

Evaluation of Acute Toxicity of Crude Plant Extracts from Kenyan Biodiversity using Brine Shrimp, *Artemia salina* L. (Artemiidae)

J.M. Nguta^{a*}, J.M.Mbaria^a, D.W.Gakuya^b, P.K.Gathumbi^c, J.D.Kabasa^d and S.G.Kiama^e

^aDepartment of Public Health, Pharmacology and Toxicology, University of Nairobi, Nairobi, Kenya

^bDepartment of Clinical Studies, University of Nairobi, Nairobi, Kenya

^cDepartment of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi, Nairobi, Kenya

^dDepartment of Physiology and Pharmacology, Makerere University, Kampala, Uganda

^eDepartment of Veterinary Anatomy and Physiology, University of Nairobi, Nairobi, Kenya

Abstract: Purpose: *Artemia salina* L. (Artemiidae), the brine shrimp larva, is an invertebrate used in the alternative test to determine toxicity of chemicals and natural products. In this study, we aimed to evaluate the acute toxicity of crude plant extracts using *A.salina*.

Materials and methods: In this study the Medium Lethal Concentrations (LC₅₀ values) of 45 antimalarial plant extracts and positive controls, cyclophosphamide and etoposide were determined using *A. salina*.

Results: Out of the 45 organic extracts screened for activity against *A. salina* larvae, 23 (51%) of the crude extracts demonstrated activity at or below 100 µg/ml, and were categorized as having strong cytotoxic activity, 18 (40%) of the crude extracts had LC₅₀ values between 100 µg/ml and 500 µg/ml, and were categorized as having moderate cytotoxicity, 2 (4.5%) of the crude extracts had LC₅₀ values between 500 µg/ml and 1000 µg/ml, and were considered to have weak cytotoxic activity, while 2 (4.5%) of the crude extracts had LC₅₀ values greater than 1000 µg/ml and were considered to be non toxic. Approximately 9 (20%) of the aqueous extracts demonstrated activity at or below 100 µg/ml and were considered to have strong cytotoxic activity, 18 (40%) of the screened aqueous crude extracts had LC₅₀ values between 100 µg/ml and 500 µg/ml and were considered to be moderately cytotoxic, 7 (16%) of the crude extracts had LC₅₀ values between 500 µg/ml and 1000 µg/ml and were considered to have weak cytotoxic activity while 11 (24%) of the aqueous extracts had LC₅₀ values greater than 1000 µg/ml and were categorized as non toxic. The positive controls, cyclophosphamide and etoposide exhibited strong cytotoxicity with LC₅₀ values of 95 µg/ml and 6 µg/ml respectively in a 24 hour lethality study, validating their use as anticancer agents.

Conclusions: In the current study, 95.5% of all the screened organic extracts and 76% of the investigated aqueous extracts demonstrated LC₅₀ values <1000 µg/ml, indicating that these plants could not make safe antimalarial treatments. This calls for dose adjustment amongst the community using the plant extracts for the treatment of malaria and chemical investigation for isolation of bioactive compounds responsible for the observed toxicity.

Keywords: Acute toxicity, *A. salina* bioassay, crude extracts, antimalarial plants, Kenyan biodiversity.

INTRODUCTION

Since ancient times people have used plants as medicines [1]. This use has great importance, because plants can provide drugs to widen the therapeutic arsenal [2]. However, during the past decade, traditional systems of medicine have become increasingly important in view of their safety [3] and for this reason, research is carried out in order to determine the toxicity of medicinal plants. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Indeed

indigenous plants play an important role in the treatment of many diseases [4] and 80% of the people worldwide are estimated to use herbal remedies [5-7].

However, few data are available on their safety, despite the fact that validation of traditional practices could lead to innovative strategies in malaria control. Natural products represent a virtually inexhaustible reservoir of molecules, most of which are hardly explored and could constitute lead molecules for new antimalarial drugs, such as artemisinin, isolated from *Artemisia annua* [8]. Although modern medicine may be available in developing countries, phytomedicines have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs [9].

*Address correspondence to this author at the Department of Public Health, Pharmacology and Toxicology, University of Nairobi, Box 29053-00625, Nairobi, Kenya; Tel: +254 735 750 278; Fax: 254-020-631325; E-mail: joseph.nguta@uonbi.ac.ke

The current study seeks to evaluate the acute toxicity of crude plants commonly used as antimalarials by the Msambweni community of Kenyan Coast using *A.salina* larvae.

