

ANTIMICROBIAL PHYTOCHEMICALS

Plants from Puerto Rico with Anti-*Mycobacterium tuberculosis* Properties

ANNE D. FRAME, MS, Ed D*[†]; EDDY RÍOS-OLIVARES, MPH, PhD[†]; LARRY DE JESÚS, BS[‡]
DAMARIS ORTIZ, BS[‡]; JUAN PAGÁN, BS[‡]; SANDRO MÉNDEZ, BS[‡]

Objective. This study assesses the antitubercular potential of natural products obtained from plants reputed to have medicinal properties and collected from the tropical flora of Puerto Rico.

Background. The increase in persons infected with *Mycobacterium tuberculosis* (MTB) the world over and the development of resistance to antibiotics by this microbe and other infectious bacteria has created the need for new drugs to replace those which have lost effectiveness.

Method. In Phase I of this study, ethanolic leaf extracts of fifty local plants were submitted to preliminary screening to assess their *in vitro* *Mycobacterium smegmatis* inhibitory activity using the Bauer-Kirby disk diffusion method. In Phase II, the definitive screening of the six most promising extracts which inhibited *M. smegmatis* were assayed for their MTB inhibitory activity using the BACTEC 460 susceptibility test method. The brine shrimp bioassay was used as a toxicity bioassay and the mice inoculation test was used to determine mice tolerance to the effect of the daily intraperitoneal inoculations of the plant extracts.

Results. MTB showed varying degrees of susceptibility to each plant extract. This effect was dependent upon the plant species, dose and time of

exposure. Evidence is provided suggesting that: (1) Six crude plant extracts (12%) tested possessed inhibitory capacity at the amount of 500 µg per disc; (2) *Mammea americana* extract yielded the strongest inhibitory effect at 50 µg per disc, followed by *Marchantia polymorpha*, *Mangifera indica*, *Callistemon citrinus*, *Syzygium jambos* and *Momordica charantia*; (3) the bactericidal inhibitory pattern of MTB growth, exposed to *Mammea americana* extract, was comparable to streptomycin; and (4) the transitory reduction pattern of MTB growth, produced by *Callistemon citrinus*, *Marchantia polymorpha* extracts at 100 µg and 250 µg, was similar to that of bacteriostatic agents.

Conclusion. Of 50 plants screened six extracts tested for their anti-MTB activity yielded positive results with varying degrees of inhibition. *Mammea americana* showed the greatest inhibitory activity suggesting that certain plant species yield valuable anti-*Mycobacterium tuberculosis* substances. The procedures employed in this study, including the BACTEC 460 modified method, are useful for *in vitro* screening of plant extracts with potential antitubercular activity. **Key words:** Medicinal plants of Puerto Rico, Anti-*Mycobacterium tuberculosis* agents, *Mycobacterium* susceptibility testing, Natural products.

Discovering plants with anti-infectious disease compounds and testing these for their comparable effects to known antibiotics represents a significant challenge. This becomes more meaningful if

we consider that some pathogenic *Mycobacterium* strains are developing resistance to known antibiotics at such a rapid pace that the need for new drugs to replace those that have lost their effectiveness is urgent (1, 2). It is estimated that 1.7 billion people, or one third of the world population, is infected with *Mycobacterium tuberculosis* (MTB) (2). Of particular concern is the high prevalence of MTB infections in Human Immunodeficient Virus (HIV) infected persons and drug users, estimated to be 10 to 15 million, who have contributed to the rapid spread of MTB in the world population. This scenario gives urgency to the call for new pharmaceuticals (3, 4).

The emergence of resistant strains of MTB has become a global health problem causing morbidity and mortality

* Inter American University of Puerto Rico, Distinguished Professor of Biology, Metropolitan Campus, Division of Sciences and Technology, PO Box 191293, San Juan, PR 00919-1293, Tel. (787) 250-0169, Fax: (787) 250-8736, E-mail: frame@inter.edu.

† Universidad Central del Caribe, School of Medicine, Professor and Director of Microbiology and Immunology Department, Tel. (787) 798-3001, Fax: (787) 740-4300, E-mail: erios@uccarib.edu.

‡ NIH-MBRS/MARC research participant.

on a worldwide scale (5,6). The re-emergence of this microbe, with a history of devastation and disease, has hastened the development of some rapid and sensitive diagnostic methods. Effective treatment of MTB requires rapid assessment of drug sensitivity. Test methods using radiometric means and others such as luciferase have helped reduce the time required for information on antibiotic sensitivity of MTB strains from weeks to days (7,8). In the case of MTB, early detection and early determination of its susceptibility to antibiotics with prompt initiation of treatment, contribute to meeting the crisis caused by both the disease and the rise in multi-drug resistant (MDR) strains of this bacterium (9). The MDR mechanism developed by microorganisms provides broad defense for survival. The MDR pump in the resistant organism expels molecules, including antibiotics, from the microbe (10), thus ensuring its survival.

Historically, natural products preparations have been a significant source of pharmaceutical agents. More than 90 percent of current therapeutic classes of drugs are derived from natural products. It is estimated that still today, two-thirds to three-quarters of the world's population rely upon medicinal plants for their primary pharmaceutical care (11). Yet, it is found that only 5 to 10 percent of the known plant species have been analyzed for their chemical content.

Some recent accounts of pharmaceutical findings and testing of natural products include the following: (a) finding of compounds with anticancer properties, initiated by the U.S. National Cancer Institute (NCI) in 1957, where natural products were isolated, identified, placed in clinical development of human cancer trials (12); (b) the search for natural products with selective antimalarial activity (13); (c) the review of taxol science and applications (14); (d) the identification of several plant-derived anti-HIV agents (15), and (e) the NIH supported program which screens drugs against MTB and *M. avium* at the Southern Research Institute (16).

In Puerto Rico and the Caribbean much of the knowledge on medicinal plants comes from folkloric information both written and verbal, rather than scientific investigation, thus, some medicinal plants widely used for health purposes have not been adequately evaluated (17). The sources of information on medicinal plants found in Puerto Rico are adequate. In addition, one can obtain information on healing plants just from conversations with the average adult citizen. The compilations by Liogier (18), Julia Morton (19) and Núñez-Melendez (20) provide adequate information on medicinal plants and were the principal references used in this study.

