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THE ANTI-MYCOBACTERIAL ACTIVITY AND SAFETY PROFILE OF SELECTED *Crinum* SPECIES IN NORTHWESTERN UGANDA.

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Abstract

Crinum species is a source of many bioactive molecules with both antimicrobial and anti-tumor properties. Chloroform, methanol and aqueous extracts of *Crinum scabrum* and *Crinum macowanii* bulbs and leaves were investigated for their anti-mycobacterial activity against *Mycobacteria tuberculosis* using agar well diffusion, and broth dilution methods. Rifampicin-streptomycin resistant (R.S), pan African sensitive (H37Rv) and wild type (Sou 14827) strains of the bacteria were used.

The investigation aimed to determine the anti-mycobacterial activity and safety profile of the crude extracts of *Crinum scabrum* and *Crinum macowanii*. The extracts had antimycobacterial activity that ranged between 1-5µg/ml. Their patterns of inhibition varied with the plant extract, solvent used for extraction and the organisms tested. Different concentrations of methanol extract were compared with similar concentrations of chloroform and aqueous extracts for their maximum zones of inhibition. All the extracts of *Crinum macowanii* were found inactive against *Mycobacteria tuberculosis*. Methanol leaf extract of *Crinum scabrum* was the most active, with minimum inhibitory concentration (MIC) less than1µg/ml and minimum bactericidal concentration (MBC) of 1µg/ml. acute toxicity test in mice for *Crinum scabrum* leaf was found to have LD50 greater than 2000mg/kg.

In conclusion, the leaf of *Crinum scabrum* is a natural source of new anti-mycobacterial compound that is tolerable and effective in treatment of tuberculosis. The study recommends herbalists to use the leaves instead of bulb of *C. scabrum* Alani Davis for treating TB patients. In future, a comparative study should be done on activity of crude methanol leaf extract of *C. scabrum* and combination of the drugs used to treat multidrug resistant MTB. The active principles in the leaves of *C. scabrum* Alani Davis should also be isolated, identified and tested on strains of *Mycobacteria tuberculosis* resistant to at least two or three drugs.

Key words: Tuberculosis, *Crinum scabrum*, *Crinum macowanii*, MIC, MBC and LD_{50}

Background

Tuberculosis is a common health problem in Uganda with incidence rate of 193 per 100,000 people and overall mortality rate of 14 per 100,000 persons (WHO 2012).

The problem has been exacerbated by the HIV/AIDS epidemic where 2/3 of the people living with HIV/AIDS also have tuberculosis (TB). The country is ranked 14th among the 22 countries

that are world's TB hot spots in which 0.5% of new cases reported are due to the Multi-drug resistant tuberculosis strain (MDR.TB), (WHO 2010).
Chemotherapy is currently the mainstay in treatment of TB. However, evolution of extensively drug-resistant tuberculosis strains (XDR.TB.) has led to treatment failures and threatened to make TB incurable. Adverse reactions of the drugs, cumbersome nature of the regimen have worsened the problem. There is need for an alternative effective drug. In rural areas many people use herbs with no data on efficacy and safety to manage TB.
Plants like *Allium sativum* (garlic), *Crinum jagus and lantana camara* are traditionally used to treat microbial infections in most African communities (Refaat et al 2013; Kirimuhuzya, et al 2009; Ashwani Kumar 2009). They contain phytochemicals that include alkaloids, glycoalkaloids, sapponins and verbacoside (Refaat 2013; Iwu 1993). Despite their extreme relevance as sources of new drugs, only about 10% of the plant species in the world have been scientifically validated to satisifiable extent (Oksman-Caldentey et al., 2004).

