ORIGINAL ARTICLE

Anti-mycobacterial activity of root and leaf extracts of *Anthocleista djalonensis* (Loganiaceae) and *Diospyros mespiliformis* (Ebenaceae)

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We screened the aqueous and methanol leaf and root extracts of *Anthocleista djalonensis*, *Diospyros mespiliformis*, and their combinations for possible anti-mycobacterial activities using *Mycobacterium smegmatis* as a surrogate screen. These plants are reputed among folk practices as potent remedy in the management of tuberculosis and leprosy cases. In the sensitivity screening study, only the methanol extracts of *A. djalonensis* and *D. mespiliformis* showed anti-mycobacterial activity, while the aqueous extracts exhibited no inhibitory activity on *M. smegmatis*. The minimum inhibitory concentration (MIC) of the methanol leaf and root extract of *A. djalonensis* against *M. smegmatis* were 125 µg/ml. The MIC of the methanol leaf and root extracts of *D. mespiliformis* is 167 and 250 µg/ml, respectively. In the interaction studies, four out of nine decimal combinations of the two medicinal plant extracts exhibited synergism with fractional inhibitory concentration indices <1 and a negative activity index values. The 8:2 ratio of *D. mespiliformis* and *A. djalonensis* exhibited the greatest degree of antimycobacterial synergy against *M. smegmatis*. The result of this study supports the claims of efficacy reported in the folk use of these plants in mycobacterial infection and the plants could therefore be investigated further and harnessed as potent antimycobacterial agents.

Key words: *Anthocleista djalonensis*, checkerboard assay, *Diospyros mespiliformis*, isoniazid, leprosy, *Mycobacterium smegmatis*, tuberculosis

INTRODUCTION

It is believed that medicinal plants and plant-derived substances could be relied upon to provide solution to the problem of multi-drug resistant mycobacteria strains.[1] The potentials of phytomedicines as safe and efficacious therapy alternatives in the treatment of tuberculosis and leprosy have been identified.[2,3] The identification and isolation of novel compounds from plant sources would prove to be beneficial in the treatment of these diseases. Tuberculosis and leprosy are the most common and most deadly infectious diseases caused by *Mycobacterium tuberculosis* and *Mycobacterium leprae*, respectively. There are other species such as *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, and *Mycobacterium microti* that can also cause mycobacteria infections, especially tuberculosis, but these species do not usually infect healthy adults.[4]

Mycobacteria are intracellular parasites that are often resisted in immunocompetent individuals. Globally, an increased number of persons are contracting mycobaterial infections. Worldwide, ~1.6 billion are infected with tuberculosis, a mycobcterial infection of global health importance. Of these, perhaps only 15 million have active disease at any given time. Case rates may vary widely by country, age, race, sex, and socioeconomic status. Age has traditionally been considered an independent risk factor because the elderly have more years of potential exposure and are more likely to have impaired immunity. This phenomenal increase is due to their weak immune system compromised by stress, immunosuppressive drugs, substance abuse, or HIV/AID.[5,6] HIV infection is the greatest single medical risk factor because cell-mediated immunity, which is impaired by HIV, is essential for defense against mycobacterial infection; other immunosuppressive illnesses (e.g., diabetes) or therapies (e.g., corticosteroids) are risks but less so than HIV. The problem of multi-drug resistant strains of mycobacteria has made the search for more efficacious, safer, cheaper, and more accessible drugs a priority. The search for new and better anti-mycobacterial drugs has informed our present study of medicinal plants with folk reputations as effective alternative therapies for mycobacteria infections. Two of such plants are *Anthocleista djalonensis* (Loganiaceae) and *Diospyros mespiliformis* (Ebenaceae).

