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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*, (Hoecst) Vatke *Carissa eduli* (Forsk) vahl, *Acassia tortilis* (Fossk) Hayne *Myrsine africana* L., *Rhamnus staddo* A. Rich, *Rhamnus prinoides* L, herit, *Psiadia arabica* Jabb and Spach and *Sansevierria 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 24 hrs. Mortality data was analysed using the probit method of Finney Computor 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 (LC_{50}) and its 95 % confidence interval. **Results:** All the aqueous extracts had an LC_{50} equal to or higher than 1000 µg/ml which is considered non cytotoxic. The extracts showing a low LC_{50} (< 1000) are likely candidates for cytotoxic or anticancer drugs and can be investigated further. The extracts showing a high LC_{50} (> 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.

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INTRODUCTION

Medicinal plants have been used for treatment of disease of humans and animals since prehistoric time (since time immemorial). The first accepted use of plants as healing agents was depicted in the cave paintings discovered in the Lascaux caves in France and radiocarbon-dated to 13,000-25,000 BC [1] (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). The uses of medicinal plant-derived medicines have been on the increase in the past few decades in many countries, which mainly stem from the perception that their "natural" condition makes them beneficial with no health risks. In developing countries, medicinal plants are extensively utilized for food, economic, and medicinal purposes. (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]. The investigation of plants bioactive secondary metabolites has become inevitable due to significant correlation between their uses in traditional medicine and the observed bioeffects of their products (It has become necessary to investigate plant secondary metabolites due to observed adverse effectsrelated to their use in in traditional medicine practice) [3]. The Brine Shrimp Lethality Test (BSLT) is a protocol thatis used to detect a wide spectrum of bioactivity in plants crude extracts.Tthis protocol can help predict the cytotoxicity and pesticidal activity of an extract. general Bioassay which is capable of detecting a broad spectrum of bioactivity in plant crude extract. The test can 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]. The brine shrimp assay basically detects substances that are cytotoxic enough to kill shrimp's larvae on exposure to solution of the sample. A product is only considered cytotoxic if it can prevent important metabolic processes from occuring 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 to kill shrimps larvae on exposure [5]. The Brine shrimp Lethality test gives LC_{50} concentrations in µg /ml which when above 1000 µg/ml is considered safe but is considered toxic when below 1000µg/ml [6,7].

Brine shrimp lethality assay is a rapid inexpensive and simple bioassay for testing plant extracts bioactivity, the result of which in most cases correlate with cytotoxic and antitumor properties of the plant. Toxicity to brine shrimps has a good correlation with anti-tumor activity in man This test can be used to detect substances with antitumor properties as the results can be well related to the substance toxicity property [7] since the brine shrimp responds similarly to the corresponding mammalian system 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]. Since the test was introduced [6], it has been used successfully for bioassay guided fractionation for active cytotoxic and antitumor agents including (This protocol has been used with great success for various antitumor and active cytotoxic agents in their assay guided fractionation which include but not limited to) Trilobacin from *asimina triloba* [9], cis annonacin from *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 plant extract can be first assayed for particular activities and the active fraction then analysed phytochemically (Crude extracts from plants can be subjected to screening for phytochemicals and the active portion analysed for chemicals) [12]. Brine shrimps have been used for various bioassay systems including analysis of pesticide residues, mycotoxins, stream pollutants, anaesthetics, dinoflagellates, morphine like substances, toxicity of oil dispersants, co-carcinogenicity of phorbol esters and toxicants in marine environments. (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 activitiy [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).

