Journal of Ethnopharmacology 126 (2009) 287–293

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Comparative acute toxicities and immunomodulatory potentials of five Eastern Nigeria mistletoes

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ARTICLE INFO

Article history:
Received 22 June 2009
Received in revised form 6 August 2009
Accepted 15 August 2009
Available online 22 August 2009

Keywords:
Comparative
Immunomodulatory
Toxicities
Loranthus micranthus
Mistletoe
Host trees

ABSTRACT

Ethnopharmacological relevance: Traditionally, mistletoes of Eastern Nigeria origin, *Loranthus micranthus* Linn. have been used as immunostimulant for the management of certain diseases with high profile immune depleting potentials. This practice has remained till date without scientific validation.
Aim of study: To obtain and validate evidence for or against its continued use as immunostimulant and afford data for further studies on this specie of mistletoe. The present work is an in vivo proof of ethnopharmacological concept of the age long immunomodulatory use of our local mistletoe.
Materials and methods: Aqueous-methanol extracts of the plant leaves from five different host trees were evaluated for immunomodulatory activity using four *in vivo* models in mice or rats, namely; total and differential leukocyte count (TLC and DLC), the cellular mediated delayed-type hypersensitivity reaction (DTHR) test, the humoral mediated antibody titration (AT) test and the cyclophosphamide-induced myelosuppression (CIM) test at different dose levels (100, 200 and 400 or 50, 100 and 250 mg kg^{-1}; depending on model) against standard controls. Phytochemical and acute toxicity tests were equally carried out on all the extracts.
Results: Results obtained indicate that all the mistletoes contained the same phytochemical constituents, although in varying amounts. The mistletoes exhibited statistically significantly different ($p < 0.05$ or $p < 0.001$, ANOVA) immunomodulatory (up-regulatory) activities in the overall order of that from *Kola acuminata* > *Citrus spp* > *Persia americana* > *Parkia biglobosa* > *Pentaclatra macrophylla*. LD_{50} values were generally greater than 5000 mg/kg.
Conclusion: The present study confirms the Eastern Nigeria mistletoe as a potent and safe alternative or complementary medicine for the management of immunodeficiency diseases.
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1. Introduction

Natural products either as pure compounds, fractions or whole plant extracts have remained useful and unmatchable sources of molecules for effective treatment and mitigation of disease burdens of man and animal (Perry, 1980). The ethno-based evidences for use of these medicinal plants must be validated through *in vitro*/*in vivo* proof of concept (Paul et al., 2006). In recent times, we have witnessed a tremendous surge in the use of medicinal plant products as well as research interest in them to generate potent bioactive molecules necessary for health enhancement (Donald, 2000). In Nigeria, natural biodiversity hold great potential for the development of novel potent drugs against viral and other microbial infections. The Eastern Nigeria mistletoe, *Loranthus micranthus* Linn. has been employed traditionally in management of various ailments, notably diabetes, high blood pressure and conditions affecting human immune system for the past many years. Aqueous decoctions or alcoholic extracts of *Loranthus micranthus* have diverse folkloric claims of effectiveness for treatment of epilepsy, diabetes, hypertension, cardiovascular diseases, menopausal syndrome, infertility, rheumatism, agglutination and in conditions generally requiring modulation of the immune system. It is equally used locally as an antimicrobial and antispasmodic agent. Contrary to the well-studied European specie of mistletoe, *Viscum album* (Bussing, 2000), some of its age-long ethno medicinal uses have not been strongly validated. *Viscum album* was considered a "heal-all" by the Druids in ancient Britain and the European continent because of its multiple medicinal properties. It was reportedly used by the Druids and the ancient Greeks in the treatment of epilepsy. Mistletoe (*Viscum album*) is reputed to possess hypotensive, cardiac depressant and sedative properties, used particularly in the aged for the treatment of symptoms of cardiovascular diseases including headache, dizziness, fatigue, and irritability (Calvin, 2006). Fortunately, some research groups in Nigeria including ours have carried out appreciable research works on this unique parasitic plant. In Nigeria, mistletoe exists in the different geopolitical

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doi:10.1016/j.jep.2009.08.024

zones as a semi-parasitic plant on different host trees and has been shown to possess some of the activities. These activities have been reported to be host tree and season dependent (Osadebe et al., 2004; Osadebe and Ukwueze, 2004). Recently too, the anti-diabetic, antimicrobial, antimotility, antioxidant and antihypertensive activities of *Loranthus micranthus* have been reported (Obatomi et al., 1997; Osadebe et al., 2004; Osadebe and Akabogu, 2005; Ofem et al., 2007). One ethnomedicinal application of our local mistletoe yet to be scientifically validated is its ability to improve on overall patients' immune system in both normal and diseased states with concomitant quicker convalescence from sickness. It is therefore the focus of the present work to evaluate the immunomodulatory potentials of our local mistletoe harvested from five different host trees. Interestingly, the stimulation of defense mechanistic pathways has been recognised as a possible means of inhibiting disease progression in humans without per se eliciting harmful effects (Kim et al., 1996; Ameho et al., 1997). Immunostimulation is a desired response if the overall process culminates in cure or quicker convalescence in diseased conditions. In light of this development, very many plant constituents with immunostimulatory activity have been isolated (Perry, 1980; Anderson, 1982; Juanita, 2005). These immune stimulating substances target specific components of the organism's immune system which are group of organs, cells, and a specialized system called the lymphatic system, designed to protect the host from invading pathogens and to eliminate diseases. The present study will provide a rationale for its continued use in phytomedicine as an alternative and/or complementary drug in the management of immune deficiency diseases.

