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Cytotoxicity of selected medicinal plants extracts using the brine shrimp lethality assay from Samburu county, Kenya

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Abstract

Aim of the study: This test was carried out to assess the cytotoxicity bioassay of selected medicinal plants from Samburu County, Kenya. **Materials and Methods:** Selected medicinal plants namely *Clerodendrum myricoides*, (Hoechst) Vatke *Carissa eduli* (Forsk) vahl, *Acassia tortilis* (Fosk) Hayne *Myrsine africana* L., *Rhamnus staddo* A. Rich, *Rhamnus prinoides* L, herit, *Psidium arabica* Jabb and Spach and *Sansevieria enhribergii* Bach were subjected to the Brineshrimp Lethality Test. Three dilutions of the aqueous, methanol/water (70/30) v/v and chloroform extracts were used five (5) tubes per dilution. Ten naupli were introduced per tube and mortality evaluated after 24hrs. Mortality data was analysed using the probit method of Finney Computer Programme. The programme uses the number of dose level, the number of brine shrimp for every concentration, percent mortality for every concentration and dose level to calculate lethal concentration (LC₅₀) and its 95 % confidence interval. **Results:** All the aqueous extracts had an LC₅₀ equal to or higher than 1000µg/ml which is considered non cytotoxic. The extracts showing a low LC₅₀ (< 1000) are likely candidates for cytotoxic or anticancer drugs and can be investigated further. The extracts showing a high LC₅₀ (> 1000) can be used as non cytotoxic drugs and hence further investigations would be necessary. **Conclusion:** The bioactivity results in this study validates the use of the plants as herbal remedies by Samburu Traditional healers.

Keywords: Brine shrimp lethality, Medicinal plants, Samburu Community, Kenya.

INTRODUCTION

Medicinal plants have been used for treatment of disease of humans and animals since time immemorial. Cave paintings discovered in Lauscaux in France and which were radiocarbon dated to between 13,000-25,000 BC showed clearly the use of plants as healing agents in early times [1]. The use of herbal medicines has increased in the past few decades in many countries and more so the developing countries. The increased use has resulted from the assumption that their being natural poses no health risk with the additional economic and nutritional benefits [2]. It has become necessary to investigate plant secondary metabolites due to observed adverse effects related to their use in in traditional medicine practice) [3]. The Brine Shrimp Lethality Test (BSLT) is a protocol that is used to detect a wide spectrum of bioactivity in plants crude extracts. This protocol can help predict the cytotoxicity and pesticidal activity of an extract [4] and hence the assay was chosen to investigate the plant extracts from Samburu County Kenya. A substance is considered to be cytotoxic if it inhibits vital metabolic processes or it causes disorders in living organisms resulting in perversion of behavior or death [3]. A product is only considered cytotoxic if it can prevent important metabolic processes from occurring in an organism on exposure. It is equally cytotoxic if it causes anomalies in the organ systems which may result in abnormal behaviour or death of the organism the test is sensitive enough to detect samples that are cytotoxic enough to kill shrimps larvae on exposure [5]. The Brine shrimp Lethality test gives LC₅₀ concentrations in µg /ml which when above 1000µg/ml is considered safe but is considered toxic when below 1000µg/ml [6,7].

This test can be used to detect substances with antitumor properties as the results can be well related to the substance toxicity property [7]. This property of brineshrimp relates well with antitumor activity in man as the test in brineshrimp responds in a similar manner as in the mammalian system [8]. This protocol has been used with great success for various antitumor and active cytotoxic agents in their assay guided fractionation [6] which include but not limited to Trilobacin from *asimina triloba* [9], cis annonacin from

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Annona maricata [10], Ent- Kaur-16-en -19-oic acid from *Elaeoselinum foetidum* [11]. Taxal from the bark of *Taxus brevifolia* was discovered this way. Crude extracts from plants can be subjected to screening for phytochemicals and the active portion analysed for chemicals [12]. The application of this protocol is seen in the analysis of pesticide residues, mycotoxins, stream pollutants, anaesthetics, dinoflagellates, among others [6].

