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# Anti-tubercular activity of *Callistemon citrinus* and *Piptadenistrum africanum* on resistant strains of *Mycobacterium tuberculosis* using Microplate alamar blue assay

# *Callistemon citrinus* ve *Piptadenistrum africanum* bitki türlerinin *Mycobacterium tuberculosis* dirençli suşları üzerine anti-tüberküloz aktivitesinin mikropleyt alamar mavisi deneyi ile değerlendirilmesi

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**ABSTRACT**

**Aim:** Tuberculosis (TB) is one of the leading causes of death among infectious diseases in the world. It is responsible for killing approximately 1.4 million people per year worldwide. This devastating situation has steadily worsened, exacerbated by the emergence of drug-resistance and Human Immunodeficiency Virus (HIV) co-infection. The objectives of the study were to determine the minimum inhibitory concentration (MIC) of selected plant species on three TB strains and to determine different phytochemicals contained in the plant species.

**Methods:** Microplate Alamar Blue Assay (MABA) was used to determine the MIC of two commonly mentioned plant species, *Piptadenistrum africanum* and *Callistemon citrinus* on resistant variant strains of *Mycobacterium tuberculosis*. Qualitative tests were used to determine the phytochemicals in the plants.

**Results:** The chloroform extract of *Callistemon spp.* had MICs of 0.048mg/ml, 0.158mg/ml and 0.19mg/ml on the pan sensitive, isoniazid resisistant and rifampicin resistant strains respectively. *P. africanum* had MICs of 0.395 mg/ml, 0.395 mg/ml and 0.78 mg/ml on the pan sensitive, rifampicin and isoniazid resistant strains respectively.

**Conclusion:** These plant species appear to be active not only on the pan sensitive strains of TB but also on resistant strains and could be developed into drugs for the treatment of Multi drug resistant (MDR) TB.

**Keywords:** Drug resistant tuberculosis; Mycobacteria; Efficacy; Medicinal plants.

**ÖZET**

**Amaç:** Tüberküloz (TB) Dünya genelinde enfeksiyon hastalıkları arasında en yaygın ölüm nedenlerinden birisidir. Her yıl Dünya genelinde yaklaşık 1,4 milyon insanın ölüm nedenidir. Bu korkunç durum ilaç direnci ve HIV koenfeksiyonundan dolayı giderek daha kötü hale gelmektedir. Çalışmanın amacı, seçilen bitki türlerinin üç TB suşu üzerine minimum inhibitör konsantrasyonunun (MIC) bulunması ve bitkilerin fitokimyasal içeriğinin ortaya konulmasıdır.

**Yöntemler:** *Piptadenistrum africanum* ve *Callistemon citrinus* bitki türlerinin *Mycobacterium tuberculosis*'in dirençli suşları üzerine MIC değerleri mikropleyt alamar mavisi testi ile (MABA) tespit edildi. Bitkilerin fitokimyasal içeriğinin analizi için kantitatif testler kullanıldı.

**Bulgular:** *Callistemon spp.*'nin kloroform ekstraktının tam duyarlı, izoniazid dirençli ve rifampisin dirençli suşlar üzerindeki MIC değerleri sırasıyla 0,048 mg/ml, 0,158 mg/ml ve 0,19 mg/ml idi. *P. africanum*'un tam duyarlı, izoniazid dirençli ve rifampisin dirençli suşlar üzerindeki MIC değerleri sırasıyla 0,395 mg/ml, 0,395 mg/ml ve 0,78 mg/ml idi.

**Sonuç:** *Piptadenistrum africanum* ve *Callistemon citrinus* bitki türleri sadece tam duyarlı TB suşları üzerine değil aynı zamanda dirençli suşlar üzerinede etkilidir. Bu bitki türlerinden çok ilaca dirençli (MDR) tüberküloz tedavisi için ilaç geliştirilebilir.

