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Cytotoxicity and *In vitro* Antimycobacterial Activity of *Vigna unguiculata* Grown in Dodoma, Tanzania

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Abstract: *Vigna unguiculata (L.)Walp (Fabaceae) is an annual herbaceous legume crop which is commonly known as "Cowpea" and locally called as "Kunde" in Tanzania. Traditionally, it is highly valued and employed in treating various ailments, including chest pain and relieve cough among others, and used as a vegetable in Tanzania. Therefore, this study aimed at screen the ethanolic leaf extract of V. unguiculata for the presence of phytochemical constituents, assess the toxicity using brine shrimp lethality test (BST) assay and in vitro antimycobacterial activity using microplate dilution method against Mycobacterium indicus pranii and Mycobacterium madagascariense. The results of phytochemical screening indicated the presence of steroids, flavonoids, alkaloids, tannins, terpenoids, proteins and reducing sugars. Toxicity using brine shrimp lethality test exhibited LC_{50} 9.827 µg/mL where minimum inhibition concentration values of in vitro antimycobacterial activity against M. indicus pranii and M. madagascariense were 0.78125 mg/mL and 1.5625 mg/mL respectively. Thus, the observed bioactivities validate the above traditional uses and also prove to be an indicative for presence of the antimycobacterial compounds which requires further phytochemical investigation.*

Keywords: *Vigna unguiculata, phytochemical constituents, brine shrimp lethality test, antimycobacterial activity*

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1. Introduction

Tuberculosis is an airborne as well as highly contagious chronic infectious disease that is transmitted by cough-propelled droplets caused by *Mycobacterium tuberculosis* and other mycobacterial species causing millions of deaths every year and still is a major worldwide health threat (Chakaya et al., 2021, Jain et al., 2007). Almost, one third of world's population has been affected by latent tuberculosis and Tanzania is among 22 countries with the highest TB burden globally (Innocent et al., 2022).Despite

efforts by the government of Tanzania to provide free anti-retrovirals (ARVs) and anti-TB drugs in all regions and districts there are still people who are using traditional medicines either alone or concomitantly with ARVs (Alphonce et al., 2021, Kisangau et al., 2007). Medicinal plants play an important part for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds. Among those medicinal plants, *Vigna unguiculata* (L.) Walp (Fabaceae) is an annual herbaceous legume crop which commonly known as "Cowpea" and locally called as "Kunde" in Tanzania. Traditionally is highly valued and employed in treating various ailments including chest pain and relieve cough among others, and also used as a vegetable in Tanzania. It is a major staple food crop in sub-Saharan Africa and widely cultivated in semiarid regions across Africa, Asia & America. It is grown in households and also on commercial basis. Ethnobotanically, Kritzinger et al., 2005 and Enyiukwu et al., 2018 reported that *V. unguiculata* seeds are used to treat amenorrhoea, roots as antidote against snake bite, treat painful menstruation, epilepsy and chest pain by the indigenous people of South Africa, leaves are applied on burns and as a snuff to treat headaches and whole plant is used to treat fever and schistosomiasis. Scientifically proven to reduce the risk of lymphoblastic leukemia & aberrant foci cyst development (Campos-Vega *et al*., 2010) and also act as an antioxidant, antimicrobial agent and used in nutraceuticals among other uses. Thus, the present study aimed to investigate cytotoxicity and *in vitro* antimycobacterial activity of *V. unguiculata* grown in Dodoma, Tanzania.

Fig. 1 Geographical distribution of *Vigna unguiculata* (Ravelombola, et al., 2017)

2. Methodology

2.1. Plant Collection

Fresh leaves of uninfected and healthy *V. unguiculata* plant were collected in May 2019 at Kikuyu area, Dodoma, Tanzania. Carefully hand plucked samples were placed in ziploc bags and transported safely to the Pharmaceutical laboratory at St John's University of Tanzania, Dodoma and then air dried for two weeks at room temperature (25-27 °C). The plant was identified by a botanist and authenticated and preserved at the herbarium of the university.

