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The *in vitro* antimycobacterial activity of medicinal plants used by traditional medicine practitioners (TMPs) to treat tuberculosis in the Lake Victoria basin in Uganda

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Introduction

- Tuberculosis (TB) is one of the dreadful infectious diseases and the leading cause of mortality worldwide
- Approximately 9 million people developing the disease and 2 million people dying annually (WHO, 2007; Sanjay 2004; Navin *et al.*, 2002).
- Globally, more than one-third of the world’s population (more than 2 billion) is infected with MTB (CDC Report, 2005; Navin *et al.*, 2002).
- Outbreaks of multi-drug resistant (MDR) and extensively drug-resistant (XDR) tuberculosis have also compounded the problem.

Introduction (cont’d)

- The emergence of XDR TB is cause for concern because it renders patients virtually untreatable with available drugs
- XDR TB is widely distributed geographically and is now in over 50 countries on all inhabited continents
- New drugs have to be developed to deal with MDR and XDR TB strains, which have become a problem especially where there is co-infection with HIV/AIDS.
- There is an urgent need to search for and develop new, comprehensive, safer and more effective quick acting and affordable anti-TB agents,
- This also includes searching for leads from natural products of plant origin.

Introduction (cont'd)

- This presentation is a report of results of a study that was carried out in Uganda as part of a wider East African regional survey in the Lake Victoria basin.

- The objectives of the study included **:**

1. documenting indigenous knowledge and the existing practices used by traditional medicine practitioners (TMPs) in the treatment of TB
2. identifying the medicinal plants used by Traditional Medicine Practitioners
3. screening selected medicinal plants against rifampicin-resistant mycobacterium tuberculosis
4. isolating , characterising and identifying the active compounds from the selected medicinal plants that have anti TB activity

Resaerch questions

This study was conducted to answer the following fundamental questions:

1. What are the traditional methods and practices used in treating TB using herbal medicines by TMPs?
2. Do the TMPs actually have appropriate knowledge about the causes and symptoms of TB?
3. How did the TMPs aquire the knowledge of treating TB?
4. What are the perceptions and attitudes of the TB patients treated by TMPs about use of herbal medicines in treatment of TB?
5. What are the medicinal plant species used in this process?

Research questions (cont'd)

6. Are crude extracts from the mentioned plant species actually active against *Mycobacterium tuberculosis*?

7. If yes, how does the activity of the active extracts compare with that of standard drugs?
8. What is the acute toxicity profile of the most active extracts?
9. What are the structures and identities of the active compounds in the plant extracts screened?
10. Could these active compounds be developed into a locally formulated herbal remedy for TB or a new and more effective anti TB drug?

Materials and Methods

- The study area included three districts of Mayuge, Mbarara and Mukono in the Lake Victoria basin part of Uganda.
- Data was mainly gathered using key informant interviews, guided questionnaire interviews and direct observation techniques.
- A total of 31 TMPs and 16 patients who had received TM treatment for TB were interviewed

Materials and Methods (cont'd)

- After the survey, a list of the most frequently mentioned plant species was prepared

- Parts of 11 selected plants were collected from various areas.
- Crude petroleum ether, chloroform and methanol extracts were sequentially prepared.
- Total crude methanol extract was also prepared.
- The extracts were cold sterilized using 0.2µm membrane filters (Acrodisc®) and screened in a bioassay on three strains of *Mycobacterium*

Susceptibility tests were carried out using the disc diffusion method on Middlebrook 7H10 on 90mm quadri divided Petri dishes

-Rifampicin and isoniazid were used as standard drugs for comparison

-Activity was reported as means of diameters of zone of in inhibition

- MIC tests for the active extracts were carried out using the Microtitre plate method where Middle brook 7H9 broth was used in 96-well Microtitre plates.
- Bacterial growth was detected from the degree of turbidity

Materials and Methods (cont’d)

- The MBC tests were carried using the Microtitre plate method by using ten-fold dilutions of contents of wells showing no apparent growth in
- Confirmation was made by using the ten fold dilutions to inoculate fresh solid medium and observations made for any growth of colonies for up to 8 weeks
- *Mycobacterium* strains used were obtained from Joint Clinical Research Centre (JCRC) Mengo in Kampala, Uganda where the bioassays were carried out.

