Evaluation of the antibacterial activity of Launaea cornuta extracts

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Abstract

Background

Medicinal plants have been used time immemorial to treat various ailments and diseases caused by pathogenic microorganisms attributed bioactive compounds they contain. Such compounds may have developed in plants as self-defense mechanism against pests and pathogens purposely to survive and perpetuate themselves in their ecosystem. A few studies have been conducted on *L. cornuta*. The study sought to determine the effect of *L. cornuta* leaf, stem bark and root extracts against selected bacteria.

Methods

Methanol, Dichloromethane and Aqueous extracts of *L. cornuta* were tested for bacteriostatic and bactericidal effects against *Escherichia coli* ATCC 27853, *Shigella dysenteriaea* ATCC 13313 and *Pseudomonas aeruginosa, Staphylococcus aureus* ATCC 25923. Phytochemical screening of the extracts was performed using thin layer chromatography (TLC) with Ethyl acetate: petroleum spirit at the ratio of (3:7) as the solvent system for dichloromethane extracts while dichloromethane: methanol ratio (9.5: 0.5) solvent system was utilized for methanol extracts. Cytotoxicity of the active extracts was determined using Vero E6 cell lines. The cell cytotoxicity levels were read using an ELISA scanning Multiwell spectrophotometer (Multiskan Ex Labssytems, Thermo Fisher Scientific, USA) at 562 nm and 620 nm as reference. The cytotoxic concentration responsible for lysis and death of 50% of the cells was determined using Microsoft Office Excel 2007 software by linear regression analysis.

Results

Stem, leave and root extracts showed weaker / little or no activity with zone diameter inhibition < 10mm against the selected bacteria. No activity was observed with *S. aureus.* Minimum inhibitory concentration (MIC) value of extracts ranged between 500mg/ml to 60 mg/ml. DCM stem bark extracts had MIC value of 62.5 mg/ml against *S. dysenteriaea* and 500mg/ml against *S. aureus.* The extracts were not cytotoxic at 1000 µg/ml which was the highest concentration tested except methanol leave extracts that were found to be moderately toxic at 493.5 µg/ml.

Conclusions

The study findings suggest that *L. cornuta* methanol, aqueous and dichloromethane extracts are moderately efficacious in the control of the selected bacteria.

Background

To counter bacterial infections, the utilization of antibiotics is mostly predominant. In the recent past, most antibiotics have lost their potency due to the emergence of resistant strains attributed to expression of resistance genes [1]. The urge to develop alternative antibacterial drugs for the treatment of infectious diseases from various sources such as medicinal plants is paramount.

Launaea cornuta (Bitter lettuce) is native to Africa predominantly Cameroon, Central African Republic, Ethiopia, Kenya, Malawi, Mozambique, Nigeria, Rwanda, Somalia, Sudan, Tanzania, Uganda, Congo, Zambia, and Zimbabwe [2]. It thrives on alluvial soils in cultivated areas, on roadsides, near rivers and bush vegetation and is widely

distributed in almost all parts of Kenya. In Marakwet community, a concoction from the boiled roots of *Launaea cornuta* locally called “Kipche” is used in the treatment of throat cancer [3]. Equally, its leaves and roots are used by the Suba people in Kenya as antimalarial and antimicrobial particularly to treat opportunistic diseases associated with HIV/AIDS [4]. Traditional medicine as an alternative form of therapy has inspired scientists to further research on the antimicrobial activity of several medicinal plants [5 and 6]. The study focused on the evaluation of antibacterial properties and cytotoxicity of methanol, aqueous and dichloromethane extracts of *L. cornuta.* This provided valuable information on the antibacterial activity and safety of *L. cornuta* extracts.

Materials And Methods

Plant materials

Launaea cornuta stem, root and leave were collected in Kapsowar, Marakwet west sub-county based on its local use in traditional medicine. The plant species was authenticated by a botanist at the Department of Biological Sciences University of Eldoret. A voucher specimen (MU/0048/89) was deposited at the University of Eldoret Herbarium.

Bacterial cultures

The bacterial strains used were *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 27853, *Shigella dysenteriaea* ATCC 13313 and *Pseudomonas aeruginosa*. All the strains were obtained from the KEMRI culture collection and maintained as stock cultures in 50% glycerol in Eppendorf® tubes at -30°C until usage.

Cell lines

Vero E6 cell lines, sourced from American Type Culture Collections (ATTC® CRL-1586™) and cultured at Centre for Traditional Medicine and Drug Research (CTMDR) KEMRI laboratories were used in cytotoxicity studies.