The plants under study have had some medicinal properties and phytochemicals from them reported as for example *Clerodendrum myricoides* is used in treatment of venereal diseases [13,14], treatment of infertility [15], it has antibacterial and antifungal activity [16], It is used in treatment of Malaria [17,18], it treats epilepsy, arthritis, diabetes, typhoid, cough/cold, eye problems, proper positioning of a foetus,

tonsillitis, rheumatism, East Coast Fever [19,20] among others. *Carissa edulis* has been reported to treat venereal disease [13,21], it is used for the treatment of headache, chest complaints, rheumatism, syphilis, rabies and as a diuretic [22], it contains as active phytochemicals lupeol, carissol, β -amyrin and oleuropein [23]. This test was carried out to assess the cytotoxicity bioassay of selected medicinal plants from Samburu County, Kenya.

MATERIAL AND METHODS

The study area

The plant materials used for Brine shrimp Lethality tests were collected from the Samburu County (Figure 1).

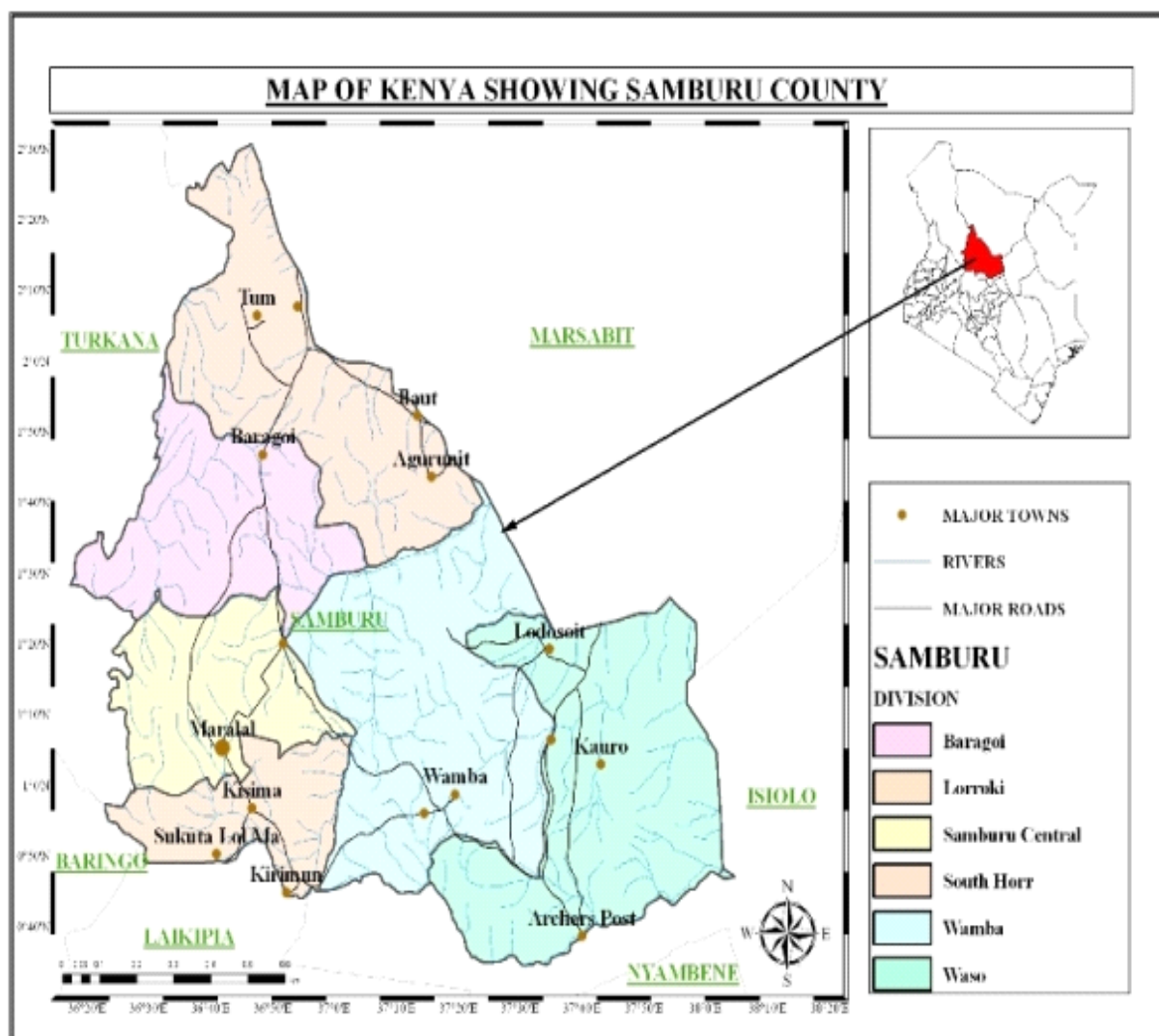


Figure 1: The map of Kenya showing the location of Samburu County and its administrative boundaries

Collection and identification of plants

A guided tour of the study area was taken during which plant samples were collected from highlands, escarpments and lowland areas of the vast dry land. The medicinal plants that were reported by the herbalists as useful for medicinal purposes were collected by the herbalists and the researchers. The medicinal plants were identified at the department of Land Resource Management and Agriculture Technology (LAMART) University of Nairobi where voucher specimens were allocated a specific number and the voucher specimen deposited.

Preparation of plants and plant extract.

The Samples were prepared for extraction according to Gakuya, [24] and these samples were extracted according to [25] and [24].

The plant material was prepared by drying them at room temperature under the shade. these were thereafter chopped with a sharp knife. an electric mill was used to grind the chopped plants to fine powder that was packed into airtight polythene bags. the weight of the packed bags was half a kilogramme. The process was carried out in a fume cupboard to avoid the fumes emitted. Further protection was through wearing of face masks.

Aqueous extraction