**Anahtar kelimeler:** İlaca dirençli tüberküloz; Mikobakteri; Etkinlik; Şifalı bitkiler.

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## INTRODUCTION

Tuberculosis (TB) is an airborne contagious disease which latently affects approximately 2 billion people in the world [1]. Despite the fact that the disease is treatable and curable, it kills approximately 1.5 million people annually [2]. Tuberculosis is considered a disease of poverty with over 80% cases occurring in Africa and Asia [3]. According to World Health Organization, the Millennium Development Goal (MDG) to halt and reverse the TB epidemic by 2015 has not been realized in the African region due to HIV co-infection and development of drug resistance [4]. Multi drug resistant TB (MDR TB) requires use of a combination of second line drugs for a minimum of twenty months [2]. Second line TB drugs used are more expensive, toxic and less effective [5]. Treatment of HIV and MDR TB is difficult since there are drug interactions involved while extensively drug resistant TB (XDR TB) is proving to be untreatable. It is much more likely that the emergence of even more resistant TB strains will be experienced in the near future; exhausting the current battery of chemical defenses available [6]. There is therefore need for development of new remedies to curtail the TB trend.

For a long time, natural products such as plants have provided unlimited opportunities for new drug discoveries because of the unmatched availability of diverse chemicals [7]. In the search for new antimycobacterial agents, *Callistemon citrinus* and *Piptadeniastrum africanum* were tested on different resistant TB variants. *Callistemon citrinus* and *Piptadeniastrum africanum* are used by local communities in Uganda for treatment of TB and TB related symptoms [8-9].

## MATERIALS AND METHODS

### Collection and preparation of extracts

Leaves and seeds of *Callistemon citrinus* (Curtis) Skeels (Mwambalabutonya in luganda language) and stem bark of *Piptadeniastrum africanum* (Hook.f.) Brenan (Mpewere in luganda) were collected from Luwero and Mukono districts in Central Uganda respectively. Voucher specimen indexed as BL014 and BL040 respectively were deposited at the Makerere University Herbarium where they were also identified. The Plant parts were dried under shade, pulverized using a wooden mortar and pestle and stored at room temperature for further use. Plant powder (200g) of both plants was weighed and soaked separately in methanol (1000ml) and chloroform (1000ml) for four days with occasional shaking. Filtration was done using Whatman's filter paper no 1. The filtrate was concentrated using a rotary evaporator (BüchiLabortechnik AG, Switzerland) at 40°c and reduced pressure. The crude extracts were stored at -2°C until further use. Percentage yields for each of the crude extracts were calculated using the formula below.

Percentage yield = $(X_1 / X_2) \times 100\%$. [Where, $X_1$ = weight of the concentrated extract, $X_2$ = weight of the dry plant powder before extraction].

### Mycobacterial testing

Susceptibility testing of extracts was determined using the Micro plate Alamar Blue Assay [10]. The method involves use of a dye to detect growth. If there is change of color from blue to pink then this indicates that there is growth with the implication that the extract/ drug at this concentration is not active and vice versa.

These tests were carried out in a bio safety level three laboratory at Joint Clinical Research Center (JCRC); located in Mengo Kampala Uganda. Three preserved strains of *M. tuberculosis* were used and they included; a rifampicin resistant strain (TMC 331/ATCC35838), an isoniazid resistant strain (TMC 301/ ATCC 35820) and a fully susceptible strain (H37Rv) as a positive control. All strains used were obtained from JCRC.

The three strains of *M. tuberculosis* were revived on Middle brook 7H10 agar (Becton Dickinson Company (DifcoTM), 7 Loveton Circle, Sparks, Maryland, USA; Lot No. 8) which was prepared according to Manufacturer's instructions. Cells were scraped from freshly growing colonies (two weeks old) on Middle brook 7H10 plates and introduced into 7H9 broth (10ml) (Difco, Detroit, Mich) supplemented with 0.2% (v/v) glycerol (Becton Dicknson Lot: B 0523384), 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase; Becton Dicknson Lot: 2065182). This was incubated at 37°C for 24 hours. The mixture was vortexed for 30 seconds in a glass bottle containing glass beads and the particles were allowed to settle. McFarland standard 0.5 was prepared using a nephrometer.

Isoniazid and rifampicin were used as positive control drugs and these were prepared in sterile water and DMSO respectively. The extracts were prepared in methanol and double dilutions made using 7H9 broth. The final concentrations tested were 0.063-16 µg/ml for both isoniazid and rifampicin and 0.012-6.250 mg/ml for the extracts.