2.2. Plant Extraction

The under-shade air dried, pulverized samples were soaked for 48 hours in ethanol. The filtered crude extracts were concentrated *in vacuo* using a rotatory evaporator while maintaining water bath temperature below 40 °C to avoid thermal decomposition of labile compounds (Begum et al., 2020, Begum et al., 2021). The weight of the crude extracts was determined and about 20 mg each of the extracts was taken to carry out antimicrobial activity.

2.3. Preliminary phytochemical screening tests

Phytochemical screenings of aqueous and ethanolic leaf extracts of *V. unguiculata* were carried out using standard procedures (Begum et al., 2021, Rajasudha and Manikandan 2019, Pradeep et al., 2014, Edeoga et al., 2005) and were conducted at pharmaceutical laboratory of St John's university of Tanzania. The results were recorded in **Table 1.**

2.3.1. Test for Alkaloids (Wagner's Test): Both the extracts were treated with few drops of Wagner's reagent

(0.5 g of iodine and 1.5 g of potassium iodide were dissolved in 5 mL of distilled water and the solution was diluted to 20 mL using water). The formation of reddish brown precipitate indicates the presence of alkaloids.

2.3.2. Test for Terpenoids (Salkowki's test): Extracts were dissolved in 2 mL of chloroform and treated with 2 mL of concentrated sulphuric acid to form a layer. Reddish brown coloration at the interface indicates presence of terpenoids.

2.3.3. Test for Flavonoids (Alkaline reagent test): Extracts were treated with few drops of 20% sodium hydroxide solution. Formation of an intense yellow color, which turns colorless on addition of dilute hydrochloric acid, indicates the presence of flavonoids.

2.3.4. Test for Sterols (Liebermann-Burchard's test): Extracts were diluted with chloroform and filtered. Few drops of acetic anhydride was added to the filtrates and boiled. The cooled filtrates were treated with few drops of concentrated sulphuric acid. Formation of a brown ring at the junction indicates the presence of phytosterols.

2.3.5. Test for Phenols (Ferric chloride test): Extracts were treated with 3-4 drops of 5% ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

2.3.6. Test for Saponins (Foam test): The extracts were diluted with distilled water and warmed on water bath. Development of persevering forth affirms the vicinity of saponins. Furthermore, the frothing was mixed with 3 drops of olive oil and then shaked vigorously. Formation of emulsion indicates the presence of saponins.

2.3.7. Test for Tannins (Braymer's test): Extracts were boiled with 20 mL water and then filtered. The filtrates were treated with 10% alcoholic ferric chloride solution and formation of brownish green to blue-black color indicates presence of tannins.

2.3.8. Test for Cardiac glycosides (Keller Kelliani's test*):* The extracts were dissolved with 4 mL of distilled water and then treated with 2 mL of glacial acetic acid containing few drops of ferric chloride solution. Further, 2 mL of concentrated sulphuric acid was added carefully without mixing the solution. Formation of a brown ring at the interface indicates the presence of deoxysugars (characterisitics of cardenolides).

2.3.9. Test for Carbohydrates (Benedict's test): Extracts were treated with Benedict's reagent (cupric citrate complex) and heated gently. Orange-red/brick red/rusty brown precipitate indicates presence of reducing sugars.

2.3.10. Test for Proteins (Biuret test): Extracts were dissolved in 4mL of distilled water and then treated with an equal volume of 1% sodium hydroxide solution followed by 3 drops of aqueous copper II sulphate solution. A color change from blue to purple/violet indicates presence of proteins.

2.3.11. Test for Quinones: Extracts were treated with concentrated hydrochloric acid. Formation of yellow coloration or precipitate indicates the presence of quinones.

2.4. *In vitro* anti-mycobacterial activity

*In vitro*antibacterial activity was evaluated by standard microdilution method (Begum 2020, Elloff 1998) using non-pathogenic *Mycobacterium madagascariense* and *Mycobacterium indicuspranii*at biological and pre-clinical laboratory of Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences (MUHAS).

