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Safety and Antimicrobial Properties of *Euclea divinorum* Hiern, Chewing Sticks Used for Management of Oral Health in Nairobi County, Kenya

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ABSTRACT

Chewing sticks from *Euclea divinorum* root have long been used in management of oral health in Kenya but various safety aspects and antimicrobial data are limited. The mineral content was determined using Total Reflection X-Ray Fluorescence (TRXF) while phytochemical composition was assessed using standard procedures. Toxicity effects of extracts on growth rate, relative organ body weight and function of kidney, liver, bone marrow and histopathological changes on major organs was assessed. Disc diffusion method was used to assess antimicrobial activity of the chewing sticks extracts against *Staphyloccocus aureus, Escherichia coli, Bacillus subtilis* and *Lactobacillus acidophilus.* The safety of chewing sticks extracts was studied by orally administering 1g/kg body weight of organic extracts daily in mice for 21 days and determining changes in body organ weight, hematological and biochemical parameters. Results indicate presence of high levels of minerals including lead and aluminium. Various groups of phytochemical components were detected. Organic extracts showed growth inhibitory effects on test organisms compared to water extracts. Oral administration of organic body weight on mice for 21 days resulted to retarded growth, increased organ body weight index and alteration of hepatocytes on liver cells. Significant ($P< 0.05$) variation of thrombocytes, creatine kinase and aspartate aminotransferase was recorded. This shows that extracts of *E. divinorum* roots have toxic effects and should be used with care; gargling of extracts is recommended instead of swallowing.

KEY WORDS; *Euclea divinorum,* Chewing sticks, toxicity, herbal materials, oral health, antimicrobial properties, safety

INTRODUCTION

Oral health is essential to overall quality of life due to several psychological problems associated with having discolored, diseased or missing teeth [1]. Furthermore, oropharyngeal colonization is associated with several life threatening systemic diseases which include heart conditions such as endorcaditis [2]. Conventional medicines used in dentistry have not been adequate for majority of developing countries since investment in oral health care is low [3]. In these countries, resources are primarily allocated to emergency oral health care and pain relief [4]. Therefore, alternative health care systems remain the only choice for majority in the populations.

Traditional methods for maintaining oral hygiene have been practiced by different populations and cultures around the world since antiquity [5]. The Babylonians recorded the use of chewing sticks in 7000 BC and its use ultimately spread throughout Greek, Egyptian, Jewish, Islamic and Roman Empires [6]. In Kenya, plants such as *Euclea divinorum* are used to manage oral health**.** *Euclea divinorum* Hiern is an evergreen shrub or a small tree usually 3-5m high with dense foliage [7]. It is one of the most important medicinal plants in Kenya. The roots and stems of *E. divinorum* are sold in Nairobi Kenya, as tooth brushes. The roots are also used for treatment of chest pain, pneumonia and internal body swelling [8].

Although herbal materials are used to treat different diseases because they are available and cheap, clinicians are often reluctant to prescribe them because of knowledge deficiency, real concerns about product safety and liability, and due to the presence of compounds that are injurious [9]. Herbal materials are known to cause toxic effects, serious allergic reactions and adverse drug interactions [10]. Therefore experimental screening methods including thorough toxicity studies are important to ascertain the safety of commonly used herbs [11].

Though the medicinal uses of *E. divinorum* Hiern are documented [8], there is scant information on toxicity and antimicrobial properties of its chewing sticks. The aim of this study was to evaluate the efficacy and safety of chewing sticks used for management of oral health in Nairobi County, Kenya.

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MATERIALS AND METHODS

Materials

Chewing sticks of *E. divinorum* were purchased from herbalists in Nairobi County. Mice for toxicity evaluation were obtained from department of Zoology, Kenyatta University. Culture media were purchased from Chemoquip, Nairobi.