Materials and Methods (cont’d)

The MTB strains used included a rifampicin-resistant strain (TMC -331strain) to serve as an indicator of MDR, a fully susceptible strain (H37Rv) as a control.

- A wild strain of *Mycobacterium avium* (MA), isolated from a Ugandan patient was also tested.
- Preliminary qualitative phytochemical analysis was carried out according to standard procedures.
- Acute toxicity tests were a done on mice using the most active extracts according to Gosh (1984).

Results and Discussion:

Table 1:Signs by which TMPs recognised TB

Characteristic	Frequency	Percentage
Persistent cough (lasting more than 2 months)	21	21.6
Laboured breathing	13	13.4
Slimy sputum	11	11.3
Weight loss	11	11.3
Loss of appetite	10	10.3
Chest pain	5	5.2
Night sweating	5	5.2
Chronic fever	4	4.1
General weakness	4	4.1
Dehydration	2	2.1
Swollen eyes	2	2.1
Heavy chest	1	1.0
Head ache	1	1.0
Burning sensation in the chest	1	1.0
Irritation in the throat	1	1.0
Painful joints	1	1.0
Pale eyes	1	1.0
Spitting a lot	1	1.0
Swelling of the chest	1	1.0
Swelling of the lymph nodes	1	1.0
Total	**97**	**100.0**

RESULTS AND DISCUSSION

Table 2: Causes of TB as reported by TMPs

Cause	Frequency	Percentage
Contamination (food, utensils)	20	28.2
Smoking	13	18.3
Droplet infection from infected people	10	14.1
Over crowding	5	7.0
Doing heavy work	4	5.6
Inherited	4	5.6
AIDS	3	4.2
Poor nutrition	2	2.8
Asthma	1	1.4
Through infected blood	1	1.4
Drug abuse	1	1.4
Eating an infecting insect	1	1.4
Excessive fried foods, too much sugar	1	1.4
Fur in the milk	1	1.4
Insects carrying the germs to food	1	1.4
Smoke from fuels	1	1.4
Stress	1	1.4
Syphilis	1	1.4
Total	**71**	**100.0**

Results and Discussion

Table 3:Signs or ways by which TMPs ascertained that their patients are healed

Sign	Number of TMPs	Percentage
Reporting back when cured	12	27.9
After laboratory tests	10	23.3
No more coughing	6	14.0
Becoming healthy	3	7.0
Gain of appetite	3	7.0
Change in sputum state	2	4.7
Good breathing	2	4.7
Checking tongue; clear and moving well	1	2.3
Eyes are no longer yellow	1	2.3
Gives them extra month to stay around after treatment -	1	2.3
Eyes becoming wet	1	2.3
When they do not come back for treatment	1	2.3
Total	**43**	**100.0**