2.4.1. Preparation of Extract and Inoculums

The stock solution was prepared by using the respective broth and solubilizationthat aided by 2% DMSO. Dilutions were made to set the concentration in the first wells of the microtitre plates at 12.5 mg/mL.Sterile wire loop was used to take some few colonies of non-pathogenic mycobacteria from sub-cultured plate into a vial containing sterile distilled water; the colonies were emulsified on the sides of the vial to make a suspension equivalent to McFarland 0.5 turbidity.

2.4.2. Testing of crude extract against non-pathogenic *Mycobacterium* species

The media was prepared according to manufacturer instructions (Akintola et al., 2013). The 7H9 broth base medium was used for Minimum Inhibitory Concentration (MIC). Iodonitrotetrazolium chloride (INT) was used as indicator to determine Minimum Inhibitory Concentration. The antimycobacterial activity of ethanolic crude extract of leaveswas determined by using 2-fold broth micro dilution method to determine their Minimum Inhibitory Concentration (Begum et al., 2020). The 96 well plate was labeled with marker pen to include date, name of organism, crude extract, negative control, positive control drug (Ciprofloxacin) and growth control. Sterilized broth was added in a clean and sterilized petri dish, then transferring 50 µL of broth and adding into each of the well of 96 well plate using multichannel pipette with sterilized tips. 50 µL (equal volumes) of crude test extract solution (in triplicate) was added to the first well of the row and the mixed well in well 1 then transferred the 50 µL to well in row B and mix, then C until you reach well in row H where 50 µL was discarded leaving only 50 µL. The same procedure above was repeated for negative control and positive control drug respectively. *Mycobacterium madagascariense*s suspension was added in a clean and sterilized petri dish and then transferring 50

μL into each well of 96 well plate containing test crude extract, negative, positive control and growth control and then incubated at 31 ˚C for overnight. Two hours before reading the result, 40 μL of the indicator (0.2% nitrotetrazoleum) was added, and then incubated the plates for a maximum of two hours. The disappearance of pink/purple color was observed and recorded minimum inhibitory concentration (MIC) where there is no color change. The same procedure explained above was repeated to test crude extract against *Mycobacterium indicus pranii* and the organism was incubated for 37 ˚C and results were recorded in **Table 2**

2.5. Brine Shrimp Cytotoxicity Test

The Brine shrimp test was carried out at the Institute of Traditional Medicine, MUHAS following standard procedures with slight modification (Begum et al., 2020). Brine shrimp (*Artemia salina*) larvae were used as indicator organism for preliminary determination of cytotoxicity of the crude extract. Artificial sea water was prepared by dissolving 3.8 g of sea salt in 1 L of distilled water and then filtered. The solution prepared was then filled into two divided compartments of a tank. Shrimp eggs were spread into the covered part of a tank, and a lamp illuminated the uncovered part to attract the hatched shrimps. After 48 hours of hatching, the matured napaulii were collected. Each sample under study was tested at concentration of 240, 120, 80, 40, 24 and 8 μg/mL dissolved in a DMSO in triplicate vials having 10 brine shrimp larvae. The fourth vial which was used as negative control had only DMSO and brine shrimp larvae whereas cyclophosphamide was used as a standard positive control. The recorded numbers of survivors were used to calculate percentage mortality and survival **(Table 3)** where by percentage mortality was plotted against log concentrations (μg/mL) to obtain regression equations appears in **figure 4**. The regression equations were used to calculate LC_{16}, LC_{50} and LC_{84} and results were tabulated in **Table 4.**

3. Results and Discussion

3.1. Qualitative phytochemical screening tests

The results of phytochemical screening of the ethanolic leaf extract of *V. unguiculata* are tabulated in table 1 which depict the presence of alkaloids, flavonoids, saponins, carbohydrates, terpenoids, tannins, steroids and proteins, and absence of cardiac glycosides and quinones when analyzed qualitatively.