This was carried out according to [25] and [26]. Forty grams of plant powder was put in a conical flask and covered with 400mls of distilled water. A hot water bath was heated to 100 °C. The mixture was placed here and boiled for thirty minutes. This is in line with the method of preparation by the Samburu Traditional healers who use hot extraction for preparation of medicinal products. A muslin gauze was used to filter the extract which was then put into clean vials. The vials were placed in a centrifuge and were centrifuged at four thousand (4,000) revolutions per minute. This was done for ten minutes. The resultant was freeze dried for a day then lyophilized in an Edward High Vacuum Model M₆B lyophilizer

Methanol /water extraction

(Forty (40) grams of powder was soaked in methanol/water 70%/30% v/v in a conical flask.) This under went cold maceration for three days during which time shaking was done. Whatmann no. 1 filter paper was used to filter the mixture which was thereafter reduced in a rotary evaporator under pressure. This gave a residue that was further put in an oven at 40 °C to completely reduce the methanol. thereafter the residue was freeze dried using the lyophilizer.

Chloroformic extraction

Forty (40) grams of powder was put in a conical flask and covered with 400 ml chloroform and cold maceration done for six days during which time continuous shaking was done. A whatmann no.1 filter paper was used to filter the mixture. the product was reduced to dryness using a soxhlet apparatus. this product was further reduced in an oven at 40 °C and the final product stored at 4 °C.

Evaluation of bioactivity for selected plants using brine shrimp lethality test

Hatching of Brine shrimp nauplii.

Thirty three gramms of salt from the seawas weighed using a weighing machine. this was then put in a one litre conical flask where distilled water was added as stirring continued which helped to dissolve all the marine salt. distilled water was used to make up one litre of water by adding it to the one litre mark. Brine shrimp eggs were acquired from the department and were hatched in a rectangular box. the box had two chambers with two holes in the partition. One chamber was dark while the other was illuminated by a 40 watt electric bulb. The box was filled with the marine salt solution and 50 grams of brineshrimp eggs were sprinkled with a spatula into the box. five grams of yeast was added that was the feed for the naupli. After 48 hours the naupli which are attracted by light were collected from the illuminated chamber and used for the brine shrimp lethality test [24].