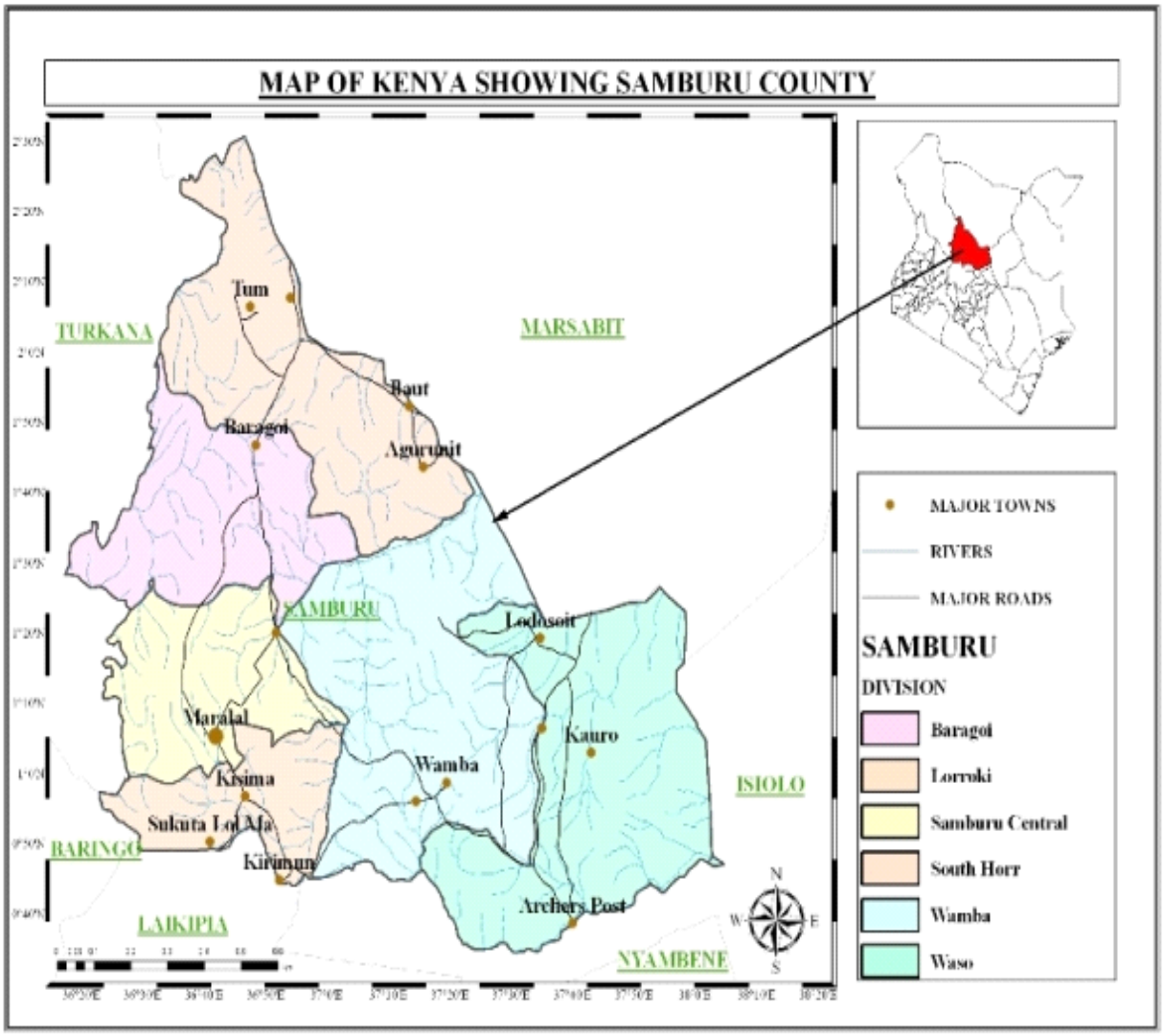

Figure 1: The map of Kenya showing the location of Samburu County and its administrative boundaries

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 in situ by the herbalists and plant specimens collected for botanical identification. The specimens were identified by a botanist at the University of Nairobi, Department of Land Resource Management and Agriculture Technology (LARMAT) (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 materials were dried under the shade at room temperature for three weeks. The dried plants were then chopped into small pieces using a sharp knife. The chopped plant material was then ground into powder using an electric mill. The powder was then packed into clean airtight polythene paper bags in portions of 500 g. (The plant material was prepared by drying them at room temperature under the shade. these were thereaftre chopped with a sharp knife. an electric mill was used to gring the chopped plants to fine powder that was packed into airtight polythene bags. the weight of the packed bags was halh 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]. Fourty grams of plant powder was put in a conical flask and covered with 400 ml of distilled water. The mixture was boiled in a hot water bath for 30 minutes at 100 ^{o}C (A hot water bath was heated to 100 ^{o}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. The resultant extract was filtered using muslin gauze into clean vials then centrifuged at 4000 revolutions per minute for ten minutes. A clear extract was obtained and put in clean vials. This was then put in a freeze drier for 24 hrs then lyophilized in a lyophilizer (Edwards High Vacuum, Model M6B). (A muslin gauze was used to filter the extract which was then put into clean vials. The vials were placed in a cemtrifuge and were centrifuged at four thousand (4,000) revolutions per minte. This was done for ten minutes. The resultant was freeze dried for a day then lyophilized in an Edward High Vacuum Model M_6B lyophilizer)

