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Cytotoxicity And Phytochemical Screening Of Potential Medicinal Plants Of Tinderet District

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Abstract
An ethnobotanical survey was conducted in Tinderet District from September 2012 to August 2013. This was to identify the medicinal plants of the Nandi people of Tinderet District. Selected plants were tested for toxicity using brine shrimp (Artemia salina) and also screened for major secondary metabolites.
Information on medicinal plants was gathered through key informant interviews with the aid of an interview guide in fifteen locations. Forty two (42) herbalists (24 males and 18 females) between the age of 18 and 92 were purposively selected and interviewed.
Five popularly used medicinal plants; Albizia coriaria (Mimosaceae), Clutia abyssinica (Euphorbiaceae), Dovyalis abyssinica (Flacourtiaceae), Ehretia cymosa (Boraginaceae) and Rhamnus prinoides (Rhamnaceae) were selected, collected, extracted in ethanol and used for toxicity studies using brine shrimp (A. salina). They were also screened to determine presence or absence of Alkaloids, Flavonoids, Saponins, Sesquiterpene lactones and Steroids using Thin Layer Chromatography (TLC). LD_{50} was determined by analysis of toxicity assay data using Finney's Probit analysis.
Albizia coriaria was toxic to brine shrimp while Dovyalis abyssinica, Clutia abyssinica, Ehretia cymosa and Rhamnus prinoides were non toxic to brine shrimp. Ethanolic extracts of the five species tested positive for alkaloids, flavonoids, sesquiterpene lactones, saponins and steroids except steroids were lacking in Ehretia cymosa ethanol extract.
Key Words: *Albizia coriaria, Clutia abyssinica, Dovyalis abyssinica, Ehretia cymosa, Rhamnus prinoides, Toxicity and phytochemical screening.*

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I. Introduction

WHO defines traditional medicine as diverse health practices, approaches, knowledge and beliefs incorporating plant, animal, and/or mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness [1]. The use of plants for treatment of various diseases, as specific antidotes against magic and for religious ceremonies is universal and has been practiced for many years [2]. These botanical medications contain curative bioactive chemical ingredients which have proven to be valuable as primary or supplemental therapies when carefully applied [3].

African traditional medicine has survived for centuries despite the burdens of time, prejudices against it and the pressure by westerners to abandon it in favour of western medicine. It has a lot to offer to the rest of the world if it is given its recognition and importance [4].

1.1 Positive aspects of Traditional Medicine

Throughout the ages, humans have relied on nature for their basic needs. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies [5]. In Africa, nearly 80% of the population live in rural areas and depend on the services of traditional healers [6]. Traditional healers are often visited by patients who recount long periods of seeking help from various sources without success, and who then interprets the healers' consultation as a 'breakthrough'. This fact contributes to their evaluation of TM as being more affordable and also avoiding operations that are often associated with fear and/or high costs [7].

1.2 Negative aspects of Traditional Medicine

The use of herbal medicines is widely practiced in Kenya [8]. While the benefits of these herbal remedies are appreciated within communities, most of them lack evidence of efficacy and safety [9]. Traditional medicine lack adequate scientific proof, unstandardized, affected by occultic practices, has no precise diagnosis

and dosage is not accurate [11]. Many plants are highly toxic. Herbal medicine presents a greater risk of adverse effects than any other complementary therapy. Serious adverse events after administration are due to self-prescription and acquisition from sources other than a registered practitioner [11]. Most trials on complementary alternative medicine have been of poor quality and the efficacies of most of the therapies have not been adequately elucidated [12].

1.3 Toxicity studies and its relevance

Among consumers, there is a widespread misconception that 'natural' always means 'safe' and a common belief that remedies from natural origin are harmless and carry no risk. However, some medicinal plants are inherently toxic. Further, as with all medicines herbal medicines are expected to have side effects which may be of an adverse nature [13]. It is therefore important to assess the bioactivity of plant extracts [14]. This is the salvation of the natural product chemist, and as such it must be performed with all useful bioactive botanicals if these products are to be accepted and incorporated into legitimate long term, health practices [15].