Crinum species
This is a genus of family Amaryllidaceae with 85 genera and 1100 species (Trease, 2009). It is a herbaceous plant with large, tunicate bulb and pseudo stem made of the sheathing bases of the old leaves. The plant's family contains about 150 different isoquinoline especially *Crinum* alkaloids (vittatine or crinine, lycorine, and norbelladine) with most noted effects such as; analgesic, anticholinergic, antitumor, antibacterial and antiviral (Delphine et *al.,* 2009; Alexander *et al.,* 2008; Robert, 2004). In Africa, members of the genus are reportedly used to treat a wide range of ailments from abscesses and allergies to rheumatism and urinary tract problems, including tumor, typhoid fever, malaria, tuberculosis and many other microbial infections (Moshi *et al.,* 2012; Gatsing *et al.,* 2009).
Previous studies have reported that *C. scabrum* Alani Davis has medicinal properties such as antitumor, immunostimulating, analgesic, antiviral, antimalarial, antibacterial and antifungal activities (Refaat et al., 2012). The same studies also revealed that the plants' leaves contain *Crinum* alkaloids such as lycoriside ($C_{38}H_{57}NO_{10}$), lycorine ($C_{16}H_{17}NO_4$) oxoassoanone ($C_{17}H_{15}NO_3$), pratorimine ($C_{16}H_{11}NO_3$). None of these compounds has been evaluated for its anti-mycobacterial activity (Refaat et al., 2012). The bulbs of the same plant are also reported to contain lycorine (4, 5-dehydroanhydrolycorine), tazettin, phenanthridine, lycorenine, galanthamine, ryllistine and cherylline, in addition to some quaternary salts isolated from them (Tahir et al., 1999; Sharon 2004). Some of the compounds in the bulb are reported to have antibacterial, antimalarial, antitumor and anti-inflammatory effect (Tahir et al., 1999; Sharon, 2004).

Objective
The purpose for this research was to determine the anti-mycobacterial activity and safety of crude extracts of *Crinum scabrum* and *Crinum macowanii.*

Materials and Methods
This was an experimental laboratory based study of bioactivity and safety profile of *Crinum scabrum* and *Crinum macowanii.* The plant extracts were tested on selected *Mycobacterium tuberculosis* (M.TB) strains using agar well diffusion and broth dilution methods

Plant Selection, Collection, Preparation and Extraction
The selection was based on Ethnobotannical survey reports on the traditional use of *Crinum* sp. in treatment of respiratory infections in particular chronic cough and TB (Moshi et al 2012, Abu

Honif et al 2009). In literature, genus *Crinum sp*. was reported to have antimicrobial properties (Moshi et al 2012, J. Ode et al 2011). However, none of the studies above validated *Crinum* sp. for anti-mycobacterium sensitivity. Interview with traditional health practitioners (THP) on Ethnobotanical uses of *Crinum* sp. in Arua, (West-Nile, Uganda) claimed one to be very effective in treatment of TB and cough. The plant was collected with the help of traditional health practitioner and identified in national herbarium with the help of a taxonomist. Bulbs and shoots of the plants were sorted and washed with clean water, and then air dried to constant weight (Ogundare et al 2006). Dry materials were then pulverized to powder using a mortar and a pestle; weighed and stored in clean dry polythene bag at room temperature.

Chloroform, methanol and water solvents were used sequentially in order of polarity. Weighed powdered plant material (500g) was soaked in solvent (1L) for four days and filtered with Whatman filter paper. Solvents were concentrated by a rotary evaporator at 40oc under reduced pressure and evaporated to constant weight in hood. Water extract was mixed with ethanol, evaporated to constant weight at 30oC and all stored at -30oC (Ameen et al, 2005).

Anti -Mycobacterium assays

Assays of the plant extracts on *Mycobacterium* were done by agar-well diffusion method in Microbiology Laboratory Makerere University. Stored strains of inoculum (Sou 14847, R.S, and H37Rv) were revived prior to susceptibility test (Pereira et al 2005). Sterile Middle brooks 7H10 agars (5ml) in 90mm diameter Petri dishes with quadrants were used for incubation. Solutions (1.0mg/ml) of extract and rifampicin were prepared from stock solutions for susceptibility tests. The petri dishes were inoculated by flooding method. Wells (2.0mm by 2mm) born in the dry inoculated medium using a sterile cork borer, and test extract (50µl) dispensed into those of the first quadrant. Equal volume of rifampicin (1.0µg/ml) for positive control was dispensed into wells of the second quadrant. Third quadrant wells were control, while fourth quadrant wells filled with the solvent of extraction (negative control). Petri dishes were left in the hood overnight, then sealed with a carbon dioxide-permeable tape and incubated at 37oC in a carbon dioxide incubator for four weeks. The sensitivity of *M. tuberculosis* (M.TB) to the extracts and rifampicin were determined by measuring the zones of inhibition surrounding the well using millimeter scale. Test for each M.TB strain was done in triplicate. MIC was taken as the concentration of the drug or extract that inhibits growth of M. tuberculosis by 90% or greater, in comparison to the positive growth control. MBC was taken as the concentration of the drug or extract that prevents growth by 99% or greater, compared with the untreated controls.