Methanol /water extraction

Fourty grams of powder was put in a conical flask and covered with methanol 70% v/v methanol /water. (Fourty (40) g of powder was soaked in methanolwater 70%/30% v/v in a conical flask.) This under went cold maceration for three days during which time shaking was done. The mixture was then filtered using whatman no. 1 filter paper and reduced in a rotary evaporator to dryness under pressure. The resultant residue was put in an oven at 40 ^{o}C for methanol to be reduced completely. The final residue was then freeze dried using the lyophiliser (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 ^{o}C to completely reduce the methanol. thereafter the residue was freeze dried using the lyophilizer).

Chloroformic extraction

Fourty (40) g 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. The mixture was then filtered using whatmans No. 1 filter paper. The filtrate was reduced to dryness using soxhlet apparatus. The final residue was further reduced in an oven at 40 ^{o}C. The final extract was stored in a refrigerator at 4 ^{o}C. (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 ^{o}C and the final product stored at 4 ^{o}C).

Evaluation of bioactivity for selected plants using brine shrimp lethality test

Hatching of Brine shrimp nauplii.

Hatching of the brine shrimp was carried out according to [24]. Thirty three (33) g marine salt was weighed on an electric machine and transferred into a 1 liter conical flask. Distilled water was added concurrently stirring to dissolve the marine salt. When all the salt dissolved distilled water was added to make the 1 liter mark to constitute the marine salt solution. Brine shrimp eggs acquired from the department of Public Health, Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi were hatched in shallow rectangular plastic double chambered box with a dividing wall with 1-2 mm holes. The box was filled with marine salt solution (33 g of marine salt in 1 liter distilled water). Using a spatula 50 mg of brine shrimp eggs were sprinkled and about 5mg of yeast to act as food for the nauplii was sprinkled in the dark compartment. The other compartment was illuminated through a hole in the lid of the box and kept under a light source using a 40 watts electric bulb. After 48 hrs the phototrophic nauplii were collected using a Pasteur pipette from the lighted compartment and subjected to a Brine shrimp lethality test. (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 hrs the naupli which are attracted by light were collected from the illuminated chamber and used for the brine shrimp letahality test.)

Plant extracts solution preparation.

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 (33 g 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 10,000 µg/ml. Serial dilutions were prepared from this stock solution

Cytotoxicity bioassay

Three dilutions were prepared by transferring 500 µl, 50 µl and 5 µl of plant extract into the set of five graduated tubes. Ten shrimps were transferred into each of the tubes using Pasteur pipettes and marine salt was added to 5ml mark to make dilutions of 1000 µg/ml, 100 µg/ml and 10 µg/ml. Five graduated vials were set for each dilution and a further five for the control. The tubes were left at room temperature and the number of live larvae counted after 24 hrs. The percent

mortality was determined for each dilution and controls. Where the deaths of controls occur within 24 hrs data was corrected using death = (test- control/control *100) [24] (Pasteur pipettes wre used to put 10 shrimps into each of the tubes. Marine salt solution was also put into the tubes to make dilutions of 1000 ug/ml, 100 ug/ml, 10 ug/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 occured the data was corrected using Gakuyas formula.

Death Test – Control x 100/Control)

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 LC_{50} 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 40 g 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* 11.13 g (27.83 %) followed by *Rhamnus staddo* 8.11 g (20.28%) while the lowest percent yield of methanolic/water extract was provided by *Acacia tortilis* 1.34 g (3.35 %) followed by *Myrsine africana* 1.51 g (18.88%) . The percent yield of the chloroformic extract was extremely low where the highest yield was observed with *Myrsine africana* 3.88 g (7.76 %) and *Ramnus staddo* 1.63 g (3.26%) while the lowest yield was provided by *Carissa edulis* 0.12 g (0.4%) and *Acacia tortilis* 0.28 g (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.