Plant extracts solution preparation.

Table 1: Extraction efficient (yield) of the plant species in water, methanol/water and chloroform

Plant name	Amount of powder (mg)	Extract yield (mg)	% extract yield	Extract yield (mg)	% extract yield	Extract yield	% extract yield
		(aqueous)	(aqueous)	(methanol/water)	(methanol/water)	(chloroform)	(chloroform)
<i>Clerodendrum myricoides</i>	40	4.30	10.75	3.62	9.06	0.64	1.28
<i>Myrsine africana</i>	40	3.60	8.99	1.51	18.88	3.88	7.76
<i>Carissa edulis</i>	40	1.38	3.45	5.33	13.33	0.12	0.4

All the aqueous and organic extracts under this study were treated in a similar manner where 0.1 g of plant extract was weighed (Mettler PM 4600, Delta Range®) and transferred into a universal bottle. Ten milliliter of marine salt solution (33g marine salt in 1 liter distilled water) was added to dissolve and stirred using an electric mixer Voltex Reamix 2789® at 2800 rpm to make a final stock concentration of 10,000µg/ml. Serial dilutions were prepared from this stock solution

Cytotoxicity bioassay

Three dilutions were prepared by transferring 500µl, 50µl and 5µl of plant extract into the set of five graduated tubes. Pasteur pipettes were used to put 10 shrimps into each of the tubes. Marine salt solution was also put into the tubes to make dilutions of 1000ug/ml, 100ug/ml, 10ug/ml. Each dilution had five vials and another five for the control. The tubes were left at room temperature for a day and live larvae counted. Percent mortality was determined for each dilution and control. In case death of the control occurred the data was corrected using Gakuyas formula;

$$\text{Death Test} - \text{Control} \times 100 / \text{Control} \quad [24]$$

Data Analysis and reporting

The extraction data was entered and handled in Microsoft Excel 2007 software where descriptive statistics were generated.

The cytotoxicity results were interpreted using the probit method of Finney computer programme which uses the number of dose level, the number of brine shrimp for every concentration, percent mortality for every concentration and dose level. The lethal concentration LC₅₀ and the 95 % confidence interval were obtained using the computer programme [7].

RESULT

Extraction efficiency (yield) results

For the aqueous extraction of the selected plants out of 40g of powder the amount extracted ranged from 0.87 to 10.92 g (2.18 to 27.3%). The highest percent yield of aqueous plant extract was provided by *Acacia tortilis* (27.3%) followed by *Rhamnus prinoides* (17.79 %) while the lowest yield was observed with *Psiadia Arabica* (2.18 %) followed by *Carissa edulis* (3.45 %). The highest percent yield of methanol/water extract was observed with *Rhamnus prinoides* 11.13g (27.83 %) followed by *Rhamnus staddo* 8.11g (20.28%) while the lowest percent yield of methanolic/water extract was provided by *Acacia tortilis* 1.34g (3.35 %) followed by *Myrsine africana* 1.51g (18.88%). The percent yield of the chloroformic extract was extremely low where the highest yield was observed with *Myrsine africana* 3.88g (7.76 %) and *Ramnus staddo* 1.63g (3.26%) while the lowest yield was provided by *Carissa edulis* 0.12g (0.4%) and *Acacia tortilis* 0.28g (0.56 %). The details of the amount of powder, the percent yield of the extract for all the plants studied are detailed in Table 1.

