Cytotoxicity of antimalarial plant extracts from Kenyan biodiversity to the brine shrimp, *Artemia salina* L. (Artemiidae)

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Abstract

Artemia salina (Artemiidae), the brine shrimp larva, is an invertebrate used in the alternative test to determine toxicity of chemicals and natural products. In this study the medium lethal concentration fifty (LC_{50} values) of 45 antimalarial plant extracts and positive controls, cyclophosphamide and etoposide were determined using *Artemia salina* (Artemiidae). 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 LC_{50} values between 100 µg/mL and 500 µg/mL, and were categorized as having moderate cytotoxicity, 2 (4.5%) of the crude extracts had LC_{50} 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 LC_{50} values greater than 1000 µg/mL and were considered to be non toxic. Approximately 20% (9) of the aqueous extracts demonstrated activity at or below 100 g/mL and were considered to have strong cytotoxic activity, 40% (18) of the screened aqueous crude extracts had LC_{50} values between 100 µg/mL and 500 µg/mL and were considered to be moderately cytotoxic, 16% (7) of the crude extracts had LC_{50} values between 500 µg/mL and 1000 µg/mL and were considered to have weak cytotoxic activity while 24% (11) of the aqueous extracts had LC_{50} values greater than 1000µg/mL and were categorized as non toxic The positive controls, cyclophosphamide and etoposide exhibited strong cytotoxicity with LC_{50} values of 95 µg/mL and 6 µg/mL respectively in a 24 hour lethality study, validating their use as anticancer agents. In the current study, 95.5% of all the screened organic extracts and 76% of the investigated aqueous extracts demonstrated LC_{50} values <1000 g/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.

Introduction

Since ancient times people have used plants asmedicines.[1] This use has great importance, becauseplants 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 andfor this reason,[3] 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 and 80% of the people worldwide are estimated to use herbal remedies.[4-7] 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] The preclinical toxicological evaluation, carried out routinely in mice, is of great importance for validation of the traditional use of medicinal plants. Mouse bioassay could provide more pharmacokinetic and pharmacodynamic data from the different types of samples such as serum and biopsy which are important to elaborate the mechanism of action of various plant extracts. There is currently a tendency to call for substitutingthe use of laboratory animals in toxicological tests, dueto the high cost and the animals' suffering caused bythese experiments. Alternative methods include procedures that could replace experiments carried out with animals; reduce the number of animals used in every testor refine the existing methodology in order to reducepain and stress, according to the *3 R-principle*.[10,11] *Artemiasalina* L. (Artemiidae), the brine shrimp, isan invertebrate component of the fauna of saline aquaticand marine ecosystems. It plays an important role inthe energy flow of the food chain and it can be used in a laboratory bioassay inorder to determine toxicity through the estimation ofthe medium lethal concentration (LC_{50} values) which have been reported for a series oftoxins and plant extracts.[12-14] In this paper, results of a screening of organic ($CHCl_3$/ MeOH) and water extracts of some important antimalarial plants used by the Msambweni community of Kenyan coast for lethality towards *Artemia salina* larvae are presented. The current study also seeks to compare the cytotoxicities of the aqueous and organic plant extracts to the *Artemia salina* larvae to the

lethality of the cytotoxic drugs, cyclophosphamide and etoposide to the brine shrimp 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 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 (Etosid™), 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 n.1. The filtrate was concentrated to dryness *in vacuo* by rotary evaporation and weighed. The dry solid extracts were stored at -20°C in airtight containers until used.

Product identification and description (*Artemia salina* L.)

Artemia cysts, batch number DE RP 33801, were purchased from JBL GmbH & Co.KG (Neuhofen, Germany) and the product was labeled as JBL ArtemioPur Brand. The *Artemia* cysts had been harvested from Great Salt Lake, Utah, USA and were identified *as Artemia salina,* based on zoogeography.[15] *A. salina* is the best studied of the *Artemia* species,[16] 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).[17]

Culture and harvesting of *Artemia salina L.*

Artemia salina cysts were stored at -20°C before use. *A. salina* cysts were incubated for hatching in a shallow rectangular dish (14 cm × 9 cm × 5 cm) filled with 225 mL 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 (0.0827 grams) 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.[18] 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, $CH_3.SO.CH_3$ M.W 78.13 (DMSO), batch number PJ/25/3496/709-05/6/16, (Thomas Baker Chemicals, PVT. Limited, Mumbai, India) followed by subsequent dilution to lower concentration of DMSO, to <1% to avoid carry over (solvent) effect.[19] 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 *Artemiasalina L.*

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 formulaas 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.[20]

Lethal concentration fifty determinations

The LC_{50}, 95% confidence interval and slope were determined from the 24 hour counts using the probit analysis method described by Finney.[21] LC_{50} is indicative of toxicity level of a given plant extract to the brine shrimp larva.