A survey carried out at Inter American University of

Puerto Rico, Metropolitan Campus, showed that 90 percent of the student's parents and grandparents in this population relied on the use of plants reputed to have healing properties to treat some health problems, in part because many could not afford medical treatment and because of the trust people have in ethnomedical use of plants (Frame, A.D. and Bendezú, P., unpublished data, 1990, Inter American University of Puerto Rico, Metropolitan Campus). Hernández and colleagues from the School of Pharmacy of the University of Puerto Rico conducted a study with an outpatient group using medicinal plants to determine the main use and frequency of use by this segment of the population. Most of the herbs were used to treat self limiting ailments rather than serious medical conditions (21). In this study the patients chose to use natural herbs in addition to prescribed medication.

The main goal in this investigation was to determine the antitubercular potential of natural products obtained from plants collected in Puerto Rico. Since screening directly against the highly infectious and slow growing MTB is hazardous and time consuming, *M. smegmatis* was used as the target organism for the *Mycobacterium* genera in the preliminary screening of this study. The saprophyte *M. smegmatis* which grows more rapidly than the tubercle bacilli, is generally not pathogenic to humans (22, 23).

Materials and Methods

The work in this investigation was divided in two parts. Phase I consisted of the collection and processing of the plant material, preparation of ethanolic extractions, and screening the extracts for their inhibitory activity against *Mycobacterium smegmatis*. Phase II consisted of screening the extracts for their inhibitory activity against MTB, as well as, conducting toxicity bioassays with *Artemia salina* Leach and mice tolerance test.

Phase I: Collection and processing of plants, preparation of ethanolic extracts and screening extracts for inhibitory activity against *Mycobacterium smegmatis*. In Phase I of this work, ethanolic extracts of leaves from 50 plants selected on the basis of medicinal use on the island and field collected in the northeastern sector of Puerto Rico, were screened for *in vitro* anti-*Mycobacterium smegmatis* activity. A voucher sample of each plant was placed at the Inter American University Herbarium to conserve the specimen for future reference. The plants screened are listed below by genus, species, family and common name. Plants were classified by staff and confirmed by Dr. Henri Alain Liogier, Taxonomist at the Experiment Station of the University of Puerto Rico and Dr. Herminio Lugo, Botanist at Inter American

University of Puerto Rico, Metropolitan Campus.

Botanical names of species screened:

Acacia farnesiana (L.) Willd C. (Leguminosae Mimosoideae) Acacia, aroma amarilla.
Annona muricata L. C: (Annonaceae) guanabana
Artocarpus altilis (S. Parkinson) Fosberg RD: (Moraceae) panapen, bread fruit.
Bougainvillea glabra Choisy C: (Nyctaginaceae) trinitaria
Bursera simaruba (L.) Sarg C: (Burseraceae) Almácigo, turpentine tree.
Caesalpinia pulcherrima (L.) Sw. C: (Leguminosae-Caesalpiniordeae), Flamboyán
Cajanus cajan (L.) Millsp. (Leguminosae-Papilioideae) gandures, gandul
Callistemon citrinus (Curtis) Skeels (Myrtaceae) bottle brush
Capsicum frutescens L. (Labiatae) ají morón
Cassia fistula L. C: (Leguminosae Caesalpinoideae), guayaba cimarrona
Catharathus roseus (L) G. Don-C: (Apocynaceae) playera, periwinkle
Cecropia peltata L. y C. schreberiana Miq. (Moraceae) Yagrumo
Cessus trifoliata L. C:(Vitaceae) bejuco de caro
Coleus amboicus Lour C:(Labiatae) oregano brujo
Crescentia cujete L. C: (Bigoniaceae) higuera
Cymbopogon citratus (DC) Stapf C: (Gramineae) limoncillo, lemon grass, fever grass
Enterolobium cyclocarpum (Jacq) Griseb C: (Leguminosae Mimosoideae) orejas de mono
Eryngium foetidus L. (Apiaceae) culantro
Fragaria vesca L. (Rosaceae) fresa, wild strawberry
Hibiscus rosa - senensis L. C: (Malvaceae) amapola
Lippia origanoides HBK (Verbenaceae) poleo
Malpighia emarginata Sessé & Mocei, DC. -C: (Malpighiaceae) acerola
Mammea americana L. -C: (Guttiferae) mamey amarillo
Mangifera indica L.: (Anacardiaceae) mangó
Marchantia polymorpha L. C: (Marchantiaceae) hepática
Marchantia conecephalus L. (Marchantiaceae) conecephalus
Melaleuca quinquenervia (Cav.) S. T. Blake-C: (Myrtaceae) cayeput
Melia azedarach L. C.: (Meliaceae) lila, alelaila
Mimosa pudica L C: (Leguminosae-Mimosoideae) moriviví

Momordica charantia L (Cucurbitaceae) cundeamor
Musa paradisiaca L. (Musaceae) plátano
Musa sapientum L. (Musaceae) banana, guineo
Nasturtium officinale R. Br. -(Capparaceae) berro
Ocimum basilicum L. C: (Labiatae) albahaca, sweet basil
Omphalea trianda L. C: (Euphorbiaceae) avellana criolla
Passiflora edulis Sims R.D. (Passifloraceae) parcha
Pothomorphe peltata Miq (Piperaceae) baquiña
Peperomia pellucida (L.) HBK -C: (Piperaceae) parietaria
Petiveria alliacea L. -C: (Phytolaccaceae) anamú
Plantago major L. (Plantaginaceae) llantén
Psidium guajava L. C: (Myrtaceae) guayaba
Randia aculeata L. -C: (Rubiaceae) tintillo
Rheo spathacea (S.W.) Stearn - C: (Commelinaceae) sanguinaria
Rosa odorata (Andr.) Sweet (Rosaceae) rosa
Ruta chalepensis L. (Rutaceae) ruda
Sambucus mexicana Presl - (Caprifoliaceae) saúco
Spinacia oleracea L. (Chenopodiaceae) espinaca
Syzygium jambos (L.) Alston (Myrtaceae) pomarrosa
Tillandsia recurvata L. -C: (Bromeliaceae) ball moss
Tropallum majus L. C: (Tropaeoaceae) jacinto

Approximately three pounds of plant leaves to be tested were oven dried at 42° Celsius and ground in a blender. Thirty grams of the ground material was extracted with 300 ml of 95% ethanol over a 24 to 36 period. The suspension was filtered with Watman filters, #4 then # 2, to filter out undissolved particles. The resultant extract was vacuum dried at 40° Celsius in a Buchii rotavapor to remove the solvent and then stored in a refrigerator in a crystal container. The lapse of time between the preparation of the extract and testing with the microorganism was approximately 48 hours.

