malaria	81.38	81.80	0.42
P26	*Bischofia javanica* Blume	Euphorbiaceae	Roots	Stomach infections	76.37	76.97	0.60

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Table 3: Zones of inhibition (mm) produced by selected plants extracts (1 mg/mL) commonly used to manage mouth infections against *P. gingivalis* ATCC 33277 (n=3).

Plant botanical name	Mean zone of inhibition (mean ± SE)
Erlangea marginata S. Moore	6.30 ± 0.33
Spilanthes mauritiana DC.	6.00 ± 0.00
Orthosiphon hildebrandtii Vatke	7.30 ± 0.33
Dichrocephala integrifolia Kuntze	7.00 ± 0.58
Rhoicissus tridentata (L. f) Wild and Drumm	6.00 ± 0.00
Croton macrostachyus Hochst ex Delile	6.20 ± 0.02
Warburgia ugandensis Sprague	6.00 ± 0.00
Cassia didymobotrya Fresen	21.70 ± 0.88
Broth (Negative control)	6.00 ± 0.00
Amoxicillin (Positive control)	40.30 ± 0.88

Table 4: Zones of inhibition (mm) produced by selected plants extracts (1 mg/mL) commonly used to manage gastrointestinal infections against *H. pylori* ATCC 700824 (n=3).

Plant botanical name	Mean zone of inhibition (mean ± SE)
Urtica dioica L.	6.20 ± 0.17
Erlangea marginata S. Moore	6.00 ± 0.00
Orthosiphon hildebrandtii Vatke	6.00 ± 0.00
Leonotis nepetifolia (L) R. Br.	15.80 ± 0.20
Rhoicissus tridentata (L. f) Wild and Drumm	6.20 ± 0.17
Clerodendrum myricoides (Hochst) R. Br. and Vatke	14.20 ± 0.44
Solanum renschii Vatke	6.00 ± 0.00
Warburgia ugandensis Sprague	6.00 ± 0.00
Acacia gerrardii Benth.	6.10 ± 0.07
Cassia didymobotrya Fresen	6.20 ± 0.17
Acacia nilotica (L.) Delile.	6.00 ± 0.00
Bischofia javanica Blume	6.10 ± 0.06
Broth (Negative control)	6.00 ± 0.00
Gentamicin (Positive control)	36.00 ± 0.58

Table 5: Zones of inhibition (mm) produced by selected plants extracts (1 mg/mL) commonly used to manage urinary tract infections against uropathogenic *E. coli* NU14 (n=3).

Plant botanical name	Mean zone of inhibition (mean ± SE)
Toddalia asiatica (L.) Lam	6.00 ± 0.00
Asparagus africanus Lam.	6.00 ± 0.00
Balanites orbicularis Sprague	6.00 ± 0.00
Clerodendrum myricoides (Hochst) R. Br. and Vatke	7.00 ± 0.58
Caesalpinia decapetala (Roth) Alston	6.00 ± 0.00
Erythrina abyssinica Lam.	6.00 ± 0.00
Rhus natalensis Bernh Ex Krauss	6.00 ± 0.00
Elaeodendron buchananii Loe	6.00 ± 0.00
Carissa edulis Vahl	6.20 ± 0.17
Broth (Negative control)	6.00 ± 0.00
Gentamicin (Positive control)	19.30 ± 0.78

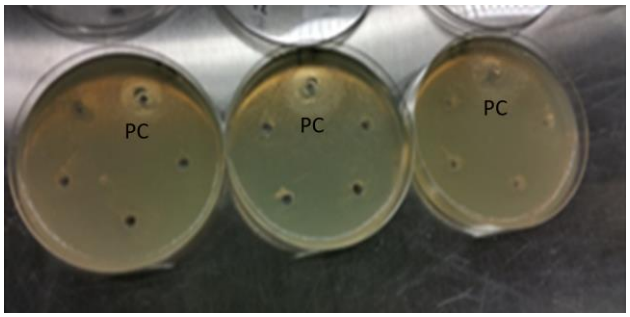

Figure 1: The zone of inhibition produced by various plant extracts that were documented to be used to manage urinary tract infections and as presented in Table 5 against uropathogenic *E. coli* NU14 (1 mg/mL). PC, positive control.