Toxicity test on the crude extract (the most active)

Acute toxicity test of *C. scabrum* Alani Davis crude methanol extract (the most active) was done on healthy mice, *Mus musculus* (obtained from department of Veterinary medicine) using guidelines and procedures described by NIH, OECD, WHO and Ghosh (OECD, 2001; Ghosh, 1984). Both sexes of mice (3 males and 3 females) were considered for each concentration. The mice were fasted overnight and four dose ranges were used. A pilot study was done on a pair of mice, using widely separated doses (10, 50, 100, and 500mg/kg) by oral rout using a syringe fitted with a cannula. Acute drug effects were observed within twenty-four hours. Lethality at any concentration below 5000mg/kg was the reference for a definitive study with doses of narrow intervals (1mg/kg) within the range determined in the pilot study.

An ogive (dose- response curve) of the result was to be used to define the acute toxicity of the plant under study by interpolating 50% of response to get the LD50. However, the results

obtained did not make it feasible. Other drug effects such as, restlessness, lacrimation on eyes, urination, defecation, raised hair and sleepiness; were also observed for the first two hours constantly then at interval of 2 hrs overnight. The mice were sacrificed and incinerated at the end of the tests.

Ethical considerations

Ethical approval was sought from the Research and Ethics Committee of Biomedical Science and the Uganda National Council for Science and Technology (HS1627). Protection of the investigators was ensured by working under aseptic conditions and carrying out the work under the guidance of the laboratory staff both at the Mycobateriology Laboratory School of Biomedical Science, Makerere University and other laboratories used. The necessary protective wears including respirators and gloves as well as safety cabinets were used to minimize the risk of exposure to *M. tuberculosis*.

Consent was obtained from those providing Ethno botanical information and the results of the study were disseminated to them.

RESULTS

Percentage yields from crude extracts of *Crinum L.* species.

The highest yield was observed in the methanol leaf extract of C. macowanii Baker (49.89%), followed by the water leaf extract (47.62%) and chloroform leaf extract (19.23%) of the same plant. While the least yield was observed in chloroform stem extract (0.76%) of *C. macowanii* Baker and chloroform stem extract of *C. scabrum* Alani Davis, (0.85%) respectively.

Only *C. scabrum* Alani Davis leaf methanol and chloroform crude extracts inhibited the growth of *Mycobacteria tuberculosis* (M.TB). Methanol and chloroform stem extracts of *C. scabrum* Alani Davis had no inhibitory effects on any of the test M.TB strains used at the selected concentration. All the water crude extracts showed no inhibitory effects on all the test strains used.

Extent of anti-mycobacterial activity of *C. scabrum* Alani Davis and rifampicin

The extent of anti-mycobacterial activity of the crude extracts of the *Crinum L. sp*. were obtained as mean diameter of zones of inhibition at concentration of 1.0µg/ml of the medium (table 4 and fig.1). The diameters of the wells were excluded.

All the selected strains of *M. tuberculosis* showed sensitivity to all the drugs used. The greatest zone of inhibition (22.0±1.2mm) was for crude leaf methanol extract of *C. scabrum* Alani Davis Chloroform crude leaf extract of the same plant showed the least zone of inhibition (1.0±1.7mm). Methanol crude leaf extract of *C. scabrum* Alani Davis proved to be the most active (22.0±1.2mm). Rifampicin had least inhibition for Rifampicin-streptomycin resistant (R.S) strain (2.0±0.6mm) and chloroform crude extract had the least activity for Pan African sensitive strain -H37Rv, (1.0±1.7mm).

4.2.2. **The MIC and MBC of leaf crude extract of *C. scabrum* Alani Davis**.

The effectiveness of the active crude extracts was ascertained by percentage inhibition of the different test strains of M.TB at selected concentrations of the leaf extracts (table 5 and figure 2). MIC (inhibition of 90% 0r more) of chloroform and methanol crude leaf extracts for all the test strains was 1µg/ml. MBC (99% inhibition or more) also occurred at 1µg/ml for both extracts, for all test strains. The MIC and MBC of both extracts for R.S strain is also1µg/ml. Complete (100%

inhibition) occurred at 4µg/ml for all the strains except, for R.S where inhibition occurred at 3µg/ml , with methanol extract (Figure 3).
No mice died at the highest doses of 2000mg/kg. The LD_{50} of *C. scabrum* Alani Davis crude leaf methanol extract was above 5000mg/Kg and so could not thus be ascertained. Full recovery occurred after four hours and the animals continued to be lively for the rest of the experimental time.