The agar-disk diffusion procedure described by Bauer, et al. (24) and included in the National Committee for Clinical Laboratory Standard Method (NCCLS), was utilized to assay the susceptibility of *M. smegmatis* (ATCC No. 607) towards the plant extracts. Sterile filter paper disks were impregnated with known amounts of plant extracts from 10, 50, 100 and 250 µg. and allowed to dry at room temperature. The impregnated discs were then placed on the agar plates inoculated with a standardized

suspension of *M. smegmatis*. After 36 to 48 hours of incubation at 35°C (± 1), the diameter (mm) of the inhibition zone was measured. Disks containing 10 μ g of streptomycin were used as reference. Disks impregnated with methanol, the diluent used to dissolve the extracts, were also tested. A blank disk with sterile water was the negative control.

Phase II: MTB susceptibility activity, plant extract toxicity and mice tolerance. The goal in Phase II of this investigation was to determine the anti-MTB activity, toxicity and tolerance of the plant extracts which demonstrated anti-*M. smegmatis* activity in the first phase of the study.

The BACTEC-460 method modified by Ríos Olivares, E. and García, V. (unpublished data) was used in this study. Although more rapid and less costly methods have recently been recommended (1), the BACTEC-460 technique is being used effectively due to its availability, reliability, safety, rapidity and reproducibility compared with conventional methods (25, 26, 27).

A bacterial suspension was prepared by transferring a small inoculum of MTB (strain ATCC #27294) known to be susceptible to streptomycin, from a Lowenstein-Jensen agar medium to a sterile tube containing a diluent composed of saline solution, tween 80 and fatty acid free solution. This solution was homogenized and allowed to stand for 30 minutes to settle the large particles. The supernatant homogeneous suspension was placed into a separate sterile test tube and turbidity adjusted to McFarland 0.5. Then, a dilution of 1:100 was obtained. All initial culture suspensions and dilutions were performed in a Class IIA Biological Cabinet with no antibiotic added.

For each experiment four different controls were prepared: negative (untreated), positive control (streptomycin) and two methanol controls. The untreated negative control was prepared by suspending 0.1 ml of the adjusted MTB suspension into 9.9 ml of diluent, yielding a 1:100 dilution. The amount of 0.1 ml from this dilution was mixed with the radiometric medium BACTEC 12B. For the positive control, 0.1 ml of the standardized bacterial suspension was added to 4 ml of BACTEC 12B containing 0.1 ml of streptomycin (2.0 mg/ml). The methanol controls were prepared by adding 100 μ l of the bacterial suspension to two separate tubes, one containing 0.1 ml and the other 0.2 ml of methanol.

To test the anti-mycobacterial activity of different plant extracts, a stock solution was prepared by diluting 50 mg of the dried extract in 5 ml of methanol. Then, BACTEC 12B culture medium was added to 10, 20, 40, 100 and 200 μ l separately of this stock solution to make preparation of 4 ml., thereby, yielding concentrations of 25, 50, 100,

250, and 500 μ g of plant extract respectively. After inoculating each with 0.1 ml of the MTB suspension, they were incubated at 37°C for 12 days in an atmosphere of 5% CO₂ of the BACTEC instrument. The result of each BACTEC test is expressed as Growth Index (GI). The GI is a measure of the ¹⁴CO₂ aspirated from the test vial. The instrument reads directly on a scale of 0-999. A GI value of 100 corresponds to approximately 0.025 micro Ci (28).

From day 4 to day 12 the growth index (GI) rate of the bacteria was measured daily at the same time each day until the negative control reached 30 or more. Change in the GI (GI) was calculated by subtracting the GI of one day from GI of the previous day. If the change in the GI of the negative control was greater than the change in the GI of the tested extract dilution, the microorganism was susceptible to the extract, if there was no difference, then it was assumed to be borderline. However, if the difference in the GI of the negative control was less than the difference in the GI of the tested extract, the bacteria was said to be resistant to the extract concentration tested (28).

The brine shrimp assay was used as a biological screen for potentially bioactive substances in the plant extracts. It is considered to be a good predictor of toxicity for living organisms (29). Toxicity of the plant extracts was tested using the procedure outlined by Meyer (30). Lyophilized eggs of *Artemia salina* Leach were incubated in artificial sea water utilizing the Instant Ocean Aquarium System. The tank was divided in two compartments by means of a perforated plastic dam. The eggs were sprinkled to one side of the tank and covered with a lid. After the 48-hour time limit, eggs hatched yielding a large number of the phototropic first stage larvae of *Artemia salina*, the nauplii. The other side of the container was illuminated and attracted the nauplii through the perforation in the dam. Three different concentrations of the plant extracts, 1000, 100 and 10 μ g/ml were added to vials of artificial sea water solutions containing a known number of nauplii. After 24 hours, the survivors were counted and the LC₅₀ values were determined with Finney computer analysis (31, 32).

This test was conducted to show the degree of toxicity or tolerance of the mice towards the plant extracts in representation of a mammalian species. The strain of mice used was from SASCO Laboratories CF-1 outbred, 50 day-old males, with an average weight of approximately 30 grams. Seven outbred CF-1 mice per cage were used for each experimental group. The six experimental groups of mice, one for each plant extract, received a daily dose of 100 μ l of an extract mixture (50 μ l of extract suspended in methanol and 50 μ l of saline solution) intra-peritoneally (IP) for 15 consecutive days. This daily dose corresponds to a concentration of 500 μ g of plant extract, which was

the highest concentration used in the MTB susceptibility testing with BACTEC 460. Two control groups were included: one of the control groups was administered 100 µl of saline solution (IP) and the other 100 µl of a mixture consisting of 50 µl methanol and 50 µl saline solution. Mice remained under observation for a period of one month and the following parameters were measured on a daily basis: changes in activity, fecal consistency, hair condition, cutaneous lesions, eye changes, appetite (water and food intake), weight loss, temperature changes and mortality.

