Journal of Biology, Agriculture and Healthcare www.iiste.org
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.4, No.12, 2014

Extracts of *Zingiber officinale* Rosc. (Ginger) and *Curcuma longa* Linn. (Turmeric) Rhizomes inhibited Nontuberculous Mycobacteria *in vitro*

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Abstract
Non-tuberculous mycobacteria (NTM) are all the other mycobacteria which can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease. The antimycobacterial activity of methanol, *n*-hexane and dichloromethane extracts of *Zingiber officinale* Rosc. (Ginger) rhizome and extract of *Curcuma longa* Linn. (Turmeric) were evaluated against four (4) species of nontuberculous Mycobacteria: *Mycobacterium abscessus*, *Mycobacterium smegmatis* ATCC 19420, *Mycobacterium phlei* ATCC 19240 *and Mycobacterium fortuitum* 684. Susceptibility tests were carried out using the agar well diffusion techniques while agar dilution method was used to determine the minimum inhibitory concentration (MIC). Bactericidal activities of bioactive extracts were evaluated by the viable counting techniques. Dichloromethane extract of *Zingiber officinale* showed the highest inhibition zone of 16 ± 0.5 and 22 ± 0.00 mm for *M. abscessus and M. fortuitum* ATCC 684 respectively; and the MIC and MBC for both species of organism were 6.25 mg/mL and 75 mg/mL. The *n*-hexane and methanol fractions of *Zingiber officinale* had no activity against tested *Mycobacteria species*. Methanol and *n*-hexane extracts of *Curcuma longa* both showed the highest inhibition zone of 16 ±0.5 and 20 ±0.0 mm respectively for *M. abscessus* while the MIC and MBC for both extracts were 25 mg/mL and 75 mg/mL respectively. The kill kinetics of the two plants against susceptible test organisms indicated a dose-dependent bactericidal activity, with decline in population (> 50%) within 4 hours of exposure followed by a total kill (100%) of the population at 8 hours exposure time at doses equivalent to 4 x MIC and 8 x MIC. Thus, it is concluded that this plants may contain compounds with therapeutic activity that could be used in treatment of diseases originating from non-tuberculous mycobacteria infection.
Keyword: *Zingiber officinale* Rosc. (Ginger), *Curcuma longa* Linn. (Turmeric), Nontuberculous Mycobacteria, Antimycobacterial activity, *In vitro*

1. Introduction

Nontuberculous mycobacteria (NTM) species, also known as environmental mycobacteria, atypical mycobacteria (Griffith *et al.,* 2007) and mycobacteria other than tuberculosis (MOTT), are mycobacteria which do not cause tuberculosis or Hansen's disease (also known as leprosy). Nontuberculous mycobacteria are widely distributed in the environment, particularly in wet soil, marshland, streams, rivers and estuaries. Different species of NTM prefer different types of environment (Grange, 2007). Human disease is believed to be acquired from environmental exposures, and unlike tuberculosis and leprosy, there has been no evidence of animal-to-human or human-to-human transmission of NTM, hence the alternative label "environmental bacteria" (ATS, 1997). Nontuberculous mycobacteria diseases have been seen in most industrialized countries, where incidence rates vary from 1.0 to 1.8 cases per 100,000 persons. Recent studies, including one done in Ontario, Canada, suggest that incidence is much higher (Marras *et al*, 2013). Pulmonary NTM is estimated by some experts in the field to be at least ten times more common than TB in the U.S., with at least 150,000 cases per year (Griffith *et al.,* 2007). Most NTM disease cases involve the species *Mycobacterium avium* complex (MAC), *M. abscessus*, *M. fortuitum* and *M. kansasii*. *Mycobacterium abscessus* is being seen with increasing frequency and is particularly difficult to treat (ATS, 1997). Rapidly growing NTMs are implicated in catheter infections, post-LASIK, skin and soft tissue (especially post-cosmetic surgery) and pulmonary infections (Nash *et al*., 2009). Nontuberculous mycobacteria have been observed to cause pulmonary disease that is very similar to *Mycobacterium tuberculosis* especially when such cases showed no clinical improvement to conventional anti-tuberculosis (TB) treatment in addition to isolation of the NTM organism during culture. The diagnosis of NTM is not possible using acid fast bacillus (AFB) microscopy which is the main stay of TB diagnosis in several developing countries. As a result, many of the pulmonary diseases caused by NTM are not identified but rather treated with conventional anti-TB treatment which eventually fails because majority of the NTM are resistant to conventional anti-TB treatment (Park *et al.,* 2010). The prevalence of NTM and NTM-associated hospitalization has been on the increase in several industrialized countries (Gopinath *et al.,* 2008). Some of the countries report a NTM prevalence rate as high as 50% among cultured mycobacteria (Park *et al.,* 2010). However, there is paucity of data from developing countries largely due to the lack of laboratory infrastructure for culture and specie identification. The increasing frequency of pathogenic NTM has become important especially with the advent of HIV/AIDS. It has been observed that HIV/AIDS patients with severe immunosuppression are at risk of NTM which can cause localized or disseminated infections as in *Mycobacterium*