Extraction Procedure

The plant parts (roots, leaves and stem bark) were air dried at room temperature (25 °C) under shade for two weeks, crushed using a laboratory mill (Christy and Norris Ltd., Chelmsford, England) and packed in airtight polythene bags at the Center for traditional medicine and drug research (CTMDR) KEMRI. At least 200g of the powdered plant material were collected and their percentage extract yield calculated. Fifty grams of the powdered plant material was extracted using three solvents: 500 ml distilled water in a shaking water bath set at 70°C for two hours, filtered, dried using a freeze dryer (Edwards freeze dryer Modulyo) then weighed and stored. Equally, soaking of the powdered plant material was done using methanol and dichloromethane for 24 h each, and the organic solvents evaporated to dryness by vacuum evaporation using a rotary evaporator (Buchi Rotavapor R-114). Percentage yield was calculated as follows:

$$\text{Percentage yield} = \frac{\text{Weight of extract obtained}}{\text{Weight of powdered material}} \times 100\ \%$$

Determination of Antibacterial Activity

Disc diffusion assay

The antibacterial activity of the extracts against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 27853, *Shigella dysenteriaea* ATCC 13313 and *Pseudomonas aeruginosa* using the Kirby-Bauer disc diffusion method [7] on

Mueller Hinton (MH) agar (Oxoid) at 37 ° C for 24h. Mueller Hinton agar test plates were set and inoculated on their surface with a cell suspension of the test bacteria (1.5 x 10^8 c.f.u ml^{-1}) in sterile normal saline. All the test assays were done in Class II Biological Safety Cabinet. Sterile Whatman's No.1 (6mm diameter) discs were infused with 20 µl of the extracts from the stock solution of 100 mg ml^{-1} all observing the disc diffusion assay protocol. Disks containing extracts, Gentamicin discs (25 µg) used as positive control and discs containing sterile distilled water used as negative control were aseptically placed on the MH agar. The test plates were incubated at 37°C for 24 h. Each assay was done in triplicates. The zones of inhibition diameters were measured in millimeters and the results expressed as mean inhibition zones ± standard deviation. Figure 1 below

Minimum inhibitory concentration (MIC) assay

The minimum inhibitory concentration (MIC) test as described by [8] was considered only on plant extracts that exhibited significant antimicrobial activity that is a mean zone diameter of inhibition of ≥10 mm. Serial dilutions of the extract were done using distilled water resulting in a working concentration range from 500 mg ml^{-1} to 30 mg ml^{-1}.

Accurately 20 µl of each plant extract was conveyed onto a 96-well titer plate and serially diluted from a concentration of 100 mg/ml to 0.05 mg/ ml using sterile tryptic soy broth. Gentamicin (20 µl), the positive control, was serially diluted from a concentration of 14 mg/ml to 0.0068 mg/ml using sterile tryptic soy broth. Each plant extract (test) and Gentamicin (control) were loaded in two rows of the micro titer plate for serial dilution respectively. The micro titer plates were incubated at 35 °C for 24 hours for observations to be made. MIC was pronounced as the lowest concentration of the extract that presented a clear zone of inhibition [9].

Phytochemical Screening

Phytochemical screening of active extracts was done to determine the phytocompounds present in different extracts separated by thin layer chromatography (TLC) (Kieselgel 60 F254 0.2 mm, Merck). TLC plates were developed with Ethyl acetate: petroleum spirit (3:7) as the solvent system for dichloromethane extracts while dichloromethane: methanol (9.5:0.5) solvent system was employed for methanol extracts [10]. Separated constituents were visualized under ultra violet light (254nm and 365 nm) then sprayed with visualizing agents for the colorimetric view

Cytotoxicity Assay

The most active plant extracts were examined for *in vitro* cytotoxicity according to the modified rapid calorimetric assay [11] using Vero E6 cancer cell lines acquired from American Type Culture Collections (ATTC) and were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 2 mol L^{-1} glutamine. Approximately 2 x 10^5 cell ml^{-1} suspensions were seeded on 96- well microtiter plates and incubated in a humified atmosphere with 5% CO_2 at 37°C for 12 hours. Test extracts were added to the cultured cells over a concentration range of 1000 µg ml^{-1} to 1.23 µg ml^{-1}. The plates were incubated at 37°C, 5% CO_2 for 48 h following which 10 µL of (3-(4, 5-dimethylthiazol-2-yr)-2, 5- diphenyl tetrazolium bromide) MTT dye was added to each well. Incubation of the plates was effected for another four hours, and subsequently, the media detached from the wells and 100 µL of Dimethyl sulfoxide (DMSO) were added [11]. The plates were read as color absorbance (abs) using an ELISA scanning Multiwell spectrophotometer (Multiskan Ex Labssytems) at 562 nm and 620 nm as reference. The percentage cell viability (C.V.) was calculated using the formula:

$$\% \text{ C. V.} = \left[\frac{(\text{Average abs of duplicate drug wells} - \text{Average abs of blank wells}) \times 100\%}{[\text{Average abs of control wells}]}\right]$$