Results

Of 50 plant extracts screened for their anti-*Mycobacterium smegmatis* activity using the Bauer-Kirby Agar Diffusion method, six (12%) yielded detectable microbial inhibitory effect during the initial Phase I. The relative antimicrobial activity of the six active plant extracts is compared in Table 1. The most effective extracts in this phase were those obtained from *Mammea americana*, *Callistemon citrinus* and *Marchantia polymorpha*, since these three extracts demonstrated inhibitory activity even at low concentrations of 50 µg and 25 µg. The least effective plant was *Mangifera indica* which at a concentration of 500 µg did not produce an inhibitory zone in the disk agar diffusion test, while at this concentration all of the other extracts showed an inhibitory zone above 10 mm. On the other hand, both *Momordica charantia* and *Syzygium jambos* showed the highest inhibitory zone of activity, 30 mm, at

concentrations of 1000 and 500 µg. Factors inherent to the Bauer-Kirby assay, that influence the diffusion capacity of the molecules involved such as size, ionic charge, concentration, etc. could account for the variation in dose-response inhibitory pattern seen in some of the results presented in Table 1. Another factor that could have bearing on this effect is the fact that, thus far, the data presented in this study is the result of experiments done with crude plant extracts and not with purified compounds. The preliminary findings shown on Table 1 prompted us to design experimental protocols for further characterization.

Table 2 shows the results obtained in Phase II after challenging a slow-growing, pathogenic strain of *Mycobacterium tuberculosis* (MTB), with each of the six selected plant extracts using the BACTEC-460 radiometric method to measure growth inhibition. Decreasing concentrations (500, 250, 100, 50, 25 µg) of the plant extracts were confronted with the MTB strain and the growth index (GI) determined for a period of 12 days. It can be seen in this table that at a concentration of 500 µg all the extracts included in the study possessed anti-MTB activity. At 250 µg, the plant extracts with a GI below the cut-off value of 30, which is an indication of inhibition of MTB, were those obtained from *Callistemon citrinus*, *Marchantia polymorpha*, *Mangifera indica* and *Mammea americana*. At 100 µg, only *Marchantia polymorpha*, *Syzygium jambos* and *Mammea americana* showed a GI below the growth indicator limit. However, at 50 µg, this growth inhibitory capacity was seen only in the plant extracts from *Mammea americana*. *Mammea americana* yielded a growth-inhibitory pattern

Table 1. Antimicrobial Activity of Different Concentrations (25-1000µg) of Plant Extract to *Mycobacterium smegmatis* (Buer-Kirby agar diffusion method)

Extract	Concentration (µg) and Inhibition zone (mm)*					
	1000µg	500µg	250µg	100µg	50µg	25µg
<i>Syzygium jambos</i> (Myrtaceae)	30	30	R†	R	R	R
<i>Callistemon citrinus</i> (Myrtaceae)	27	25	15	13	10	8
<i>Momordica charantia</i> (Cucurbitaceae)	30	30	R	R	R	R
<i>Marchantia polymorpha</i> (Marchantiaceae)	10	12	8	9	7	R
<i>Mangifera indica</i> (Anacardiaceae)	12	0	R	R	R	R
<i>Mammea americana</i> (Guttiferaceae)	12	10	12.5	12	14	10

* = (\bar{x} of 6 readings)
† = Resistance
Ampicillin control (10µg)

Table 2. Effect of Different Concentrations (µg) of Plant Extract on the Growth of *Mycobacterium tuberculosis*

Plant Extract from	Concentration (µg)				
	500µg	250µg	100µg	50µg	25µg
<i>S. jambos</i>	S*	R†	R	ND‡	ND
<i>C. citrinus</i>	S	S	S	R	R
<i>M.charantia</i>	S	R	R	R	R
<i>M. polymorpha</i>	S	S	S	R	R
<i>M. indica</i>	S	S	R	ND	ND
<i>M. americana</i>	S	S	S	S	R

n = 3-6 readings
Negative Control, Growth Index Indicator (Δ GI) > 30
Positive Control, Streptomycin (50 µg/ml, Δ GI = + 2.8
Methanol 0.1 ml, Δ GI = + 40
Methanol 0.2 ml, Δ GI = -2
* = Susceptible
† = Resistant
‡ Not Done

similar to that exhibited by the antibiotic streptomycin (50 µg/ml), which was included in this work as control of growth inhibition (positive control). At a concentration of 25 µg, none of the plant extracts was effective in reducing MTB growth. There was no interference caused by the use of 0.1 ml of methanol as the vehicle to suspend the plant extracts, since at this concentration there was no growth inhibition of MTB.

The data in Figure 1 (A and B) indicate that all plant extracts used here were able to inhibit MTB at a concentration of 500 µg and that this growth reducing effect was effective throughout the experimental period. However, there were differences in the intensity of inhibition as shown by individual growth-reducing curve. It should be mentioned that the growth index (GI) decrease depends on the speed of action of the drug and the

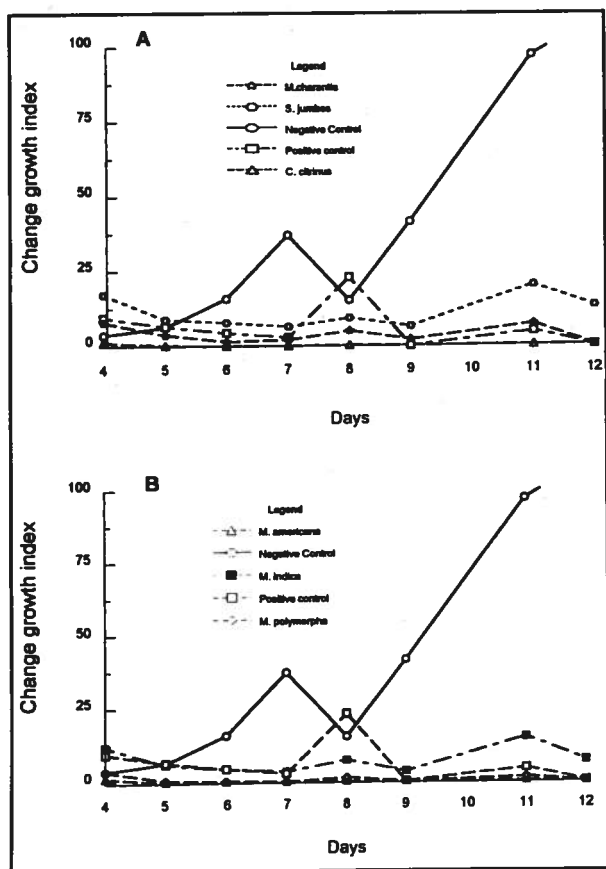


Figure 1. Effect of 500 µg of different plant extracts on the growth index of *Mycobacterium tuberculosis*. A. Comparison of extracts from *Momordica charantia*, *Syzygium jambos* and *Callistemon citrinus* to positive control (medium) and negative control (streptomycin). B. Comparison of extracts from *Mammæ americana*, *Mangifera indica* and *Marchantia polymorpha* to positive and negative controls. Susceptibility cut-off point, GI=30.