Table 1: Phytochemical classes of ethanol extract of *Vigna unguiculata* leaves extract

SN	Chemical constituent	Plant extract
1.	Alkaloids	+
2.	Flavonoids	+
3.	Cardiac glycosides	-
4.	Saponins	+
5.	Carbohydrates	+
6.	Terpenoids	+
7.	Tannins	+
8.	Steroids	+
9.	Quinones	-
10.	Proteins	+

(+): presence of phytochemical constituents (-): absence of phytochemical constituents

3.2. *In vitro* anti-mycobacterial activity

Vigna unguiculata leaf extract was tested for antimycobacterial activity against non- pathogenic *Mycobacterium indicus pranii* (MIP) and *Mycobacterium madagascariense* (MM). The minimum inhibitory concentrations (MICs) were 0.78125 mg/ml and 1.5625 mg/ml against MIP and MM respectively and tabulated in **Table 2**. The good activity was observed in MIP when compared to MM **(Figures 2 and 3).**

Table 2: The MIC ofleaves crude extract against *Mycobacterium madagascariense* (MM) and *Mycobacterium indicus pranii* (MIP)

SN		Concentration in mg/ml	
	Sample	**MM (n=6)**	**MIP (n=6)**
1.	Leaf extract	1.5625	0.78125
2.	Negative control	No inhibition	No inhibition
3.	Positive control (CP)	< 0.00391	< 0.00391
4.	Positive control (ETH)	No inhibition	No inhibition

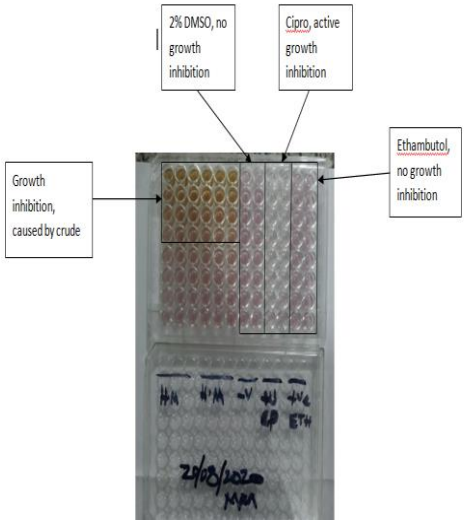

Figure 2

Figure2: Microtitre plate showing results of *Vignaunguiculata*leaf crude extract against *Mycobacterium madagascariense.* Purplish colour means active growing organism, clear or yellow colour means growth inhibition. -Ve = Negative control and +Ve = Positive.

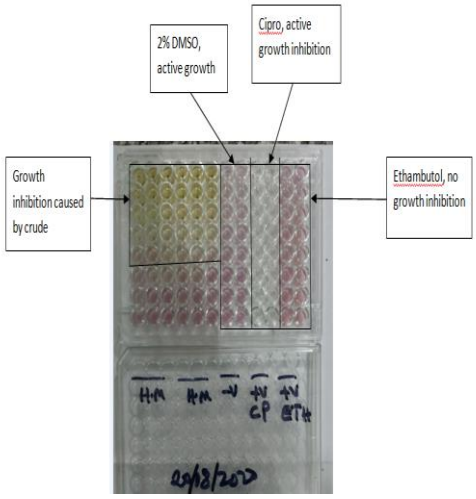

Figure 3

Figure 3: Microtitre plate showing results of *V. unguiculata*leaf crude extract against *Mycobacterium indicuspranii.* Purplish colour means active growing organism, clear or yellow colour means growth inhibition. -Ve = Negative control and +Ve = positive

3.3. Cytotoxicity

Table 3.Nauplii % mortality and survival in different concentrations of extract

Vol. (µL)	Conc. (µg/mL)	Vial set No.	Extract		
			Number of Survival	% Survival	%Mortality
30	240	1.1	6	65	35
		1.2	7		
15	120	2.1	7	70	30
		2.2	7		
10	80	3.1	9	85	15
		3.2	8		
5	40	4.1	9	90	10
		4.2	9		
3	24	5.1	10	95	5
		5.2	9		
1	8	6.1	10	100	0
		6.2	10		
Solvent control	None	7.1	10	100	0
		7.2	10		