Methods

The collected herbal materials were kept at room temperature away from direct sunlight in closed dry plastic bags. The sticks were cut into small pieces, dried and ground to make a powder. The powdered material was sequentially extracted [12] using dichloromethane (DCM), for 72 hours, DCM: Methanol (1:1) and finally water. The organic extracts were concentrated by use of Rota evaporator at 40 ºC to give DCM and DCM: MeOH extracts while the water extract was subjected to freeze drying for 48 hours. The freeze dried powder was then weighed in an air tight container and stored at -20 ºC until used for bioassay.

Determination of mineral element

Mineral element profile was determined by use of Total Reflection X-ray Fluorescence (TXRF). Triplicate 1.0g weights of each sample were placed in a clean digestion flask. A volume of 10 mls of double distilled water and 30 mls of concentrated nitric acid were added and the sample heated to boiling until the entire sample was digested. The digests were transferred into clean vials and double distilled water used to top up the aliquots to 10mls. A volume of 20µl of 1000mg/kg Gallium stock solution was added into each sample (as internal standard) resulting into a concentration of 2mg/kg Ga in each sample. Each sample was shaken for a minute for homogenization. Aliquots of 10µl of each sample in triplicates were pipetted onto clean quartz carrier using a micro-pipette. Triplicate sub-samples were prepared for each sample. The carriers were then dried in an oven to evaporate the solvent. Each sample carrier was irradiated for 300 seconds using an S2 PICOFOX TXRF Spectrometer which was operated at 50kV and a current of 1000µA. Evaluation of the measured spectra was done using S2 PICOFOX software on the basis of the chosen elements. The concentrations were calculated based on the net intensities of the analyte peak elements and that of the internal standard.

Phytochemical analysis:

The plant extracts were screened for presence of biologically active compounds like, alkaloids, flavanoids, saponins steroids, glycosides and tannins using standard methods [13] [14] [15].

Determination of Antimicrobial activity

American type cultures collections (ATCC) from Kenya Medical Research Institute and Clinical isolates from Kenyatta hospital and department of Plant and microbial sciences of Kenyatta University were used. The test organisms were *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 14579), *Escherichia coli* (ATCC 25922) and *Lactobacillus acidophilus.* Agar disc diffusion method was used as described by various authors [16] [17] [18]). The Muller Hinton medium was prepared by dissolving a weighed amount in distilled water and subjected to sterilisation in an autoclave at 121ºC. About 20ml of the molten media was poured onto sterile Petri dishes and allowed to set. Sterile paper discs 6mm diameter were titrated with plant extracts at a concentration of 200mg/ml while positive and negative controls were set using Septrin and DMSO respectively. The plates were allowed to stand for about 30 minutes before incubation for 18 hours.

Experimental design and treatment of animals

A total of 12 male Swiss albino mice aged 4 weeks old of average weight 18-22.5g were acclimatized to the laboratory conditions for one week before commencement of the experiment as described by Watthanachaiyingcharoen et al [19]. The animals were fed by standard pellet diet and water was given *ad libitum*. The food was provided by local manufacturers. At the end of one week the mice were weighed and randomly divided into 2 groups (control, treatment group). The mice in control group were orally given physiological saline while those in treatment groups were administered orally with 1g/kg body weight [20, 21) of the extract for 21 days. The 1g/kg body weight dose was selected on the basis that the recommended doses for preliminary studies with plant extracts ranges between 50 and 300mg/kg body weight of animal and toxicity is induced by at least three times the highest test dose (0.9g/kg body weight [22]. The extracts were orally administered on a daily basis for a period of 21days. Extract dosage was chosen using the equation as describe by Oyedemi et al [20]. The animals were observed continuously for the first 2 hours for any gross change in behavioural, neurological and any other symptom of toxicity and mortality. The body weight of each mouse was assessed during acclimatization period,

before commencement of dosing, weekly during the dosing period and on the day of sacrifice. The animals were euthanized, different organs namely the heart, liver, lungs, spleen, kidneys, brain and testis were carefully dissected out and weighed. Relative organ weight was determined as described by Yakubu et al [23] Necropsy samples were collected and stored in 10% formalin. The tissues were processed using standard protocols of histopathology as described by Saha et al [11].