Brine shrimp lethality assay has proven to be a convenient system for monitoring biological activities of plant species that are used in traditional medicine [14]. They give the lethal dose (LD_{50}) i.e the dose required to kill 50% of a population under stated conditions [16]. *A. salina* is the most important test organisms for toxicity studies because of its unique advantages over all other zooplankton as a bioassay organism since it does not require any maintenance or stock culturing [17]. They have a significant potential for being cost effective and rapid [18].They have short generation times, high reproductive capacity, can be easily manipulated and also favoured by flexibility to nutrient sources, temperature, and salinity tolerance [19].

The study of toxicity serves the society in many ways, not only to protect humans and the environment from deleterious effects of toxicants but also to facilitate the development of more selective toxicants such as anticancer and other clinical drugs and pesticides [16].

II. Materials and methods

2.1 Area of study

Tinderet District's topography is rugged with lots of natural forest and several rivers draining to Lake Victoria. It is made up of 15 locations which include, Kamelil, Meteitei, Kapkoros, Chemamul, Tinderet, Kabolebo, Kapkitony, Kamelilo, Songhor, Kabutie, Ainapngetuny, Soba, Tach-asis, Kabirer, Chepkemel. It is one of the Districts in Nandi County that borders Kisumu County to the West, UasinGishu County to the East, Kericho County to the South and Nandi Hills constituency to the North. The District is dominated by the Nandi ethnic community, one of the Southern Nilotic languages within the Kalenjin.

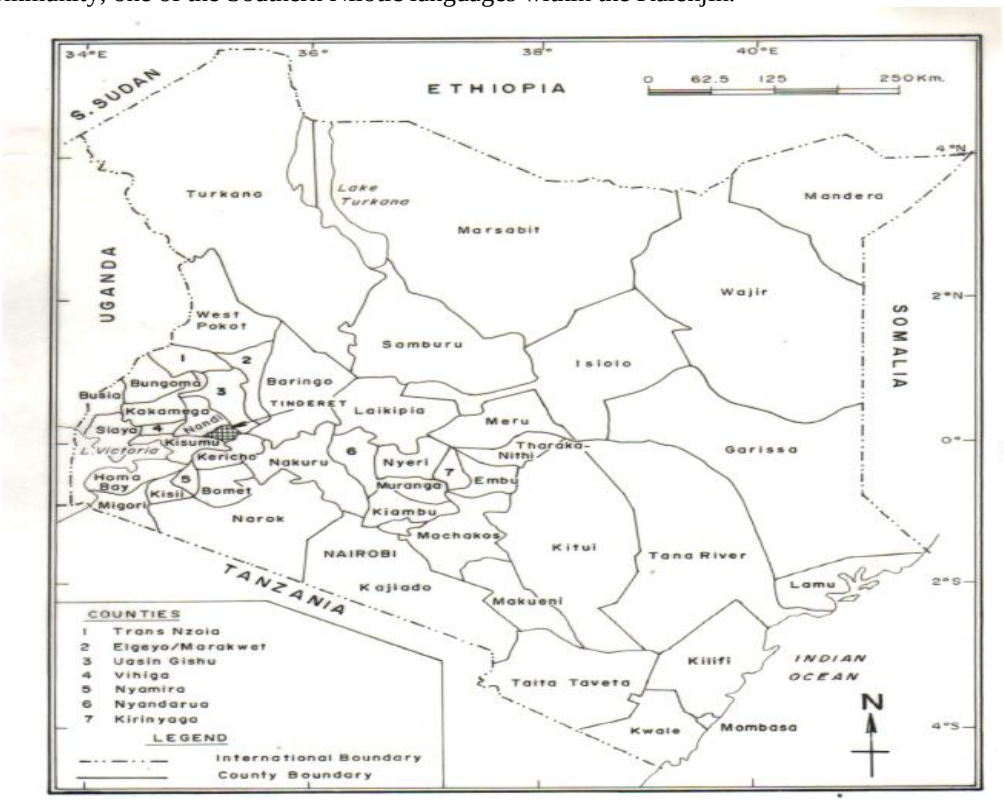

Figure 1: Map of Kenya showing the location of Tinderet District

2.2. Collection of ethnobotanical data

Ethnobotanical information was collected from all the fifteen locations in the District. Forty two (42) herbalists (24 males and 18 females) regardless of their gender and social background between the age of 18 and 92 were purposively selected and interviewed on the use of plants in treating diseases. They were identified with assistance of the local administration for observations and interviews. Permission using Prior Informed Consent (PIC) was sought from the community.

