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

Joseph Mwanzia Nguta,1 James Mucunu Mbara,1 Daniel Waweru Gakuya,2 Peter Karuri Gathumbi,3 John David Kabasa,4 Stephen Gitahi Kiama5
1Department of Public Health, Pharmacology and Toxicology, University of Nairobi; 2Department of Clinical Studies, University of Nairobi; 3Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi, Nairobi, Kenya; 4Department of Physiology and Pharmacology, Makerere University, Kampala, Uganda; 5Department of Veterinary Anatomy and Physiology, University of Nairobi, Nairobi, Kenya

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 (LC50 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 cytotoxicity, 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 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 LC50 values between 100 µg/mL and 500 µg/mL and were considered to be moderately cytotoxic, 16% (7) of the crude extracts had LC50 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 LC50 values greater than 1000µg/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. In the current study, 95.5% of all the screened organic extracts and 76% of the investigated aqueous extracts demonstrated LC50 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 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 and/or 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. Indigenous plants play an important role in the treatment of many diseases and 80% of the people worldwide are estimated to use herbal remedies.1-7 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, pharmaceuticals 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 substituting the use of laboratory animals in toxicological tests, due to the high cost and the animals’ suffering caused by these experiments. Alternative methods include procedures that could replace experiments carried out with animals; reduce the number of animals used in every test or refine the existing methodology in order to reduce pain and stress, according to the 3 R-principle.10,11 Artemisia annua L. (Artemiidae), the brine shrimp, is an invertebrate component of the fauna of saline aquatic and marine ecosystems. It plays an important role in the energy flow of the food chain and it can be used in a laboratory bioassay in order to determine toxicity through the estimation of the medium lethal concentration (LC50 values) which have been reported for a series of toxins and plant extracts.12-14 In this paper, results of a screening of organic (CHCl3/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

Correspondence: Joseph Mwanzia Nguta, Department of Public Health, Pharmacology and Toxicology, University of Nairobi, Box 29053-00625, Nairobi, Kenya.
Tel. +254 735.750278 - Fax: +254 020.631325
E-mail: joseph.nguta@uonbi.ac.ke

Key words: Cytotoxicity, Artemia salina bioassay, crude extracts, antimalarial plants, Kenyan biodiversity.

Acknowledgements: the authors would like to thank BecA-ILRI hub, Kenya, for technical support in lyophilization of plant extracts.

Funding: the authors acknowledge financial support from the Carnegie Corporation of New York through Regional Initiative in Science and Education African Natural Product Training Network (RISE-AFNNET).

Contributions: JMN participated in study design and in study execution, drafting of the manuscript and in proof reading. All the other authors contributed equally in the study design, manuscript preparation and proof reading.

Conflict of interests: the authors report no potential conflict of interests.

Received for publication: 2 August 2011. Revision received: 24 April 2012. Accepted for publication: 3 May 2012.

This work is licensed under a Creative Commons Attribution NonCommercial 3.0 License (CC BY-NC 3.0).

lethality of the cytotoxic drugs, cyclophosphamide and etoposide to the brine shrimp larvæ.

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 ethnopharmaceutical 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 PJ 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 (30 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 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. A. salina is the best studied of the Artemia species, 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. 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 sulfoxide, CH3.SO.CH3 M.W 78.13 (DMSO), batch number PJ/25/4969/709-05/6/16, (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 nm 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 follows: % deaths = [(Test-control)/control] x 100. The surviving nauplii were killed by the addition of 100 µL of 5% (w/v) phenol to each vial.20

Lethal concentration fifty determinations
The LC50, 95% confidence interval and slope were determined from the 24 hour counts using the probit analysis method described by Finney.21 LC50 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 different extracts 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 relationship of every extract and determination of the LC50 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 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

[Drugs and Therapy Studies 2012; 2:e12]
values greater than 1000 µg/mL and were considered to be non toxic (Figure 1).

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

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 crude extracts had LC50 values between 100 µg/mL and 500 µg/mL and were considered to be moderately cytotoxic, 16% (7) of the crude extracts had LC50 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 LC50 values greater than 1000 µg/mL and were considered as non toxic (Figure 2). The LC50 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 is indispensible in order to consider a treatment safe; it enables the definition of the intrinsic toxicity of the plant and the effects of acute overdose. The current study aimed at screening the lethality of crude plant extracts commonly used as antimalarial phytotherapies in Msambweni district, Kenya against brine shrimp, Artemia salina larvae. The procedure of Meyer et al 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. The LC50 values of the brine shrimp obtained for extracts of the screened medicinal plants and that of the cytotoxic drugs, cyclophosphamide and etoposide have been presented in Supplementary Files. The degree of lethality was found to be directly proportional to the concentration of the extract. In the evaluation for general toxicity, brine shrimp, maximum mortality was placed at a concentration of 1000 µg/mL whereas least mortalities were at 10 µg/mL concentration. The cytotoxic activity was considered weak when the LC50 was between 500 and 1000 µg/mL, moderate when the LC50 was between 100 and 500 µg/mL, strong when the LC50 ranged from 0 to 100 µg/mL and designated as non toxic when the LC50 value was greater than 1000 µg/mL.23 In toxicity evaluation of plant extracts by brineshrimp bioassay, an LC50 value lower than 1000 g/mL is considered bioactive. In the current study, 95.5% of all the screened organic extracts exhibited strong cytotoxic activity (LC50 < 1000 µg/mL), while 51% of the organic crude extracts exhibited strong cytotoxic activity (LC50 < 1000 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 isolation of the cytotoxic compounds. The current observation is in agreement with the findings of Cantrell et al., 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 (LC50 = 6 g/mL), with toxicity comparable to that of the antitumor agent, etoposide. Literature suggests that the species had been previously investigated for antimalarial activity.

**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 LC50 values <1000 μg/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 this. Further investigations into the in vivo toxicological profile of these crude extracts is recommended.

References