MATERIALS AND METHODS

Plant Materials

The plant samples used in the current study were collected in August 2009 from Msambweni district of Kenya based on ethnopharmacological use through interviews with local communities and traditional health practitioners.

The collected species were: *Aloe vera* L. ex Webb (JN07), *Aloe secundiflora* Engl. (JN06), *Aloe macrosiphon* Baker (JN05), *Aloe deserti* A. Berger (JN04), *Flacourtia indica* (Burm.f) Merr. (JN019), *Harrisonia abyssinica* Oliv. (JN023), *Fagaropsis angolensis* (Engl.) Dale (JN017), *Clausena anisata* (Willd.) Hook.f. ex Benth. (JN013), *Canthium glaucum* Hiern (JN011), *Agathisanthemum globosum* Hochst. ex A. Rich.) Bremek. (JN03), *Flueggea virosa* (Roxb.ex Willd.) Baill. (JN049), *Bridelia micrantha* (Hochst.) Baill. (JN010), *Ficus bussei* Warb. ex Mildbr. & Burret (JN018), *Cissampelos mucronata* A.Rich (JN047), *Azadirachta indica* A. Juss. (JN09), *Grewia trichocarpa* Hochst. ex A.Rich.(JN022), *Grewia hainesiana* Hole (JN021), *Adansonia digitata* L.(JN02), *Clerodendrum myricoides* R. Br. (JN050), *Harungana madagascariensis* Lam. Ex Poir. (JN 053), *Dichrostachys cinerea* (L.) Wight & Arn. (JN016), *Acacia seyal* Delile (JN01), *Albizia anthelmintica* Brongn. (JN046), *Cassia occidentalis* hort.ex Steud. (JN012), *Gerrardanthus lobatus* C. Jeffrey (JN020), *Combretum molle* Engl. & Diels (JN 059), *Combretum padoides* Engl and Diels.(JN014), *Commiphora schimperi* (O.Berg) Engl. (JN015), *Carissa edulis* (Forrsk.) (JN042), *Centella asiatica* (L.)Urb. (JN043) and *Amaranthus hybridus* L.(JN08).

The information gathered included part of the plant used and the method of preparation of the herbal antimalarial remedies. The plants were identified by taxonomists at the University of Nairobi and the National Museums of Kenya Herbaria, Nairobi, where voucher specimens were deposited. Species nomenclature follows the international code of botanical nomenclature. The plant parts were chopped into small pieces; air dried at room temperature (25°C) under shade and pulverized using a laboratory mill (Christy & Norris Ltd., England).

Cytotoxic Drugs

Cyclophosphamide, Mfg. Lic. No.: DD/140 and batch number KB 791001, was purchased from Biochem Pharmaceutical Industries Limited (Mumbai, India). Etoposide (Etoposid™), batch number J8 05 26, a semi synthetic derivative of podophyllotoxin, was purchased from CIPLA Limited, plot No.S-103 Verna.

Preparation of Extracts

Considering that people in Msambweni usually use hot water to prepare their herbal remedies as decoctions and sometimes concoctions, aqueous hot infusions of each plant part was prepared (50 grams of powdered material in 500 ml

of distilled water) in a water bath at 60°C for 1 hour. The extracts that were obtained were filtered through muslin gauze and the filtrate kept in deep freezer for 24 hours, which was then lyophilized. The lyophilized dry powder was collected in stoppered sample vials, weighed and kept at -20°C until used. Organic extracts were prepared by maceration of 50 grams of the dried and powdered plant material with the organic solvent [(Chloroform (CHCl₃): Methanol (MeOH)] (1:1) for 48 hours. The extract was then filtered through Whatman filter paper No.1. The filtrate was concentrated to dryness in vacuo by rotary evaporation and weighed. The yields of the water extracts were higher than the corresponding organic extracts and ranged between 1.74 and 17.02%, while those of the organic extracts ranged between 1.72 and 13.4%. The dry solid extracts were stored at -20°C in airtight containers until used.