Clear-bottomed, 96-well micro plates (Falcon 3072; Becton Dickinson, Lincoln Park, N.J) were used in the study. Two hundred microliters of sterile distilled water was added to all outer-perimeter wells of sterile 96-well plates. This was done to minimize evaporation of the medium in the test wells during incubation. The remaining wells received 100µl of 7H9 broth. One hundred microliters of drug solutions were added to the wells in rows B to G in column 2. By using a pipette, 100µl was transferred from column 2 to column 3, and the contents of the wells were mixed well. Identical serial 1:2 dilutions were continued through column 10 and 100µl of excess medium was discarded from the wells in column 10. The wells in 11 were drug free and therefore acted as negative control wells. The inoculum (100µl) was added to each of the wells. The plates were sealed with carbondioxide permeable tape and they were incubated at 37°C for 24 hours.

Thirty micro liters of a freshly prepared Alamar Blue (Accumed International, Westlake, Ohio) reagent was added to well B11. The plates were re-incubated at 37°C for 24 hours. Well B11 had not turned pink, the reagent mixture was added to another control well and the microplates were incubated for an additional 24 hr at 37°C. After 24 hours the control well had turned color from blue to pink and the dye was added to all the wells and re-incubated for 24 hours.

A few wells appeared blue after 24hr of incubation, but they invariably changed to pink after another day of incubation and thus were scored as growth. The Minimum Inhibitory Concentration was defined as the lowest drug concentration which prevented a color change from blue to pink.

The tests were prepared in triplicate; the mean value and standard deviation of the mean were calculated using Microsoft excel.

### Qualitative phytochemical testing

Phytochemical screening of the plant extracts were carried out to determine presence of Alkaloids (Dragendorff's, Mayer's, and Wagner's reagents), Flavonoids (Lead acetate test, Ferric chloride test, Shinoda's test), Saponins (Froth test and Legal test), Tannins (Ferric chloride solution test), Terpenes (Liebermann-Burchard's test) in each plant sample using standard methods [11].

The study was approved by the Uganda National council of Science and Technology (HS1288) and the Makerere University College of Health Sciences Institutional Review Board.

## RESULTS

### Antimycobacterial testing

A total of six crude extracts from *P. africanum* and *C. citrinus* were tested on resistant variant strains of *Mycobacterium tuberculosis* using MABA. Five of the six tested extracts were active on the pan sensitive, isoniazid resistant and rifampicin resistant strains

The leaf Chloroform extract of *C. citrinus* had the highest activity with MICs of 0.19mg/ml on the Isoniazid resistant strain, 0.158mg/ml on the rifampicin resistant strain and 0.048mg/ml on the pan sensitive strain (Table 1). It was followed by the seed chloroform extract of the same plant with MICs of 0.195mg/ml,0.395mg/ml and 0.256mg/ml on the pan sensitive, rifampicin resistant and isoniazid resistant strains respectively. The *C. citrinus* seed methanol extract was active on the pan sensitive (MIC= 0.195mg/ml) and rifampicin resistant strain (MIC= 0.395mg/ml) however it was not active on the isoniazid resistant strain.

The *P. africanum* chloroform extract showed moderate activity on all the three strains with MICs of 0.395mg/ml on both TMC 301 and 331 and 0.78mg/ml on H37Rv. The methanol extract of *P. africanum* was not active on all tested strains.

The pan sensitive strain showed the highest susceptibility to the extracts followed by the rifampicin resistant and Isoniazid resistant strains. Isoniazid and rifampicin drugs had MICs of 4µg/ml and 2µg/ml respectively on the pan sensitive strain.

### Preliminary phytochemical testing

The plant extracts were qualitatively tested for alkaloids, terpenoids, tannins, flavonoids, saponins and phenols (Table 2). All the extracts tested positive for presence of phenols except for the chloroform extract of *P. africanum*. Saponins were present in very high concentrations in the methanol extract of *P. africanum* and they were also present in the methanol extracts of *C. citrinus*.

While tannins were present in all the extracts except the chloroform extracts of *C. citrinus*, alkaloids were only present in the methanol extract of *P. africanum*. Flavonoids were present in all the extracts except *P. africanum* methanol extract.

