Food Research International 44 (2011) 304–309

Contents lists available at ScienceDirect

Food Research International

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

Toxigenic microorganisms in medicinal plants used for ritual protection of infants

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ARTICLE INFO

Article history:
Received 15 July 2010
Accepted 8 October 2010

Keywords:
Medicinal plants
Bacillus spp.
Moulds
Mycotoxins

ABSTRACT

Plants used as a part of infant protective rituals in some countries of South Eastern Africa and which use has been associated to food poisoning episodes were submitted to a microbiological analysis to investigate potential microbiological hazards. This characterization led to the detection of a high load of moulds and aerobic spore-formers microorganisms. Isolates of *Bacillus cereus* and *Bacillus thuringiensis* were observed to contained different toxin-encoding genes, and the production of diarrheal enterotoxin was confirmed in some of them. The production of aflatoxin B_1 and cyclopiazonic acid by strains of *Aspergillus flavus*, and citrinin and penicillic acid by *Penicillium citrinum* was revealed by HPLC. The toxicity of these isolates was also showed by the *Artemia salina* lethality test. The results indicate the presence of microorganisms with toxigenic potential in plants used as folk medicine in South Eastern Africa. The traditional use of these preparations should be carefully reconsidered due to the microbiological risks associated with their ingestion.

1. Introduction

Mankind has made use of a wide spectrum of plants as medicines to alleviate or prevent a high number of diseases due to their antimicrobial, anti-inflammatory, antioxidant, psychotropic and/or neurotropic properties (Fennell et al., 2004). Herbal medicines still play an important role within the culture and traditions of many developing countries since they usually offer a more widely available and more affordable alternative to pharmaceutical drugs; therefore, it is not strange that a major part of the sub-Saharan African population is reliant on such traditional practices for their health care needs. As an example, herbal medicine is the first choice for home treatment of nearly two thirds of children with high fever in Ghana, Mali, Nigeria or Zambia (Aschwanden, 2001).

Although many people (both in developing and developed countries) perceive that herbal medicines are safer or "more natural" than pharmaceuticals, different studies have shown that many plants used in traditional medicine are potentially toxic, mutagenic and carcinogenic (Deciga-Campos et al., 2007; Efuntoye, 1999; Elgorashi et al., 2003; Halt, 1998; Reif & Metzger, 1995). As a consequence, they can lead to serious illnesses, including allergy, poisoning, cancer, blindness, liver or kidney malfunction, and even death (Aschwanden, 2001). Mortality and morbidity rates associated to acute poisoning due to traditional medicines are high in many African countries (Thomson, 2000) and, actually, the actual figures may be notably higher because many cases remain unrecorded (Popat et al., 2001). Even in the reported cases, the plant or plants implicated are seldom identified or remain undisclosed because the use of herbal medicines is sometimes secret or frowned upon by Western medicine. Most of the traditional medicine-related poisoning cases are paediatric (Popat et al., 2001; Stewart & Steenkamp, 2000) and, therefore, affect a particularly sensitive population, often suffering from malnutrition, infectious and/or parasitic diseases. In addition to the problems cited above, plants may constitute an important source of toxigenic microorganisms. Herbs and other plants are often harvested, manipulated and stored in poor sanitary conditions in developing countries. As a result they became heavily contaminated with potentially toxigenic moulds and bacteria (particularly heat-resistant spores belonging to the genus *Bacillus*) (Baxter & Holzapfel, 1982; Malmsten, Pääkkönen, & Hyvönen, 1991).

In some areas of South Eastern Africa (and as a part of a "protective" ritual), infants receive a herbal preparation when they are just a few months old but, paradoxically, such practice frequently leads to food poisoning episodes and growth retard. In this context, the objective of this study was to characterize the microbiological risks associated to plants used for such purposes.