Inquiries regarding the ethomedicinal use of the plants were made during the structured interviews that each lasted around one hour. Guided tours with respondents were done to observe and collect the plants cited for identification and laboratory studies. For each plant mentioned; the local (vernacular) name, part used, preparation, disease treated and mode of administration of the drug was recorded. Ethnobotanical information of individual plant species was compiled from field notes, herbarium sheets and available literature. Only those species confirmed through two independent reports were considered to be potentially medicinal.

The plant materials were collected in duplicates and herbarium specimens prepared. All plant materials collected were deposited at Nairobi University Herbarium (NAI) for identification. Identification was done by comparing the plants with permanently prepared herbarium collections at the NAI herbarium and with the help of floras with the aid of a taxonomist [20, 21].

2.3. Selecting priority species

Plant species for chemical and toxicity analysis were identified by selecting those species which were mentioned by five or more respondents. Their plant parts used medicinally were collected.

2.4. Preparation of crude extracts

The selected species were cleaned, chopped in small pieces and air dried at room temperature for 3 weeks after which they were grounded into uniform powder separately using an electric mill [22]. Extraction was performed by continuous soaking of 100 grams of powdered plant material in a 1 litre beaker covered with aluminium foil with 250 ml absolute ethanol due to its polarity that provides total extraction of secondary metabolites. The solvent was drained out after 72 hours and the procedure repeated twice [23]. The extracts obtained were filtered with Whatman® filter paper (no 1) and the filtrate obtained were combined and then evaporated to dryness under reduced pressure using a rotary evaporator with water bath set at 40^{o}C until ethanol crude extract formed [23]. The extracts were then dried in an oven in preparation for further analysis.

2.5. Brine shrimp (*Artemia salina*) toxicity tests (BST)

Brine shrimp-Leech (*A. salina*) eggs were used to determine the toxicity of selected plants. This was based on the method developed by with slight modifications [24]. Artificial sea water was prepared by dissolving 20 grams of sea salt in 1000 ml of distilled water. Brine shrimp eggs were hatched in a hatching tank containing artificial sea water within 24-36 hrs at room temperatures of 25-30 degrees Celsius. The tank measured 14 by 8 by 5 cm and had two compartments; one large one and one small one. Between the two compartments were three 2 mm holes to allow hatched brine shrimps to pass through. The larger compartment was then covered with aluminium foil to prevent light exposure because Brine shrimp eggs hatch in the dark. The smaller compartment was exposed to light. Hatching occurred within 24 to 36 hours. Various concentrations of the crude extract in sea water containing Dimethylsulphoxide (DMSO) were used: 10, 100, and 1000 µg/ml in testing toxicity. A stock solution for each crude extract of 10,000 µg/ml was first prepared by dissolving 0.1 g of each crude extract in 10 mls of sea water. 0.1 g of each sample was first dissolved in DMSO then further diluted using artificial water to 10 mls to make stock solution. DMSO content in the stock solution was less than 0.05% to make it nontoxic to brine shrimp larvae.

10 Brine shrimp larvae were transferred from the hatching tank to plastic tubes with the aid of Pasteur pipettes. The volume of artificial sea water in each plastic tube containing 10 Brine shrimp larvae was increased to 5ml except the tubes for 1,000 µg/ml which were topped to 4.5 mls with artificial sea water. Using micropipettes, 0.5 mls, 0.05 mls and 0.005 mls were transferred from the stock solution to the plastic tubes containing 5 mls artificial sea water to make experimental solutions containing 1000 µg/ml, 100 µg/ml and 10 µg/ml respectively. A control containing artificial sea water and Brine shrimp larvae only denoted a concentration of 0 µg/ml which had no larvicidal effect. Each of the experimental solutions was replicated thrice with survivors counted after 24 hours using a magnifying glass [25].