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A. djalonensis (Loganiaceae) is a medium-sized flowering plant growing up to 10-m tall and 12-cm wide, with grayish outer stem bark which are green below. The plant produces tibular white flowers in May and green smooth fruits in October/November. Previous studies showed that the cold water and ethanol extract of the roots have remarkable activities against *S*aphylococcus *aureus* and *Escherichia coli*.[7] The root decoction is also taken against chest pains for constipation, dysentery, and other gastrointestinal diseases [8]. Aqueous extracts of the leaves mixed with lemon juice is used by the Abros of Ghana to cure epilepsy.[9]

D. mespiliformis (Ebenaceae) commonly called African ebony or jackal tree is a tall, upright tree that can reach a height of 25 m, with a trunk circumference of >5 m. It has a dense evergreen canopy and a rough textured black to grey bark. Leaves are simple, alternate, leathery, and dark green. The fruit is a fleshy berry, with an enlarged calyx, yellow to orange when ripe.[10] It is found to be growing in woodlands, Savannas, along river banks and it is also found in Ethiopia, North to South Swaziland, Sudan, Tanzania, Uganda, Zimbabwe, and Senegal.[11] The plant has been shown to possess a number of medicinal uses; the leaves are used to treat fever, as wound dressings, and as an antidote for a variety of poisonous substances. The roots and bark are used to treat diseases such as malaria, syphilis, leprosy, and to stop purging. The antihelminthic and insecticidal properties have also been reported. The methanol extract of the leaves, stem, and roots have also been reported to show a broad spectrum of antibacterial activity.[12]

Prompted by their folk uses and claims of efficacy in mycobacterial infections, we investigated the aqueous and methanol leaf and root extracts of *A. djalonensis*, *D. mespiliformis*, and their combinations for anti-mycobacterial activities.

For this pilot screening study, we employed *M. smegmatis*, which has been shown to be a suitable surrogate screen for selecting compounds with potential activities against multi-drug resistant *M. tuberculosis*.[13,14]

MATERIALS AND METHODS

Preparation of Plant Materials

Fresh leaves and roots of *A. djalonensis* and D. mespiliformis were collected from Nsukka area, Enugu State, Nigeria, in April 2007. The plant materials were authenticated by Mr. A.O. Ozioko of the Bioresources Diversity and Plant Conservation Programme Center, Nsukka, Nigeria. The roots and leaves were cut into tiny pieces, dried under shade at ambient temperature, and then pulverized in a milling machine.

The root and leaf powder of *A. djalonensis* and *D. mespiliformis* were divided into two portions (200 g each) and then extracted with either hot water or methanol by maceration. A total of eight extracts were obtained and screened for anti-mycobacterial potentials-aqueous leaf extract of *A. djalonensis* (AdL/Aq), methanol leaf extract of *A. djalonensis* (AdL/MeOH), aqueous root extract of *A. djalonensis* (AdR/Aq), methanol root extract of *A. djalonensis* (AdR/MeOH), aqueous leaf extract of *D. mespiliformis* (DmL/Aq), methanol leaf extract of *D. mespiliformis* (DmL/MeOH), aqueous root extract of *D. mespiliformis* (DmR/Aq), methanol root extract of *D. mespiliformis* (DmR/MeOH). A stock solution (40 mg/ml) of aqueous and methanol extracts were prepared in water and in DMSO, respectively. Phytochemical screening of the dried powdered leaves and roots of *A. djalonensis* and *D. mespiliformis* were carried out for the presence of alkaloids, glycosides, tannins, saponins, flavonoids, terpenoids, steroids, reducing sugars, and resins using standard procedures outlined by Trease and Evans, 1989.[15]

Test Microorganisms

Mycobacterium smegmatis obtained from the Department of Veterinary Microbiology, University of Nigeria, Nsukka was used in the studies. The stock culture was maintained on nutrient agar slants at 4°C. Fresh subcultures were made in brain–heart infusion agar (Antex)® at 37°C for 18–24 hours as reported earlier.[16] Standard suspensions (10^5 cfu/ml) of the test organism was made by transferring colonies from the subculture into 5 ml of sterile nutrient broth (Antex)® and then adjusting and comparing with McFarland's 0.5 standard.