Product Identification and Description (*A. Salina*)

Artemia cysts, batch number DE RP 33801, were purchased from JBL GmbH & Co.KG (Neuhofen, Germany) and the product was labeled as JBL Artemio Pur Brand. The *Artemia* cysts had been harvested from Great Salt Lake, Utah, USA and were identified as *A. salina*, based on zoogeography [10]. *A. salina* is the best studied of the *Artemia* species [11], estimated to represent over 90% of studies in which *Artemia* is used as an experimental test organism (very often using material sourced from Great Salt Lake, Utah, USA) [12].

Culture and Harvesting of *A. Salina*

A. salina cysts were stored at -20°C before use. *A. salina* cysts were incubated for hatching in a shallow rectangular dish (14 cm x 9 cm x 5 cm) filled with 225 mls of a 3.3% solution of artificial sea water. A plastic divider with several 2 mm holes was clamped in the dish to make two unequal compartments. The cysts (1.11 grams) and yeast (0.0827grams) were sprinkled into the larger compartment which was darkened. The smaller compartment was illuminated by a tungsten filament light and gently sparged with air. After 24 hours, hatched *A. salina* cysts were transferred to fresh artificial seawater and incubated for a further 24 hours under artificial light with air sparging [13]. The phototropic nauplii were collected by pipette from the lighted side, having been separated by the divider from the shells.

Preparation of Test Extracts

Stock solutions of aqueous extracts (10,000 µg/ml) were made in distilled deionized water and filter sterilized using 0.22 µm membrane filters in a laminar flow hood. The organic extracts were dissolved in dimethyl sulphoxide, CH₃.SO.CH₃ M.W 78.13 (dmsO), batch number PJ/25/34-96/709-05/6/16, (Thomas baker chemicals, pvt. Limited, Mumbai, india) followed by subsequent dilution to lower concentration of DMSO, to <1% to avoid carry over (solvent) effect [14]. Test extracts at appropriate amounts (5 µl, 50 µl, and 500 µl for 10 µg/ml, 100 µg/ml, and 1000 µg/ml, respectively) were transferred into 10 ml vials (5 vials for each dose and 1 for control). Five replicates were prepared for each dose level.

Preparation of Cytotoxic Drugs

Stock solutions of cyclophosphamide and etoposide (10,000 µg/ml) were prepared in distilled deionized water and filter sterilized using 0.22 µm membrane filters in a laminar flow hood. Test solutions at appropriate amounts (5 µl, 50 µl, and 500 µl for 10 µg/ml, 100 µg/ml, and 1000 µg/ml, respectively) were transferred into 10 ml vials (5 vials for each dose and 1 for control). Five replicates were prepared for each dose level.

Bioassay of *A. Salina*

For toxicity tests, ten *A. salina* nauplii (larva) were transferred into each sample vial using 230 mm disposable glass Pasteur pipettes (Ref. D812) (Poulten & Graf Ltd, Barking, UK) and filtered brine solution was added to make 5 ml. The nauplii were counted macroscopically in the stem of the pipette against a lighted background. A drop of dry yeast suspension (Red star) (3 mg in 5 ml artificial sea water) was added as food to each vial. All the vials were maintained under illumination. The surviving nauplii were counted with the aid of a 3x magnifying glass, after 24 hours, and the percentage of deaths at the three dose levels and control were determined. In cases where control deaths occurred, the data was corrected using Abbott's formula [15] as follows: % deaths = [(Test-control)/control x 100. The surviving nauplii were killed by the addition of 100 µl of 5% (v/v) phenol to each vial.

LC₅₀ Determinations

The lethal concentration fifty (LC₅₀), 95% confidence interval and slope were determined from the 24 hour counts using the probit analysis method described by Finney [16]. LC₅₀ is indicative of toxicity level of a given plant extract to the brine shrimp larva.

RESULTS

Out of the 45 organic extracts screened for activity against *Artemia salina* larvae, 23 (51%) of the crude extracts demonstrated activity at or below 100 µg/ml, and were categorized as having strong cytotoxic activity, 18 (40%) of the

crude extracts had LC₅₀ values between 100 µg/ml and 500 µg/ml, and were categorized as having moderate cytotoxicity, 2 (4.5%) of the crude extracts had LC₅₀ values between 500 µg/ml and 1000 µg/ml, and were considered to have weak cytotoxic activity, while 2 (4.5%) of the crude extracts had LC₅₀ values greater than 1000 µg/ml and were considered to be non toxic (Fig. 1).