intracellulare (Park *et al.,* 2010). The main reason for eradication failure can be NTM resistance to some of the antibiotics used for treatment. Also chemical drugs have side effects such as diarrhoea and colitis. Therefore it is necessary to introduce alternative remedial regimens. One of these resources is medicinal plants, the therapeutic properties of some of which have been recognized in traditional medicine. Most people have positive attitude toward natural products due to their natural origin and lesser toxicity (Naik *et al.,* 2003). Study of anti-*Mycobacterial* effects of these plants, especially those that are traditionally used for pulmonary infections are important. The present study was carried out to evaluate *in vitro* anti-Mycobacterial activity of some spices of medicinal plants [*Zingiber officinale* Rosc. and *Curcuma longa* Linn. (Zingiberaceae)] that have been traditionally used in folk medicine.

2. Materials and Methods

2.1 Plant collection, extraction, and preparation of extracts

Edible rhizome of *Zingiber officinale* Rosc (Ginger) and *Curcuma longa* Linn. (Turmeric) were purchased from Bodija Market, Ibadan, Oyo State, South–West of Nigeria; between the months of April 2012 and May 2012. They were identified and authenticated by a botanist at the Department of Botany and Microbiology, University of Ibadan, Oyo State. Voucher specimens were deposited at the Herbarium for reference and plant samples were assigned voucher specimen number UIH 22362 for *Z. officinale* and UIH 22363 for *C. longa*. The rhizomes were dusted and air dried at room temperature for 5 to 7 weeks and then grounded to coarse powder using a dry electric mill (Moulinex, France). The pulverized plants material (1.8 kg of *Z. officinale*) and (2.3 kg of *C. longa*) were subjected to exhaustive Soxhlet extraction with *n*-hexane, dichloromethane and methanol in succession. Extracts were collected, dried under reduced pressure (lyophilized) at International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria. The lyophilized extracts were weighed and stored at 4 ℃ before use. Stock solutions of lyophilized extracts were reconstituted in 20% ethanol with final concentrations of 20 to 100 mg/mL prepared for the initial screening. Concentrations in the range of 6.25 and 200 mg/mL were also prepared to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the bioactive extracts.

2.2 Antimicrobial Agents

The chemotherapeutic agents used in the test as positive control was Rifampicin (Nicholas Laboratories Limited, England), while the negative control was 20% Ethanol.

2.3 Phytochemical screening

Phytochemical screening was carried out to detect the presence of secondary metabolites such as anthraquinones, tannins, saponins, alkaloids, and phenol using methods described by Harborne (1991).

2.4 Strains of Nontuberculous Mycobacteria (NTM)

Four (4) non-tuberculous mycobacteria isolates used for this investigation were *Mycobacterium abscessus*, *Mycobacterium smegmatis* ATCC 19420, *Mycobacterium phlei* ATCC 19240 *and Mycobacterium fortuitum 684*.