Table 1: Brine shrimp bioassay set up for each plant extract

Tubes	Volume (ml) of artificial sea water	No of Brine shrimp larvae	Volume of stock solution (ml)	Concentration (µg/ml)	Nature of experiment	Final volume in the tube (ml)
1	4.5	10	0.5	1,000	Trial	5
2	4.5	10	0.5	1,000	Repeat	5
3	4.5	10	0.5	1,000	Repeat	5
4	5	10	0.05	100	Trial	5
5	5	10	0.05	100	Repeat	5
6	5	10	0.05	100	Repeat	5
7	5	10	0.005	10	Trial	5
8	5	10	0.005	10	Repeat	5
9	5	10	0.005	10	Repeat	5
10	5	10	0	0	Control	5
11	5	10	0	0	Control	5
12	5	10	0	0	Control	5

2.6. Phytochemical screening of secondary metabolites using Thin Layer Chromatography

Secondary metabolites were screened based on previously methods [26, 27, 28, and 29] (Refer to Table 2). The TLC plates measured 6.6 cm by 5 cm. On each plate 1 cm mark was drawn using a pencil on either sides of the plate to show the base line and solvent front. Each of the crude extract were first dissolved in ethanol. Using micropipettes 2 mm spots of each extract were made on the TLC plates then placed on a chamber with appropriate chromatographic solvents. The developed plates weres prayed using specific spray reagents to determine the presence or absence of alkaloids, flavonoids, saponins, sesquiterpene lactones and steroids.

Table 2: Screening for alkaloids, flavonoids, saponins, sesquiterpene lactones and steroids

Class of Secondary metabolites	**Solvent system**	**Positive test**
Alkaloids	Dichloromethane: Methanol (85:15) [26]	Dragendorff's reagent was sprayed on the developed plates. Orange colors indicated the presence of alkaloids. Spraying with sodium nitrate intensified the orange colors [26]
Flavonoids	n-hexane: ethyl acetate: acetic acid (6:3:1) [27]	Flavonoids at 254 nm appeared as dark blue zones on a yellow background on the developed plates intensifying when sprayed with ammonia. At 365 nm flavonoids fluoresced yellow, blue or green [27]
Saponins	Dichloromethane: Ethyl acetate (9:1) according to [28]	Saponins appeared as black spots when plates were sprayed with a mixture of ethanol and H_2SO_4 (9:1) and then heated at 110^0C for 10 minutes [28]
Sesquiterpene lactones	n-hexane: ethylacetate (9:1) [27]	Brown spots on the developed plates in presence of iodine indicated the presence of sesquiterpene lactones [27].Also detected as brown, yellow spots when plates were sprayed with concentrated H_2SO_4 and heated for 5 minutes at 100-110^0C [26]
Steroids	Dichloromethane : methanol (9:1) [29]	Detection evident by green coloured spots. The developed plates were sprayed with vanillin in concentrated sulphuric acid then heated at 120^oC till spots attain maximum colour intensity [29]

2.7 Statistical analysis

Applications in MS Excel 2007 spread sheet were utilized to make simple calculations. The LD_{50} of the selected plants was determined using Finney's Probit analysis; a type of regression analysis [30].

III. Results And Discission

3.1. Estimation of lethal dose (LD_{50})

The LD_{50} of the plant extracts on the brine shrimp larvae was done using Finney Probit analysis Program. Average mortalities of the three concentrations (1000 µg/ml, 100 µg/ml and 10 µg/ml) for each plant were fed into the program to give an estimate of the LD_{50}.

3.2. Toxicity of the organic crude plant extracts

Albizia coriaria was found to be highly toxic at a concentration of 86.34µg/ml. The other four plants; *Clutia abyssinica*, *Dovyalis abyssinica*, *Ehretia cymosa* and *Rhamnus prinoides* were non toxic with *Dovyalis abyssinica*, having the least toxicity (1149.56µg/ml). The results summarized in Table below.

Table 3: Acute toxicity of crude extracts

Plant species	**1,000 µg/ml**	**100 µg/ml**	**10 µg/ml**	**0 µg/ml**	**LD_{50}**
Albizia coriaria	8.33	3.66	1.66	0.00	86.34
Clutia abyssinica	3.33	2.00	1.00	0.00	1043.23
Dovyalis abyssinica	3.33	0.66	0.33	0.00	1149.56
Ehretia cymosa	3.66	1.66	0.66	0.00	1005.13
Rhamnus prinoides	3.00	1.33	0.66	0.00	1102.89

LD_{50} ranging between 0-500 µg/ml implied high toxicity while LD_{50} between 500-1000 µg/ml implied moderate toxicity and LD_{50} over 1000 µg/ml implied non toxic nature of the extract [31].

3.3. Phytochemical analysis of crude plant extracts for secondary metabolites

Extracts of each priority species were screened for secondary metabolites using TLC.

Table 4: Phytochemical screening of the crude extracts for secondary metabolites.

