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Antimicrobial Activity of *Euclea divinorum* Hern (Ebenaceae) Leaves, Tender Stems, Root Bark and an Herbal Toothpaste Formulated from Its Ethanolic Root Bark Extract

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Authors' contributions

This work was carried out in collaboration among all authors. Authors IM, PW and IOK designed the study. Author IM collected the samples and performed laboratory analyses. Authors PW and IOK supervised the work and provided technical support. Authors IM, PW and IOK analyzed the collected data. Author IM wrote the first draft of the manuscript. All authors revised and approved the final manuscript.

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ABSTRACT

Aim: *Euclea divinorum* Hern (Ebenaceae) is a treasured East African medicinal plant which has a long use in the management of dental caries. However, there are few reports on the antimicrobial activity of its different parts used in oral care. The aim of this study was to compare the antimicrobial activity of organic extracts of *E. divinorum* leaves, tender stems and root bark against some oral pathogens and formulate an herbal toothpaste from its most active extract.

Place and Duration of the Study: Leaves, tender stems and root bark of *E. divinorum* were collected from Elgueyo Marakwet Rift Valley located in the North Rift region of Kenya. The samples were analyzed at Moi University Chemistry Laboratory, Kenya between January 2020 and April 2020.

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Methodology: Shade-dried samples were ground into powder and successively extracted with hexane, dichloromethane and ethanol. Antimicrobial activity of the extracts were determined by agar disc diffusion method. Minimum inhibitory concentration assay was performed for microorganisms that exhibited the highest sensitivity to ethanolic root bark extract (which had the highest recorded zone of inhibition diameters). The ethanolic root bark extract was used for the formulation of an herbal toothpaste.

Results: The ethanolic extract of *E. divinorum* root bark was found to be the most active with minimum inhibitory concentration of 25, 50, 25 and 25 µg/ml for *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Herbal toothpaste formulated with the ethanol extract of *E. divinorum* root bark had higher antimicrobial activity against the tested microorganisms compared to Colgate herbal toothpaste formulated with fluoride as the active ingredient.

Conclusion: The current study supports the use of this plant in traditional management of dental caries and as chewing sticks in Kenya.

Keywords: Chewing sticks; dental caries; oral hygiene; traditional medicine.

1. INTRODUCTION

Euclea divinorum Hern (Ebenaceae) is a deciduous or evergreen medicinal shrub which thrives at lower altitudes in various countries in Africa [1]. The plant is popularly known as magic guarri or diamond leaved *Euclea*. Its stem bark decoction is taken with meat soup for various ailments or for good health [2-4]. The branches (twigs) and the bark of its roots have been used by the local people of Kenya and Uganda; they are chewed as a disinfectant, used to impart red colour to the lips and to clean and whiten the teeth (as toothbrushes) as well as treat gum bleeding and toothache [3,5]. The plant root extract with those of *Carissa edulis* (Forsk.) Vahl and *Carica papaya* L. are used for treatment of venereal diseases [6,7]. The root bark is traditionally used to treat snakebites, headache, chest pain, ulcers, gastrointestinal disturbances, arthritis, miscarriage, jaundice, diarrhea, convulsions, cancers, skin diseases, malaria, and gonorrhoea [2,3,8-17]. Outside East Africa, the roots of this plant is used for oral care, toothaches, fungal diseases, sores, wounds and abscesses [18-21]. It is also used in South Western Ethiopia to purify drinking water in which the branches are added to the gourds or pots, and left to soak in the water for several hours [3].

Though different parts of this plant has some investigated bioactivities [10,20-24], no study has compared the antimicrobial potential of its leaves, tender stems and root bark which are commonly used in oral care. In the present study, we compared the antimicrobial activity of hexane, dichloromethane (DCM) and ethanol extracts of *E. divinorum* leaves, tender stems (twigs) and root bark against some common oral pathogens.