2.5 Susceptibility testing

Susceptibility was determined using the agar cup diffusion technique. A 0.1 mL aliquot of logarithmic phase broth culture of each bacterium (optical density equivalent to 10^7-10^8 cfu/mL) was used to seed sterile molten Mueller-Hinton agar (Oxoid) medium maintained at 45 ℃ and supplemented with 5% sterile horse blood. The seeded plates were allowed to dry in a sterile incubator at 37 ℃ for 20 min. A standard cork borer (8 mm diameter) was used to cut uniform wells on the surface of the agar, into which was added 100 μL of the test extracts reconstituted in 20% ethanol. A pre-incubation diffusion of the extracts into the seeded medium was allowed for 1 hr. Plates were incubated at 37 ℃ for 2-3 days after which diameters of zones of inhibition (mm) were measured. 20% ethanol was included in each plate as a solvent control while Rifampicin (10 μg/mL) was used as positive control. This method is similar to previous published procedures (Adeniyi *et al*., 2006).

2.6 Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of bioactive extracts were determined by a modification of standard agar dilution method procedures as previously described (Adeniyi *et al*., 2009). Extracts were tested at various concentrations. The positive control antibiotic included was rifampicin. The MICs were determined after 2-3 days of incubation at 37 ℃. The MIC was regarded as the lowest concentration that prevented visible growth of test organisms from a duplicate experiment.

2.7 Determination of minimum bactericidal concentrations

Minimum bactericidal concentration (MBC) of active plant extracts was determined by a modification of the method of Aibinu *et al.,* (2007). To a 0.5 mL extract at different concentration as used in the MIC assay that showed no visible growth on the agar plates, was added 0.5 mL of test organism in a tube. These were incubated at 37°C for about 24-48 hours. Samples were streaked out from the tubes on the surface of sterile extract-free Mueller-Hinton agar (Oxoid) medium supplemented with 5% sterile horse blood in Petri dishes to determine the minimum concentration of the extract required to kill the

organisms. These concentrations were indicated by the inability of the organisms to grow on transfer to the extract-free agar. The lowest concentration that prevented bacterial growth after 48 hr of incubation was recorded as the minimum bactericidal concentration (MBC). The entire tests were carried out in duplicates to ensure accuracy. Agar plates without extracts and another agar plate without any inoculated organism were also incubated to serve as organism and extract control plates respectively. Minimum bactericidal concentration (MBC) was also determined for the drug control.

2.8 Determination of bactericidal activity of the methanol extract of Z. officinale and C. longa

The viable counting technique was employed for this purpose (Lajubutu *et al.,* 1995). An overnight broth culture in 4.5 mL of Tryptic Soy broth inoculated in a static growth condition of each organism was made. *Mycobacterium fortuitum* ATCC 684 and *M. abscessus* were used for this experiment. A 0.5 mL of each culture was sub-cultured into a warm (37 ℃) 4.5 mL Tryptic Soy broth and incubated for 90 min using a Gallenkamp orbital incubator to give a logarithmic phase culture. A 0.1 mL of the logarithmic phase culture was then inoculated into a warm 4.9 mL of Tryptic Soy broth containing the test compound to give 1 in 50 dilution of the culture (equivalent to approximately 1×10^7 colony forming units) and the required concentration of the extract. An appropriate quantity of the test sample (extract-culture mixture) was withdrawn immediately, diluted out in Tryptic Soy broth and 0.2 mL of 1:1000 dilution plated on an oven dried Mueller-Hinton agar supplemented with 5% sterile horse blood in Petri dishes to give control time 0 minute count. Samples were taken at 30 min, 1, 2, 4, 8 and 24 h. The procedure was carried out in duplicate. Plates were incubated at 37 ℃ for 24 h before counting the colonies. Control plates for negative and positive controls were also incubated. The number of colony forming unit (cfu) were counted after the period of incubation. The numbers of surviving bacterial cells per ml were calculated by taking into consideration the dilution factor and the volume of the inoculum. All the procedure was repeated for MIC, 2 × MIC, 4 × MIC and 8 × MIC. A graph of percentage viable count against time in hour was plotted to show the rate of kill of the test organisms after duplicate experiments.