Anti-mycobacterial Screening of the Extract

The sensitivity of the test microorganism (*M. smegmatis*) to the various aqueous and root extracts (AdL/Aq, AdL/MeOH, AdR/Aq, AdR/MeOH, DmL/Aq, DmL/MeOH, DmR/Aq, and DmR/MeOH) was determined by the cup plate agar diffusion method.[17] A sterile cork borer of 8-mm diameter was used to bore holes into the seeded solidified nutrient agar. A 0.1 ml volume each of a 20 mg/ml dilution of each of the extracts was added into the labeled hole in the seeded agar plate using a sterile pipette. The test was performed in triplicate and the plates incubated at 37°C for 24 hours. Growth of *M. smegmatis* was observed after the incubation and the inhibition zone diameter was measured. The sensitivity of test microorganism to isoniazid and dimethylsulfoxide was also determined as standard and control treatments.

Determination of the Minimum Inhibitory Concentrations (MICs) of the Extracts

The MICs of the various aqueous and methanol root extracts (AdL/Aq, AdL/MeOH, AdR/Aq, AdR/MeOH, DmL/Aq,

DmL/MeOH, DmR/Aq, and DmR/MeOH) were determined separately against the test isolate using the agar-diffusion method described by Esimone *et al*. 2003. [18] Two-fold serial dilution of the stock solutions were prepared and introduced into each of 8-mm wells bored on the *M. smegmatis*-seeded nutrient agar plates and allowed to pre-diffuse. Thereafter, the plates were incubated at 37°C for 18–24 hours and the inhibition zone diameters (IZDs) surrounding each well was measured. Triplicates determinations were made and mean values obtained. The MICs were extrapolated from a linear regression plot of IZD^2 versus log concentartion values as previously described.[18]

Evaluation of the Combined Antimycobacterial Effect of the Extracts of *D. mespiliformis* and *A. djalonensis*

Based on the result of the prior sensitivity screening and MIC values previously obtained, the alcoholic root extracts of *D. mespiliformis* (Dm) and *A. djalonensis* (Ad) which showed the highest inhibitory effects were investigated for combined antimycobacterial activities against *M. smegmatis* using the continuous variation checkerboard protocol.[19,20] Stock solutions of the two agents were prepared and mixed in varying ratios ranging from 0:10 of Dm:Ad to 10:0 of Dm:Ad in accordance with the continuous variation checkerboard method. Each of the 11 combinations of these agents was serially diluted 2-fold in distilled water.

A standard inoculum of the test isolate (10^5 cfu/ml) was seeded into sterile molten nutrient agar and allowed to set. Holes of known diameter (7 mm) were bored in the nutrient agar plates and each of the dilutions introduced into the holes and incubated at 37°C for 24 hours. The IZDs were measured and used to estimate the MIC of extracts and their various combinations. The combined effect of the extracts of *D. mespiliformis* and *A. djalonensis* against *M. smegmatis* was determined by estimating the fractional inhibitory concentration (FIC) indices according to a relation previously reported:[18, 20]

RESULTS AND DISCUSSION

Mycobacterial infections, especially tuberculosis (TB) is believed to be a disease of antiquity, which is still a major global public health problem. Current estimates suggest that one-third of world's population is infected resulting in some 2 million deaths per year. Pulmonary TB, the most common type of the disease, is usually acquired by inhalation of the bacillus and causes irreversible lung destruction, although other organs are sometimes involved. The introduction, some 50 years ago, of the first drugs for TB treatment (streptomycin, para-aminosalicylic acid, isoniazid) led to optimism that the disease could be controlled if not eradicated. However, since the late 1980s there is resurgence in the disease driven by variety of changes in social, medical, and economic factors. Concomitant with the resurgence of TB has been the occurrence of multidrug-resistant mycobacterium strains MDR, which has exposed the limitations of the current drug armamentarium. These facts have made the development of indigenous therapeutic antimycobacterial agents, which are based on renewable, relatively inexpensive materials, as an urgent necessity.[6]

In our study of antimycobacterial effects of two medicinal plants, *A. djalonensis* and *D. mespiliformis*, the minimum inhibitory concentrations of the methanol leaf and root extract of *A. djalonensis* against *M. smegmatis* were 125 µg/ml. The MICs of the methanol leaf and root extracts of *D. mespiliformis* are 167 and 250 µg/ml, respectively [Table 1]. The blank control, dimethylsulfoxide, had no activity, while the standard control, isoniazide, had an MIC of 5 µg/ml. In the study, only the methanol extracts of *A. djalonensis* and *D. mespiliformis* showed antimycobacterial activity, while the aqueous extracts exhibited no inhibitory activity on *M. smegmatis*.