Further, an herbal toothpaste was formulated using its ethanol extract of the root bark which showed the highest bioactivity.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preparation

Leaves, tender stems and root bark of *E. divinorum* (Fig. 1) were collected from Elgueyo Marakwet Rift Valley located in the North Rift region of Kenya (0°58'56.0" N and 35°35'16.5 "E) near St. Joseph's Lawich Catholic. Purposive and random sampling method were used during the collection of samples from the field where the first plant of *E. divinorum* was purposively selected and the rest were selected at random. The sampling method was also purposive because of the previous knowledge about the availability of *E. divinorum* plants in this location. The samples were obtained in clean sterilized manila bags and transported to Moi University Chemistry Laboratory, Eldoret (Kenya).

The leaves, tender stems and root barks of *E. divinorum* were washed under running tap water followed by distilled water and then shade-dried for 4 weeks. After, the plant materials were then warmed for 45 minutes in an oven at 30 °C before grinding them separately into fine powder (Fig. 2).

Serial extraction method was used starting with hexane, DCM and finally ethanol. For leaves, weighed aliquots (200 g) of the dry powder were placed in a 250 ml conical flask and then macerated at room temperature for 48 hours in 800 ml of 98.5% hexane with occasional shaking. The samples were filtered through Whatman No.

1 filter paper. The filtrates were concentrated on a rotary evaporator set at 45°C while the residues (labelled R1) were used in the subsequent extraction procedure. The concentrated extracts were collected in labelled 50 ml sample vials, dried, weighed and masses of the extracts recorded [25]. The extracts were sealed and kept at 4°C.

Residues (R1) were air dried, and then macerated with 800 ml of 99.5% DCM at room temperature for 48 hours with occasional shaking. These were then filtered through Whatman No. 1 filter paper, and the second set of residues (labelled R2) were kept for further extraction. Filtrates obtained were concentrated on a water bath set at 35°C. The concentrated extracts were collected in 50 ml sample vials, dried, weighed and the respective weights recorded. The extracts were sealed in labelled sample vial tubes and kept at 4 °C for further studies.

Residues (R2) were air dried, then macerated in 800 ml of absolute ethanol at room temperature

for 48 hours with occasional shaking. They were filtered and the filtrates were concentrated on a rotary evaporator at 65 °C. The concentrated extracts were collected in labelled sample vials, dried, weighed, sealed and kept at 4 °C. The tender stem and root bark powders were subjected to the same treatment as described for the leaves to obtain the respective hexane, DCM and ethanol extracts.

2.2 Formulation of Herbal Toothpaste and Antimicrobial Activity Evaluation

2.2.1 Antimicrobial activity of *E. divinorum* extracts

The antimicrobial activity of the extracts was assessed using disk diffusion method with surface plating to check the efficacy of the plant extracts against four microorganisms namely: *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 25923, *Candida albicans* and *Escherichia coli* ATCC 25922. The microbes were obtained from Egerton University Microbiology Laboratory, Nakuru, Kenya. The



Fig. 1. Fresh samples of *E. divinorum* (a) leaves, (b) root barks and (c) tender stems

