Buruli Ulcer: A Review of *In Vitro* Tests to Screen Natural Products for Activity against *Mycobacterium ulcerans*

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Key words
- natural products
- antimycobacterial
- screening
- Buruli ulcer

Abstract

Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, has recently been recognized by the World Health Organization (WHO) as an important emerging disease. It is largely a problem of the poor in remote rural areas and has emerged as an important cause of human suffering. While antimycobacterial therapy is often effective for the earliest nodular or ulcerative lesions, for advanced ulcerated lesions, surgery is sometimes necessary. Antimycobacterial drugs may also prevent relapses or disseminated infections. Efficient alternatives different from surgery are presently explored because this treatment deals with huge restrictive factors such as the necessity of prolonged hospitalization, its high cost, and the scars after surgery. Traditional treatment remains the first option for poor populations of remote areas who may have problems of accessibility to synthetic products because of their high cost. The search for efficient natural products active on *M. ulcerans* should then be encouraged because they are part of the natural heritage of these populations; they are affordable financially and can be used at the earliest stage. This review provides a number of tests that will help to evaluate the antimycobacterial activity of natural products against *M. ulcerans,* which are adapted to its slow growing rate, and lists active extracts published up to now in Medline.

Introduction

Buruli ulcer (BU) is a skin disease caused by *Mycobacterium ulcerans*. It usually begins as a painless nodule or papule and may progress to a massive skin ulceration. If untreated, BU may lead to extensive soft tissue destruction that may extend to the deep fascia and sometimes to the bones. BU was recently recognized by the World Health Organization (WHO) as an important emerging disease [1].

The disease has been reported in many countries, mostly tropical, in Africa, North America (Mexico), South America, Southeast Asia, and Oceania. Recent reports have suggested increased incidences of BU in, for example, some areas of Bénin [2], Australia [3], and Ivory Coast [4].

Early, limited *M. ulcerans* infection can be safely and effectively managed by antimycobacterial treatment alone, without surgical debridement. The drug regimen proposed by WHO, consisting of 8 weeks of streptomycin and rifampicin, is effective for early lesions [5–7]. Surgery seems, however, necessary for some severe forms of the disease (large ulcerated forms, disseminated forms, and osteomyelitis) [8]. This surgical treatment can only be used in a few medical centers with proper and adequate equipment and is neither affordable nor accessible to an important part of the population [9].

Traditional treatment remains the first option for patients with generally low resources [9,10]. Two previous studies performed in Bénin have described this kind of treatment; notably its cultural context and different forms. We recently observed that most components in this treatment belong to the vegetal kingdom [11]. However, *in vitro* evaluation of the activities of the natural products used is not documented enough. To our knowledge, only one previous study performed in the Ivory Coast evaluated the *in vitro* antimycobacterial activity of total aqueous extracts of *Sacoglottis gabonensis* (Humiriaceae) and *Okoubaka aubrevillei* (Octoknemataceae) versus seven strains of *M. ulcerans* from different geographical origins [12].

The purpose of this review is to inventory *in vitro* tests that can be used to screen natural products

received July 14, 2010
revised Nov. 25, 2010
accepted Nov. 27, 2010

Bibliography
DOI http://dx.doi.org/10.1055/s-0030-1250642
Published online January 14, 2011
Planta Med 2011; 77: 641–646

ISSN 0032-0943

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for their antimycobacterial activity against *M. ulcerans* and present our first results. We focused our literature search on publications on Medline PubMed (NCBI) database dealing with the evaluation of activities of natural products against *M. ulcerans* and limited it to papers published in English or in French. Key words used were "natural products", "antimycobacterial screening", "Buruli ulcer", "*Mycobacterium ulcerans*", "drugs susceptibility test", "mycobacteria", or/and "plant". All retrieved titles and abstracts were scrutinized for relevant studies that are discussed here. We only found one publication [12] dealing with the evaluation of the activity of natural products against *M. ulcerans*.

Natural antimycobacterial products may be of great importance in the early treatment of BU, preventing the spread of the disease at an affordable cost for the local population. Antimicrobial activity of natural products and pure compounds can be detected by observing the growth response of various microorganisms that are placed in contact with them. Several methods for detecting activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method [13].