Hematological analysis

At the end of the experimental period (3 weeks) all animals were exsanguinated and blood samples were taken by cardiac puncture of each sacrificed animal following methods described by [21]. This blood was divided into two parts. One part was collected in plastic vacutainers treated with EDTA. Heamatological parameters white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (Hb), packed cell volume (PCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and mean cell volume (MCV) were determined using a haemoanalyse Melet Schoesing Machine MS4 from France. Calibration was done using RD system CBC3D.

Biochemical analysis

Blood samples were centrifuged at 3000 rpm for ten minutes and the clear plasma aspirated off and stored frozen at -20º C. The collected plasma was analyzed for the following analytes: alanine aminotransferase (ALT), Aspartate aminotransferase (AST), creatine kinase (CK), lactate dehydrogenate (LDH), Alkaline phosphatase (ALP) and blood urea nitrogen (BUN) using Clinical Chemistry Autoanalyzer Olympus 640. All reagents for the auto analyzer machine were commercially prepared to fit the required volumes and concentration. All the assays were performed based on the standard operating procedures (SOPs) written and maintained in the Department of Laboratory Medicine, Kenyatta National Hospital. An internal quality control (IQC) serum for a specific parameter was included in each analytical session throughout the study period.

Statistical analysis

Diameter zones of inhibitions, organ, body weight and haematological and biochemical parameters were presented as mean ±standard error. Data generated from two groups used in the toxicity study (normal, normal treated with 1g/kg body weight of chewing stick extract) was done using *t* test using Sigma plot soft ware. The level of significance for all the analyses was set at a value of $P < 0.05$.

RESULTS

Elemental profile

Results indicate that *E. divinorum* roots have a number of mineral elements that are essential for healthy bones and teeth. The highest elemental concentration reported was calcium (7087.24±167.27 mg/kg) followed by iron (826.88±28.82 mg/kg), phosphorous (600.74 mg/kg), strontium (345.19±28.32 mg/kg) and manganese with concentration of 87.8±2.10 mg/kg. All the other elements had concentration levels of less than 10mg/kg. Toxic elements detected were aluminium (487.45±78.09 mg/kg) and lead (3.79±1.42 mg/kg).

Photochemistry

Table 1 shows the phytochemical components of *E. divinorum* extracts. Triterpenoids and amino acids, resins and tannins, were present in the dichloromethane extracts of *E. divinorum*. The dichloromethane methanol extracts had all the phytochemical components investigated apart from flavonoids. The water extracts lacked resins, tannins and diterpenes.

Table 1. Phytochemical compositions of organic and aqueous extracts of chewing sticks

Phytochemicals	Dichloromethane extract	Dichloromethane/ Methanol extract	Water extract
Alkaloids	-	+	+
Reducing sugars	-	+	+
Saponins	-	+	+
Triterpenoids	+	+	+
Resins	+	+	-
Phenols	-	+	+
Tannins	+	+	-
Flavonoids	+	-	+
Amino acids	+	+	+
Diterpenes	-	+	-

Key: **(+)** present (-) absent

Antimicrobial activity

Table 2 shows the antimicrobial activity of the various extracts of chewing sticks. The dichloromethane extracts of *E. divinorum* had inhibitory effects on all the test organisms apart from *L. acidophilus.* Dichloromethane /methanol extracts of chewing sticks had antimicrobial activity ranging from 6.7mm on *L. acidophillus* to 10.8mm on *B. subtilis*. The water extracts showed very poor antimicrobial activity compared to organic extracts.