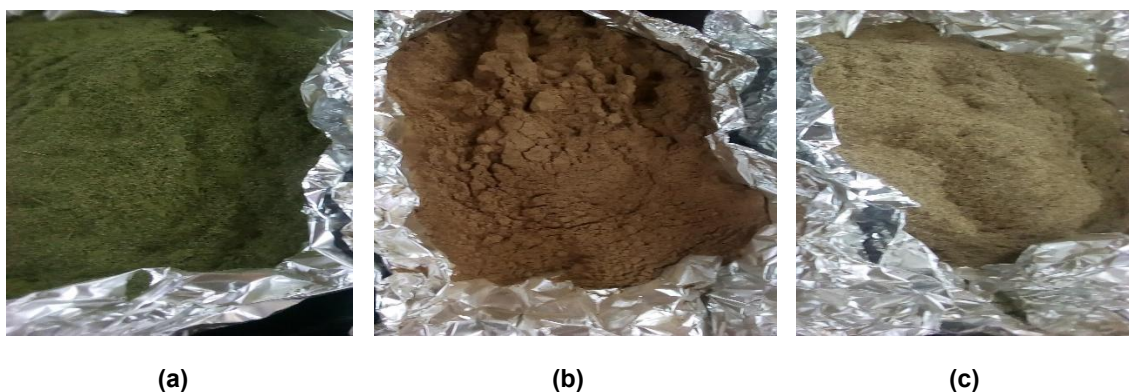


Fig. 2. Dry ground powder of *E. divinorum* (a) leaves, (b) root barks and (c) tender stems

extracts were prepared by dissolving 0.05 g of the powders separately in 100 ml of 10% dimethyl sulfoxide (DMSO). The microbial media were prepared according to the manufacturer's instructions.

Briefly, Mueller Hinton Agar (19 g) or Subouraud Dextrose Agar (20 g) was dissolved in 500 ml of distilled water, autoclaved (at 121 °C, 1.5 bars) for 30 minutes and allowed to cool to 45 °C. The agar plates were prepared by pouring the prepared media onto sterilized plates and then allowed to solidify. The plates were seeded with respective microorganisms (0.1 ml) which were spread uniformly using a sterilized L-shaped rod. Whatman discs (3) impregnated with 0.5 ml of different extracts were placed aseptically on the agar surface. Discs soaked in 10% DMSO were used as the negative control and penicillin G (10 µg/ml) was used as the positive control. The plates were incubated at 37 °C for 24 hours [26, 27]. Positive antimicrobial activities were established by the presence of measurable zones of inhibition.

2.2.2 Determination of the minimum inhibitory concentration of the extracts

Minimum inhibitory concentration (MIC) was performed using microorganisms that exhibited highest sensitivity to ethanolic root bark extract (which had the highest recorded zone of inhibition diameters). The ethanol root bark extract was prepared by dissolving the dry solid extract in 250 ml of 10% DMSO to obtain solutions of 25, 50, 100, 200 and 400 µg/ml. The MIC was determined using the disk diffusion method. MIC values were taken as the lowest concentration of extracts that produced no visible bacterial growth when compared with the control plates after 24 hours of incubation at 37 °C.

2.2.3 Formulation of toothpaste and evaluation of its antimicrobial activity

Methylcellulose gum (2.0 g) was slowly added to water (2 ml) and vigorously agitated until a smooth uniform mixture was obtained. Glycerol (4 ml) was added into the mixture and stirred thoroughly. Calcium carbonate (7.0 g), menthol crystals (0.5 g), hydrated silica (1.0 g), calcium phosphate (2.0 g), extract (0.5 g), sodium hydrogen carbonate (2.0 g), and sodium lauryl sulphate (2.0 g) were added in the order one at a time while mixing with a stirring rod thoroughly [28]. This was labelled T1. Another

formulation was prepared without the herbal extract (T2).

The formulated toothpastes were diluted with 250 ml of distilled water to make respective solutions of 400 µg/ml which were tested for their activity against *S. pyogenes*, *S. aureus*, *C. albicans* and *E. coli*. Mueller Hinton Agar, Subouraud Dextrose Agar and agar plates were prepared as described for the extracts. The plates were seeded with microorganisms (0.1 ml) separately which were spread uniformly using a sterilized L-shaped rod. Whatman discs (3) which were separately impregnated with toothpaste solutions (0.5 ml) were placed onto surface of each agar plate containing the test microorganisms. Discs soaked in 10% DMSO were used as the negative control while Colgate herbal fluoride toothpaste (Colgate Palmolive, China) was used as a positive control. The plates were later incubated at 37°C for 18 hours. The zone of inhibition diameter (ZOI) was measured for each after incubation.