Results

Forty five crude extracts belonging to thirty one species in 26 genera and 22 families were evaluated in the current study (see Supplementary Files).

The yields of the water extracts ranged between 1.54 and 17.02 % W/w, while those of organic extracts were between 1.72 and 13.98% W/w (see Supplementary Files). Mortality (percentage) for every organic plant extract and cytotoxic drugs, after testing the differentextracts and cytotoxic agents with brine shrimp, is shown in Supplementary Files. Increase in mortality was observed to be proportional to increase in concentration, which provided linearity in the dose-effect relationshipof every extract and determination of the LC_{50} value.

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 LC_{50} values between 100 µg/mL and 500 µg/mL, and were categorized as having moderate cytotoxicity, 2 (4.5%) of the crude extracts had LC_{50} 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 LC_{50}

values greater than 1000 μg/mL and were considered to be non toxic (Figure 1).

The results from screening 45 aqueous extracts of 31 differentplant species against *A. salina* larvae are shown in Supplementary Files.

Approximately 20% (9) ofthe aqueous extracts demonstrated activity at or below 100 g/mL and were considered to have strong cytotoxic activity, 40% (18) of the screened crude extracts had LC_{50} values between 100 μg/mL and 500 μg/mL and were considered to be moderately cytotoxic, 16% (7) of the crude extracts had LC_{50} values between 500 μg/mL and 1000 μg/mL and were considered to have weak cytotoxic activity while 24% (11) of the aqueous extracts had LC_{50} values greater than 1000 μg/mL and were categorized as non toxic (Figure 2). The LC_{50} values of crude plant extracts and those of the positive controls, cyclophosphamide and etoposide, are compared in Supplementary Files.

Discussion

The evaluation of the toxic action of plant extracts isindispensable in order to consider a treatment safe; itenables the definition of the intrinsic toxicity of theplant and the effects of acute overdose.[22] The current study aimed at screening the lethality of crude plant extracts commonly used as antmalarial phytomedicines in Msambweni district, Kenya against brine shrimp, *Artemia salina* larvae. The procedure of Meyer *et al.*[23] was adopted to determine the lethalityof 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. The LC_{50} values of the brine shrimp obtainedfor extracts of the screened medicinal plantsand that of the cytotoxic drugs, cyclophosphamide and etoposide have been presented in Supplementary Files. Thedegree of lethality was found to be directlyproportional to the concentration of the extract. In the evaluation for general toxicityusing brine shrimp, maximum mortalitiestook place at a concentration of 1000 g/mLwhereas least mortalities were at 10 g/mL concentration. The cytotoxic activity was consideredweak when the LC_{50} was between 500 and 1000 μg/mL, moderate when the LC_{50} was between 100 and 500 μg/mL, as strong when the LC_{50} ranged from 0 to 100 μg/mL and designated as non toxic when the LC_{50} value was greater than 1000 μg/mL.[23,24] In toxicity evaluation of plant extracts by brineshrimp bioassay, an LC_{50} value lower than 1000 g/mLis considered bioactive.[23] In the current study, 95.5% of all the screened organic extracts and 76% of the investigated aqueous extracts demonstrated LC_{50} values <1000 g/mL, indicating the presence of cytotoxic compounds responsible for the observed toxic activity. This calls for dose adjustment amongst the community using the plant extracts for the treatment of malaria.The most promising aqueous extracts ($LC_{50} = <100$ g/mL) were the leaves and stem bark of *Adansonia digitata* L. (Malvaceae); leaves of *Bridelia micrantha* (Hochst.) Baill. (Phyllanthaceae); leaves and stem bark of *Flacourtia indica* (Burm.f) Merr. (Salicaceae); leaves of *Aloe macrosiphon* Baker (Xanthorrhoeaceae); leaves of *Aloe secundiflo-ra* Engl.(Xanthorrhoeaceae); roots of *Dichrostachys cinerea* (L.) Wight &Arn. (Fabaceae); leaves of *Grewia hainesiana* Hole (Malvaceae); roots of *Canthium glaucum* Hiern (Rubiaceae) and the leaves of *Cassia occidentalis* hort. Ex Steud. (Fabaceae). Pharmacological properties of these plants have been demonstratedin preclinical studies, including those of *Adansonia digitata* L. as an antiplasmodial agent;[25] *Bridelia micrantha* (Hochst.) Baill. as an antiplasmodial agent;[26] *Flacourtia indica* (Burm.f) Merr. as an antiplasmodial agent;[26] *Aloe secundiflora* Engl. as an antimalarial agent and *Grewia hainesiana* Hole, where triterpenoids have been isolated.[27,28] These plant species have demonstrated bioactivity low enough ($LC_{50} = <100$ g/mL) to merit chemical investigation for isolation of bioactive compounds responsible for the observed toxicity. Approximately 96% of all the organic extracts were active (LC_{50} values < 1000 g/mL), while 51% of the organic crude extracts exhibited strong cytotoxic activity ($LC_{50} = <100$ g/mL). These results indicate that majority of the bioactive constituents in the screened plant species are non polar, and merit further phytochemical analysis for the isolation of the cytotoxic compounds. The current observation is in agreement with the findings of Cantrell *et al.,*[29] who found organic extracts to be more active than aqueous extracts in a brine shrimp bioassay.The organic extract of the root bark of *Harrisonia abyssinica* Oliv. was highly active ($LC_{50}= 6$ g/mL), with toxicitycomparable to that of the anticancer agent, etoposide. Literature suggests that the species had been previously investigated for antimalarial activity.[30]