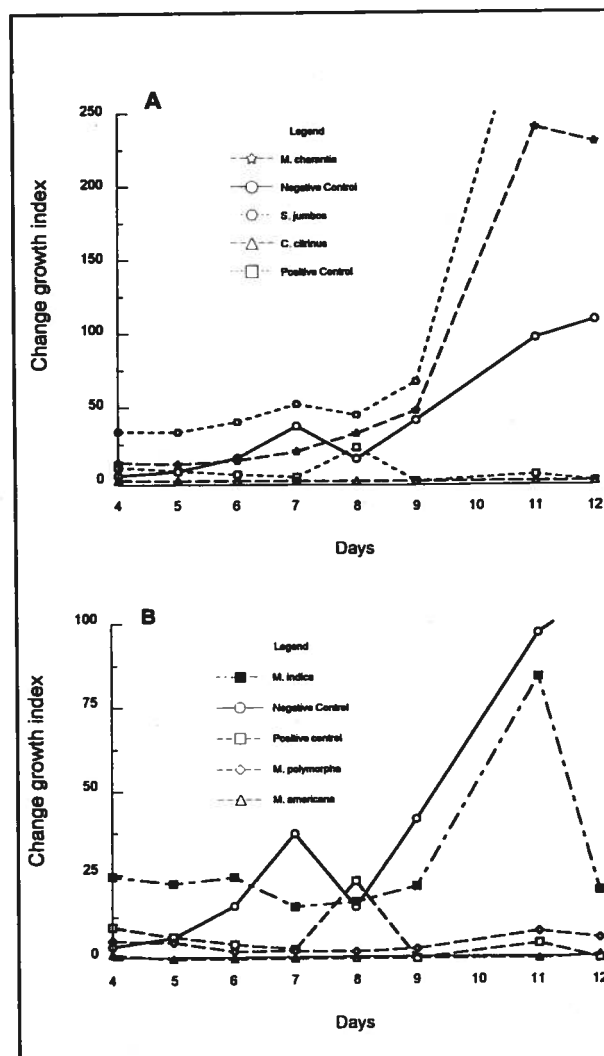


Figure 2. Effect of 250 µg of different plant extracts on the growth index of *Mycobacterium tuberculosis*. A. Comparison of extracts from *Momordica charantia*, *Syzygium jambos* and *Callistemon citrinus* to positive control (medium) and negative control (streptomycin). B. Comparison of extracts from *Mammæ americana*, *Mangifera indica* and *Marchantia polymorpha* to positive and negative controls. Susceptibility cut-off point, GI=30.

susceptibility of the bacteria. The irregularities of the curves (sudden decrease or increase pattern) could be explained by the presence of mixed populations of susceptible and resistant MTB microorganisms in the same culture. Therefore, it could be expected that the curve of GI values versus time could vary depending on the percentage of the population that is susceptible. The weakest capacity to maintain a constant growth reducing effect on MTB, throughout the observation period, was seen with *Syzygium jambos*, *Mangifera indica* and

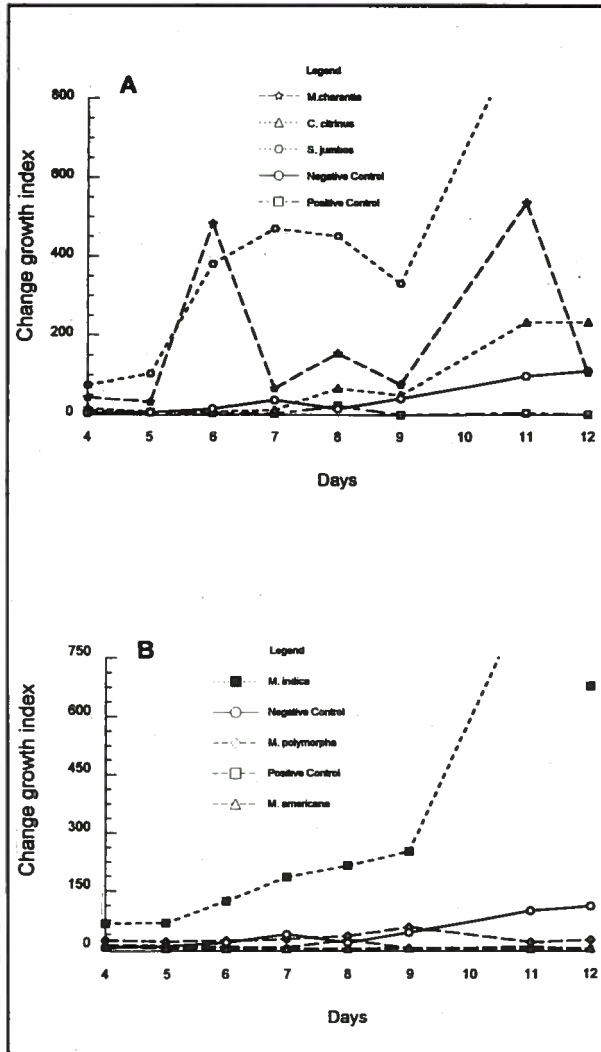


Figure 3. Effect of 100 µg of different plant extracts on the growth index of *Mycobacterium tuberculosis*. A. Comparison of extracts from *Momordica charantia*, *Syzygium jumbos* and *Callistemon citrinus* to positive control (medium) and negative control (streptomycin). B. Comparison of extracts from *Mammee americana*, *Mangifera indica* and *Machantia polymorpha* to positive and negative controls. Susceptibility cut-off point, GI=30.