3. Results and Discussion

The anti-Mycobacteria activity of extracts of *Zingiber officinale* Rosc.(Ginger) and *Curcuma longa* Linn. (Turmeric) rhizomes on nontuberculous mycobacteria were evaluated. The yield and macroscopic characteristics of *Zingiber officinale* Rosc. (Ginger) and *Curcuma longa* Linn. (Turmeric) rhizomes are presented in Table 1. The percentage yield of the methanol extracts of the dried rhizome of *Zingiber officinale* Rosc. (Ginger) and the dried rhizome of *Curcuma longa* Linn. (turmeric) was highest. This is contrary to the report of Cowan (1999) that ranked methanol second next to dichloromethane in terms of yield in extraction of plant active components. Phytochemical screening for secondary metabolites revealed the presence of high concentration of alkaloids and tannins as well as a moderate amount of flavonoids in *Zingiber officinale* Rosc. (Ginger) rhizome while saponins, anthraquinones and phenols were not detected. High concentrations of tannins and anthraquinones and a moderate amount of saponins and phenols were present in *Curcuma longa* Linn. (Turmeric) rhizome. Alkaloids and flavonoids were absent. These various plant metabolites have earlier been reported to possess medicinal, antimicrobial and physiological activities (Shula & Singh, 2007; Surh *et al*., 1998; Surh 2002). The presence of these secondary metabolites could be the reasons for the observed antimicrobial activities of these plants. Many phytomedicines exert their effects through the additive or synergistic action of several compounds acting at a single or multiple target sites associated with physiological process (Tyler, 1999). The results of the anti-*Mycobacteria* screening of the extracts by the use of agar well diffusion technique are presented in Tables 2. The zone of inhibition as presented in Table 2, clearly indicated that dichloromethane extract of *Zingiber officinale* Rosc. (ginger) had the highest inhibitory activity as indicated for *Mycobacterium fortuitum* ATCC 684 and *M. abscessus* with zones of inhibition of 22 ±0.0 mm and 16 ±0.5 mm respectively at a concentration of 100 mg/mL. *Mycobacterium abscessus* was susceptible to methanol and *n*-hexane extracts of *Curcuma longa* Linn. (turmeric) as well as the dichloromethane extract of *Zingiber officinale* Rosc. (ginger) at the test concentrations. *Mycobacterium smegmatis* ATCC 19420 and *M. phlei* ATCC 19240 were not susceptible to extracts of both ginger and turmeric. Dichloromethane extract of *Curcuma longa* Linn. (turmeric) as well as methanol and *n*-hexane extracts *Zingiber officinale* Rosc. (ginger) had no antibacterial activity on the test organisms, and so the results were not tabulated. The factors responsible for the high susceptibility of *M. fortuitum* ATCC 684 and *M. abscessus* to ginger extracts are not exactly known but may be attributed to the secondary metabolites (inhibins) and phytochemicals (gingerol and shagelol, flavonoids) isolated from ginger (Stewart *et al*., 1991). The results obtained in this study agree with that of Ficker *et al.,* (2003), Grange and Davey (1990) and Zahra *et al*., (2009).