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

Plant name	Amount of powder (mg)	Extract yield (mg) (aqueous)	% extract yield (aqueous)	Extract yield (mg) (methanol/ water)	% extract yield (methanol/water	Extract yield (chloroform)	% extract yield (chloroform)
Clerodendrum myricoides	40	4.30	10.75	3.62	9.06	0.64	1.28
Myrsine africana	40	3.60	8.99	1.51	18.88	3.88	7.76
Carissa edulis	40	1.38	3.45	5.33	13.33	0.12	0.4
Rhamnus staddo	40	4.50	11.46	8.11	20.28	1.63	3.26
Rhamnus prinoides	40	7.12	17.79	11.33	27.83	0.61	1.22
Acassia tortilis	40	10.92	27.3	1.34	3.35	0.28	0.56
Psiadia arabica	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 10 µ/ml *Clerodendrum myricoides* had a mortality of 4% while at 1000 µ/ml it had a mortality of 34%. The LC_{50} of the aqueous extract of the selected medicinal plants from Samburu County was greater than 1000 µ/ml except for *Psiadia arabica* which had an LC_{50} lower than 1000 µ/ml. The LC_{50} ranged from as low as 499.9 for *Psiadia arabica* to as high as 6921.05 for *Rhamnus prinoides*. 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 LC_{50} ranging from as low as 191.10 µg/ml for *Sansevierria 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 LC_{50} greater than 1000 µg/ml while the rest had an LC_{50} 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. For example At 10 µ/ml C*lerodendrum myricoides* had no mortality while at 1000 µ/ml it had a mortality of 26%. The LC_{50} of the selected medicinal plants from samburu showed LC_{50} as low as 110.40 µ/ml for *Rhamnus staddo* and as high as 8553.47 µ/ml for *Carissa edulis*. Three medicinal plants namely *Clerodendrum*

myricoides, *Carissa edulis* and *Acasia tortilis* had an LC_{50} higher than 1000 µ/ml while the others had LC_{50} lower than 1000 µ/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*
Clerodndrum myricoides	*0.4*	*1.2*	*3.4*	*4242.15*	-	-
Myrisine africana	*0.2*	*2.0*	*4.2*	*1507.15*	-	-
Rhamnus staddo	*0.6*	*2.4*	*4.6*	*1261.27*	-	-
Carissa edulis	*0.2*	*1.0*	*3.0*	*4885.50*	-	-
Acacia tortilis	*0.2*	*1.4*	*3.4*	*3158.48*	-	-
Sansevierra enrhenbergii	*0.4*	*2.0*	*4.4*	*1462.95*	-	-
Rhamnus prinoides	*0.0*	*1.4*	*2.6*	*6921.05*	-	-
Psiadia Arabica	*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*
Clerodndrum myricoides	*1.6*	*3.2*	*7.6*	*204.66*	*3052.25*	*43.39*
Myrisine africana	*1.0*	*1.4*	*7.0*	*441.94*	*12865.23*	*122.84*
Rhamnus staddo	*1.0*	*2.4*	*3.8*	*3883.55*	-	-
Carissa edulis	*0.6*	*1.6*	*3.8*	*3195.14*	-	-
Sansevierra enrhenbergii (roots)	*1.2*	*4.6*	*7.0*	*191.10*	*3215.14*	*37.80*
Sansevierra enrhenbergii (Shoots)	*0.4*	*2.8*	*6.4*	*421.29*	*8068.53*	*120.74*
Rhamnus prinoides	*0.4*	*2.8*	*8.8*	*214.33*	*624.14*	*78.49*
Psiadia Arabica	*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*
Clerodndrum myricoides	*0.0*	*0.6*	*2.6*	*5584.44*	-	-
Myrisine africana	*0.6*	*0.2*	*7.6*	*320.55*	*2063.35*	*102.22*
Rhamnus staddo	*0.6*	*3.8*	*10.0*	*110.40*	*284.92*	*40.14*
Carissa edulis	*0.0*	*1.0*	*2.4*	*8553.47*	-	-
Acacia tortilis	*0.2*	*2.2*	*4.2*	*1463.01*	-	-
Rhamnus prinoides	*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 LC_{50} higher than 1000 µ/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 LC_{50} 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 [28,18]. 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 LC_{50} (< 1000) are likely candidates for cytotoxic or anticancer drugs and can be investigated further. The extracts showing a high LC_{50} (> 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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