Momordica charantia. Similar results were obtained at a concentration of 250 µg (Figure 2, A and B), where it can be observed that the inhibitory effect of *Syzygium jumbos*, *Mangifera indica* and *Momordica charantia* was only transitory (for 8 to 10 days). In comparison, *Callistemon citrinus*, *Marchantia polymorpha* and *Mammee americana* were able to maintain the GI of MTB at 250 µg well below the growth indicator mark and a comparable growth pattern with that shown by streptomycin. This phenomenon was also seen at a concentration of 100 µg

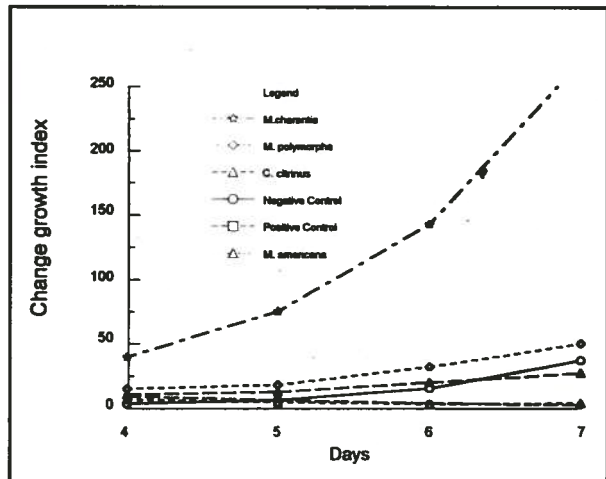


Figure 4. Effect of 50 µg of different plant extracts on the growth index of *Mycobacterium tuberculosis*. Comparison of extracts from *Momordica charantia*, *Marchantia polymorpha*, *Callistemon citrinus* and *Mammee americana* to positive control (medium) and negative control (streptomycin). Susceptibility cut-off point, GI=30.

(Figure 3, A and B), but repeated only for *Callistemon citrinus* and *Mammee americana* at 50 µg (Figure 4) and not for any of the extracts at 25 µg (Figure 5). Overall, the growth inhibitory patterns by the plant extracts determined by the use of the BACTEC-460 system correlate adequately with those described for *Mycobacterium smegmatis* in Table 1.

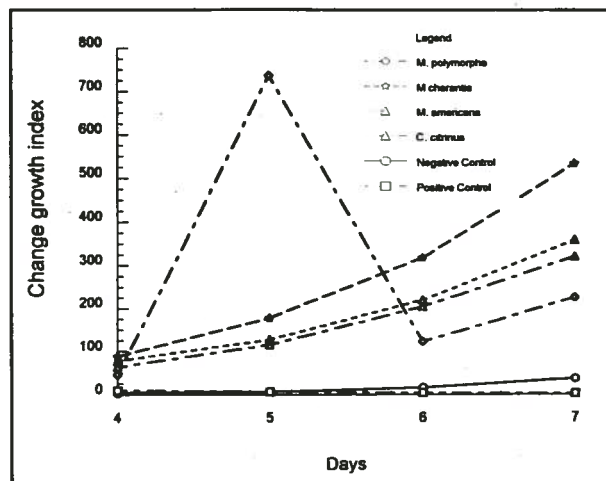


Figure 5. Effect of 25 µg of different plant extract on the growth index of *Mycobacterium tuberculosis*. Comparison of extracts from *Momordica charantia*, *Marchantia polymorpha*, *Callistemon citrinus* and *Mammee americana* to positive control (medium) and negative control (streptomycin). Susceptibility cut-off point, GI=30.

The results of the Toxicity-Brine Shrimp bioassay can be seen in Table 3. These results are expressed as 50 percent lethal concentration (LC₅₀ µg/ml) for each plant extract. All six extracts from the plants included in the study presented different degrees of toxicity to the nauplii, the shrimp first stage larva. However, *Syzygium jambos*, *Callistemon citrinus*, *Momordica charantia* and *Mammea americana* showed the greatest degree of toxicity LC₅₀ µg/ml of 93, 168, 33 and 224 µg respectively. It is interesting to note that *Syzygium jambos* and *Momordica charantia* were able to kill the nauplii displaying high

Table 3. Toxic Effect of Selected Plant Extracts Using the Brine-Shrimp Bioassay

Plant	LC ₅₀ µg/ml*
<i>Syzygium jambos</i> (Myrtaceae)	93
<i>Callistemon citrinus</i> (Myrtaceae)	168
<i>Mormodica charantia</i> (Cucurbitaceae)	33
<i>Marchantia polymorpha</i> (Marchantiaceae)	>1000
<i>Mangifera indica</i> (Anacardiaceae)	>1000
<i>Mammea americana</i> (Guttiferaceae)	224,6

n = 3 observations

* LC-50 = Lethal concentration 50%

degree of toxicity, but their effect was relatively weak against MTB in the BACTEC-460 susceptibility test. The degree of toxicity of the other plant extracts correlated well with the results obtained in the anti-MTB susceptibility test.

In general, all six extracts derived from the plants included in the present work were well tolerated in the mice toxicity test. One mouse inoculated with *Marchantia polymorpha* extract died on day 13 due to sepsis caused by perforation of the intestine during inoculation. Another mouse injected with *Callistemon citrinus* died on day 15 after experiencing loss of appetite and weight for three consecutive days. The result from the necropsy performed one day after this mouse was found dead showed an undetermined cause of death.

Discussion

In the course of our search for plants with anti-tuberculosis properties we surveyed the ethanolic extract of leaves from fifty local plants and found that six of these inhibited the growth of *Mycobacterium smegmatis* by the disk diffusion method. Subsequently, these same six plant extracts showed varying inhibitory patterns to the growth

of *MTB* using the BACTEC 460 system. The extracts were obtained from the following plants: *Syzygium jambos*, *Callistemon citrinus*, *Momordica charantia*, *Marchantia polymorpha*, *Mangifera indica* and *Mammea americana*. These six plant extracts were further characterized for toxicity and tolerance in biological systems to meet sound criteria of good antimicrobial agents.

The growth inhibitory effect of the six plant extracts tested against *M. smegmatis* in the agar disk diffusion method was shown to depend on the plant utilized and the concentration of the extract applied to the disk. The best results were obtained with *Callistemon citrinus* and *Mammea americana*, followed by the bryophyte *Marchantia polymorpha*. This inhibitory effect correlated positively with the toxic activity observed in the brine-shrimp test as was the case with *Callistemon citrinus* and *Mammea americana*. However, none of the extracts demonstrated potential toxicity to mammals as seen in the mice tolerance bioassay.