DISCUSSION OF RESULTS

Anti-mycobacterial activity of *Crinum spp*.

The extensive survey of literature presents genus *Crinum* as an endless source of bioactive principles. They produce huge number and diverse classes of phytoconstituents, and are best known biofactories for Amaryllidaceae alkaloids (Refaat et al., 2013). The antimicrobial potential of ethanol and aqueous extract of *Crinum L. sp*. has been investigated against some human pathogenic bacteria like *Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi* (Refaat et al., 2013; Ilavenil et al., 2010). In this study, *C. scabrum* Alani Davis was found to be active against the test strains of M.TB used. Methanol and chloroform Leaf extracts of the active plant inhibited the growth of all the selected strains of M.TB, with the highest activity shown by methanol leaf crude extract of *C. scabrum* Alani Davis implying the plant has anti-mycobacterial activity. The chloroform leaf extract of the same plant showed a similar magnitude of activity but the inhibitions were less than that of methanol at increased concentrations for all the test strains. This implies methanol crude leaf extract is more active (efficacious) in inhibiting M.TB growth than the chloroform crude leaf extract of the same plant. The MIC of the leaf extract (1µg/ml) turned to be the MBC for all test strains of M.TB with its corresponding percentage inhibition greater than 99%.
It is thus possible that lower concentration of the extract can give percentage inhibitions equal to or greater than 90% and hence MIC of the extracts less than 1µg/ml.
This implies, comparatively the leaf extracts of *C. scabrum* Alani Davis are more effective than rifampicin. The extracts' complete clearance of rifampicin-streptomycin resistant strain at a concentration of 3µg/ml for methanol extract and 4µg/ml for chloroform extract implies the plant is active against MDR.TB. It can be used to treat both drug naïve and drug experienced TB patients. The multidrug resistant M.TB inhibition property of the plant extract also indicates that, pure compounds isolated from this plant can provide a lead compound for development of TB drug with better efficacy than rifampicin and streptomycin. There is need for fractionation, Isolation and test for the active pure compounds from leaf extracts of the plant. This may also lead to development of a single drug to replace the cumbersome drug dose regimens for TB patients which intern will improve patient compliance and shorten period for TB treatment.

Water leaf extract of *C. scabrum* Alani Davis was not active against the MTB strains used. This implies the non-polar compounds in *C. scabrum* Alani Davis leaf have no anti-mycobacterial activity. The use of water in the treatment of TB patient with the plant will produce minimum or no therapeutic effect. Users are advised to use alcohol for extraction or form powder of the leaf that can be leaked. Chewing of a fresh piece of the plant leaf washed with clean water may even be a better way to use the plant than water extract for TB treatment if patients do not get irritated. Stem (bulb) extracts of the same plant were inactive.

The sensitivity tests of the *C. scabrum* Alani Davis leaf extract on selected M.TB strains in this study showed that the plant has anti-mycobacterial activity in addition to other medicinal properties. This also implies that some of the *Crinum* alkaloids have antimycobacterial activity. All extracts of the bulb had no effect on the M.TB strains tested. The therapeutic effects of the bulbs on TB patients claimed by the traditional health practitioner could be due to immunostimulatory properties of some compounds in it which produce synergic effect with ingredients of leaf extracts to treat the patients.

Crinum macowanii Baker is reported to have anticholinesterase and anticholinergic activity which is useful in management of myasthenia gravis, myopathy and CNS disorders like Alzheimer's disease (Refaat et al., 2013). It is possible that in a mixture, the plant could enhance the therapeutic effects of *C. scabrum* Alani Davis hence the need for scientific evaluation of this effect.

Safety of the extract in mice

The alkaloids in *Crinum* spp. make the plant toxic. The bulbs are reported to be more toxic than the leaves (Refaat et al., 2013).