Organic extracts	Alkaloids	Flavonoids	Saponins	Sesquiterpene lactones	Steroids
Albizia coriaria	+ve	+ve	+ve	+ve	+ve
Clutia abyssinica	+ve	+ve	+ve	+ve	+ve
Dovyalis abyssinica	+ve	+ve	+ve	+ve	+ve
Ehretia cymosa	+ve	+ve	+ve	+ve	-ve
Rhamnus prinoides	+ve	+ve	+ve	+ve	+ve

Key: +ve= Present, -ve= Absent

Among the five species *Albizia coriaria* was found to be toxic with a LD_{50} value of 86.34 µg/ml, while four of them were non toxic to brine shrimp which include; *Clutia abyssinica*, *Dovyalis abyssinica*, *Ehretia cymosa* and *Rhamnus prinoides* with LD_{50} values of 1043.23, 1149.56, 1005.13 and 1102.89 µg/ml respectively. In other studies, methanol and aqueous extracts of *Albizia coriaria*were found to have antileshmanial effects on *Leshmania major* promastigotes and moderately high toxicity (CC50 >500_g/ml) against human embryonic lung fibroblast (HELF) cells which is in line with the toxic bioactivity results on *Albizia coriaria* obtained from the current study [32]. Despite the high toxicity of *Albizia coriaria*, it has been widely used in treatment of various diseases in the District. It was noted that the extract from the plant is given in small amounts to avoid any toxic effect. From other studies, the toxicity of a plant extract could be due to the presence of biologically active compounds which are found in high concentration [15].

From the results, *Clutia abyssinica* was found to have the least toxicity on brine shrimp. Similar toxicity results were obtained when ethanolic extracts from *Clutia abyssinica* were tested against HIV-1 virus which gave an effective concentration value (EC_{50}) of >128.01 [33]. Aqueous and organic extracts from *Rhamnus prinoides* has been reported to have low toxicity [34].

Phytochemical screening of *Albizia coriaria* indicated presence of several secondary metabolites; flavonoids, sesquiterpenes lactones, alkaloids, steroids and saponins. The high toxicity observed for *Albizia coriaria* on brineshrimp can be possibly attributed to saponins present in high concentrations, as reported in other studies with *Albizia* spp [35]. Conversely, non toxic nature of the other four plants: *Clutia abyssinica, Dovyalis abyssinica, Ehretia cymosa* and *Rhamnus prinoides* on brine shrimp can be attributed to the fact that some of the secondary metabolites identified in these plants could be found in only low concentrations and their toxic threshold can only be achieved at high doses [15]. This non toxic nature justifies the wide usage of *Clutia abyssinica, Dovyalis abyssinica, Ehretia cymosa* and *Rhamnus prinoides* in traditional treatment of various ailments in Tinderet District and in other geographical regions at large.

Majority of plant species are rich in secondary metabolites which include flavonoids, sesquiterpenes lactones, tannins, anthraquinones, flavonoids, terpenes and saponins [36]. The ethanolic extracts of the four species which include: *Albizia coriaria, Clutia abyssinica, Dovyalis abyssinica* and *Rhamnus prinoides* tested positive for alkaloids, flavonoids, saponins, sesquiterpene lactones and steroids. Ethanolic extract of *Ehretia cymosa* tested positive for alkaloids, flavonoids, saponins, and sesquiterpene lactones except for steroids which gave a negative test. Plants existing in different geographical regions exhibit varying concentrations of secondary metabolites. In addition, plant secondary metabolites differ from plant to plant and also within the same plant they differ from one part to the other [37]. Plant secondary metabolites offer plants protection from animals, diseases, pests and adverse environmental conditions making them grow well in their environment. From the pharmacological point of view, plant secondary metabolites have various bioactivities which include

antibacterial, antifungal, antioxidant, antiplasmodial, hepatoprotective activity, wormicicidal and molluscicidal activities and most of all they are responsible for toxicity of most plants [37].

IV. Conclusion

From the study, *Albizia coriaria* is toxic to brine shrimp while *Dovyalis abyssinica*, *Clutia abyssinica*, *Ehretia cymosa* and *Rhamnus prionoides* were non toxic to brine shrimp. Ethanolic extracts of the above five species tested positive for alkaloids, flavonoids, sesquiterpene lactones, saponins. Steroids were present in the other four species except for *Ehretia cymosa* ethanol extract.

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