The minimum inhibitory concentration (MICs) and minimum bactericidal concentration (MBCs) of the bioactive extracts against the susceptible non-tuberculous mycobacteria species are summarized in Table 3. The MIC, MBC and kill kinetics of methanol and *n*-hexane extracts of *Curcuma longa* Linn. (turmeric); and dichloromethane extracts of *Zingiber officinale* Rosc. (ginger) rhizomes were determined using *M. fortuitum* ATCC 684 and *M. abscessus.* The MIC values confirmed the existence of inhibitory effects of *Zingiber officinale* Rosc. (ginger) and *Curcuma longa* Linn. (turmeric) dried rhizome with MIC values ranging from 6.25 mg/mL to 25 mg/mL for both extracts on selected susceptible species except *M. smegmatis* ATCC 19420

and *M. phlei* ATCC 19240 which were not susceptible to the extracts at the tested concentrations. The MICs of dichloromethane extract of *Zingiber officinale* Rosc. (Ginger) on the entire test *Mycobacterium species* in Table 3 were observed to be lower than that of *Curcuma longa* Linn. (turmeric) extracts. The observed results revealed the presence of more potent antibacterial compounds in *Zingiber officinale* Rosc. (Ginger) than in *Curcuma longa* Linn. (turmeric). This may be attributed to the fact that the two plants though in the same family belong to different genus and also possess different phytochemical components to which the observed anti-mycobacterial activity had been attributed.

The time-kill study of the extracts of *Zingiber officinale* Rosc. (Ginger) and *Curcuma longa* Linn. (Turmeric) dried rhizome on *M. fortuitum* ATCC 684 and *M. abscessus* as shown in figures 1- 4 revealed a dose-dependent bactericidal activity, with decline in population after 4 hours of exposure to the extracts at doses equivalent to MIC, 2 x MIC, 4 x MIC and 8 x MIC followed by a total kill of the population at 8 hours of exposure to concentrations equivalent to 4 x MIC and 8 x MIC, and at 24 hours of exposure to concentrations equivalent to MIC and 2 x MIC. The higher kill rate by the extract at higher concentration (8 x MIC) observed, suggested resistance of the *Mycobacterium species* to lower concentrations. This observation is supported by the reports of Yang *et al.*, (2003) who noted that nontuberculous mycobacteria are usually more resistant to antimycobacterial agents. This was also observed during the susceptibility testing where the test organisms were resistant to low concentration (5 μg/mL) of Rifampicin. The bactericidal activity was observed to be dependent on time and dose/concentration as the percentage reduction in viable count of surviving population increased with increase in exposure time and concentration of the extracts. This is similar to previous kinetics study (Funatogawa *et al*., 2004). Nontuberculous Mycobacteria infection (NTM) is said to be opportunistic in nature, infecting both humans and animals. Infection is acquired by consuming water, inhaling aerosols, or through penetrating injuries of the skin. Three principal classes of disease due to NTM are lymphadenitis, post-inoculation lesions and pulmonary disease. There have been few major controlled clinical trials of the therapy of pulmonary disease due to NTMs. Those that have been conducted have been based on triple regimens of rifampicin, isoniazid, and ethambutol (BTS 2000). However, these regimens may have side effects, poor compliance, and antibiotic resistance. Therefore, alternative antimicrobial agents sourced from natural products such as *Zingiber officinale* Rosc. (ginger) and *Curcuma longa* Linn. (turmeric) rhizomes with fewer disadvantages are necessary for the treatment of nontuberculous Mycobacteria infection in developing countries, especially as they are edible and readily available.

Table 1: Yield and Macroscopic Characteristics of *Zingiber officinale* Rosc. (Ginger) and *Curcuma longa* Linn. (Turmeric)

Name of Plant	Plant Part	Solvent Used	Weight of sample extracted (g)	Yield of extract (g)	Percentage yield of extract (%)	Macroscopic Characteristics of extract
Zingiber officinale Rosc. (Ginger)	Rhizome	Methanol	2290.92	107.60	4.70	Dark-brown solid
		Dichloro-methane	2290.92	37.00	1.62	Black Semi-solid
		n-Hexane	2290.92	32.86	1.43	Dark-brown semi solid
Curcuma longa Linn. (Turmeric)	Rhizome	Methanol	1819.20	70.40	3.87	Brown coloured solid
		Dichloro-methane	1819.20	57.73	3.17	Dark-brown solid
		n-Hexane	1819.20	66.97	3.68	Reddish-brown semi-solid

Table 2: Antimicrobial susceptibility of *Mycobacteria species* to lyophilized extracts of *Zingiber officinale* Rosc. (Ginger) and *Curcuma longa* Linn. (Turmeric). Mean diameter of zone of inhibition (mm) ± SEM.