<i>Rhamnus staddo</i>	40	4.50	11.46	8.11	20.28	1.63	3.26
<i>Rhamnus prinooides</i>	40	7.12	17.79	11.33	27.83	0.61	1.22
<i>Acassia tortilis</i>	40	10.92	27.3	1.34	3.35	0.28	0.56
<i>Psiadia arabica</i>	40	0.87	2.18	7.23	18.23		

Cytotoxicity of aqueous extract

The percent mortality of the brine shrimp larvae increased with increase in concentration of solution. For example at 10µ/ml *Clerodendrum myricoides* had a mortality of 4% while at 1000µ/ml it had a mortality of 34%. The LC₅₀ of the aqueous extract of the selected medicinal plants from Samburu County was greater than 1000µ/ml except for *Psiadia arabica* which had an LC₅₀ lower than 1000µ/ml. The LC₅₀ ranged from as low as 499.9 for *Psiadia arabica* to as high as 6921.05 for *Rhamnus prinooides*. The results are detailed in Table 2.

Cytotoxicity of methanol water extract.

The percent mortality of the brine shrimp larvae increased with increase in concentration of the solution. For example at 10 µ/ml *Clerodendrum* had a 16% mortality while at 1000µ/ml it had a mortality of 76%. The methanol/water extract of the selected medicinal plants from Samburu County had an LC₅₀ ranging from as low as 191.10 µg/ml

for *Sansevieria erhenbergii* roots to as high as 3883.55 µ/ml for *Rhamnus staddo*. Three medicinal plants namely *Rhamnus staddo*, *Carissa edulis* and *Psiadia arabica* had an LC₅₀ greater than 1000 µg/ml while the rest had an LC₅₀ lower than 1000 µg/ml. the results are detailed in table 3.

Cytotoxicity of chloroformic extract.

The percent mortality of the brine shrimp increased with an increase in concentration of the solution. At 10µ/ml *Clerodendrum myricoides* had no mortality while at 1000µ/ml it had a mortality of 26%. The LC₅₀ of the selected medicinal plants from samburu showed an LC₅₀ as low as 110.40µ/ml for *Rhamnus staddo* and as high as 8553.47µ/ml for *Carissa edulis*. Three medicinal plants namely *Clerodendrum myricoides*, *Carissa edulis* and *Acasia tortilis* had an LC₅₀ higher than 1000µ/ml while the others had an LC₅₀ lower than 1000µ/ml. The results are detailed in table 4.

Table 2: Results of Brine shrimp Lethality Assay on the crude aqueous extracts

Plant/conc.µ/ml	10 µg/ml	100 µg/ml	1000 µg/ml	LC ₅₀	UCL	LCL
<i>Clerodndrum myricoides</i>	0.4	1.2	3.4	4242.15	-	-
<i>Myrisine africana</i>	0.2	2.0	4.2	1507.15	-	-
<i>Rhamnus staddo</i>	0.6	2.4	4.6	1261.27	-	-
<i>Carissa edulis</i>	0.2	1.0	3.0	4885.50	-	-
<i>Acacia tortilis</i>	0.2	1.4	3.4	3158.48	-	-
<i>Sansevieria enrhenbergii</i>	0.4	2.0	4.4	1462.95	-	-
<i>Rhamnus prinooides</i>	0.0	1.4	2.6	6921.05	-	-
<i>Psiadia Arabica</i>	1.0	2.4	6.2	499.90		

Table 3: Results of Brine shrimp Lethality Assay of the crude methanol/water extract

Plant	10 µg/ml	100 µg/ml	1000 µg/ml	LC ₅₀	UCL	LCL
<i>Clerodndrum myricoides</i>	1.6	3.2	7.6	204.66	3052.25	43.39
<i>Myrisine africana</i>	1.0	1.4	7.0	441.94	12865.23	122.84
<i>Rhamnus staddo</i>	1.0	2.4	3.8	3883.55	-	-
<i>Carissa edulis</i>	0.6	1.6	3.8	3195.14	-	-
<i>Sansevieria enrhenbergii</i> (roots)	1.2	4.6	7.0	191.10	3215.14	37.80
<i>Sansevieria enrhenbergii</i> (Shoots)	0.4	2.8	6.4	421.29	8068.53	120.74
<i>Rhamnus prinooides</i>	0.4	2.8	8.8	214.33	624.14	78.49
<i>Psiadia Arabica</i>	0.0	1.0	3.2	3272.64	-	