Results

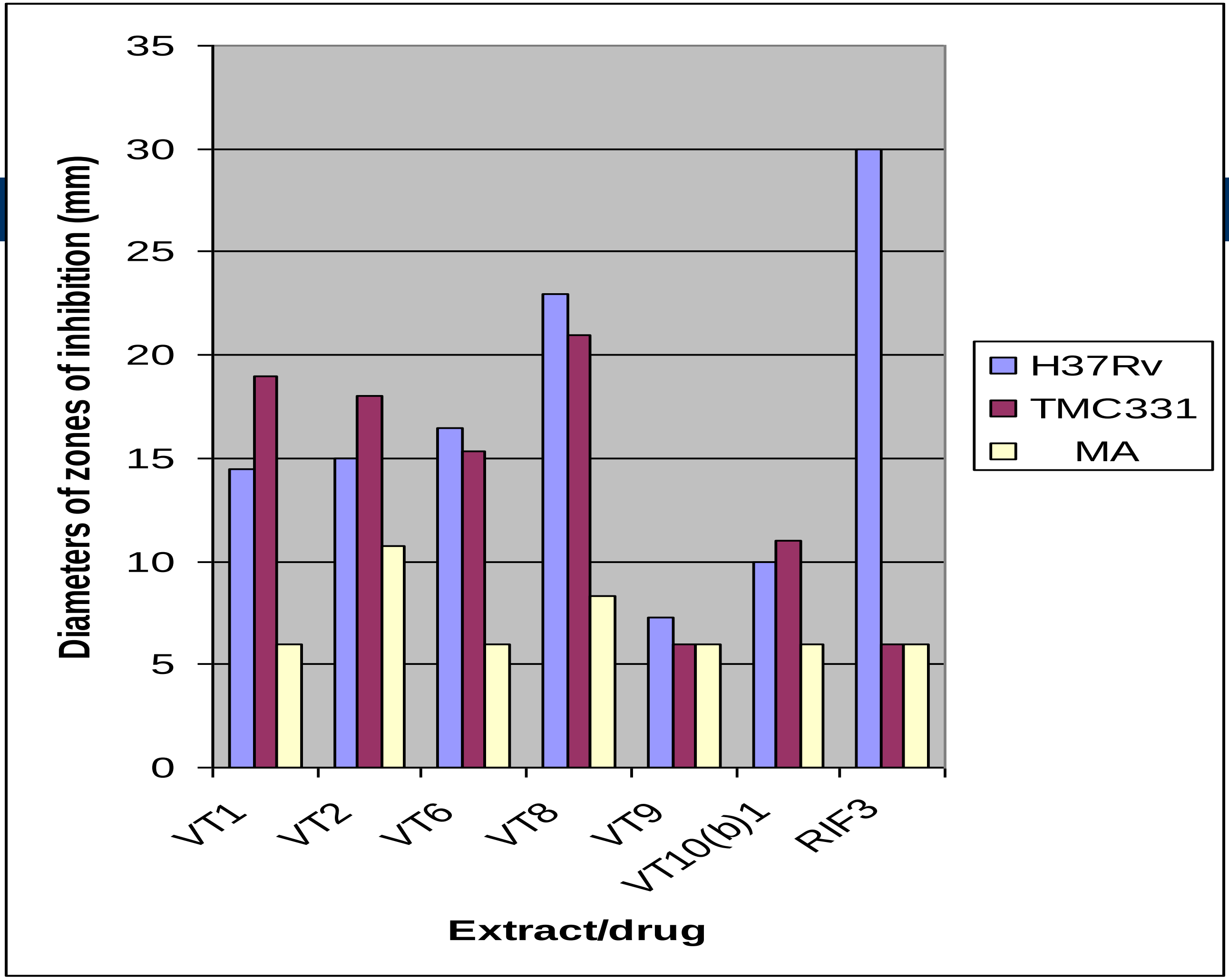
Table 4: Plant species most commonly used by TMPs to treat symptoms of TB

Species	Local name	Healers #	Districts #
Warbugia ugandensis	Omwiha/Abaki	9	3
Albizia coriaria	Omusisa/Omugavu	8	3
Mangifera indica	Muyembe	6	3
Eucalyptus grandis	Kalitunsi	5	3
Erythrina abyssinica	Ekiko/Ejirikiti	4	3
Garcinia buchananii	Omusaali	4	3
Solanum incanum	Ntengotengo	4	3
Solanum aculeastrum	Entengo Enene	3	3
Rubia cordifolia	Kasalabagesi	8	2
Zanthoxylum chalybeum	Ntale ye dungu	5	2
Cryptolepis	Kafulu	4	2
Dracaena steudneri	Kajolyenjovu	4	2
Cissus quadrangularis	Kiwondowondo	3	2
Psidium guajava	Omupera	3	2
Sarcocpahalus latifolius	Mutamatama	4	1
Azadirachta indica	Neem	3	1
Zingiber officinale	Ntangawuzi	3	1
Total		**80**	**39**