Table 2. Zones of inhibition (mm) against various microorganisms by organic and aqueous extract of *E. divinorum* roots

Extract	*E. coli*	*S. aureus*	*B. subtilis*	*L. acidophilus*
Co-Trimoxazole (septrin)	27.5±0.7	8.5±0.5	24±0.41	22.8±0.8
Dichloromethane extracts	10.3±0.63	10.75±1.6	10± 0.41	0
Dichloromethane/ Methanol extracts	0	9.13±0.72	10.8±0.25	6.7±0.0.48
Water extracts	0	0	0	0
DMSO	0	0	0	0

Results are expressed as means ± standard error mean (SEM) of four replicates per sample

Body weight gain

Results indicate that the *E. divinorum* gave retarded growth at week 3 compared to the control (Figure. 1). At day seven and fourteen the weight gain was higher than that of the control.

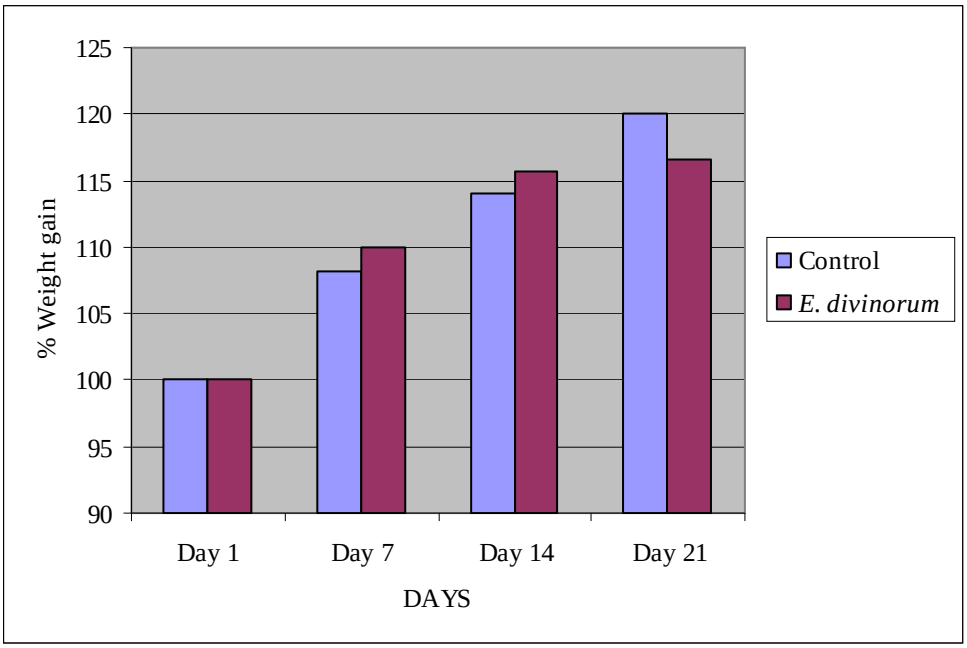


Figure 1. Percentage body weight gain in mice treated with1000mg/kg body weight of dichloromethane /methanol extracts of *E. divinorum* roots.

Relative organ weight

The current study indicates significant hypertrophy of liver, kidney, lungs and testis of mice treated with chewing stick extract compared to control ($P<0.05$) (Figure 2).

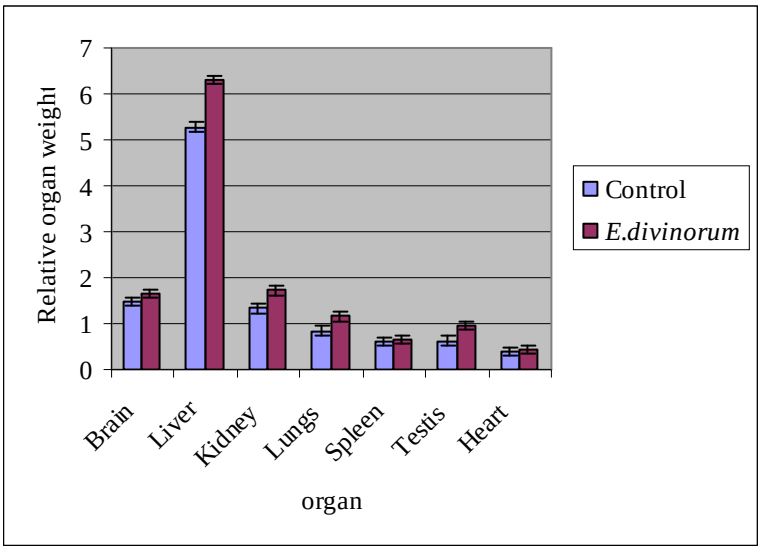


Figure 2. Relative organ weight of mice treated with 1000 mg/kg body weight of dichloromethane/ methanol extract of *E. divinorum* used in management of oral health in Nairobi County, Kenya.