2. Materials and methods

2.1. Plant material and isolation of the microorganisms

In South Eastern Africa many breastfed infants undergo a traditional preventive treatment to protect them following the suggestion of a healer. The treatment consists on the administration of herbal preparations made from complex mixes of plants (of up to 50 different species) including, at least, the following six species:

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doi:10.1016/j.foodres.2010.10.015

Cardiogyne africana, *Artabotrys brachypetalus*, *Catharanthus roseus*, *Senna petersiana*, *Celosia* sp. and *Ficus platyphylla*. The infusions can be made either from dry leaves or from roots. Both kinds of infusions are usually prepared with hot (but not boiling) water and kept uncovered at room temperature.

Two random samples of two different plant materials (A: dry leaves; B: roots) were analyzed. The samples were suspended in sterile 0.1% peptone water (Oxoid, Basingstoke, UK) incubated at 45 °C for 30 min and homogenized in a stomacher (Seward, Worthing, UK) for 3 min. Then, aliquots were serially diluted and plated by triplicated onto agar plates of the following media: *Bacillus cereus* selective agar base supplemented with polymyxin B (100,000 IU/l) (BCSA, Oxoid), reinforced clostridial medium (RCM, Oxoid), violet red bile glucose (VRBG) (Difco, Detroit, USA) and Sabouraud dextrose chloramphenicol (SDC, bioMérieux, Marcy l'Etoile, France). VRBG and BCSA plates were incubated at 37 °C for 24–48 h in aerobic conditions while those of RCM were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) in a MACS-MG-1000-anaerobic workstation (DW Scientific, Shipley, UK). Finally, SDC plates were incubated aerobically at 25C° for 3 days. Once microbial counts were obtained, between 5 and 10 different colonies (including, at least, one representative of each morphology) were selected from those media where growth was observed for further assays. The selected isolates were routinely grown in brain heart infusion (BHI) medium (Oxoid).

2.2. Identification of the bacterial and fungal isolates by PCR sequencing and RAPD genotyping

Bacterial isolates were submitted to microscope observation (including Gram-staining), catalase and spore-formation tests and, parallel, they were identified at the species level by 16S rRNA gene sequencing. For this purpose, bacterial genomic DNA was extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany), and used as a template for PCR amplification of a 16S rRNA gene fragment (470 bp) using primers pbl16 (5′–AGAGTTTGATCCTGGCTCAG-3′) and mlb16 (5′-GGCTGCTGGCACGTAGTTAG-3′) (Kullen, Sanozky-Dawes, Crowell, & Klaenhammer, 2000). The PCR conditions were as follows: 96 °C for 4 min (1 cycle); 96 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s (30 cycles); and a final extension at 72 °C for 4 min.

Fungal isolates were grown on BHI broth for 72 h at 25 °C. The cells were collected by centrifugation (14,000 rpm, 5 min), washed with a 0.9% saline solution and resuspended in 400 µl of a 20 mM Tris–HCl pH 8, 1.2% Triton X100, and 2 mM EDTA solution. After the addition of 3 M sodium acetate pH 5.5 (100 µl), the solutions were transferred to 2 ml tubes containing glass beads (106–450 µm; 0.1 g) and a single zirconium bead (0.1 mm) (Biospec, Bartlesville, USA). The tubes were agitated vigorously for 3 min and submitted to 60,000 rpm for 45 s (3 successive cycles separated by 45 s periods on ice) using a FastPrep instrument (QBioGene, Irvine, USA). Then, mould DNA was extracted from the suspensions (360 µl) using the DNeasy Tissue kit (Qiagen) and served as template (50 ng/µl) for the identification of the isolates by PCR sequencing of a 550 bp fragment of the eukaryotic rRNA operon encompassing the 5.8S rRNA gene and the flanking internal transcribed spacers ITS1 and ITS2, using the primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAGTGCTAA-CAAGG-3′) and the conditions described by White, Burns, Lee, and Taylor (1990).