2.3 Statistical Analysis

All quantitative data were reported as means ± standard deviations of triplicates. Data were subjected to one-way ANOVA test at $P = .05$ using SPSS for Windows (Version 20).

3. RESULTS AND DISCUSSION

3.1 Percentage Yield of *E. divinorum* Leaves, Tender Stems and Root Bark

The extraction yields shown in Table 1 were expressed as the percentages of initial mass of the sample macerated. As shown, extraction using ethanol gave the highest yields in comparison to hexane and DCM with the root barks having the highest yield of 7.60% followed by tender stems (3.30%). For the leaf extracts, a high yield was obtained using ethanol (2.51%) than using DCM (1.92%) and hexane (1.13%). This trend was also observed for the extracts of tender stems and root bark. However, these differences were not statistically different ($P = .07$). This result indicates that ethanol is a good solvent for extraction as compared to hexane and DCM probably because most compounds in the parts of *E. divinorum* extracted are polar, thus were able to dissolve in the more polar ethanol than other non-polar solvents used. Differences in solvent polarities used for extraction is known to play a key role in

increasing the solubility of phytochemical compounds [25,29,30]. Further, differences in the structure of phytochemical compounds also determine their solubility in solvents of different polarities [31]. Indeed, the three solvents used had different polarities arranged as hexane < DCM < ethanol. This change appear to be related not only to the differences in the polarity of extract components but also to the solvents used, which also plays a vital role in increasing the solubility of phytochemical compounds. Therefore, the results of the current study confirmed the effect of solvent extraction and the plant organ on the yield and consequently confirms the richness of this plant in polar substances.

3.2 Antibacterial Activity of *E. divinorum* Leaves, Stem and Root Bark Extracts

The antimicrobial activity of leaves, tender stems and root bark extracts of *E. divinorum* are shown in Table 2. The results of antimicrobial assay showed that the crude extracts of *E. divinorum* had inhibitory effect on the growth of the tested microorganisms. The activity was higher for ethanol extracts of leaves and root barks. The results showed no inhibition activity of solvent used in dilution of the plant extracts (DMSO) against the test bacteria and fungus. The positive

control (penicillin) had comparable bioactivity to that of the crude extracts.

In a preceding study, the ethanol extract of *E. divinorum* leaves and barks were tested for their inhibitory activity against oral pathogens [23]. The authors reported that the extracts did not inhibit the growth of *C. albicans* while *S. mutans* was inhibited with a ZOI of 6.0 mm and 2.4 mm at 2 mg and 3 mg of the plant extracts per disc [23]. In another study, DCM extracts of *E. divinorum* roots showed the highest antifungal activity with ZOI of 30 mm against *Trichophyton mentagrophytes*. It also exhibited comparable antifungal activity as ethyl acetate and methanol extracts of the root barks against the tested fungal strains [32]. Similarly, aqueous extracts of *E. divinorum* twigs were reported to inhibit 50% of proteolytic activity of three strains of *Bacteroides gingivalis*, *Bacteriodes intermedius* and *Treponema denticol* at concentrations of 10 µg/ml up to 200 µg/ml [24].

In another investigation [18], the root methanolic extract of *E. divinorum* exhibited antimicrobial activity against *S. aureus*, *Bacillus subtilis*, *Micrococuss flavus*, *E. coli*, *Pseudomonas aeruginosa* and *Candida maltosa* (with ZOI of 24, 12, 18, 11, 15 and 10 mm respectively) and three multiresistant *Staphylococcus* strains

Table 1. Organic extract yield (%) of different parts of *E. divinorum*

Part used	Hexane extract	DCM extract	Ethanol extract
Leaves	1.13	1.92	2.51
Tender stems	0.57	1.74	3.30
Root bark	0.51	2.35	7.60

Table 2. Zone of inhibition diameter (mm) of leaf, tender stem and root bark extracts of *E. divinorum* against the tested oral pathogens