The results from screening 45 aqueous extracts of 31 different plant species against *A. salina* larvae are shown in Fig (2).

Approximately 9 (20%) of the aqueous extracts demonstrated activity at or below 100 µg/ml and were considered to have strong cytotoxic activity, 18 (40%) of the screened crude extracts had LC₅₀ values between 100 µg/ml and 500 µg/ml and were considered to be moderately cytotoxic, 7 (16%) of the crude extracts had LC₅₀ values between 500 µg/ml and 1000 µg/ml and were considered to have weak cytotoxic activity while 11 (24%) of the aqueous extracts had LC₅₀ values greater than 1000 µg/ml and were categorized as non toxic (Fig. 2).

The positive controls, cyclophosphamide and etoposide had LC₅₀ values of 95 µg/ml and 6 µg/ml respectively.

DISCUSSION

The evaluation of the toxic action of plant extracts is indispensable in order to consider a treatment safe; it enables the definition of the intrinsic toxicity of the plant and the effects of acute overdose [17]. The current study aimed at screening the lethality of crude plant extracts commonly used as antimalarial phytomedicines in Msambweni district, Kenya against brine shrimp, *A. salina* larvae. The procedure of Meyer *et al* [18] was adopted to determine the lethality of crude plant extracts to brine shrimp. *Artemia* bioassay has been demonstrated to provide a viable alternative to the mouse bioassay, which is expensive and associated with ethical constraints.

In bioactivity evaluation of plant extracts by brine shrimp bioassay, an LC₅₀ value lower than 1000 µg/ml is considered cytotoxic, Meyer *et al* [3]. In the current study, 98 (97.6%) of all the screened organic extracts and 85 (73%) of the in-

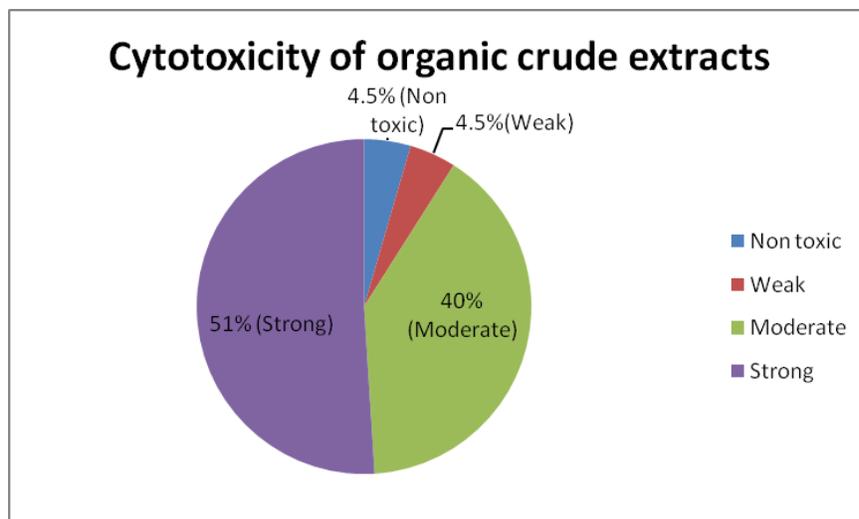


Fig. (1). Lethality of organic (CHCl₃/MeOH) crude extracts against *A. salina* (n=45).