The methods used to test antibacterial activity are classified into three main groups, i.e., diffusion, dilution, and radiorespirometry method [14]. These methods are discussed in this review. As *M. ulcerans* has a slow growing rate and a tendency to clump in liquid medium, these general methods must be adapted to give sensitive and reliable results in an acceptable period of time. In our laboratory, we adapted the proportion method on Löwenstein Jensen (LJ) medium, using Middlebrook 7H11 agar and Resazurin Microtiter Assay (REMA), to investigate the antimycobacterial activity against *M. ulcerans* of 44 plants (see **Table 1**) employed in traditional medicine in Bénin to treat BU.

Compound Handling and Storage

The most frequently used solvents to prepare test products solutions include dimethyl sulfoxide (DMSO), methanol, and ethanol. The latter two, however, have the disadvantage of rapid evaporation whereby the stated concentration of stock solutions cannot be maintained. They also have an inhibitory effect on the growth of mycobacteria. Solutions in 100% DMSO have become the standard. Added advantages of stock solutions in 100% DMSO are: (1) elimination of microbial contamination, thereby reducing the need for sterilization by autoclaving which can affect the quality of the test product, and (2) good compatibility with test automation and integrated screening platforms, assuring, for example, good solubility during the serial dilution procedures [13]. It is important to note that DMSO is potentially toxic for cells and many microorganisms including *M. ulcerans*. Then, in order to avoid later interference in the biological test systems, the in-test concentration of DMSO should not exceed 0.625%. In practical terms, this entails the need for inclusion of an intermediate dilution step in water. Because of the variability of individual compounds, there are no general storage conditions that guarantee sample integrity [15]. A practical recommendation for storage of natural products is either without solvent for long-term storage or in 100% DMSO at −20 °C with minimal exposure to freeze-thaw cycles and humidity.

Growth medium

LJ is a conventional growth culture medium for *M. ulcerans*. Middlebrook 7H11 agar, Middlebrook 7H9, and Middlebrook 7H12 liquid media are also convenient for the tests. *M. ulcerans* optimally grows between 30 and 33 °C. The incubation period depends on the culture medium used. Tubes are read within 2 to 3 weeks (with Middlebrook 7H12), after 28 and 42 days of incubation (with Middlebrook 7H11 agar), or 72 to 81 days (with LJ), and plates are read after 15 days (with Middlebrook 7H9).

Inoculum

The standardization of the bacterial cell number used for susceptibility testing is of critical importance for obtaining accurate and reproducible results. Inoculum concentration can have a profound influence on the antimycobacterial potency of a natural product, endorsing the need for standardization of inoculates [16]. Fresh colonies of *M. ulcerans* are collected from the LJ medium and suspended in distilled water; the turbidity of the resulting suspensions is then adjusted with distilled water to match that of a standard 1 mg/mL suspension of *M. bovis* BCG (containing approximately 10^8 CFU per mL), after which the suspensions are further diluted to 10^{-1} and 10^{-2} mg/mL. The inocula are 0.1 mL of diluted solutions [17].

Antimycobacterial Assays

Agar diffusion

The agar diffusion assays were first developed for bacteria [18]. Agar diffusion techniques have been widely used to assay plant extracts for antimicrobial activity but have limitations. Disk methods consist of the placing of filter paper disks containing test compounds, at a known concentration, on agar plate surfaces previously inoculated with the bacteria. Plant extracts diffuse into the agar and inhibit growth of the bacteria. After incubation, the diameter of the clear zone (growth inhibition) is measured at the end of the incubation period.

In order to enhance the detection limit, the inoculated system is kept in an incubator at 30–33 °C during 42 days concerning *Mycobacterium ulcerans*.

The common disc or well-diffusion assays employed in many antimicrobial assays of natural products are not quantitative when used to evaluate new natural products, but are merely an indication that there is growth inhibition at some unknown concentration along the concentration gradient [19]. However, the agar disk diffusion technique can only be used for drug susceptibility testing of pure substances because when it is applied to mixtures, results may be unreliable [20].

Recently, a *M. marinum* inhibition zone assay was developed as a model to evaluate antitubercular or antimycobacterial activity of natural products from marine organism extracts [21].