Similarly, the data obtained as a result of challenging *MTB* with the six plant extracts, indicate a highly diverse degree of susceptibility of this bacterium to the extracts employed. Again here, it can be stated that the susceptibility of this microorganism depended on the plant utilized, dose and time of exposure. The extract with the strongest anti-*MTB* activity was that obtained from *Mammea americana*. This extract was effective even when used at low concentration during the entire period of observation. We suggest that it is a potential bactericidal agent and that it could be as effective as streptomycin against *MTB*. On the other hand, we also detected anti *MTB* activity in the following plant extracts in decreasing order of magnitude: *Marchantia polymorpha*, *Mangifera indica*, *Callistemon citrinus* and *Syzygium jambos*. The transitory reduction, exerted by the extracts from *Callistemon citrinus* and *Marchantia polymorpha* on the growth rate of *M. tuberculosis*, suggests that these agents may possess potential bacteriostatic effect. In addition, the extracts from *Syzygium jambos*, *Momordica charantia* and *Mangifera indica* demonstrated potential bacteriostatic activity to *MTB* at a concentration of 250 µg.

Combined use of the two bioassays (shrimp toxicity and mice-tolerance) provided us with adequate information about the potential antimicrobial toxicological and tolerance properties of the plant extracts. The shrimp toxicity test has been employed successfully in other studies evaluating plant extracts (29).

The spectrum of susceptibility of *MTB* to the six plant extracts tested in this study verified the discriminatory capability of the laboratory methodology employed and confirmed its appropriateness reported in previous studies

(33,34,35). However, particular concerns have been expressed as to the use of BACTEC-460 for the study of plant extracts due to the costs, complexity and handling of radioactive biohazards. Mitscher and colleagues (1) propose a screening fluorescence method, based on transfection of luciferase genes into strains of recombinant *Bacillus Calmette et Guerin* (BCG) and *Mycobacterium intracellulare* as an alternative to the BACTEC 460. Whatever *in vitro* method is used to measure antimicrobial activity, the decision to include in clinical trials agents, such as those described in the present work, will require extensive experimental testing in infected animals. Currently, efforts are directed at purifying and characterizing the physical and chemical properties of the promising anti-mycobacteriological agents discovered in this study.

In summary, data reported in this study showing that six leaf extracts selected from 50 tropical plants and screened for their *M. smegmatis* growth inhibitory activity, presented strong susceptibility to anti-*MTB* activity. This anti-*MTB* effect was dependent on the species of the plant, doses used and time of exposure. The data presented here shows that: (a) all six extracts tested possess anti-*MTB* growth inhibitory capacity at concentration of 500 µg; (b) the *Mammea americana* extract yielded the best inhibitory effect, even at 50 µg; (c) this was followed in decreasing order by *Marchantia polymorpha*, *Mangifera indica*, *Callistemon citrinus*, *Syzygium jambos* and *Momordica charantia*; (d) the bactericidal inhibitory pattern on *MTB* growth displayed by *Mammea americana* extract was comparable to that of streptomycin; (e) the transitory reduction pattern on *MTB* growth produced by the extracts at a concentration of 100 µg and 250 µg of *Callistemon citrinus* and *Marchantia polymorpha*, suggests similarity to the growth inhibitory effects for known bacteriostatic agents; (f) the BACTEC-460 modified method is useful for *in-vitro* screening of plant extracts with potential anti-*MTB* activity.

Resumen

En este trabajo científico se reportan datos que demuestran que seis extractos seleccionados entre 50 plantas, las cuales fueron ensayadas preliminarmente para determinar su capacidad de inhibir el crecimiento de *Mycobacterium smegmatis*, presentaron actividad contra *Mycobacterium tuberculosis*. Este efecto contra el *M. tuberculosis* fue dependiente de la especie, dosis y tiempo de exposición al extracto utilizado. Se presenta evidencia de que: 1) los seis extractos de etanol a una concentración de 500 µg resultaron efectivos contra *M. tuberculosis*; 2) el extracto de *Mammea americana* resultó el más efectivo

a 50 µg; 3) en orden decreciente, el efecto inhibitorio de *Mammea americana* fue seguido en intensidad por *Marchantia polymorpha*, *Mangifera indica*, *Callistemon citrinus*, *Syzygium jambos* y *Momordica charantia*; 4) el patrón bactericida del extracto de *M. americana* sobre el crecimiento de *M. tuberculosis* es similar al del antibiótico estreptomycin; 5) el efecto inhibitorio transitorio desplegado por los extractos de *C. citrinus* y *M. polymorpha* es comparable al que se observa en agentes bacteriostáticos; 6) el método modificado BACTEC-460 resulta muy útil en la detección *in vitro* de actividad potencial anti-*M. tuberculosis* en extractos de plantas.

Acknowledgement

The authors gratefully acknowledge support from Inter American University of Puerto Rico, Metropolitan Campus through the Office of Chancellor, Manuel J. Fernós, J.D., and grants from NIH through NIGMS-MBRS (S06GM08159), NIGMS-MARC (T34GM 08462), and NCRR-RCMI (G12RR03034) facilities at Universidad Central del Caribe, School of Medicine. Acknowledgment is also extended to Virginia García, M.T. from CLENDO Reference Laboratory for technical assistance, Zilka M. Ríos Orraca for graphic work and secretarial assistance from María Rodríguez. Special appreciation to Dr. José Carrasco, Microbiologist at the UCC, School of Medicine, for reviewing the manuscript.