Preliminary phytochemical screening

The chemical identity of the extract of *C. didymobotrya* that showed outstanding antimicrobial activity against *P. gingivalis* was preliminary investigated by TLC to deduce the most probable families of phytochemicals present (Supplementary data S2). *Leonotis nepetifolia* and *C.* myricoides did show mild antimicrobial activity against *H. pylori* but not comparable with the positive control and as such they were not subjected to phytochemical determination. To this end, alkaloids, tannins, flavonoids, anthracenes, saponins, cardiac glycosides and triterpenes were screened, and its results are summarized in Table 7 below.

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Table 6: Summary of antimicrobial activity (MIC and MBC) for selected plant extracts (n=3).

Plant botanical name	Microorganism	MIC (mg/mL)	MBC (mg/mL)
Cassia didymobotrya Fresen	*P. gingivalis*	0.13 ± 0.00	0.50 ± 0.00
Gentamicin	*P. gingivalis*	0.50 ± 0.00	0.50 ± 0.00
Leonotis nepetifolia (L) R. Br.	*H. pylori*	-	-
Clerodendrum myricoides (Hochst) R. Br. and Vatke	*H. pylori*	-	-
Gentamicin	*H. pylori*	0.02 ± 0.00	0.02 ± 0.00

Table 7: Preliminary TLC results of the *C. didymobotrya* extracts.

Alkaloids	Tannins	Flavonoids	Anthracenes	Saponins	Cardiac glycosides	Triterpenes
-	+	+	+	-	-	+

+, present; -, absent.

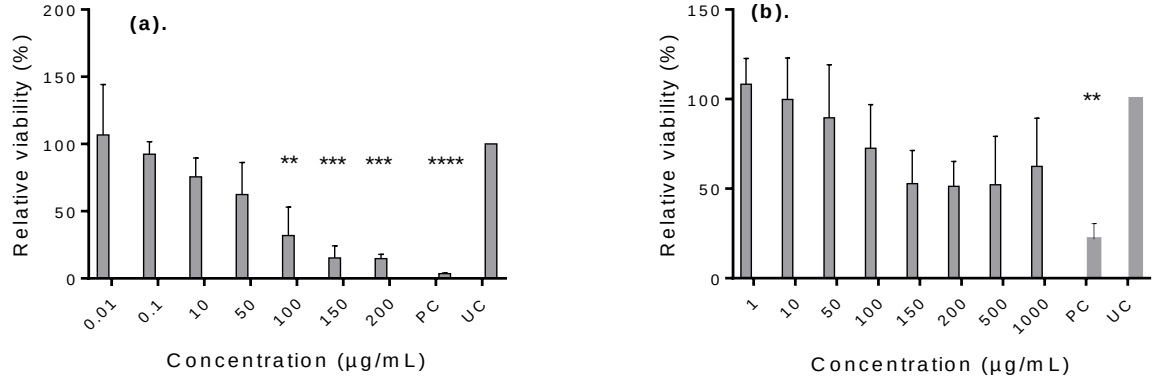

Figure 2: Cytotoxicity of ethanol 50% (v/v) extracts of *C. didymobotrya* against (a) KB, and (b) TR146 cells in 96-well plates determined using the MTT assay (n=3). UC, negative control (untreated cells); PC, positive control – 4% Triton X100; * indicates significant different to UC.

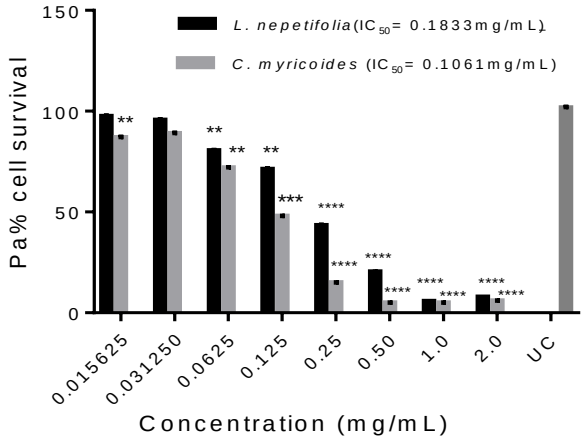

Figure 3: Cytotoxicity of ethanol 50% (v/v) extracts of *L. nepetifolia* and *C. myricoides* against AGS cell line (n=3). UC, untreated cells (negative control); * indicates significant different to UC.