Data were entered into Microsoft Office Excel 2007 software and expressed as a percentage of the untreated controls. Cytotoxic concentration responsible for lysis and death of 50% of the cells was determined using Microsoft Office Excel 2007 software by linear regression analysis.

Results

Extraction of the Plant

The percentage yields resulting from different solvent extracts are summarized in Table 1. Water extracts presented relatively higher extract yields followed by methanol and DCM the least. Stem bark produced the higher extract yield, followed by roots and leaves the least. Aqueous stem bark presented the highest yields of 12.1% while dichloromethane leaves presented the least yield of 2.2% (Table 1).

The percentage yields resulting from different solvent extracts are summarized in Table 1. Dichloromethane leave extracts presented relatively higher extract yield 3.9 % followed by its root bark 2.7 % and the least crude extract yield displayed by aqueous stem bark 1.3 % (Table 1).

Antibacterial bioassay

Table 2 shows the results of bacterial growth inhibition by *L. cornuta* against *S. dysenteriaea, P. aeruginosa, E. coli,* and *S. aureus*. The root and stem bark methanol and DCM extracts were moderately active against *S. dysenteriaea* and *S. aureus* with average IZD of 11.00±1.00mm and 10.67±0.58mm respectively.

Table 3 illustrates the minimum inhibitory concentration (MIC) values that were considered for extracts showing IZD ≥ 10 mm and that this featured DCM stem bark and root extracts against *S. dysenteriaea* and *S. aureus* with MIC values of 62.5mg/ml, 125 mg/ml and 500 mg/ml respectively.

Cell cytotoxicity studies

Cell cytotoxicity studies of the selected extracts indicated by their MIC values ≤ 125 mg/ml. As the concentration of the selected extracts decline following serial dilutions on the 96-well microtiter plates, the viability of the Vero E6 cells increase. This is depicted by the deduction of MTT quantified by measuring absorbance of the colored solution using spectrophotometer. Methanol leaf extracts were found to be moderately toxic under fifty percent inhibitory concentration (IC_{50}) of 493.5 mg/ml while DCM root extracts were less toxic with IC_{50} value of 801.43 mg/ml (Table 5).

Phytochemical screening

This was carried out to determine the bioactive compounds present in the extracts which tested abundance for tannins, phenols, saponins, terpenoids, and anthraquinones. Apparently, flavonoids were totally absent for methanol and aqueous extracts in all parts and likewise traces of the extracts were observed in all plant parts for alkaloids (Table 4).

The positive control was Gentamicin standard antimicrobial discs 10µg ml^{-1}. Plant extract concentration was 100 mg/ml. *p-values* of ≤0.05 demonstrate statistical significance.

Discussion

The antibacterial activity of *Launaea cornuta* against the bacteria tested was determined for each of the three extracts under scrutiny. Greater activity was observed with methanol root extracts and DCM stem extracts with inhibition zone diameters (IZD) of 11.00 ± 1.00 mm, MIC value of 125mg/ml and 11.67 ± 0.58 mm, MIC value of 62.5 mg/ml respectively. The little or no activity of the water extracts against most bacterial strains is in tandem with previous studies propagating that aqueous extracts displayed little or no antimicrobial propensities [12].

In Kenya, the Suba people utilize a decoction of the leaves and roots as antimalarial and antimicrobial particularly to treat opportunistic diseases linked with HIV/AIDS [4].

Phytochemical constituents proved the existence of phenols, tannins, flavonoids, alkaloids and anthraquinones in which case the antimicrobial tendencies are squarely attributed to these secondary metabolites.

Toxicity studies are vital in determining the effectiveness of medicinal plants. In this regard, MTT [3-(4, 5-dimethylthiazol-2-yr)-2, 5-diphenyltetrazolium bromide] colorimetric assay established by [11] was used. Extracts whose MIC values were ≤ 1.25×10^{5} µg ml^{-1} were considered and their potency was investigated. The *in vitro* cytotoxicity of the four crude extracts of *L. cornuta* are displayed in Table 5. The inhibitory concentration at 50% (CC_{50}) is the concentration of the extracts that inhibits 50% proliferation of the Vero E6 cell lines. The extracts showed wavering degrees of toxicity on the Vero E6 cell lines with IC_{50} values ranging from 300 µg ml1 to 900 µg ml1 (Table 5).