In this study, the result of acute toxicity tests of *C. scabrum* Alani Davis leaf methanol crude extract (the most active) in mice showed no deaths at dose of 5000mg/kg weight of mice. The plant leaf is safe according to Gosh and World Health Organisation recommendations for acute toxicity (Ghosh, 1984; WHO, 1992; OECD, 2001). This could be due to the animals' ability to rapidly metabolize or modify the *Crinum* alkaloids into nontoxic metabolites leading to their toleration of the high dose of the plants' crude methanol extract. However, at dose of 5000mg/kg weight of mice, the duration for sedation and micturition increased, accompanied with brief moments of pilorection. This is a sign of toxicity. It needs to be confirmed by sub-acute or chronic toxicity tests of the plant leaf extract and sacrifice of the test animal(s) to determine its effects on the internal organs.

The methanol crude leaf extract from *C. scabrum* Alani Davis showed effects like sedation, diarrhoea, increased micturition and decreased motor activity on the white albino mice at doses greater than 700mg/kg weight of mice, though disappeared after four hours. This suggests that methanol crude leaf extract of the plant contains compounds that can depress CNS, cause diuresis and increase gastrointestinal motility. It further indicates the possible side effects a patient may experience when treated with leaf extracts of *C. scabrum* Alani Davis. The methanol crude leaf extract of the same plant had the greatest overall activity, hence was considered for further tests.

Conclusion

From the results of this study it is evident that *C. Scabrum* Alani Davis is a potential source of new compounds with activity against multi-drug resistant tuberculosis. This supports its use in the treatment of tuberculosis by traditional health practitioners in west Nile. *C. macowanii* Baker has no anti-mycobacterial activity. *C. scabrum* Alani Davis leaf is the most useful part of the plant for treatment of TB. The plant leaf is safe for clinical use since its LD50 is greater than 5000mg/kg according to Gosh and WHO (Gosh 1984; WHO 1992). However, this has to come after thorough investigation of all the properties of the leaf extracts in different solvents and isolation of active principles.

Recommendations

The study recommends herbalists to use the leaves instead of bulb of *C. scabrum* Alani Davis for treating TB patients. However clinical trials should be carried out with the help of a clinician to prove efficacy of the leaves. In addition, methanol should be used for extraction and the solvent be evaporated to dryness to obtain a dry sample of the extract. This can be dissolved in ethanolic water for patient consumption. Total crude extract of the plant should also be tested on the MTB strains used.

The plant should also be protected for medicinal purpose. A comparative study should be done on activity of crude methanol leaf extract of *C. scabrum* and combination of the drugs used to treat multidrug resistant MTB. The active principles in the leaves of *C. scabrum* Alani Davis should also be isolated, identified and tested on strains of *Mycobacteria tuberculosis* resistant to at least two or three drugs.

Sub-acute and chronic toxicity tests on the plant leaf extracts should be done with sectioning of the internal organ for complete evaluation the toxic effects of *C. scabrum* Alani Davis.

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TABLES

Table 1: Summary of Petri dishes set during sensitivity test.

Quadrant	Content
1	Medium + inoculum + test extract
2	Medium + inoculum + rifampicin
3	Growth positive control
4	Control for solvent activity

Table 2: The percentage yield of crude extracts from *C. scabrum* Alani Davis and *C. macowanii* Baker.

Solvent	Plant yield (%)			
	C. scabrum		*C. macowanii*	
	Leaf	stem	leaf	Stem
Chloroform	4.19	0.85	19.23	0.76
Methanol	7.93	9.80	49.89	11.80
Water	5.49	7.53	47.62	9.42

Table 3: The antimycobacterial activity of *C. scabrum* Alani Davis and *C. macowanii* Baker crude extracts

	Growth								
Solvent	Chloroform			Methanol			Water		
Strains	R.S	H37Rv	Sou. 14827	R.S	H37Rv	Sou. 14827	R.S	H37Rv	Sou. 14827
***C. scabrum* leaf**	-	-	-	-	-	-	+	+	+
***C. scabrum* stem**	+	+	+	+	+	+	+	+	+
***C. macowanii* leaf**	+	+	+	+	+	+	+	+	+
***C. macowanii* stem**	+	+	+	+	+	+	+	+	+

Key; + growth colonies observed (inactive); - growth colonies absent (active); R.S, rifampicin-streptomycin resistant MTB strain; H37Rv, Pan-African sensitive MTB strains; Sou14827, wild strain MTB.