The major disadvantage of using diffusion assays to evaluate activity of natural products against *M. ulcerans* is that mycobacteria, having a very lipid-rich, hydrophobic cell wall, are often more susceptible to less-polar compounds [22]. Nonpolar compounds will diffuse more slowly than polar compounds in the agar medium and, thus, give a weaker activity. So the diffusion method is not appropriate for testing nonpolar samples or samples that do not easily diffuse into agar. In general, the relative antimicrobial potency of different samples may not always be compared, mainly because of differences in physical properties, such as solubility, volatility, and diffusion characteristics in agar. Further-

Table 1 Plant materials used for antimycobacterial activity screening and MIC values of their ethanol extracts.

Voucher specimen	Family name	Scientific name	Plant parts	MIC (µg/mL)
Yemoa 1	Anarcadiaceae	*Lannea kerstingii* Engl. et K. Krause	leaves	> 250
Yemoa 2	Anarcadiaceae	*Spondias mombin* Linn	stem bark	> 250
Yemoa 3	Annonaceae	*Xylopia aethiopica* (Dunal) A. Rich	fruit	> 250
Yemoa 4	Annonaceae	*Monodora myristica* (Gaertn) Dunal	seeds	> 250
Yemoa 5	Apocynaceae	*Strophanthus hispidus* DC	root	> 250
Yemoa 6	**Apocynaceae**	***Holarrhena floribunda* (G. Don) T. Durand et Schinz**[a]	**leaves**	**125**
Yemoa 7	Araceae	*Anchomanes difformis* Engl	rhizome	> 250
Yemoa 8	Asteraceae	*Launaea taraxacifolia* (Wild.) Schum	leaves	> 250
Yemoa 9	Asteraceae	*Vernonia amygdalina* Del	leaves	> 250
Yemoa 10	Bignoniaceae	*Spathodea campanulata* (P. Beauv)	stem bark	> 250
Yemoa 11	Bignoniaceae	*Stereospermmum kunthianum* (Cham)	root	> 250
Yemoa 12	Bignoniaceae	*Newbouldia laevis* (P. Beauv) Seeman	root	> 250
Yemoa 13	Caesalpinaceae	*Erythrophleum suaveolens* (Guill et Perr.) Brenan	stem bark	> 250
Yemoa 14	Caesalpinaceae	*Piliostigma thonningii* (K. Schum.) Milne-Redh	leaves	> 250
Yemoa 15	Capparaceae	*Ritchiea capparoides* (Andrews) Britten	root	> 250
Yemoa 16	Chenopodiaceae	*Chenopodium ambrosioides* Linn	leaves	> 250
Yemoa 17	Clusiaceae	*Garcinia kola* Heckel	root	> 250
Yemoa 18	Combretaceae	*Anogeissus leiocarpus* (DC.) Guill et Perr	leaves	> 250
Yemoa 19	Combretaceae	*Terminalia glaucescens* Planch	bark/root	> 250
Yemoa 20	Crassulaceae	*Bryophyllum pinnatum* (Lam.) Okem	leaves	> 250
Yemoa 21	Cucurbitaceae	*Kedrostis foedissima* (Jacq.) Cogn.	leaves	> 250
Yemoa 22	Euphorbiaceae	*Euphorbia kamerunica* Pax	bark	> 250
Yemoa 23	Euphorbiaceae	*Hymenocardia acida* Tul	bark	> 250
Yemoa 24	Euphorbiaceae	*Bridellia ferruginea* Benth	bark	> 250
Yemoa 26	**Euphorbiaceae**	***Jatropha* curcas Linn**[a]	**leaves**	**250**
Yemoa 27	Euphorbiaceae	*Jatropha gossypifolia* Linn	leaves	> 250
Yemoa 28	Fabaceae	*Lonchocarpus cyanescens* (Schum. et Thonn.) Benth	root	> 250
Yemoa 29	Lamiaceae	*Ocimum gratissimum* Linn	leaves	> 250
Yemoa 30	Lamiaceae	*Ocimum canum* Sims	leaves	> 250
Yemoa 31	Liliaceae	*Allium cepa* Linn	bulb	> 250
Yemoa 32	Liliaceae	*Aloë buettneri* A. Berger	leaves	> 250
Yemoa 33	Melastomataceae	*Dissotis rotundifolia* (Sm.) Triana	leaves	> 250
Yemoa 34	Mimosaceae	*Tetrapleura tetraptera* (Schum. et Thonn.) Taub.	fruit	> 250
Yemoa 35	Moraceae	*Ficus exasperata* Vahl.	leaves	> 250
Yemoa 37	Myrtaceae	*Eugenia aromatica* (Linn.) Baill	fruit	> 250
Yemoa 38	Nyctagynaceae	*Boerrhavia erecta* Linn	leaves	> 250
Yemoa 39	Periplocaceae	*Parquetina nigrescens* (Afzel.) Bullock	leaves	> 250
Yemoa 40	Piperaceae	*Piper guineense* Schum. et Thonn.	seeds	> 250
Yemoa 41	Poaceae	*Eleusine indica* Linn	plant	> 250
Yemoa 44	Rutaceae	*Clausena anisata* (Wild.) Hook. f.	root	> 250
Yemoa 45	Sapindaceae	*Paullinia pinnata* Linn	leaves	> 250
Yemoa 46	Sapotaceae	*Vitellaria paradoxa* Gaertner	leaves	> 250
Yemoa 48	Zingiberaceae	*Aframomum melegueta* K. Schum	fruit	> 250
Yemoa 49	Zingiberaceae	*Curcuma longa* L.	leaves	> 250