Results and Discussion:
Table 5: Plant species used for the bioassay

Scientific name	Family	Part collected	Collected from
Erythrina abyssinica	Papilionaceae	Root bark	Mukono
Solanum incanum	Solanaceae	Fruit	Mukono
Cryptolepis sanguinolenta	Periplocaceae	Root	Kayunga
Albizia coriaria	Leguminosae	Stem bark	Kayunga
Dracaena steudneri	Liliaceae	Stem bark	Mukono
Garcinia buchananii	Guttiferae	Root bark	Mukono
Mangifera indica	Anarcardeceae	Stem bark	Mukono
Psidium guajava	Myrtaceae	Leaves	Mukono
Rubia cordifolia	Rubiaceae	Whole aerial part	Mukono
Warburgia ugandensis	Carnellaceae	Root bark	Mukono
Zanthoxylum chalybeum	Rutaceae	Root bark	Kayunga

Fig 1:Anti-mycobacterial activity of total crude methanol extracts at concentrations of 50 mg/ml using the disc diffusion method.



Results(cont'd)

Key:

- VT1= *Zanthoxylum chalyeum*

- VT2=*Warburgia ugandensis*
- VT3= *Solanum incanum*
- VT4= *Rubia cordifolia*
- VT5= *Psidium guajava*
- VT6= *Mangifera indica*
- VT7= *Garcinia buchananii*
- VT8= *Erythrina abyssinica*
- VT9= *Dracaena steudneri*
- VT10= *Cryptolepsis sanguinolenta*
- VT11= *Albizia coriaria*
- 1-Concentration of VT10(b) used was 25mg/ml instead of 50mg/ml used in case VT10(a)
- 2- Concentration of isoniazid was 2.5mg/ml
- 3-Concentration of rifampicin was 5mg/ml

Results (cont'd)

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Table 6: The MICs in mg/ml of methanol and chloroform extracts against *M.Tb strains*.

	Mtb (H37Rv)	Mtb (TMC 331)	*M. avium* (MA)
Methanol extract			
VT1	4.69	6.25	ND
VT2	4.69	2.35	0.59
VT6	3.13	0.59	ND
VT8	0.20	1.17	0.20
VT10 (a)	1.17	1.56	ND
Chloroform Extract			
VC2	2.35	0.59	0.39
VC8	1.17	0.20	ND
Isoniazid*	0.25	9.38	ND
Rifampicin*	0.25	ND	ND

Key: ND = not done because the strains were resistant

*- For isoniazid and rifampicin the MICs are in µg/ml while for the extracts the vlues are in mg/ml.

Results

Table 7: Minimum Bacericidal Concentrations (MBCs) in mg/ml on *Mycobacterium* sp.

	Methanol extract			Chloroform extract		
Extract /Strain	**Mtb (H37Rv**	**Mtb (TMC-331)**	***M.avium* (MA)**	**Mtb (H37Rv)**	**Mtb (TMC-331)**	***M. avium* (MA)**
VT1	1.17	6.25	0.78	ND	ND	ND
VT2	1.56	3.13	0.78	0.098	1.17	0.40
VT6	1.56	1.56	0.78	ND	ND	ND
VT7	ND	ND	ND	0.98	ND	ND
VT8	6.25	4.69	0.80	0.98	1.56	ND
VT10(a)	ND	ND	ND	0.20	ND	ND
Isoniazid*	0.25	0.30	ND	ND	ND	ND
Rifampicin*	1.0	ND	ND	ND	ND	ND