Table 4: The mean diameter of zones of inhibition by *C. Scabrum* Alani Davis and rifampicin

Strain	TLM	TLC	RF
R.S	22.0±1.2	16.0±2.1	2.0±0.6
H37Rv	15.0±1.0	1.0±1.7	9.0±1.2
Sou.14827	18.0±4.0	14.0±3	10.0±2.6

Note: The numbers are mean diameter of zones of inhibition ± standard deviation (mm)
Key: TLM, TB plant leaf methanol extract; TLC, TB plant leaf chloroform extract; RF, rifampicin.

Table 5: Mean Percentage inhibition of the test strains at the selected concentrations.

Concentration (µg/ml)	Percentage inhibition (%)					
	TLC			TLM		
	R.S	H37Rv	Sou. 14827	R.S	H37Rv	Sou. 14827
1	99.85	99.85	99.85	99.85	99.85	99.85
2	99.99	99.91	99.90	99.99	99.90	99.91
3	99.99	99.91	99.95	100.00	99.91	99.98
4	100.00	100.00	100.00	100.00	100.00	100.00
5	100.00	100.00	100.00	100.00	100.00	100.00

Note: Percentage inhibition=(α-β)/α×100 Where; α is the number of CFU in 100µl of inocula prepared with opacity (turbidity) adjusted to McFarland No. 1 standard. β is the colony count on each plate (Appendix 4, table 3); TLM, TB plant leaf methanol extract; TLC, TB plant leaf chloroform extract; RF, rifampicin; H37Rv, Pan-African sensitive MTB strains; Sou14827, wild strain MTB.

Table 6: Drug effects in acute toxicity test of C. scabrum Alani leaf extract in mice (n = 6 per dose)

Study parameter	Dose (mg/Kg)					
	100	200	300	500	700	2000
Locomotion	N	N	N	N	N-	N-
Pinching	N	N	N	N	N	N
Sedation	N	N	N	N	S	S
Micturition	N	N	N	N+	N+	N+
Pilorection	N	N	N	N	N	N+
Diarrhoea	N	N	N	D	D	D
Mortality	0	0	0	0	0	0

Key: N Normal; N- reduced; N+ increased; S Sedation; D Diarrhoea.

GRAPHS

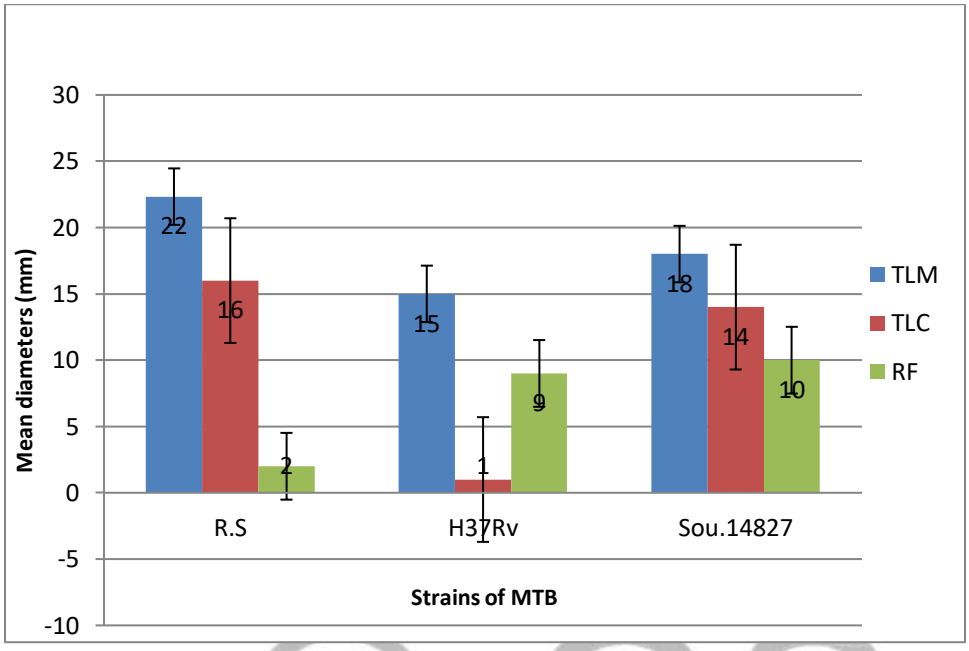

Figure 1: Graph showing mean diameter of Zones of inhibition by C. scabrum leaf crude extract.
Key: TLM, TB plant leaf methanol extract; TLC, TB plant leaf chloroform extract; RF, rifampicin; H37Rv, Pan-African sensitive MTB strains; Sou14827, wild strain MTB.