[a] Yemoa 36, 42, 43, 47, are not tested here for their antimycobacterial activity

more, agar-diffusion methods are difficult to run on a high-capacity screening.

Radiorespirometry method

The growth or inhibition of *M. ulcerans* can be determined in a liquid Middlebrook 7H12 medium within 2 to 3 weeks by the extent of oxidation of [1-^{14}C] palmitic acid to $^{14}CO_2$ which is measured in the automated radiometric BACTEC 460 instrument [23, 24]. Because of the quantitative nature of the data obtained in this assay, the relative activity of various samples can be compared by testing at only 1 or 2 concentrations and determining a percent inhibition of $^{14}CO_2$ production compared to product-free controls [25]. Alternatively, multiple concentrations can be tested and MIC calculated [26]. Newer non-radiometric clinical automated systems use indicators of oxygen consumption [27], carbon dioxide production [28], or head space pressure [29] to determine growth/inhibition.

Tests performed in the BACTEC 460 system are costly and not suited for the evaluation of large numbers of compounds. The major disadvantages of these assays are the cost and the isotope disposal in low-income countries.

Dilution methods

Dilution tests can be applied in solid (agar dilution) or liquid (broth dilution) media. Agar dilution and broth dilution are the most commonly used techniques to determine the minimal inhibitory concentration (MIC) of antimicrobial agents, including antibiotics and other substances that kill (bactericidal activity) or inhibit the growth (bacteriostatic activity) of bacteria. The results obtained allow a quantitative estimate of antimicrobial activity.

In the dilution methods, test products are mixed with a suitable medium that will be inoculated with the test organism. It can be carried out in liquid (7H9) as well as on solid media (7H11, LJ). On solid media, growth of the microorganism can be measured by counting the number of colonies. In the agar-dilution method, the minimal inhibitory concentration (MIC) is defined as the lowest concentration able to inhibit any visible microbial growth. In liquid or broth-dilution methods, turbidity and redox-indicators are most frequently used. Turbidity can be estimated visually or obtained more accurately by measuring the optical density. However, test products that are not fully soluble may interfere with turbidity readings, emphasizing the need for a negative control or sterility control, i.e., natural products dissolved in blank medium without microorganisms. Measurements can be obtained with a microplate-reader, but visual reading may also be used in cases where spectrophotometry is not available. Another assay exploits the principle that only living cells convert fluorescein-diacetate to fluorescein, producing a yellowish-green fluorescence under UV light [30]. However, it requires a more significant investment in equipment, and validation is not easy. Fluorescent constituents present in crude natural products extracts may also interfere [31].

In general, dilution methods are appropriate for assaying polar and nonpolar products for determination of MIC on *M. ulcerans*.

Agar dilution: Agar dilution involves the incorporation of different concentrations of extract into a nutrient agar medium followed by the application of a standardized inoculum of bacteria to the surface of the agar tube. After incubation, the presence of bacterial colonies on the medium indicates growth of the organism. Testing of known concentrations of extracts, fractions, or compounds in an agar medium allows for the quantitation of activity and the determination of an MIC.