Table 4: Results of the Brine shrimp Lethality Assay of crude chloroform extract

Plant	10 µg/ml	100 µg/ml	1000 µg/ml	LC ₅₀	UCL	LCL
<i>Clerodendrum myricoides</i>	0.0	0.6	2.6	5584.44	-	-
<i>Myrsine africana</i>	0.6	0.2	7.6	320.55	2063.35	102.22
<i>Rhamnus staddo</i>	0.6	3.8	10.0	110.40	284.92	40.14
<i>Carissa edulis</i>	0.0	1.0	2.4	8553.47	-	-
<i>Acacia tortilis</i>	0.2	2.2	4.2	1463.01	-	-
<i>Rhamnus prinoides</i>	1.6	4.6	10.0	133.33	426.48	41.77

DISCUSSION

In the present study the yield of the extract varied widely depending on the solvent used. The percent yield of the various extracts was most likely influenced by the polarity of the solvent together with the phytochemical present in the medicinal plant. This was also observed by Nguyen and Eun who observed that the percent yield of phenols and flavonoids in selected medicinal plants in Vietnam varied with the solvent used [27].

Clerodendrum myricoides aqueous and chloroform extracts had an LC₅₀ higher than 1000µ/ml, which is quite safe and which hence infers they may be used as non toxic drugs. The plants methanol extract however had a much lower LC₅₀ which suggests that it is quite toxic and may find use as a cytotoxic drug. These can be further investigated to verify their use as such. This plant; which is used by the traditional healers as the aqueous form can possibly be termed as safe to use as a non cytotoxic drug decoction. These results agree with what [28] was found in Uganda that the methanol extract of *Clerodendrum myricoides* was more toxic than the aqueous extract. This plant has been reported to contain various phytochemicals including terpenes, saponins, tannins, phenols glycosides among others [18,28]. These phytochemicals have been reported to exhibit medicinal properties including antibacterial anti-inflammatory antifungal, antiviral among others [29-32]. These phytochemicals may have been responsible for the bioactivity of the extracts which further validates the plants use by the Samburu traditional healers.

Carissa edulis is safe in all the three types of extracts (aqueous, methanol/water and chloroform) and can be used as a non cytotoxic drug. This plant has been reported to contain phytochemicals including alkaloids, sterols and resins [33]. These phytochemicals are reported to have medicinal value [18, 33-35] and could be attributed to the bioactivity shown by this plant. *Acassia tortilis* is safe as chloroform and aqueous extract but toxic as the methanol/water extract. This plant has been reported as containing tannins as secondary metabolites [36]. These may have contributed to the bioactivity shown by it. *Myrsine africana*, *Rhamnus staddo* and *Rhamnus prinoides* are only safe as an aqueous extract and toxic as a chloroform and methanol/water extract. *Myrsine africana* has been reported to contain various Phytochemical including tannins, saponins flavonoids [37] which may have caused the biological activity shown by the plant. *Psiadia arabica* is safe in methanol/water extract but toxic in aqueous extract. This plant has been reported to contain flavones and kaulene as bioactive compounds [38,39] which may be attributed to the bioactivity shown in the study. *Sansevierria enhribergii* is safe in aqueous and methanol/water extract. This plant has been reported to have antifungal activity which could be attributed to the bioactivity seen [40]. The extracts showing a low LC₅₀ (< 1000) are likely candidates for cytotoxic or anticancer drugs and can be investigated further. The extracts showing a high LC₅₀ (> 1000) can be used as non cytotoxic drugs and hence further investigations would also be needed.

CONCLUSION

The reported phytochemicals in the selected medicinal plants together with the bioactivity results in this study validates the use of the plants as herbal remedies by Samburu Traditional healers.

Conflict of Interest

The authors have no conflict of interest to declare.

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