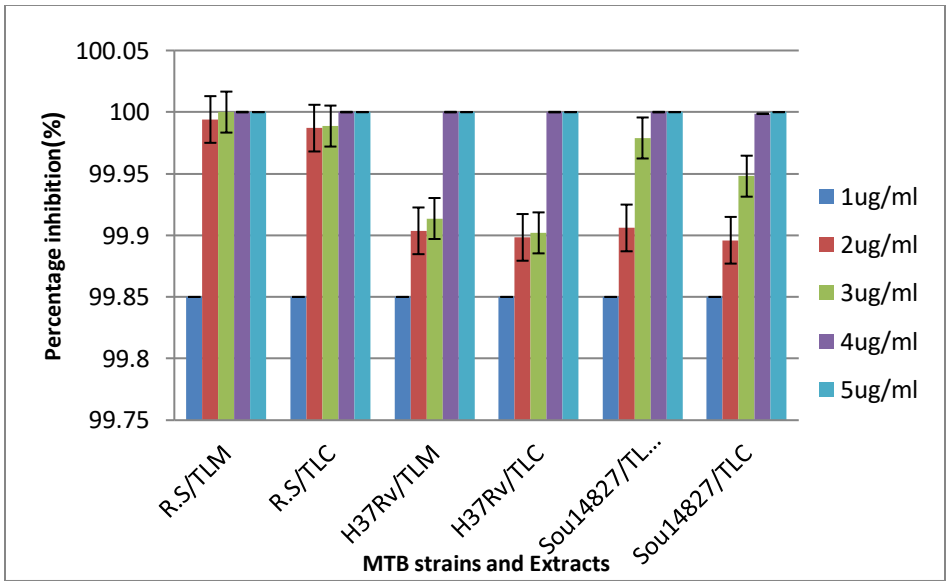

Figure 2: Graph showing percentage inhibition of MTB strains per extract concentration

Key: R.S, rifampicin-streptomycin resistant MTB strain; Sou 14827, wild MTB stain; H37Rv, Pan African sensitive MTB strain; TLM, methanol extract; TLC, chloroform extract

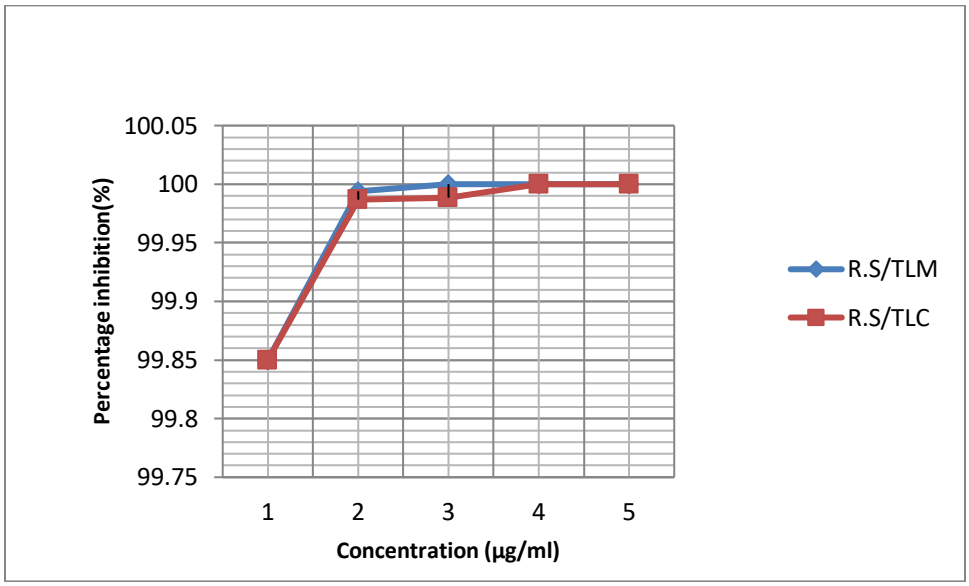

Figure 3: Graph of variation of percentage inhibition of R.S with extract concentration
Key: R.S, rifampicin-streptomycin resistant MTB strain; TLM, methanol extract; TLC, chloroform extract.