References

1. Mitscher LA, and Baker, W. Tuberculosis: A search for novel therapy starting with natural products. *Med Res Rev* 1998;18:1-17.
2. McCutcheon AR, Stokes RW, Thorson LM, Ellis SM, Hancock REW, Towers GHN. Anti-mycobacterial screening of British Columbian medicinal plants. *Int J Pharmacol* 1997;35:77-83.
3. Reyes J.C., Robles RR, Colón H, Marrero CA, Castillo X, Meléndez M. *Mycobacterium tuberculosis* infection among crack and injection drug users in San Juan, Puerto Rico. *P R Health Sci J* 1996;13:233-236.
4. Perlman DC, Salomon N, Perkins MP, Yancovitz S, Paone D, Des Jarlois DC. Tuberculosis in drug users. *Clin Infect Dis* 1995;21:1253-1264.
5. Cohn DL, Flavia B, Raviglione MC. Drug-resistant tuberculosis: Review of the worldwide situation and the WHO/UATLD global surveillance project. *CID* 1997; 24(Suppl 1): S-121-130.
6. Raviglione MC, Snider DE Jr., Kochi A. Global epidemiology of tuberculosis, morbidity and mortality of a worldwide epidemic. *JAMA* 1995;273:220-2206.
7. Jacobs WR Jr., Barletta RG, Udani R, Chan J, Kalkut G, Sosne G, Kieser T, Sarkis GJ, Hatfull GF, Bloom BR. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science* 1993;260:819-22.
8. Martin-Casabona N, Xairó Mimó D, González T, Roselló J, Arcalis L. Rapid Method for Testing Susceptibility of *Mycobacterium*

- tuberculosis* by Using DNA Probes. *J Clin Microbiol* 1997;35:2521-2525.
9. Turrett GS, Telzak EE, Torian LV, Blum S, Alland D, Weisfuse I., Fazel, BA. Improved outcomes for patients with multidrug-resistant tuberculosis. *Clin Infect Dis* 1995;21:1238-44.
 10. Lewis K, Hooper DC, Ouellete M. Multidrug resistant pumps provide broad defense. *ASM News* 1997;63:605-610.
 11. McChesney JD. The promise of plant-derived natural products for the development of new pharmaceuticals and agrochemicals. *Chemistry of the Amazon. ACS Symposium Series, American Chemical Society, D.C.* 1995.
 12. Pettit G. Progress in the discovery of biosynthetic anticancer drugs. *J Nat Prod* 1996;59:812-821
 13. Amorin CZ, Marquez D, Cordeiro RS. Screening of antimalarial activity of plants of the cucurbitaceae family. *Mem Inst Oswaldo-Cruz* 1991; Suppl 2:177-80.
 14. Suffness M. *Taxol: science and applications* (Suffness M, ed). CRC Press 1995.
 15. Kinghorn DA. *Anti-AIDS drug development: Challenges, strategies and prospects*. Mohan P. and Masanori B, eds. Langhorne, PA, Harwood Academic Publishers 1995.
 16. Tuberculosis Antimicrobial Acquisition Coordinating Facility (TAACF) NIAID. Southern Research Institute/GWL Hansens's Disease Center. Birmingham, AL, 35255-5305, 1998, Testing Center supported by NIH
 17. Towards a Caribbean Pharmacopoeia. Scientific Research and popular use of medicinal plants in the Caribbean. *Tramit 4 Workshop Tela, Honduras* 1989. Enda Caribe. National Autonomous University of Honduras. L Robineau, ed.
 18. Liogier HA. *Plantas medicinales de Puerto Rico y del Caribe*. 2nd. Ed. San Juan, Iberoamericano de Ediciones, Inc. 1990.
 19. Morton, JF. *Atlas of medicinal plants of middle America: Bahamas to Yucatan*. Springfield, IL, Charles C. Thomas Publisher 1981;3-1333.
 20. Núñez-Meléndez E. *Plantas medicinales de Puerto Rico*. San Juan, Editorial de la Universidad de Puerto Rico. 1982.
 21. Hernández L, Muñoz RA, Miró G, Martínez M, Silva-Parra J, Chavez P. Use of medicinal plants by ambulatory patients in Puerto Rico. *Amer J Hosp Pharm* 1984;41:2060-2064.
 22. Madigan MT, Martinko JM, Parker J, Brock. *Biology of microorganisms*. 8 th Ed. New York, Prentice Hall. 1997.
 23. Champoux JJ, Drew WL, Falkow S, Neidhardt FC, Plorde H, Ray CG. Spread and control of infection. In *Sherris Medical Microbiology: An introduction to infectious diseases* 3rd. Ed. Norwalk, Connecticut, Appleton and Lange 1994;169-221.
 24. Bauer AW, Kirby WMM, Sherris JC, Turk M. Antibiotic susceptibility testing by a standardized single disc method. *Amer J Clin Path* 1966;45:493-496.
 25. Tarrant J J, Groschel DHM. Evaluation of the Bactec radiometric method for detection of 1% resistant population of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1985;21:941-946.
 26. Rayner A, Harris G, Croughan M, Watt B. Use of a radiometric technique for rapid sensitivity testing of *Mycobacteria* in Scotland: The first year's experience, Published erratum appears in *Scott Med J* 1991;36:54-57.
 27. Luquin M, Gamboa F; Barcelo MG, Manterola JM, Matas L, Gimenez M, Ausina V. Comparison of a biphasic non-radiometric system with Lowenstein-Jensen and Bactec-460 system for recovery of mycobacteria from clinical specimens. *Tuber Lung Dis* 1996; 77:449-453.
 28. Siddiqi S. *Bactec TB system: Product and procedure manual*. Becton Dickinson Diagnostic Instrument System, Towson, MD, USA. 1989.
 29. Chavez PI, Sánchez LA, González FA, Rodríguez JI, Axelrod F. Cytotoxicity correlations of Puerto Rican plants using a simplified brine shrimp lethality screening procedure. *Int J Pharmacog* 1997;35:222-226.
 30. Meyer BN, Ferrigni NR, Putnam JE, et al. Brine shrimp: A convenient general bioassay for active plant constituents. *Planta Med* 1980; 45:31-34.
 31. Finney, DJ. *Probit Analysis*. 3rd. Ed. Cambridge, Cambridge University Press 1971.
 32. Guerrero RO and Robledo I. Endemic plants of Puerto Rico: Brine shrimp lethality and antibacterial activity. *P R Health Sci J* 1993;12:259-262
 33. Strand CL. Routine subcultures shown to be unnecessary in radiometric detection of bacteremia using three media. *Am J Clin Pathol* 1983;77:328-332
 34. Gangulio LA, Keaney MG, Hyde WA, Fraser SB. More rapid identification of bacteraemia by manual rather than radiometric method. *J Clin Pathol* 1985;38:1146-1149.
 35. Kyerematen GA, Ogunlana EO. An integrated approach to the pharmacological evaluation of traditional materia medica. *J Ethnopharmacol* 1987;20:191-207.
-
-