Plant part	Sample	<i>S. pyogenes</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
Leaves	Hexane	5.67 ± 0.58	4.33 ± 0.58	0.00 ± 0.00	4.67 ± 0.58
	DCM	7.00 ± 1.00	1.00 ± 0.00	2.33 ± 0.58	2.33 ± 0.58
	Ethanol	21.00 ± 6.08	0.00 ± 0.00	0.00 ± 0.00	10.00 ± 1.00
Tender stems	Hexane	5.33 ± 0.57	2.00 ± 0.00	0.00 ± 0.00	2.00 ± 0.00
	DCM	7.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	8.00 ± 0.00
	Ethanol	7.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	4.33 ± 0.58
Root bark	Hexane	8.00 ± 0.00	7.67 ± 0.58	5.67 ± 1.15	11.33 ± 0.57
	DCM	3.33 ± 0.58	4.00 ± 0.00	0.00 ± 0.00	6.67 ± 1.53
	Ethanol	25.67 ± 1.53	11.00 ± 3.61	10.33 ± 0.58	4.00 ± 0.00
Colgate herbal toothpaste (positive control)		23.00 ± 2.65	17.67 ± 1.53	6.00 ± 1.00	4.00 ± 0.00
DMSO (negative control)		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

(*S. epidermidis* 847, *S. haemolyticus* 535 and *S. aureus* North German Epidemic strain with ZOI of 24, 16 and 26 mm respectively). Another study [20] evaluated the antifungal activities of selected Venda medicinal plants against *C. albicans*, *Candida krusei* and *Cryptococcus neoformans* isolated from South African AIDS patients in which *E. divinorum* was one of the 76 medicinal plants tested. It was reported that hexane extract of *E. divinorum* leaves was more active against *Cryptococcus neoformans* with ZOI of 8 mm at 10 µl. No bioactivity was observed against the other tested fungi. Further, Ngari et al. [22] reported that aqueous and DCM/methanol extracts of *E. divinorum* roots had no antimicrobial activity against *E. coli* while its DCM extract had ZOI of 10.30 ± 0.63 mm. DCM extracts had ZOI of 10.75 ± 1.60 , 10.00 ± 0.41 and 00.00 ± 0.00 mm against *S. aureus*, *Bacillus subtilis* and *Lactobacillus acidophilus* respectively. The DCM/methanol extracts had ZOI of 9.13 ± 0.72 , 10.80 ± 0.25 and 6.70 ± 0.48 mm respectively while the aqueous extracts had no bioactivity. In another study, crude root extracts of *E. divinorum* had ZOI of 9 mm and 13 mm for *S. mutans* and *S. sanguinis* respectively [21].

The antimicrobial activity of the different parts of *E. divinorum* could be due to the presence of antimicrobial compounds reported in our previous study (Mbabazi et al., 2020, unpublished). We identified squalene, tetradecyl acrylate, palmitic acid and ethyl ester in *E. divinorum* leaves and these compounds were previously reported to possess antimicrobial activities [33]. Further, Ethyl-9- α -linolenate,

Ethyl-9,12-Octadecadienoate, Ethyl palmitate and other fatty acids and their esters which have been reported to have antibacterial activities [34] were identified in our extracts of tender stems. Similarly, 7-Ethoxycoumarin, 1,4-Naphthoquinone derivative and squalene were identified in the root bark extracts and these have been reported to have antibacterial activity [35,36].

3.3 Results of Minimum Inhibitory Concentration Assay

The ethanolic extract of *E. divinorum* root bark was found to be the most active and its MIC for the tested microorganisms was determined. It was found to have the highest activity against *S. pyogenes*, *S. aureus* and *E. coli* (Table 3). The MIC of the ethanolic extract of *E. divinorum* root bark against *S. pyogenes* was 25 µg/ml, 50 µg/ml for *S. aureus*, 25 µg/ml for *E. coli* and *C. albicans*. An earlier study [23] reported MIC ranging from 3.1 to 25.0 µg/ml for ethanolic extracts of *E. divinorum* root bark and leaves against a panel of Gram positive and Gram negative bacteria (*Actinomyces naeslundii*, *Actinomyces israelii*, *S. mutans*, *Actinobacillus actinomycesetem comitans* and *Porphyromonas gingivalis*). Nyambe [21] reported MICs of 1250 and 2500 µg/ml for *E. divinorum* root fraction against *Streptococcus mutans* and *S. sanguinis*.