	Methanol extract of *Curcuma longa* (Turmeric) (mg/mL)		*n*-Hexane extract of *Curcuma longa* (Turmeric)		Dichloromethane extract of *Z. officinale* (Ginger) (mg/mL)		Rifampicin (µg/mL)
Mycobacteria species ↓	20	100	20	100	20	100	10
M. fortuitum ATCC 684	-	-	-	-	19±0.0	22±0.0	24±0.5
M. smegmatis ATCC 19420	-	-	-	-	-	-	26±0.0
M. phlei ATCC 19240	-	-	-	-	-	-	30±0.0
M. abscessus	15±0.5	16±0.5	15±0.0	20±0.0	15±0.0	16±0.5	28±0.0

*Result is average of duplicate experiment; - = No activity (Resistant), Diameter of cork borer = 8 mm

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of lyophilized methanol extracts of *Zingiber officinale* Rosc. (Ginger) and *Curcuma longa* Linn. (Turmeric) on susceptible *Mycobacteria species*

	Methanol extract of *C. longa* (mg/mL)		*n*-Hexane extract of *C. longa* (mg/mL)		Dichloromethane extract of *Z. officinale* (mg/mL)		Rifampicin (µg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
M. fortuitum ATCC 684	ND	ND	ND	ND	6.25	75	6.25	6.25
M. abscessus	25	75	25	75	6.25	75	6.25	6.25

Note: Result is average of duplicate experiment; ND: Not determined

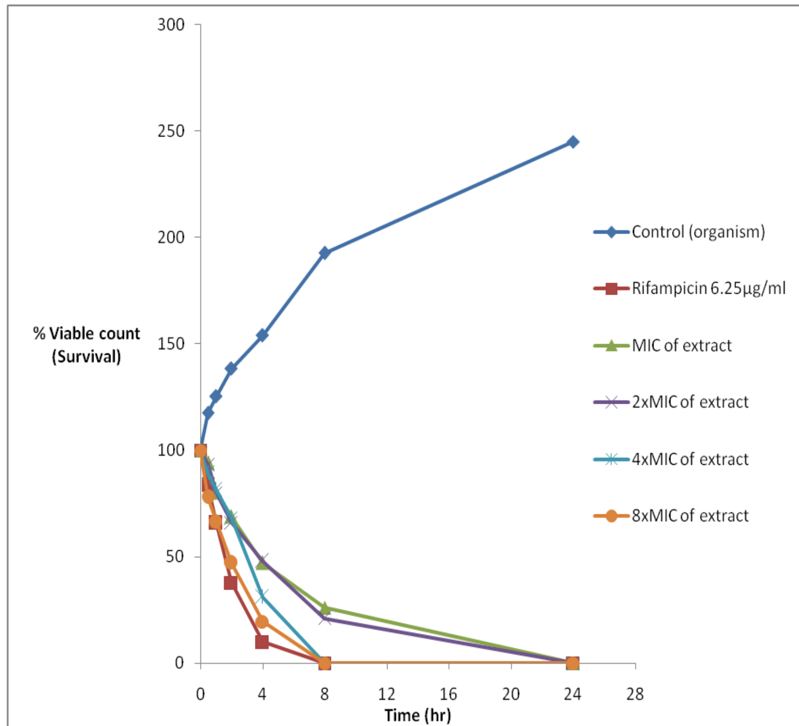

Fig. 1: Percentage viable count (Survival) vs Time (hr) of dichloromethane extract of *Zingiber officinale* Rosc. (Ginger) on *Mycobacterium fortuitum* ATCC 684 showing the rate of kill of the organism at the different concentrations of the extract.