Heamatology

Table 3 shows the effects of chewing stick extracts on heamatological parameters. Results indicate that red bloods cells (4.89±0.19), heamatocrate (23.4±1.01) and mean cell volume (48.17±1.55) were lower than those of the control. However the white blood cells count (4.41±0.42) was slightly higher than those of the control. The organic extracts of *E. divinorum* chewing sticks significantly ($P<0.05$) lowered levels of thrombocytes (179.17±12.03) compared to control (215.17±27.84).

Table 3 The effect of oral administration of 1000mg/kg body weight of extracts *E. divinorum* on some end point hematological parameters in mice

ANALYTE	Control	Extracts
Red Blood cells (M/mm^3	6.18±0.33	4.89±0.19
Mean cell volume (fl)	55.55±6.46	48.17±1.55
Haematocrit %	35.67±12.95	23.4±1.01
Mean cell volume (pg)	11.55±0.99	13.88±0.36
Mean cell heamoglobin concentration (g/dl)	22.93±2.75	29.03±1.14
Thrombocytes (m/mm3	215.17±27.84	179.17±12.03*
Heamoglobin (g/dl)	6.42±0.0.7	6.78±0.18
White blood cells (10^9/l)	4.05±0.73	4.41±0.42

Results are expressed as means ± standard error mean (SEM). *P<0.05 significantly different from control (N=6)

Biochemical parameters

Table 4 shows the effects of extracts on various biochemical parameters. In this study extracts of *E. divinorum* roots extracts had significantly ($P<0.05$) higher ALT and CK but lower LDH levels compared to control.

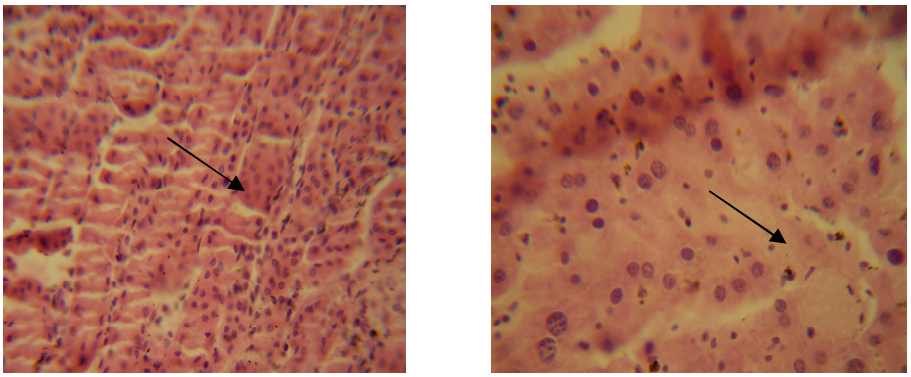
Table 4. Effect of oral administration of 1000mg/kg body weight of *E.divinorum* used in cleaning of the oral cavity for 21 days on some end point biochemical parameters in mice.

ANALYTE	BUN (mmol/L)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	LDH (IU/L)	CK (IU/L)
CONTROL	9.1±0.9	30.83±6.89	30.83±3.73	166.68± 24.1	1726.6±95.9	128.65±24.8
Extracts	8.95±0.49	37.33±3.7	45.83±3.7*	234.16± 30.19	1350.1±131*	235.8±26*

Results are expressed as mean ± SEM (N=6) (Key: BUN blood urea nitrogen, ALT= Alanine aminotransferase; AST=Aspartate aminotransferase; ALP=Alkaline phosphatise; LDH- lactate dehydrogenase CK-creatine kinase

Histopathology

The livers of the animals treated with *E. divinorum* root extracts were characterized by hepatocytes degeneration and cellular boundaries are lacking (Plate 1).