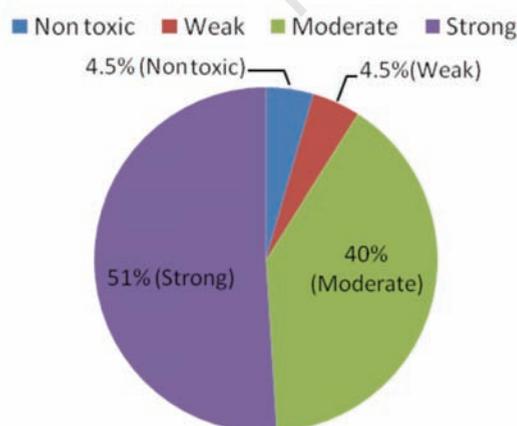
Figure 1. Lethality of organic (CHCL3/MeOH) crude extracts against *Artemia salina* (n=45).
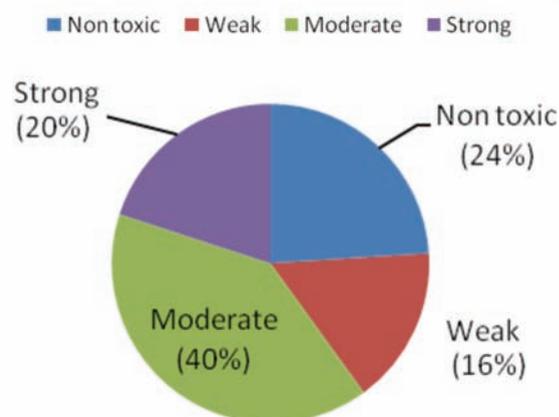

Figure 2. Cytotoxicity of aqueous crude extracts against Artemia salina (n=45).

Conclusions

The current study evaluated the cytotoxicity of crude plant extracts and cytotoxic drugs against *A.salina*. The standard *A.salina* bioassay is a useful screen for the toxicity based detection of plant extracts and could replace the more ethically challenged mouse bioassay for this purpose. Results from this study indicate that while plant species with LC_{50} values <1000 *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.Advanced technology might be used to increase the purity of crude plant extracts, and thus minimise the impurity influence. Besides parasitology, brine shrimp lethality test needs more experiments to validate its application in screening new drugs in other areas. Further investigations into the the *invivo* toxicological profile of these crude extracts is recommended.

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