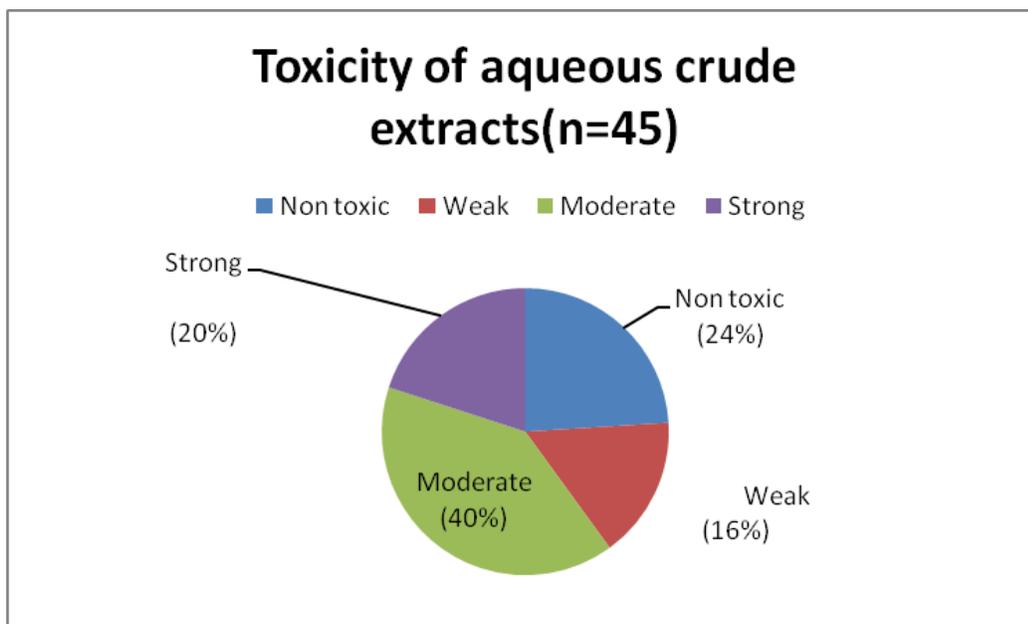


Fig. (2). Cytotoxicity of aqueous crude extracts against *A. salina* (n=45).

vestigated aqueous extracts demonstrated LC_{50} values < 1000 $\mu\text{g/ml}$, indicating the presence of cytotoxic compounds responsible for the observed toxicological activity. The current observation indicates that some of the antimalarial plants could not make safe herbal remedies. This calls for dose adjustment amongst the community using the plant extracts for the treatment of malaria.

The most toxic aqueous extracts (LC_{50} < 100 $\mu\text{g/ml}$) were the leaves and stem bark of *Adansonia digitata* Linn., leaves of *Bridelia micrantha* Baill. (Hochst), leaves and stem bark of *Flacourtia indica* (Burm.f) Merr., leaves of *Aloe macrorhizon* Bak., leaves of *Aloe secundiflora* Engl., roots of *Dichrostachys cinerea* (L) Wight and AM. and the leaves of *Grewia hexaminta* Burret. Pharmacological properties of these plants have been demonstrated in preclinical studies, including those of *Adansonia digitata* Linn. as an antipyretic [16], bioactive [10] and antiplasmodial activity [14], *Bridelia micrantha* Baill. (Hochst) as an antiplasmodial agent [4], *Flacourtia indica* (Burm.f) Merr. as an antiplasmodial agent [12], *Aloe secundiflora* Engl. as an antimalarial agent [9], and *Grewia hexaminta* Burret. where triterpenoids have been isolated [6]. These plant species have demonstrated cytotoxicity low enough (LC_{50} < 100 $\mu\text{g/ml}$) to merit chemical investigation for isolation of cytotoxic compounds responsible for the observed toxicity.

The degree of lethality was found to be directly proportional to the concentration of the extract. In the evaluation for general toxicity using brine shrimp, maximum mortalities took place at a concentration of 1000 $\mu\text{g/ml}$ whereas least mortalities were at 10 $\mu\text{g/ml}$ concentration. The cytotoxic activity was considered weak when the LC_{50} was between 500 and 1000 $\mu\text{g/ml}$, moderate when the LC_{50} was between 100 and 500 $\mu\text{g/ml}$, as strong when the LC_{50} ranged from 0 to 100 $\mu\text{g/ml}$ [19] and designated as non toxic when the LC_{50} value was greater than 1000 $\mu\text{g/ml}$ [20]. Cyclophosphamide and etoposide had LC_{50} values of 95 $\mu\text{g/ml}$ and 6 $\mu\text{g/ml}$ respectively, validating their use as potent anticancerous drugs.

CONCLUSION

Results from this study indicate that while plant species with LC_{50} values < 1000 $\mu\text{g/ml}$ may not make good antimalarial remedies due to their inherent toxicity, this study calls for further work aimed at isolating the cytotoxic compounds responsible for the observed activity. This compounds could serve as novel scaffolds in search for new drugs against cancer. Further investigations into the the *in vivo* antimalarial and toxicological profile of these crude extracts is recommended.

CONFLICT OF INTERESTS

The author(s) declare that they have no competing interests. We all seek for a new safe molecule effective against *Plasmodium falciparum* malaria and we are not part of drug industry companies.

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