The reference standards to authenticate the activity of *L. cornuta* extracts against Vero E6 cell lines was centered on CC_{50} values modified from those of National Cancer Institute (NSI) and [13] as follows: IC value ≤ 20 µg ml1 = extremely toxic (very active), CC_{50} 21–200 µg ml1 = highly toxic (moderately active), IC_{50} 201–500 µg ml1 = moderately toxic (weakly active), CC_{50} ≥ 501–1000 µg ml1 = lowly toxic (inactive), CC_{50} ≥ 1000 µg ml1 = particularly non-toxic.

Grounded on this, DCM stem bark and root extracts were weakly toxic and lowly toxic against Vero E6 cell lines with CC_{50} of 546 µg ml^{-1} and 800 µg ml^{-1} respectively. In the same way, methanol root extracts were moderately toxic with CC_{50} values of 493.5 µg ml^{-1}. The findings tend to agree with studies by [4] on the use of medicinal plants among persons immunocompromised in Suba District, Kenya.

Conclusion

The methanol and DCM extracts of *L. cornuta* assayed exhibited a promising activity against *Staphylococcus aureus, Shigella dysenteries, Escherichia coli, Pseudomonas aeruginosa.* The need to clarify which particular phytocomponent responsible for one or both activities is essential.

Abbreviations

DCM, Dichloromethane

MIC, Minimum Inhibitory Concentration

IZD, Inhibitory Zone Diameter

Declarations

Ethics approval and consent to participate

Approval to carry out this work was obtained from the KEMRI Scientific and Ethics Review Unit (SERU) (KEMRI/SERU/CTMDR/091) and the Board of Postgraduate Studies Scientific Steering Committee, University of Eldoret, Kenya SC/PGB/002/14.

Authorization was sought and was granted from the Ward Administrator, Kapsowar Marakwet West, ElgeyoMarakwet County before the plant material was collected.

The collection of plants adhered to the guidelines stipulated by the Convention on the Trade in Endangered Species of Wild Fauna and Flora (CITES).

No live animals or specimens for humans were used thereof in the study.

Consent for Publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Not applicable.

Authors' contributions

TC - Study design, literature search, data collection, writing manuscript, performing antimicrobial assays and phytochemical analysis.

LM - Research supervision, Study design, editing manuscript, provision of reagents and materials used in the study.

KL - Study design, supervision of acute toxicity, research supervision, data analysis, data interpretation and editing the manuscript.

All authors read and approved the final manuscript.

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Tables

Table 1. Percentage yield of the crude extract

Plant part	Crude extract percentage yield (%)		
	Dichloromethane	Methanol	Water
Leaves	3.9	2.3	2.3
Root bark	2.7	1.8	2.3
Stem bark	1.95	2.1	1.3

Table 2: Antibacterial bioassay

Bacteria	Part	Average IZD ± Std. dev. of triplicates (mm)				
		Methanol	DCM	Water	Gentamicin	*p-values*
S. aureus	L	6.67±0.58	6.00±0.00	6.00±0.00	15.33±0.58	0.0000
	R	7.33±0.33	7.67±0.58	9.67±0.58	15.00±1.00	0.0000
	SB	8.00±0.00	10.67±0.58	6.67±0.58	15.33±0.58	0.0000
S. dysenteriaea	L	6.00±0.00	6.00±0.00	6.33±0.47	16.33±0.58	0.0000
	R	11.00±1.00	8.00±0.00	7.00±0.00	16.00±1.00	0.0000
	SB	6.00±0.00	11.67±0.58	6.33±0.58	16.33±0.58	0.0000
E. coli	L	8.67±0.58	6.33±0.58	6.33±0.58	18.67±0.58	0.0000
	R	7.67±1.53	6.67±0.58	7.67±0.58	20.00±0.00	0.0000
	SB	9.00±0.00	6.67±0.58	6.67±0.58	18.68±0.58	0.0000
P. aeruginosa	L	6.00±0.00	6.67±0.58	6.00±0.00	16.33±0.58	0.0000
	R	6.00±0.00	6.33±0.58	7.00±1.00	15.00±1.00	0.0000
	SB	6.67±0.58	6.67±0.58	6.00±0.00	14.33±0.58	0.0000