The alcoholic root extracts of the plants that showed the highest inhibitory activities against the test organism were investigated for their combined antimycobacterial effects using the continuous checkerboard technique. The FIC index cut-off limits were used for the evaluation of interaction according to reported recommendations.[19,20] FIC_{index} values <1 were considered as synergy and the degree of synergy increases as the value tends toward zero. FIC-index values of 1 show additivity, values >1, but <2 represent indifference while values >2 represent antagonism.

In the interaction studies, four out of nine decimal combinations of the two medicinal plant extracts exhibited synergism with FIC indices <1 and a negative activity index values [Table 2]. The ratio (8:2) of *D. mespiliformis* and *A.*

Table 1: Sensitivity of *Mycobacterium smegmatis* to aqueous and methanol leaf and root extracts of *A. djalonensis* and *D. mespiliformis*

Medicinal plant	Extract	Inhibition zone diameter of 20 mg/ml extract (IZD±SEM)	Minimum inhibitory concentration (MIC in µg/ml)
A. djalonensis	AdL/Aq	- (NS)	-
	AdL/MeOH	13.0 ± 0.50	125
	AdR/Aq	- (NS)	-
	AdR/MeOH	16.0 ± 0.15	125
D. mespiliformis	*DmL/Aq*	- (NS)	-
	DmL/MeOH	12.0 ± 0.35	167
	DmR/Aq	- (NS)	-
	DmR/MeOH	21.3 ± 0.50	250
Standard	Isoniazid	13.0 ± 0.60	5.0
Blank control	DMSO	- (NS)	-

N = 3; NS = not sensitive.

Table 2: The combined effect of the alcoholic extract of *Diospyros mespiliformis* and *Anthocleista djalonensis* against *Mycobacterium smegmatis*

Combination ratio (Dm: Ad)	MIC of Dm (mg/ml)	MIC of Ad (mg/ml)	FIC of Dm	FIC of Ad	FIC index	Activity index (A.I.)	Effect
10:0	2.50	-	-	-	-	-	-
9:1	1.49	0.17	0.59	0.40	0.99	- 0.004	SYN
8:2	0.33	0.08	0.13	0.19	0.32	- 0.495	SYN
7:3	0.29	0.12	0.11	0.28	0.39	- 0.410	SYN
6:4	0.17	0.25	0.06	0.59	0.65	- 0.190	SYN
5:5	0.42	0.42	0.16	1.00	1.16	0.064	IND
4:6	-	-	-	-	-	-	-
3:7	-	-	-	-	-	-	-
2:8	-	-	-	-	-	-	-
1:9	0.33	0.49	0.13	1.16	1.29	0.111	IND
0:10	-	0.42	-	-	-	-	-

Dm=*Diospyros mespiliformis*; Ad = *Anthocleista djalonensis;* MIC=minimum inhibitory concentration; SYN=synergism; IND=indifference; = the absence of mycobacterial growth at tested extract concentration.

djalonensis exhibited the greatest degree of antimycobacterial synergy against *M. smegmatis*.

The inhibitory activity shown by the extracts were quite comparable to those of a standard anti-TB drug, isoniazide. The use of combined antimycobacterial drugs is believed to be very crucial in overcoming the problems of multidrug resistant (MDR) strains of mycobacteria.[6] In this study, we have evaluated the two medicinal plants for *in vitro* antimycobacterial activities and their combined antimycobacterial activities. This study indicates that the combinations of *D. mespiliformis* and *A. djalonensis* root extract at the ratio of 8:2 could be of possible clinical benefit in the treatment of mycobacterial infections.

The results of the phytochemical studies showed the strong presence of alkaloids, glycosides, saponins, and tannins in both the leaves and roots of *D. mespiliformis* and the presence of glycosides, terpenoids, steroids, reducing sugars, and resins in both the leaves and roots of *A. djalonensis*. A further study is needed to associate the antimycobacterial activities of these plants with any of these phytoconstituents.

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