M. ulcerans grows well on Middlebrook 7H11 agar supplemented with oleic acid, albumin, dextrose, and catalase, if incubated with CO_2. The main disadvantage with such assays is the requirement of at least 28 days to visually detect the growth of colonies [17].

Micro broth dilution: The growth of *M. ulcerans* can be quantitated by measuring turbidity in a liquid medium; the tendency of mycobacteria to clump makes this test difficult. In addition, crude extracts may also impart some turbidity to the medium, making interpretation of results difficult. The use of alamar blue (an oxidation/reduction indicator dye) makes this test rapid and sensitive. Microplate alamar blue assay (MABA) results can be read visually without the use of instrumentation [32]. The reduced form of alamar blue can be quantitated colorimetrically by measuring absorbance at 570 nm or fluorimetrically by exciting at 530 nm and detecting emission at 590 nm [33]. Non-fluorometric readouts can also be performed by using resazurin [34–36] or tetrazolium dyes [37–39]. These methods are also performed on *M. tuberculosis* as well as non-tuberculous mycobacteria (NMT) [40].

The resazurin microtiter assay (REMA) allows for the detection of microbial growth in a small volume of solution in microtiter plates. We can associate the use of a spectrophotometer (Biotrak II) to suppress the possible interference of color of natural products extracts with the resazurin color. Plates are read with a spectrophotometer at 620 nm. The 96-well microplates offer the advantage of using small volumes of reagents. Plates can be read visually without the need of instrumentation. The MIC is defined as the lowest concentration of extract that prevents a color change of resazurin (blue to pink). The plant extracts that could not prevent growth of *M. ulcerans* up to a concentration of 250 µg/mL are considered inactive.

For screening antibacterial activities of natural products, it is essential to use an *in vitro* antibacterial assay that is simple, rapid, sensitive, and cost-effective. Usually, small quantities of natural products are available for antibacterial screening, and this can be a limiting factor. Dilution method performed on LJ is time consuming, very slow and requires significant quantities of materials. When performed on 7H11, it requires a CO_2 incubator because *M. ulcerans* is a microaerophilic bacterium. With the BACTEC 460 system, microaerobic conditions (2.5 to 5% oxygen) are needed to promote the growth of *M. ulcerans*. Furthermore, the BACTEC 460 system requires significant, heavy equipment and is very expensive. We therefore decided to focus on the REMA test to screen plant extracts for antimycobacterial activity against *M. ulcerans.* The resazurin assay using a microtiter plate, described here, is modified to determine the MIC values of natural products against *M. ulcerans*. Resazurin sodium salt powder (Acros Organic N.V.) is prepared at 0.02% (w/v) in distilled water, filter sterilized and stored at 4 °C for no more than 2 weeks. A total of 100 µL of *M. ulcerans* suspensions is added to each well of a microtiter plate together with the plant extracts in Middlebrook 7H9 broth to obtain a final volume of 200 µL in each well. It is necessary to include 3 positive control wells (containing 100 µL of Middlebrook 7H9 broth and 100 µL of a mycobacterial suspension each) and 3 negative control wells (containing 200 µL of Middlebrook 7H9 broth). After 15 days incubation, 30 µL of resazurin 0.02% are added to the first positive control well. Dye color changing to pink indicates bacterial growth. The dye is then added to all remaining wells in the plate. The results are read 48 hours later. If no color change is observed until the 17th day (thus 2 days after the addition), results are considered inconclusive. The results are considered if, and only if, the negative control wells become blue by the addition of resazurin. We used this test to screen the activity of 44 plants used traditionally to treat BU in Bénin [11]. Results are given in **Table 1**. Out of the 44 plant extracts tested, two plants inhibited the growth of *M. ulcerans* at concentrations ≤ 250 µg/mL: *Holarrhena floribunda* (G. Don) T. Durand and Schinz and *Jatropha curcas* Linn showed inhibitory activity against *M. ulcerans* at concentrations of 125 and 250 µg/mL, respectively. Control experiments showed that 0.625% dimethyl sulfoxide (DMSO, solvent used for extract dissolving) or less in each well did not have any inhibitory effect on the growth of *M. ulcerans* ATCC 19423.