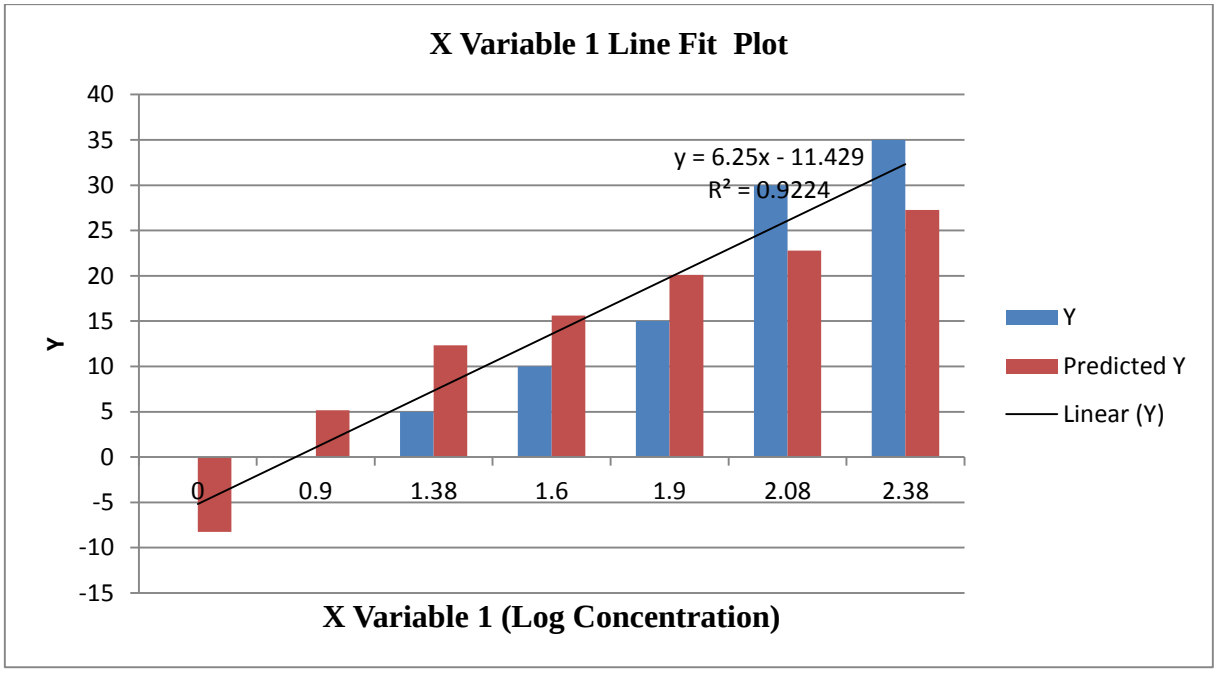

Fig.4. A plot of percentage mortality of nauplii larvae against log concentration of *Vigna unguiculata* leaf crude extract

The determined regression equation from the graph y = 6.25x – 11.42 (Fig.4)
The LC_{84}, LC_{50} and LC_{16} were calculated by using the above regression equation;

$y = m(\log X) + C$ Where by m = Slope, C = Constant.

Then, **For LC_{84}** $LC_{84} = (84 - C) \div m$, where, m = 6.25, and C = - 11.42

LC_{84} = (84 + 11.42) ÷ 6.25

LC_{84} = 15.267 µg/ml

For LC_{50}

$LC_{50} = (50 - C) \div m$

LC_{50} = (50 + 11.42) ÷ 6.25

LC_{50} = 9.827 µg/ml

For LC_{16}

LC_{16} = (16 - C) ÷ m

LC_{16} = (16 + 11.42) ÷ 6.25

LC_{16} = 4.387 µg/ml

Then;

$$S_1 = \frac{LC_{84}}{LC_{50}}$$

S_1 = 15.267 µg/ml ÷ 9.827 µg/ml

S_1= 1.553

$$S_2 = \frac{LC_{50}}{LC_{16}},$$

S_2 = 9.827 µg/ml÷ 4.387 µg/ml

S_2 = 2.24

Then;

$$\text{Average } S = (S1 + S2)/2$$

Average S = (1.553 + 1.25) ÷ 2

Average S = 1.4015

Then;

$$f = Average S^{2.77/\sqrt{n}}, \text{ where } n = \text{number of nauplii per test}, n = 10 \text{ nauplii}.$$