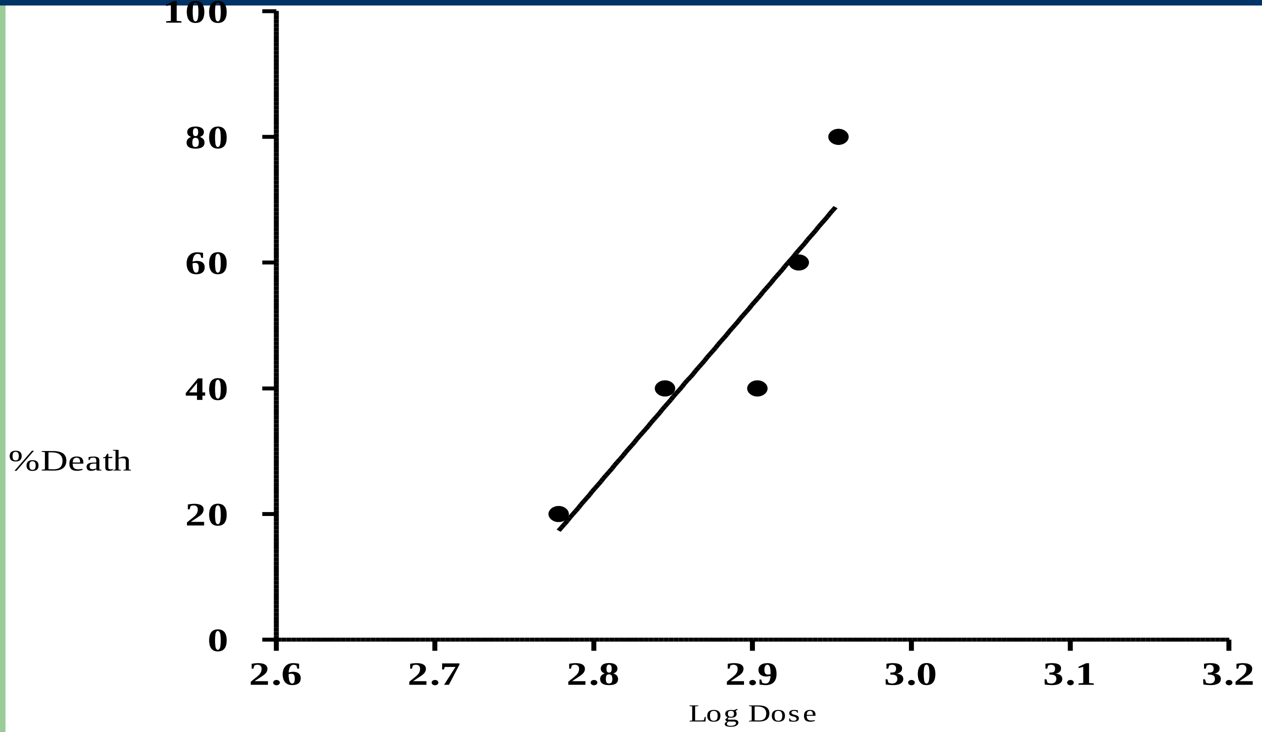
Key: ND = not done because the strains were resistant during sensitivity testing.

- *- For isoniazid and rifampicin, the MBCs are in µg/ml while for the extracts the vlues are in mg/ml.

TABLE 8: Behavioral changes made during the first 6 h of acute toxicity studies

Characteristic	VT10	VT8
↑sed motor activity	-	-
Tremors	+	-
Pilo erection	-	-
↓sed motor activity	+	+
Sedation	+	+
Analgesia	-	-
Lacrimation	-	-
Diarrhea	-	-