3.4 Antimicrobial Activity of the Formulated Toothpaste

The antimicrobial activity of the two toothpaste formulations are shown in Table 4.

Table 3. Minimum inhibitory concentration of ethanolic extract of *E. divinorum* root bark on the tested oral pathogens

Concentration (µg/ml)	<i>S. pyogenes</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
25	0.67 ± 0.58	0.00 ± 0.00	5.67 ± 2.08	6.00 ± 0.00
50	3.33 ± 0.58	3.00 ± 0.00	12.00 ± 1.73	7.67 ± 1.53
100	4.00 ± 0.00	7.33 ± 2.31	14.00 ± 2.00	8.00 ± 1.73
200	6.00 ± 1.00	12.67 ± 1.53	13.67 ± 1.53	13.0 ± 1.73
400	25.0 ± 1.53	11.00 ± 3.61	10.33 ± 0.58	7.00 ± 1.00

Table 4. Zone of inhibition diameter (mm) of the toothpaste formulations against the selected oral pathogens

Sample	<i>S. pyogenes</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
T1	22.67 ± 2.52	13.67 ± 0.58	7.67 ± 1.15	10.67 ± 1.15
T2	2.00 ± 1.00	4.33 ± 0.58	1.00 ± 0.00	0.00 ± 0.00
Colgate herbal toothpaste	16.33 ± 2.08	13.67 ± 1.15	0.00 ± 0.00	5.0 ± 0.00

The toothpaste formulated with the ethanol extract of *E. divinorum* root bark (T1) was found to have the highest activity against the tested microorganisms compared to Colgate herbal toothpaste that is formulated with fluoride as the active ingredient against selected microorganisms.

Colgate herbal toothpaste showed no activity against *E. coli* but had comparable antibacterial activity against *S. aureus*. Toothpaste formulated without the herbal extracts showed no inhibition of *C. albicans* growth and very small ZOI against the other oral pathogens tested. This could be because it had no active ingredients to inhibit the growth of the microorganisms. The little inhibition observed against the other pathogens could possibly have been due to the ingredients used in the toothpaste formulation. These results are similar to those obtained by Sharma et al. [37] where polyherbal toothpastes were formulated, and evaluated for their activity against *S. mutans*, *S. aureus* and *C. albicans*. The results from their study showed that the methanol extract of polyherbal formulation exhibited more activity against all the selected oral pathogens than the commercial herbal toothpaste comparatively used in the investigation. A previous study on preparation of herbal toothpastes with *Aloe vera* and Red Betel extracts revealed that the formulated toothpaste had comparable antibacterial activity against *S. mutans* (15 cm) to the commercial product with a ZOI of 19 cm whereas no activity was observed for the toothpaste formulated without the herbal extract [38].

4. CONCLUSION

The present study showed that ethanolic root bark extract of *E. divinorum* exhibited the highest activity against *S. pyogenes* and *S. aureus* and its formulated herbal toothpaste has antimicrobial potential against the oral pathogens. Thus, *in vivo* experiments need to be conducted on patients with dental caries to determine the efficacy of the formulated herbal toothpaste. The formulated herbal toothpaste should be evaluated for its physical and chemical characteristics to ascertain if it is safe to be used in the control of oral pathogens and thus dental caries.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was approved by the Department of Chemistry and Biochemistry, Moi University, Eldoret, Kenya for Immaculate Mbabazi (Approval No. MSC/ACH/9/18).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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