Key: *L – Leaves, R – Roots and SB- Stem bark. (IZD) Zone diameter of microbial inhibition.*

Table 3: MICs of plants' crude extracts for antibacterial assays

Plant	Plant part	Test sample (solvent)	Test organism	MIC (mg/ml)
Standard	*Gentamicin			
L. cornuta	Stem bark	DCM	*S. aureus*	500
L. cornuta	Roots	DCM	*S. aureus*	125
L. cornuta	Roots	Methanol	*S. dysenteriaea*	125
L. cornuta	Stem bark	DCM	*S. dysenteriaea*	62.5

Table 4. Phytochemical constituents present in *L. cornuta* extracts

Solvent	Part	Tannins	Flavonoids	Phenols	Saponins	Terpenoids	Alkaloids	Anthraquinones
Water	L	++	-	+++	+++	+++	++	++
	R	+++	-	+++	+++	+++	+	+++
	SB	+++	-	+++	+++	++	++	++
DCM	L	++	++	+++	+++	+++	-	++
	R	+++	+	+++	+++	+++	-	+
	SB	++	+	+++	++	+++	-	+
Methanol	L	++	-	++	+	++	-	+
	R	+	-	++	+	+	+	-
	SB	+	-	+	++	-	+	-

Key: L: Leaves, R; Roots, S.B; Stem bark, DCM; Dichloromethane, +++ Abundant, ++ Moderate, + Trace, ⊠ Absent

Table 5: Effect of various plant extract on proliferation rate of Vero cell line

Concentration (µg/ml)	Y1	Y2	Y3
0.00	1.00±0.02^{a}	1.00±0.02^{a}	1.00±0.04^{a}
1.23	0.96±0.04^{a}	0.93±0.00^{a}	0.99±0.05^{a}
3.70	0.92±0.03^{a}	0.89±0.01^{a}	0.96±0.05^{a}
11.11	0.88±0.02^{a}	0.93±0.00^{a}	0.97±0.02^{a}
33.33	0.84±0.02^{a}	0.86±0.00^{a}	0.84±0.04^{a}
111.11	0.79±0.01^{a}	0.73±0.00^{a}	0.80±0.06^{a}
333.33	0.69±0.01^{a}	0.60±0.03^{a}	0.69±0.03^{a}
1000.00	0.05±0.01^{b}	0.54±0.05^{b}	0.29±0.01^{b}

All values are expressed as Mean±SEM. Means that do not share a letter are significantly different (*P>0.05*)

Y1- *L. cornuta* leaves, Y2- *L. cornuta* roots, Y3- *L. cornuta* stem

Figures

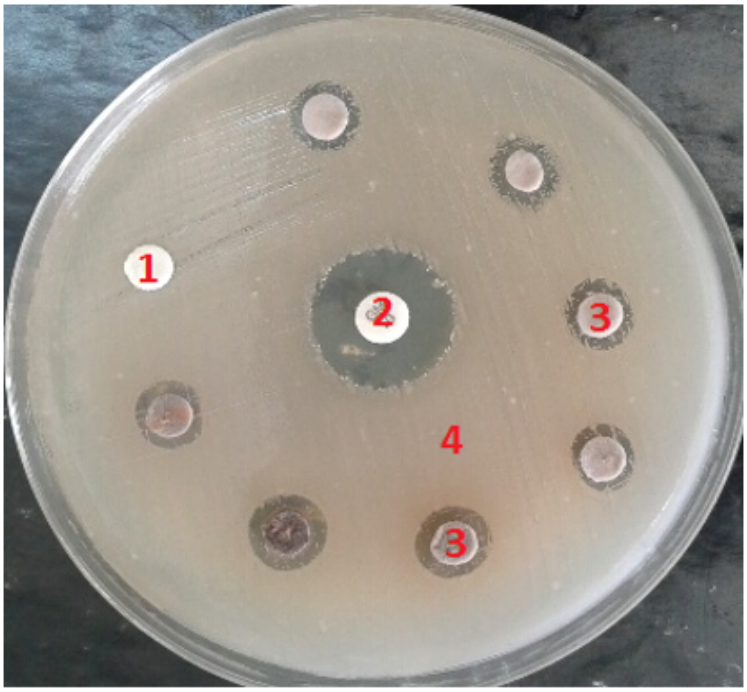

Figure 1

Disks bearing *L. cornuta* seeded against *Staphylococcus aureus* Petri plate showing clear zones of inhibition

Key: 1- Negative control 2 - Positive control, 3 extract, 4 - Staphylococcus aureus