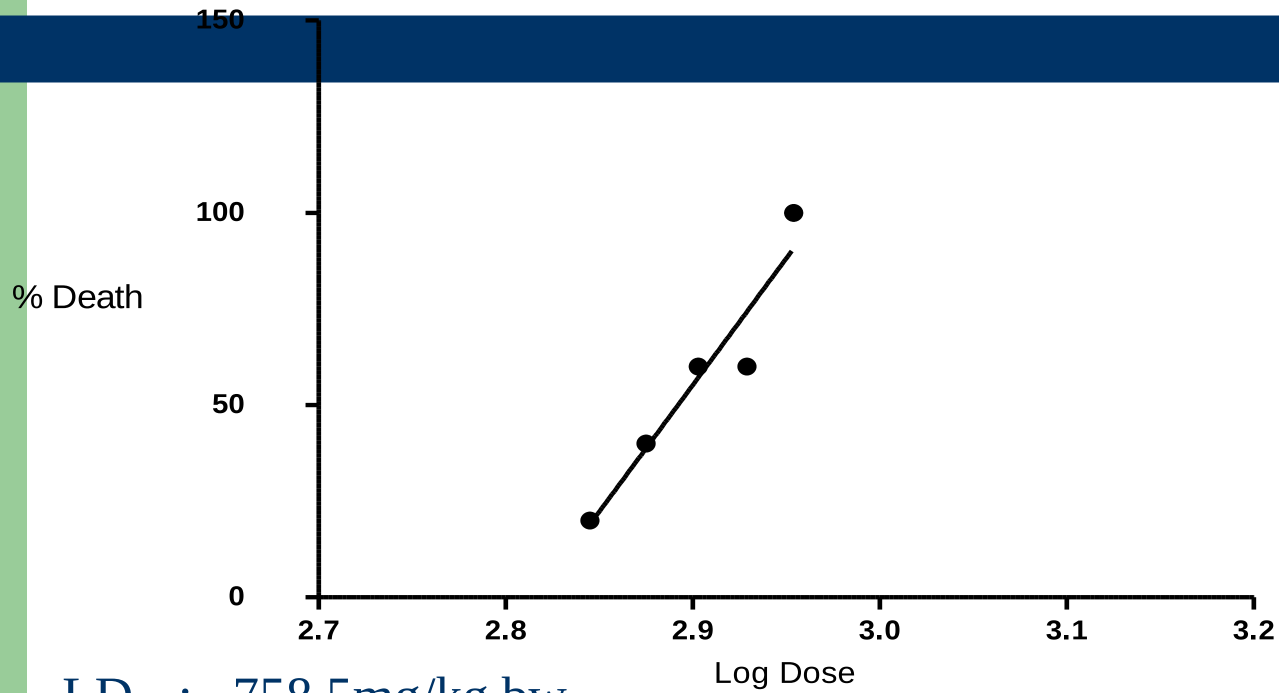
Graph 4 shows a plot of %death agnaist log dose for *Erythrina abyssinica*



LD_{50} : 758.5mg/kg bw.

Results (cont'd)

Graph 3 showing a plot of % death agnaist log dose for *Cryptolepis sanguinolenta*



LD_{50} :   758.5mg/kg bw.

Table 11: Phytochemical analysis of plant extracts from *E. abyssinica* and *C. sanguinolenta*

	saponins	alkaloids	tannins	terpenoids	flavonoids
VT10	-	+	+	-	+
VT 8	-	+	+	+	+
VM 8	-	+	-	+	+
VC 8	-	-	-	+	-

Flavonoids were found in all the four crude extracts except chloroform extract of *E. abyssinica (*VC8) while terpenoids were present in all crude extracts except *C. sanguinolenta* total crude extract(VT10).

Tannins were found in only *E. abyssinica* total crude extract.

Findings and discussion

- The practice of treating TB using medicinal plants exists in the region surveyed;
- The TMPs have substantial knowledge about the causes and symptoms of TB;
- TMPs knowledge correlated well with standard medical knowledge about TB;
- The TMPs acquired the knowledge mainly from their parents; very few had learnt from others;
- Mainly the rural semiliterate or illiterate people got treatment from TMPs;

Findings and discussion (cont'd)

- The patients that visited the TMPs believed that they could be cured and it was not the money factor that was the driving force;
- There was an established, though imperfect mechanism of making follow-up to treatment- an important principle in pharmacotherapy;
- There was some collaboration beginning between TMPs and conventional Medical practitioners, with some TMPs using lab results in their work;
- No rituals were reported to be used in the process of treatment.