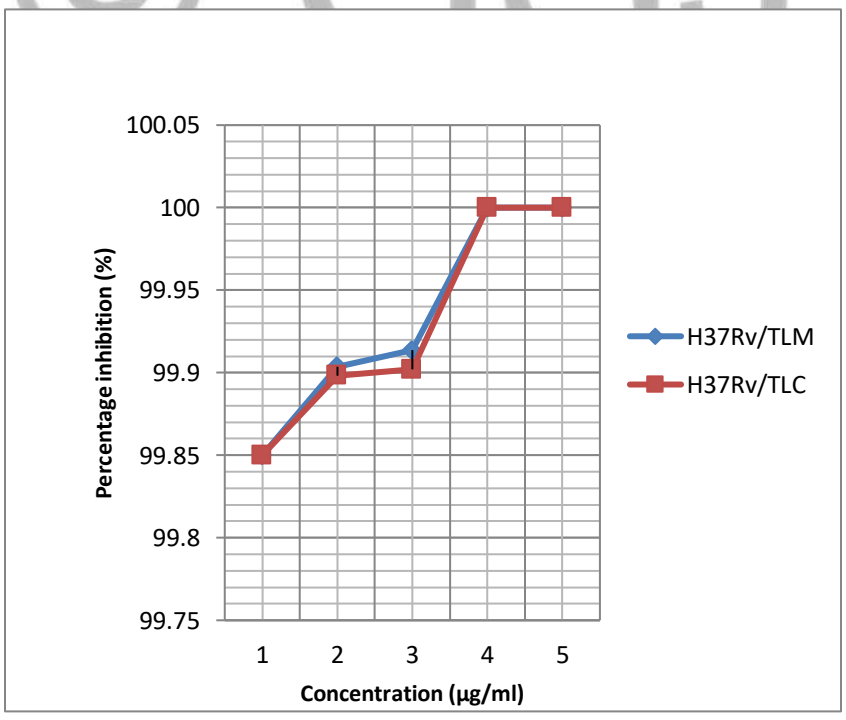

Figure 4: Graph showing variation of percentage inhibition of H37Rv with extract concentration

Key: H37Rv, Pan African sensitive MTB strain; TLM, methanol extract; TLC, chloroform extract.

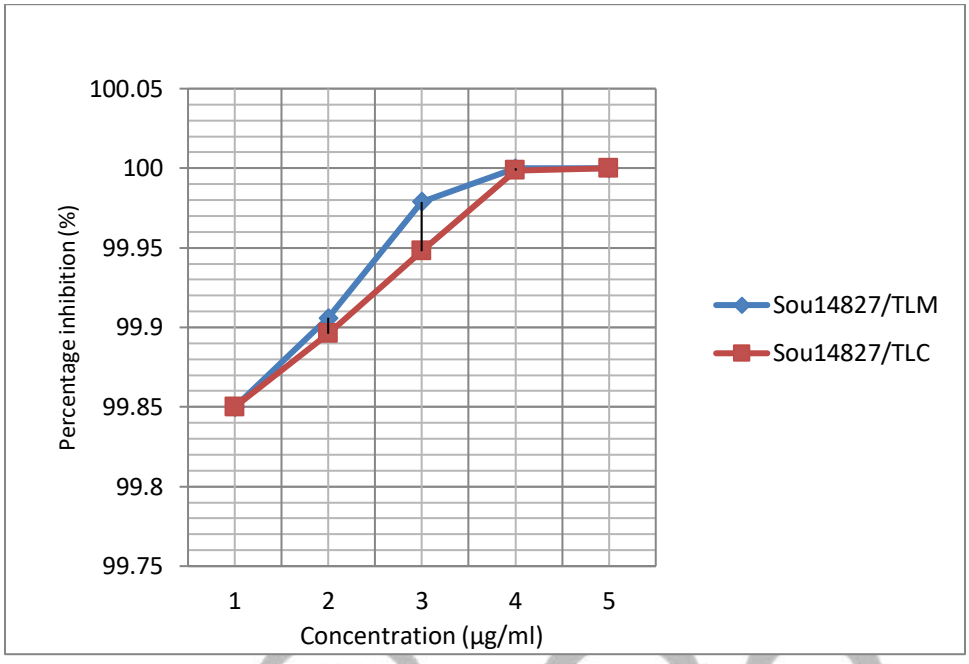

Figure 5: Graph showing percentage inhibition of Sou14827 against extract concentration
Key: Sou 14827, wild MTB stain; TLM, methanol extract; TLC, chloroform extract