A B

Plate 1.intact hepatocytes arranged in cords are observable (A); hepatocytes destruction (B) (arrow) (magnification X100 and 400 respectively).

DISCUSSION

Elemental profile of the chewing stick shows presence of elements that are necessary for bone formation and healthy teeth. Presence of high levels of phosphorous and calcium in *E. divinorum* 487.45mg/kg shows that it can be a good source of these elements. Strontium is thought to prevent tooth decay and calcify bone. Manganese is required in small amount but it is necessary for bone and cartilage synthesis. Zinc enhances wound healing and is also essential for growth and development of bones among other structures. However presence of aluminum and lead raises safety concerns to the consumers using this product. The probable source of these two mineral elements could be environmental pollution due to poor harvesting and storage conditions.

Antimicrobial activity of the chewing stick was more pronounced in organic extracts than water extracts. The results agree with antiperiodontopathic bacteria activity of this species as reported by Homer *et al* **[24]**. The antimicrobial activity could be due to presence of biologically active components such as alkaloids which are known to have pharmacological activity. The organic extracts can be used to formulate herbal tooth paste.

The current study showed decreased weight gain in animals treated with *E. divinorum* chewing stick extracts. This indicates that the extracts might have interfered with food and water intake as argued by Ilodigwe et al [25]. This is probably due to presence of anti nutritional substances such as tannins and saponins found in *E. divinorum* extracts which are reported to cause nutritional mal-absorption [26]. The slow growth rate could also be attributed to high levels of iron (826.88mg/kg) which is known to cause hemochromatosis, characterized by weakness, weight loss among other symptoms. The extracts might be having bloating phytonutrients and hence decreased food and water intake and therefore reduced weight. The toxic effects on growth rate were apparent on week three compared to week one and two. This shows that toxicity is probably due to cumulative effects.

The hypertrophy of organs reported in this study could be an indication of inflammation as observed by Moore and Dalley [27]. The increased relative organ weight observed can be attributed to oedema which might have resulted from inflammation. This shows that the extracts might have increased capillary permeability leading to accumulation of fluid in the tissues. Enlargement of organs is normally accompanied with weight gain which is contrary to the findings reported in this study. The extracts could be having degenerative effects such as decalcification on bones hence low body weight.

The heamatopoietic system is very sensitive to toxic compounds and it serves an important index of physiological and pathological status for both animals and humans **[28]**. The low red cell indices reported might have been caused by the failure of haemohepatic tissue to supply cells for circulation in the blood. Hence the plant extracts might have interfered with the liver functions. The reduction could also be attributed to possible destructive effect on erythrocyte by the plant extracts. The increase in white blood cells counts were probably due to animal's normal responses to foreign bodies or stress associated with chronic toxicity.

It is known that an increase in concentration of ALT, AST and ALP in the serum directly reflects a major permeability, congestion or cell rupture [29]. The significant high levels of ALT reported in animals treated with the plant extracts shows that the extracts might have destructive effects on hepatocytes leading to linkage of enzyme to the surrounding tissue fluids. This was also evident in the histopathology of liver tissue (Plate 1). Urea and creatinine are used to assess the renal functional capacity [30]. In this study there were no remarkable changes in blood urea levels meaning that the extracts had no toxic effects on the kidney. The significant increase of creatine

kinase (CK) observed was probably due to myocardial infarction, muscular dystrophies or rhabdomylosis as observed by Mayne [31].

CONCLUSION

The presence of toxic elements such aluminium and lead, retarded growth and alteration of biochemical parameters indicate that prolonged use of chewing stick of *E. divinorum* may be a health hazard to the consumers. However chewing and gargling would be appropriate as only small amounts of the product is swallowed.

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