Findings and discussion (cont’d)

- Over 50 plant species were identified as being used by TMPs to treat TB.

- Herbal drugs were prepared as mixtures of four or more plants.
- The most frequently used plant parts were leaves roots, root bark, stem bark and fruit.
- Of the screened plants, four were found active against TB,
- Two of the extracts were active on all the three strains of *Mycobacterium* used, including the rifampicin-resistant strain.

Findings and discussion (cont'd)

- All the active extracts were bactericidal although their activity was lower compared with isoniazid and rifampicin.

- However, they had an advantage over rifampicin, one of the first-line anti TB drugs, by being active against rifampicin – resistant TB.
- With regard to susceptibility tests the highest activity was registered with *Erythrina abyssinica* (VT8), *Cryptolepis sanguinolenta* (VT10), *Warburgia ugandensis* (VT2) (Wube *et al*., 2005),and *Mangifera indica* (VT6) with zones of inhibition ranging between 10.7 and 23mm (including diameter of the disc which was 6mm).

Findings and discussion (cont'd)

- The concentrations of the extracts were at 50 mg/ml (25 mg/ml for *C.sanguinolenta*).

- Rifampicin was not active on *Mycobacterium avium* complex and a rifampicin resistant strain TMC-331 but it showed a zone of inhibition of 26 mm for H37Rv (a pan sensitive strain) at a concentration of 0.1 mg.
- Isoniazid cleared the quadrant for two strains at a concentration of 0.05mg but it was also not effective on the *M.avium* strain used.

Findings and discussion (cont'd)

- The study revealed that *Warbugia ugandensis* , *Erythrina abyssinica* , *Mangifera indica* and *Cryptolepis sanguinolenta* had reasonable in vitro activity against M.TB;
- The activity of the crude extracts was much lower than in the case of standard drugs – rifampicin
- The extracts had the advantage of being active against rifampicin-resistant tuberculosis;
- The acute toxicity tests done on mice using *E. abyssinica* and *Cryptolepis sanguinolenta* showed that the extracts from the extracts from these were relatively safe with regard to lethality (but chronic toxicity tests are also necessary.

Findings and discussion (cont'd)

- The MICs of the active crude extracts ranged between 1.17 and 6.25 mg/ml .
- For rifampicin and isoniazid they were between 0.25 and 9.38µg/ml.
- The MBCs for the active crude extracts were between 0.20 and 6.25 mg/ml .
- For rifampicin and isoniazid they were between 0.25 and 1.0 µg/ml (but rifampicin was inactive on TMC-331).

Findings and discussion (cont'd)

- Alkaloids were found mainly in *C. sanguinolenta* (Gibbons *et al.*,2003) and flavonoids in the extracts of *E.abyssinica.*
- Acute toxicity tests on *E. abyssinica* and *C. sanguinolenta* gave LD50 between 700 and 800mg/kg body weight which were in the relatively safe range.

Findings and discussion (cont'd)

- **The last objective is being executed by Phase III of the project which is on going and has so far done the following:**

1.Harvesting, drying of root bark from *E. abyssinica* and *W. ugandensis;*

***2.* Extractions and concentration of crude extracts;**

3. Partitioning of the crude extracts to get various active fractions;

4.Preliminary analytical thin layer chromatography to estimate the number of compounds in the fractions.

Discussion (cont'd)

- Previous studies done on the antimycobacterial activity of *C. sanguinolenta* by Gibbons *et al*., (2003) are in agreement with the findings of this study though activity was on fast growing non virulent mycobacteria.

- Gibbons *et al*., (2003) went ahead to isolate an alkaloid (cryptolepine), which was responsible for the activity.

- This could explain why the minimum inhibitory concentrations of *C. sanguinolenta* in this study werere much lower than those in the above study.
- Presence of alkaloids in *C. sanguinolenta* is in agreement with the simple phytochemical tests done on the plant in this study.