In traditional medicine, practitioners use different plant combinations to treat BU, but in our study plants were investigated individually. This could explain the low number of plants found to be active against *M. ulcerans*. It is also probable that these plants are used to treat the symptoms of the disease rather than actually to kill the bacteria. Some plant species may not contain compounds which inhibit the growth of or kill *M. ulcerans* but may have anti-inflammatory, analgesic, anesthetic, antiseptic, anti-edema, or healing properties. Further investigations are now required to isolate active compounds and assess their *in vitro* and *in vivo* activities against *M. ulcerans*. The adapted REMA method is simple, sensitive, and rapid and could be a method of choice to successfully assess antibacterial properties of plant extracts against *M. ulcerans* at a relatively low cost. This is the first application of the REMA to screen plants extracts against *M. ulcerans*.

Additional methods not described in this review are bioautographic methods and a conductimetric assay detecting microbial growth as a change in the electrical conductivity or impedance of

the growth medium [14]. But there are no references using these methods for *M. ulcerans*.

Test Validation

Reference compounds

It is evident that each test should contain at least one reference drug to ascertain test performance and proper interpretation of the screening results. Those listed here are commercially available and should be preferred: rifampicin, streptomycin, amikacin, moxifloxacin, and clarithromycin. The activity of these products has already been tested [17, 29]. In addition, each test should be replicated, preferably as independent repeats. A practical solution is to combine negative and positive controls (i.e., a strain susceptible and a strain resistant to the compound) in each assay.

Test organisms

A well-characterized strain, *Mycobacterium ulcerans* ATCC 19423 has a known drug susceptibility profile. It is used in many drug susceptibility tests. *M. ulcerans* is a microorganism that can cause severe disease in humans and constitutes a danger for the employees directly exposed. Personnel handling this microorganism must wear protective gloves and, most importantly, should work in biosafety cabinets class II.

Discussion and Conclusion

Natural products constitute an important source of new drugs, and antimycobacterial susceptibility testing methods are necessary to evaluate different extracts and find new active compounds. A number of methods are in current use, ranging from the classical disk diffusion and broth dilution assay to the radiorespirometric method (BACTEC 460). There are several factors that may affect the outcome of susceptibility tests. Reproducibility of laboratory results may be considerably influenced by the method used, and the procedures have to be standardized otherwise the results will vary widely under different test conditions [41]. Standard criteria and standardization of methods for evaluation of plant antimicrobial activity are lacking, and results can differ between authors. Sometimes it is difficult to compare results obtained with plant extracts with published results in the literature because several parameters influence the results, such as the choice of plant extracts, the choice of extraction method, and the choice of antimicrobial method [42]. It is also important to take into account that the evaporation of components could affect the antimicrobial activity and to avoid this, the assays should be carried out under sealed conditions to prevent loss by evaporation.

In general, dilution methods are appropriate for assaying polar and nonpolar extracts or compounds to determine MIC values [13].

We showed that REMA is a method of choice to compare MIC values in *M. ulcerans*. The use of microtiter plates has led to significant reductions in test compound quantities; furthermore, by using the oxidation/reduction indicator dyes, such as resazurin, the growth/inhibition can be read visually; and the reduced form of these dyes can also be quantitated colorimetrically, by measuring absorbance, or fluorimetrically and have high throughput possibilities. REMA is also able to detect partial inhibition, making it ideal for determining the relative activity of fractions using one or two concentrations.

This review has highlighted some methods which can be successfully used to investigate antimycobacterial activities of natural products against the microorganism responsible for BU, an important emerging disease. This could stimulate interest in research of active natural products against *M. ulcerans*. Phytomedicine is the first treatment used by the poor local population, and studies are required to investigate the possible efficacy of some natural products for the treatment of BU when the WHO recommended treatment cannot be applied immediately. Up to now, only one publication deals with the evaluation of the activity of natural products against *M. ulcerans*. The development of sensitive, rapid, and inexpensive assays allows researchers in low-income countries to become players in the utilization of their natural product resources. We hope that this review will help researchers to develop these tests.

Acknowledgements

Achille Yemoa is a doctoral student of the Wallonie Bruxelles International (WBI). His doctoral research was financially supported by the WBI and the BURULICO Project No. INCO-CT-2005-051476.

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