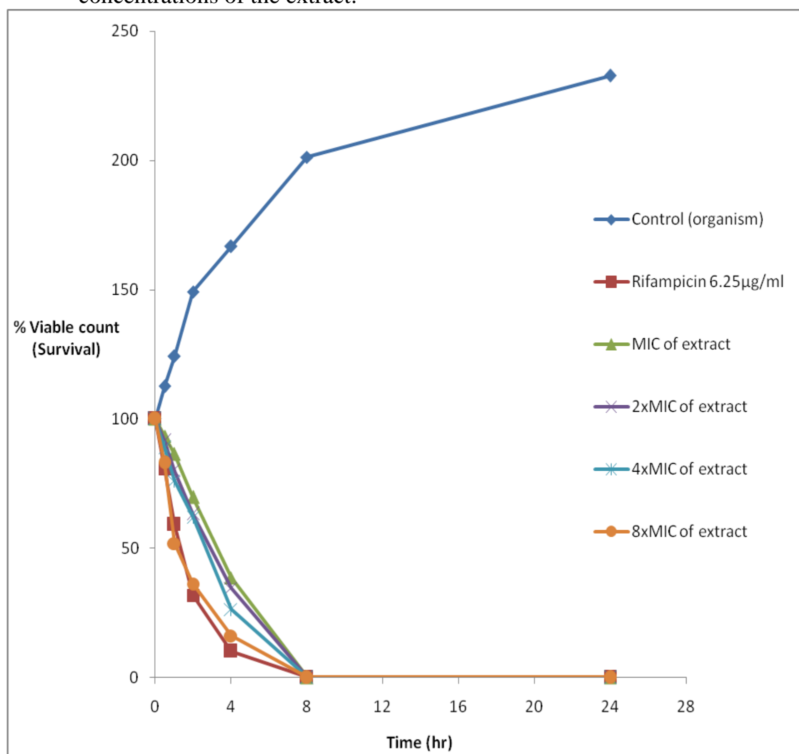

Fig. 2: Percentage viable count (Survival) vs Time (hr) of dichloromethane extract of *Zingiber officinale* Rosc. (Ginger) on *Mycobacterium abscessus* showing the rate of kill of the organism at the different concentrations of the extract.