- Though saponins and flavones have been identified in *C. sanguinolenta* total crude extract, the compound which could have caused the antimycobacterial activity could have been the cryptolepine alkaloid however more studies are needed to confirm this.

Discussion (cont'd)

- The results of Ansah *et al (*2009) agree with the acute effects of CNS disturbances of C. sanguinolenta in animals, which were observed as tremors, sedation and decreased motility in this study

- A study done by Ansah *et al,* (2008) also concluded that *C. sanguinolenta* could synergize with hypno-sedatives or other CNS depressants and therefore caution needs to be taken in the concomitant administration of the plant with other CNS depressants.
- Ansah *et al* 2009 also agrees that the extract is safe for use at doses less than 500mg/kg. In vitro studies have reported cytotoxicity at the molecular level however this may not be reflected in vivo (Ansha and Gooderham 2002).

Discussion (Cont'd)

- The results of antimycobacterial activity of *E. abyssinica* are comparable to findings on *E. indica,* a species from the same genus by waffo *et al* (2000).
- In their study *E. indica* showed antimycobacterial activity against *E. smegmatis*. Indicanine B, an isoflavonoid was responsible for the activity.
- Further studies are needed to isolate and identify the antimycobacterial compounds in *E. abyssinica*.
- The results further show that the search for new bioactive products using the ethno botanical criterion increases chances of finding active compounds which could be leads for new drug development against TB (Newton *et al*., 2000).

Conclusion

- The bioassays conducted on the selected plant species further vindicated some of the claims by the TMPs
- Results show activity against *M. tuberculosis* although more research is required especially in the area of standardization.
- Isolation and screening of active compounds and more *in vitro* and *in vivo* studies on the toxicity of the plants are needed before declaring them safe for use in humans.

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References

- Ansah C., Gooderham, N.J. (2002). The popular herbal antimalarial extract *Cryptoleps sanguinolenta* is potentially cytotoxic. Toxicological sciences, 70, 245- 251
- Ansah, C., Otsyina, R., Duwiejua, M., Woode, E., Aboagye, [redacted] medicine. Journal of Veterinary Medicine and Animal Health, 1, 011-016
- Ansha, C., Mfoafo, E.A., Woode, E., Opoku-Okrah, C. and Owiredu , W.K.B.A.(2008). Toxicological Evaluation of the Anti-Malarial Herb *Cryptolepis sanguinolenta* in Rodents. Journal of pharmacology and toxicology, 5, 335-343
- Centre for Disease Control (2005). Worldwide emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs. *Morbidity and Mortality Weekly Report,* 55, 250-253
- Edeoga HO, Okwu DE and Mbaebie BO (2005). Phytochemical constituents of some Nigerian medicinal plants. *Afri. J. Biotechnol.* 4: 685-688.

References (cont’d)

- Gibbons S, Fallah F and Wright C W (2003). Cryptolepine Hydrochloride: A Potent Antimycobacterial Alkaloid Derived from *Cryptolepis sanguinolenta. Phytotherapy research,* 17, 434-436
- Ghosh MN (1984).*Fundamentals of Experimental Pharmacology.*2nd Edition;153-190; Scientific Book Agency , Culcutta.
- Navin TR, Mc Nab SJ, Crawford JT (2002). The continued threat to tuberculosis. *Emerg Infec Dis*. 8: 1187.
- Parish T and Stroker NG (1998). *Mycobacteria Protocols: Methods in molecular Biology*. (vol 101) (eds,) Humana Press, Totowa, NJ. 395-422
- Sanjay MJ (2004). Natural Products: An important source for Anti-tubercular Drugs. *CRISP.* 5 :1.
- World Health organization (2007*).* Global Tuberculosis Database. [Online Tuberculosis Database as of 21st March 2005].
- Wube AA, Bucar F, Gibbons S, Asres K (2005). Sesquiterpenes from *Warburgia ugandensis* and their antimycobacterial activity. *Phytochemistry* 66(19): 2309-15.