Amplicons were purified using the Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany) and sequenced using the primers cited above. Sequencing reactions were prepared using the PRISM ABI BigDye Ready Reaction Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase according to the manufacturer's instructions (Applied Biosystems, Foster City, USA) and were run on an ABI 377A automated sequencer. The resulting sequences were used to search sequences deposited in the EMBL database using BLAST algorithm. The identity of the strains was determined on the basis of the highest scores. The partial 16S RNA gene sequences of the different bacterial species were deposited in the EMBL nucleotide sequence database under accession numbers AM745107–AM745111, whereas sequences corresponding to ITS1, 5.8S rRNA gene and ITS2 of the different fungal species were deposited under accession numbers AM745112–AM745115.

The isolates were submitted to randomly amplified polymorphic DNA (RAPD)-PCR analysis using the Ready to go® RAPD kit (GE Healthcare, Little Chalfont, UK) and one representative of each RAPD genotype was selected for further assays.

2.3. Detection of toxin-encoding genes and production of diarrheal enterotoxin among the Bacillus isolates

Detection of *hblA*, *nheA* and *bceT* genes (encoding hemolysin, nonhemolytic enterotoxin and diarrheal enterotoxin, respectively) was performed by PCR using the primers described by Hansen and Hendriksen (2001). To amplify the *hblA* gene, the following conditions were used: 94 °C for 5 min (1 cycle); 94 °C for 15 s, 65 °C for 45 s and 72 °C for 2 min (30 cycles); and a final extension at 72 °C for 2 min. On the other hand, the *nheA* and *bceT* genes were detected in a multiplex PCR format with the conditions cited above with the exception of the annealing temperature (62 °C). Diarrheal enterotoxin was detected by a commercial immunoassay kit (BCET-RPLA; Oxoid) used according to the manufacturer's instructions. Briefly, BHI culture supernatants of the strains were collected by centrifugation, serially diluted and assayed for toxin content in microtitre plates containing latex particles covered with antibodies (rabbit IgG) specific against the *B. cereus* diarrheal enterotoxin. If enterotoxin is present, agglutination occurs.

2.4. Preparation of fungal extracts

Preparation of fungal extracts was performed basically as described by Sosa et al. (2002). Each fungal strain was grown to confluence on the surface of five yeast extract medium plates (5 g/l yeast extract, 20 g/l glucose and 15 g/l bacteriological agar). Then, the content of the five plates was transferred to a plastic bag and macerated with 200 ml of chloroform (Chromasolv® plus for HPLC, Sigma-Aldrich, St. Louis, USA) in the stomacher for 5 min. After 1 h at room temperature, the slurry was filtered through anhydrous sodium sulphate with Whatman no. 1 filter paper. Then, the filtrate was evaporated in a rotatory evaporator at 45 °C. The residue was resuspended in 5 ml of chloroform and evaporated to dryness under a gentle stream of nitrogen. The final dried extracts were stored at −20 °C in the dark and resuspended in 500 µl of chloroform when required for the lethality test or in 500 µl of acetonitrile just before liquid chromatography analysis.

2.5. Artemia salina lethality test

The fungal extracts were evaluated for lethality to brine shrimp (*Art. salina*) larvae according to the procedure described by Harwig and Scott (1971). This assay is considered a useful tool for preliminary assessment of toxicity and has been used for monitoring the potential effect of mycotoxins (Sam, 1993). Briefly, dried *Art. salina* eggs (Super HI Group, Carpeneto, Italy) were bred in brine shrimp medium (BSM; Harwig & Scott, 1971) at 30 °C for 30 h in a rotary shaker (120 rpm). One-day-old larvae (~30 per vial) were transferred into vials containing 5 ml of BSM medium and exposed to the extracts (50 µl). Then, larvae were incubated as described above and, after exposures of 24 and 48 h, the numbers of survivors were counted and percentage of death determined as absence of motility. Each extract was assayed in duplicated and chloroform (50 µl) was used as a negative control.