F = $1.4015^{2.77/\sqrt{10}}$

f = 1.344

Hence;

$$\text{Lower limit} = LC_{50}/f$$

Lower limit = 9.827 µg/ml ÷ 1.344

Lower limit = 7.3117 µg/ml

$$\text{Upper limit} = LC_{50} \times f$$

Upper limit = 9.827 µg/ml x 1.344

Upper limit = 13.207 µg/ml

Therefore;

95% CI = Lower limit - Upper Limit

95% CI is 7.3117 µg/ml to 13.207 µg/ml

Table 4.Summarized toxicity results

SN	Property	Extract
1.	LC_{84}(µg/mL)	15.267
2.	LC_{50} (µg/mL)	9.827
3.	LC_{16} (µg/mL)	4.387
4.	S_1	1.553
5.	S_2	2.240
6.	Average S	1.4015
7.	F	1.344
8.	Lower limit (µg/mL)	7.3117
9.	Upper limit (µg/mL)	13.207

Discussion

In this study we have investigated the antimycobacterial activity of *Vigna unguiculata* ethanolic leaf extract against two non-pathogenic strains *Mycobacterium indicus pranii* (MIP) and *Mycobacterium madagascariense* (MM), while cytotoxicity was investigated by using Brine Shrimp Cytotoxicity Test. The results showed that ethanolic leaf extract have significance activity against both tested mycobacterial strains which indicates that the plant may contain one or a mixture of active ingredients responsible for the antimycobacterial activity, this is justified due to the presence of secondary metabolites such as alkaloids, flavonoids, saponins, phenolics, terpenoids, tannins and steroids which have been identified in **Table 1**. These may contribute to the bioactivities and are also in line with Lattanzio*et al*. (1997). However, a previous study reported that the ethanolic leaf extract of *V.unguiculata* have potential activity that inhibit the growth of certain bacterial and fungal pathogens (Kritzinger et al., 2005, Nielsen, 2009 and Dinore et al., 2021). This is likely to occur since it is known that *V.unguiculata* leaves do contain flavonoids and the same flavonoids, isolated from other plant species, have shown antimicrobial activity (*Lattanzio et al*. 1997).

Regarding cytotoxicity test, the National Cancer Institute classified chemicals according to their cytotoxicity as follows: $IC_{50} \leq 20$ μg/mL highly cytotoxic, IC_{50} 21–200 μg/ mL moderately cytotoxic, IC_{50} 201–500 μg/mL weakly cytotoxic, and $IC_{50}>$ 501 μg/mL noncytotoxic (Erhirhie et al., 2018). The plant extract with $LC_{50}<$ 100 µg/mL in the brine shrimp bioassay is classified to be cytototoxic (Khaled A, 2005). The *V. unguiculata* ethanolic leaf extract was not only toxic but is a suggestive of possible anti-cancer activity since its LC_{50} is 9.827 µg/ml whereby it is less than 20µg/ml. This suggests that the ethanolic leaf extract of *V. unguiculata* contains toxic phytochemicals that can destroy brine shrimp larvae cells at normal concentrations. Since *V. unguiculata* is used in the management of chest infections and TB, which is a chronic disease that develops and heals slowly as reported in other literatures (Kritzinger et al., 2005 and Chidebelu et al., 2019), therefore cytotoxicity evaluation by using mammalian cells lines, subacute and subchronic toxicity studies are needed to determine the long-term effects.

4. Conclusion and Recommendations

This study shows that the ethanolic leaf extract of *Vigna unguiculata* exhibited appreciable inhibitory activities against non-pathogenic *Mycobacterium madagascariensis* and *Mycobacterium indicus pranii*. The findings support the use of *V. unguiculata* by traditional health practitioners in the treatment of coughs, tuberculosis and chest infections. However, there is still a need of further evaluation of this plant against drug sensitive *Mycobacterium tuberculosis* and Multi Drug Resistance *Mycobacterium tuberculosis*. More exploration for the constituents such as isolation of bioactive metabolites for antimycobacterial from *V. unguiculata* leaves growing in Dodoma and toxicological studies in order to know its adverse effects to the human should be done.

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