DISCUSSION

The majority of the plants (23) screened did not show any antimicrobial activity against the test bacterial pathogens. These strains were selected based on the diseases they can cause and were tested with the medicinal plants as per their use by the local inhabitants of Borabu sub-county, Nyamira county-Kenya (Omwenga *et al.*, 2015). Agar well diffusion test indicated that the ethanolic (50% v/v) extract of *C. didymobotrya* produced an inhibitory effect of 21.70 ± 0.88 mm against *P. gingivalis* ATCC 33277 (Table 3) at a concentration of 1 mg/mL. To the best of our knowledge, this is the first report of the activity of the extracts of *C. didymobotrya* against a mouth cavity pathogen. Out of the portfolio of medicinal plants reported to manage gastrointestinal (GIT) infections, only two of them showed moderate activity against the gastric pathogen *H. pylori* ATCC 700820 (Table 4), namely *L. nepetifolia* (15.80 ± 0.2 mm) and *C. myriacoides* (14.20 ± 0.44 mm) at 1 mg/mL. However, no activity was detected for the examined plant extracts at 1 mg / mL dosage

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against the *E. coli* NU14 strain chosen as the representative pathogen of the urinary tract.

According with our results, the majority of the traditional medicinal plants reported to treat ailments of the mouth cavity, GIT and urinary tract did not show significant antimicrobial activity against representative bacterial pathogenic strains upon dosing at 1 mg/mL. These results may explain why the local healers often mix the medicinal plants to make a concoction for management of the various diseases. The scientific basis of this could be related to the synergistic effects that the phytochemicals present in medicinal plants achieve when they are mixed together. Alternatively, the passage of the active compound through the various components of the Gram-negative cell wall could be inhibited. It is known that the activity of antimicrobial drugs against Gram-negative bacteria is reduced due to the low permeability of the outer membrane lipopolysaccharides (LPS) that contains bilayers that are more rigid than normal bilayers. This slows passive diffusion of hydrophobic compounds, whereas narrow pores limit by size the penetration of hydrophilic drugs/compounds (Li *et al.*, 2015; Delcour, 2009; Zgurskaya *et al.*, 2015). Also, the slow influx of drugs/compounds across the outer membrane is further opposed by the action of efflux and multidrug efflux transporters. Multidrug efflux transporters are structurally and functionally diverse, with some transporters pumping antibiotics across the inner membrane and reducing concentrations of antibiotics in the cytoplasm, whereas others expel antibiotics from the periplasm into the external medium (Zgurskaya, 2009; Zgurskaya *et al*., 2015).

Yet another consideration to explain the observed differences could result from the doses used in this study. In addition, microorganisms show variable sensitivity to chemical substances related to different resistance levels between strains (Subasri and Ahmed., 2016).

Based on the ethnobotanical survey preceding this study (Omwenga *et al*., 2015), it was found that the selected medicinal plants have been used for centuries by the healers of the Kisii communities at Borabu region, Kenya. The discrepancy between the results of the antimicrobial assays of this study and the documented traditional use could stem on several causes. One of these could be associated with the mode of action mediated not directly by antimicrobial activity but other such as immunomodulating abilities. For instance, Mwitari *et al*. (2013) reported that *Withania somnifera* up-regulated IL-7 cytokine by two-fold in IEC- 6 (ATCC) mammalian cell line. This group of cytokines has been found to stimulate the immune response by producing and releasing more CD4+ and CD8+ lymphocytes leading to enhanced clearance of invading microbes. Therefore, further studies in this direction are necessary towards a better elucidation of the specific mode of action of the studied medicinal plants.