2. Materials and methods

2.1. Collection of plant materials

Loranthus micranthus Linn. (Loranthaceae) leaves parasitic on the selected host trees were collected in April 2007 from different locations in Nsukka LGA, Enugu state. The leaves were identified and certified by Mr. A.O. Ozioko, a taxonomist of the Bioresources Development and Conservation Programme, Nsukka, Enugu state. Voucher specimens were kept at the centre with the number BDC-532-07 for reference purposes.

2.2. Chemicals/reagents used

Methanol (BDH Ltd., Poole, England), distilled water, normal saline (DANA Ltd.) and tween 20 solution were used. Phytochemical reagents were freshly prepared.

2.3. Animals used

Batches of albino mice (20–33 g) or rats (120–165 g) of both sexes were procured from the animal house, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, and from the animal house, Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The blood specimens used in the study were collected from sheep in the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The animals were kept in standard laboratory conditions and fed with pelletized rodent commercial diet (vital feed Nig., Ltd.) and water *ad libitum* throughout the study. They were exposed to 12 h light-dark cycle. This investigation was conducted following an approval by the relevant ethical Committee on laboratory animal use and international rules were observed.

2.4. Preparation of crude aqueous-methanol extract

The leaves of *Loranthus micranthus* parasitic on *Kola acuminata* were cleansed and dried under shade for 8 days. They were pulverized in mechanized laboratory grinder to fine powder. A total of 200 g of the powder was extracted in batches with 95% aqueous methanol; first wetted with 200 ml of methanol and the actual extraction carried out with another 300 ml of aqueous methanol using soxhlet extractor for 24 h. The resulting aqueous-methanol extract was evaporated to dryness under vacuum at $40 \pm 5\,^{\circ}C$ to afford dry extract which was weighed and its percentage yield was calculated. The dry extract was placed in a clean plastic container and stored in a refrigerator until use. This process was repeated for all other host trees.

2.5. Acute toxicity tests (LD_{50}) of extracts

Acute toxicity tests were performed using the Lorke's method with the aqueous-methanol extracts (Lorke, 1983). Essentially, this method involves an initial dose range determination stage in which nine mice were used (three animals per treatment group). The aqueous-methanol extract was dissolved in 3% tween 20 solution and doses of 10, 100 and 1000 mg/kg were administered intraperitoneally to the respective groups of mice. The animals were then observed for 24 h. No deaths occurred in any of the animal after 24 h and the second stage was carried out. Doses of 5000 and 10,000 mg/kg were administered to two groups of one animal per group. The animals were again observed for 24 h. LD_{50} was then calculated as geometric mean of the highest dose that did not kill any animal and the largest dose that killed all the animals. This was done with all the extracts.

2.6. Phytochemical test

This was carried out as described by Harbourne (1984). All reagents for the phytochemical test were freshly prepared following standard procedures.

2.7. Antigen

Fresh blood was obtained from a male sheep in the animal farm of the Faculty of Veterinary Medicine, University of Nigeria. The sheep red blood cells (SRBCs) were washed three times in a large volume of pyrogen-free sterile normal saline by centrifugation at $3000 \times g$ for 10 min on each occasion. The washed SRBCs were adjusted to a concentration of 10^9 cells/ml with normal saline and used for immunization and challenge (Dan et al., 1990).

2.8. Determination by total and differential leukocyte count (TLC and DLC)

The methods of Baker et al. (1985) and Jain (1986) were adopted. In summary, the mice were grouped into five (four mice each). The first, second and third groups were treated with the extracts while the fourth and fifth groups served as the controls. Starting with the methanol extract, the different groups received the extract at doses of 100, 200, and 400 mg/kg intraperitoneally (i.p.) respectively on the first, third, sixth and ninth days. On the twelfth day, blood was collected from the tail vein of mice in each group with the aid of haematocrit tubes into EDTA heparinized tubes. Thin blood films were prepared on clean glass slides for each dose level for TLC and then counting with the aid of an improved Neaubuer electric microscope at $20\times$ magnification. The DLC was determined by preparing thin slide blood film of each dose level and then staining with Leishman's stain. The films were drained and dried at room temperature and were then microscopically examined in an oil immersion at

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