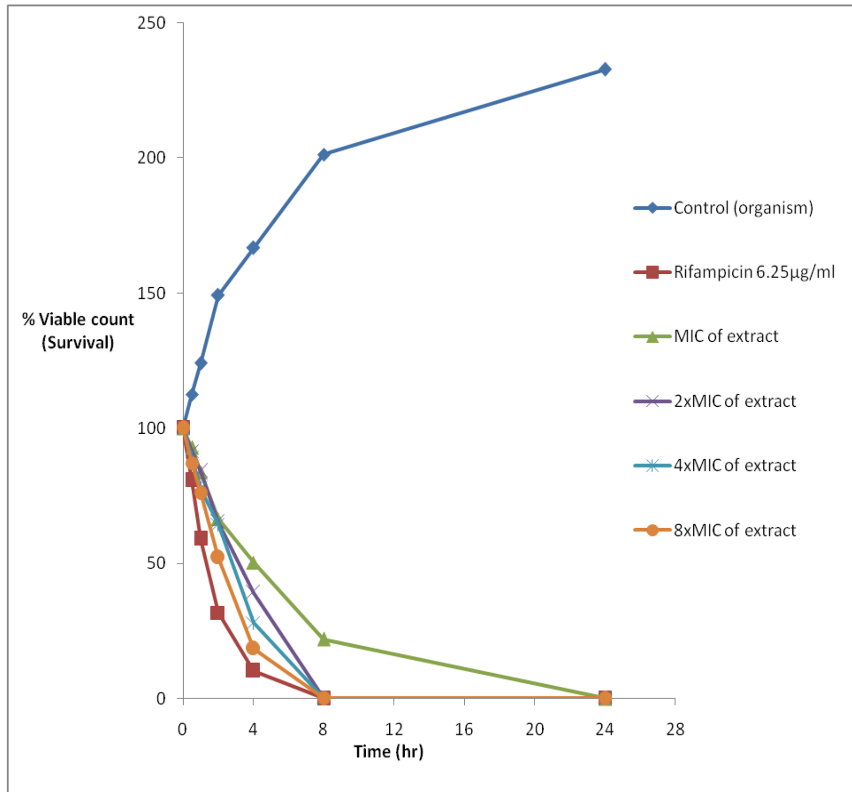

Fig. 3: Percentage viable count (Survival) vs Time (hr) of *n*-hexane extract of *Curcuma longa* Linn. (Turmeric) on *Mycobacterium abscessus* showing the rate of kill of the organism at the different concentrations of the extract.

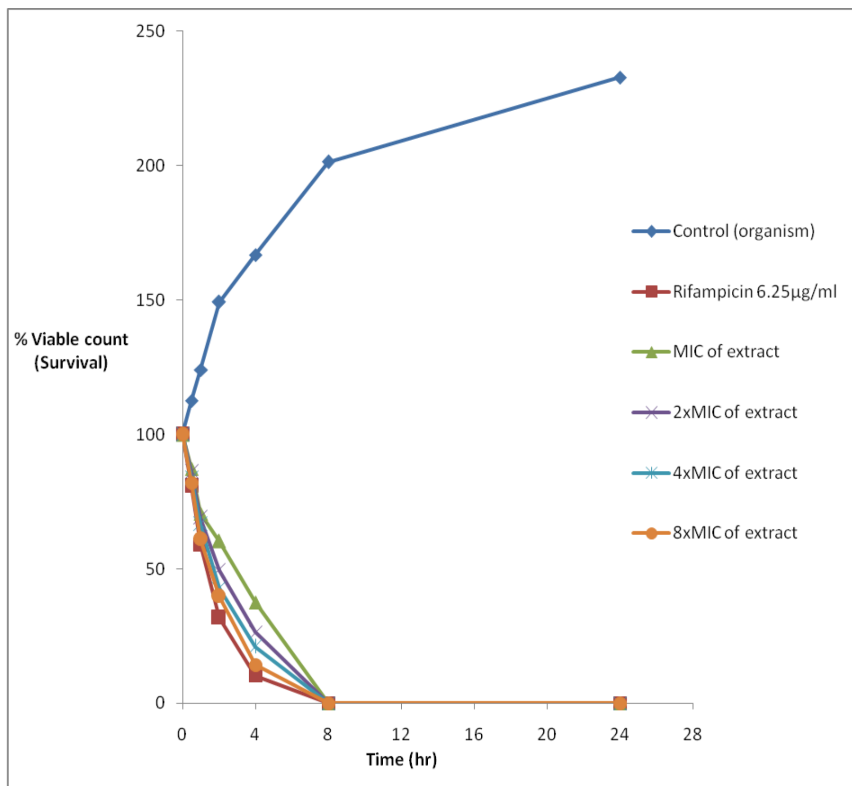

Fig. 4: Percentage viable count (Survival) vs Time (hr) of methanol extract of *Curcuma longa* Linn. (Turmeric) on *Mycobacterium abscessus* showing the rate of kill of the organism at the different concentrations of the extract

4. Conclusion

The antimycobacterial activities of *Zingiber officinale* Rosc. (ginger) and *Curcuma longa* Linn. (turmeric) were investigated on selected nontuberculous mycobacteria and the study revealed the antimycobacterial effects of ginger and turmeric on some of the test organisms. Although the active components in the ginger and turmeric samples were not isolated and characterized in this study, the results showed that the plant samples tested exhibited a high degree of antimycobacterial activity on the susceptible test microbes. It can be concluded that these plants possess bioactive compounds with good antimycobacterial properties that may be of therapeutic importance in the treatment of infections caused by the test organisms.

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