Our assays on *L. nepetifolia* and *C. myriacoides* plant extracts did not show either bactericidal or bacteriostatic effects against *H. pylori* as they had only moderate inhibitory effects (Table 4). The positive control with antibiotic Gentamicin, however, confirmed a bacteriostatic and bactericidal effect at a concentration of 0.02 ± 0.00 mg/mL. Perhaps this could be the reason of NCCLS that recommends any Gram-negative bacteria with such a range of inhibitory effects (agar well diffusion test – Table 4) should be declared as resistant or intermediate to the test antibiotic/agent. However, *C. didymobotrya* produced a bacteriostatic activity at 0.13 ± 0.00 mg/mL and bactericidal activity at 0.50 ± 0.00 mg/mL (n=3) against *P. gingivalis.* These doses are at least approximately an order of magnitude greater than that of the positive control (gentamicin) that produced a bacteriostatic and bactericidal effect at a concentration of 0.02 ± 0.00 mg/mL against *H. pylori*. These results appear to confirm the antibacterial potential of the *C. didymobotrya* investigated and its usefulness in the treatment of mouth cavity infections. However, for an antibiotic to be effective, the infective agent must be eradicated at the site of the infection. The antibiotic effectiveness will be influenced by the route of administration, the dose, and frequency of administration, rate of absorption and distribution of the antibiotic (Omwenga *et al*., 2009), and by the resistance of the pathogenic strain to the specific compound (Zgurskaya *et al*., 2015).

From these findings, it is possible therefore, to hypothesize that either the plant extracts had a higher diffusion rate (i.e., permeability) across the cell wall of the tested Gram-negative species, or there is ingrained resistance to the active compounds in some of the strains that showed no sensitivity. In addition, plant extracts could be host-specific in their antibacterial activity, since the zones of inhibition varied for each tested strain. This was observed with *C. didymobotrya*, which showed promising antibacterial activity against *P. gingivalis*, but not against *H. pylori* ATCC 700820. The difference in the activity, therefore, cannot be attributed to the cell wall/membrane properties as both are Gram-negative, but could be related to the inhibition of specific important biological pathway(s) that is/are crucial to a given species of bacteria that could be absent on the other species. The different rates of inhibition with these extracts might also be attributed to the concentration of the phytochemical compounds present in the extracts. Antagonistic activity between the occurring phytochemicals cannot be ruled out either, especially in the extracts that showed no activity against the test isolates. Therefore, such medicinal plants may not offer to be a real alternative medicine against diseases caused by such pathogens (Ruttoh *et al.*, 2009).

The active medicinal plants also showed activity against mammalian cell lines of the buccal cavity (namely KB and TR146 cell lines) and the stomach (namely AGS cell line). As shown in Figures 2 and 3 there was a significant increase ($P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$) in cell viability with the various concentrations of the plant extracts involved compared with the control group. *C. didymobotrya* produced an IC_{50} of 47.64 µg/mL with the KB cell line, while for the TR146 cell line, the extract seemed to exhibit an increased metabolic competence (as per the MTT assay) especially at higher

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concentrations. This effect has been reported before for some plant extracts. For instance, Peng *et al*. (2005) reported that quercetin and luteolin, flavonoid secondary metabolites well-known to occur in plants reduced themselves the MTT reagent into formazan (i.e., as they are known to be potent antioxidants). Bruggisser *et al*. (2002) also did indicate that the application of the tetrazolium assays in screening systems of the natural products to detect their influence on the cell viability demands precautions as they may produce false-positive results. Therefore, it seems plausible to suggest that the extract of *C. didymobotrya* could have such phytochemicals that influence the MTT assay at higher concentrations as shown in Figure 2 against TR146 cell line. *L. nepetifolia* and *C. myriacoides* also produced an IC_{50} of 0.1833 and 0.1061 mg/mL respectively, with the AGS cell line.

From the results of the preliminary phytochemical screening by use of TLC analysis, it was realised that *C. didymobotrya* extract could be rich in various phytochemicals such as tannins, flavonoids, anthracenes and triterpenes. Such phytochemicals have been proved before to possess antimicrobial activities and could be responsible for the activity evidenced against *P. gingivalis*. It has been indicated that tannins have antimicrobial effects that could be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, and mineral uptake (Scalbert, 1991; Min *et al*., 2008). The presence of tannins in *C. didymobotrya* was preliminary evidenced. Tannins and other phytochemicals may account for the activity exhibited against the *P. gingivalis* isolate. This plant has also been reported to contain other compounds, such as α-amyrin, β-amyrin, arachidonic acid, chrysophanic acid, kaempferol, lauric acid, myristic acid, myristoleic acid, oleic acid, palmitic acid, rhein, glycoside, β-sitosterol stearic acid 5, 1,4-anthoquinone chrysophanic acid, daucosterol, physcion, knipholone and several anthroquinine. These great phytochemicals diversity could be key to its antimicrobial properties (Mahadevan *et al*., 2002).

CONCLUSION

The present study has contributed to expanding the scientifically based knowledge about the library of validated traditional medicinal plants used by the local inhabitants of Borabu sub-county, Nyamira county- Kenya for management of oral cavity, gastrointestinal tract and urinary tract ailments. Most of the identified plants had no activity against representative pathogenic strains of such diseases, namely *P. gingivalis* ATCC 33277, *H. pylori* ATCC 700820 and *E. coli* NU14. However, the ethanolic (50% v/v) extract of *C. didymobotrya* showed promising antibacterial activity against *P. gingivalis*. Further research is recommended on this extract so that the individual active phytochemicals can be mined and screened against this bacterial isolate and their structure be elucidated. *L. nepetifolia* and *C. myriacoides* extracts did show a moderate antimicrobial effect against *H. pylori* and further research is encouraged against other bacterial isolates. As regards the potential toxicity of the *C. didymobotrya* extracts, not conclusive evidence could be gathered, due to the potential interference of the phytochemicals with the MTT assay. Further alternative assays to probe cell viability are necessary.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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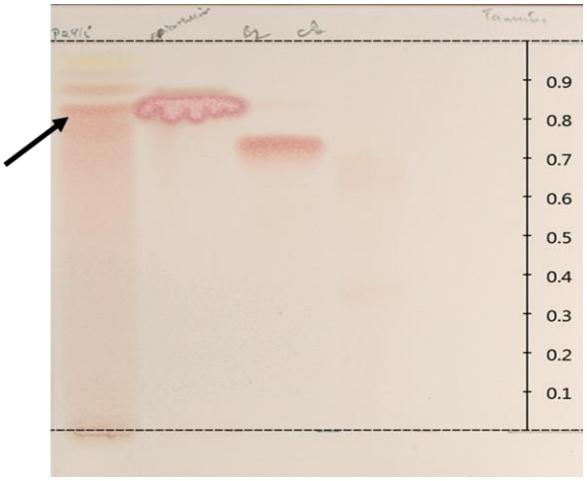
SUPPLEMENTARY DATA

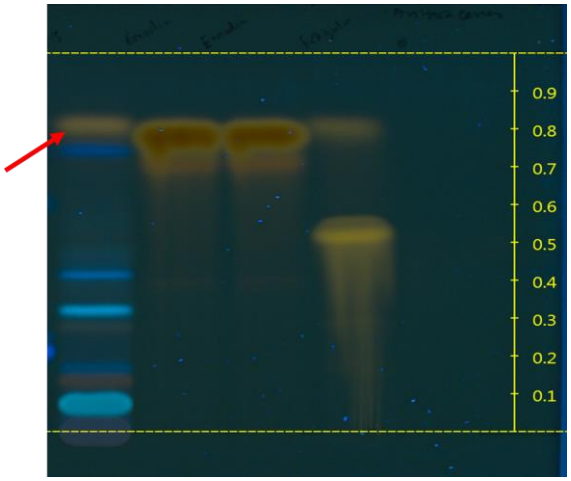
Supplementary data S1: Preparation of 1 L of medium for *P. gingivalis* culturing

Weigh the following reagents: 16 g of agar, 15 g of trypticase peptone, 5 g neutralised soy peptone (dehydrated), 5 g yeast extract, 0.5 g L-cysteine (non-animal source), 1000 µL stock solution of 100 µL Vitamin K1 in 9.9 mL 96 % ethanol (stored at 4 ºC, dark), 10 mL Hemin stock solution (50 mg Hemin (bovine) dissolved in 1 mL of 1 mol/L sodium hydroxide, pro analysis), 50 mL sheep blood (defibrinated). Add 1 L of distilled water. N/B: For broth, avoid adding 16 g of agar.

Supplementary data S2: The TLC chromatograms



S2 (i): Tannins test chromatogram. 50% ethanol extract of *C. didymobotrya* (P24/5) showing presence of tannins (as indicated by black arrow). Epicathecin (Lane 2), Procyanidin B2 (Lane 3) and procyanidin C1 (Lane 4) were used as controls.



S2 (ii): Anthracenes test chromatogram. 50% Ethanol extract of *C. didymobotrya* (P24/5) showing presence of anthracenes (as indicated by the red arrow). Emodin (Lane 2 and 3) and fragulin (Lane 4) were used as controls.

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