**Table 1.** Percentage yields and Minimum Inhibitory Concentrations of plant species used on H37Rv, TMC331 and TMC 301

| Plant spp | Part used | Extract | % yield | H37Rv MIC mg/ml | TMC 331 MIC mg/ml | TMC 301 MIC mg/ml |
|---|---|---|---|---|---|---|
| *C. citrinus* | leaves | methanol | 15.34 | 0.325±1.2 | 0.78±0.6 | 0.39±0.0 |
| *C. citrinus* | leaves | chloroform | 25.60 | 0.048±0.0 | 0.158±0.0 | 0.19±0.0 |
| *C citrinus* | seeds | methanol | 7.98 | 0.195±1.7 | 0.395±0.0 | NA |
| *C. citrinus* | seeds | chloroform | 3.68 | 0.195±1.0 | 0.395±0.0 | 0.256±0.7 |
| *P. africanum* | stem bark | methanol | 0.75 | NA | NA | NA |
| *P. africanum* | stem bark | chloroform | 14.00 | 0.395±0.0 | 0.395±0.0 | 0.78±0.0 |
| Rifampicin | | | | 0.002±0.0 | NA | 0.002±0.0 |
| Isoniazid | | | | 0.004±0.0 | 0.004±0.0 | NA |

NA= Not active, MIC= Minimum inhibitory concentration, H37rv=pan sensitive strain, TMC331 = rifampicin resistant strain and TMC 301= isoniazid resistant strain, ± standard deviation

**Table 2.** Phytochemicals present in *P. africanum* and *C. citrinus* extracts

| Plant extract | Alkaloids | Triterpenoids | Tannins | Flavonoids | Saponins | Phenols |
|---|---|---|---|---|---|---|
| P. africanum methanol extract | ++ | + | + | - | +++ | + |
| P. africanum Choloform extract | - | - | + | + | - | - |
| C. citrinus Leaf methanol extract | - | + | + | ++ | ++ | ++ |
| C. citrinus Leaf Chloroform extract | - | + | - | + | - | ++ |
| C. citrinus seeds methanol extract | - | + | + | + | ++ | ++ |
| C. citrinus seeds Chloroform extract | - | - | - | + | - | + |

+++ = very high concentrations, ++= high concentrations, +=Trace, - = absence

## DISCUSSION

Development of TB resistance to conventional first line and second line drugs has necessitated the search for novel drugs that are more effective, cheaper and less toxic. This study determined anti TB activity of *C. citrinus* and *P. africanum* using MABA. Compared to other extracts tested in this study the chloroform leaf extract of *C. citrinus* showed the highest activity on both the Isoniazid and rifampicin resistant strains. This could mean that the extract could be active on MDR TB because it confers an advantage over the most efficacious first line drugs. The high activity could be attributed to presence of phenols, triterpenoids and flavonoids which could have conferred synergistic effects. The seeds of the *C. citrinus* were less active than the leaves. This could be due to presence of higher concentrations of phytochemicals in the leaves than the seeds

Sensitivity of the leaf ethanol extract of *C. citrinus* on fast growing *M. aurium* was documented using the disc diffusion method by Frame *et al* [13]. The zones of inhibition were 27mm and 8mm at 1000µg/ml and 8µg/ml respectively. Measurement of zones of inhibition is not a reliable method for quantitative comparison [14]. This is perhaps the first study to test *C. citrinus* seeds and leaves on resistant strains of slow growing virulent *M. tuberculosis* which causes TB in humans. Several studies have documented the plant's antibacterial, antifungal, spasmolytic and anticancer activities [15-16].

Previous studies done on the chloroform and methanol leaf extract of *C. citrinus* have demonstrated presence of alkaloids, saponins, tannins and phytosterols [17]. This is in agreement with what we document except that alkaloids were not present in the *C. citrinus* tested in this study. This might be due to the fact that the studies are from different geographical regions. This study has also documented

presence of terpenoids and is in agreement with previous studies [16, 18]. The most abundant terpenoids such as α pinene, 1,8 lineole, spathlenol , limonene and essential oils in the leaf extract were characterized [16].

The chloroform extract of *P. africanum* showed minimal activity on the three tested species of *Mycobacteria*. The activity could be attributed to presence of flavonoids in the chloroform extract. This is one of the first studies to document antimycobacterial activity of the plant however, its activity on different species of fungi and bacteria has been investigated [10, 19]. These studies have documented presence of triterpenes, polyphenols, tanins, flavonoids and saponins. In this study, flavonoids were only present in the chloroform extract and abscent in the methanol extract. The difference in the results could be due to the different methods of extraction and parts used. Saponins like oleanolic acid and echinocystic acid were isolated from *P. africanum* [20].

Generally the chloroform extracts showed higher activity on TB than the methanol extracts. This does not differ from what [21] observed that chloroform extracts of *Erythrina abyssinica* were more active than the methanol and ether extracts on TB. This is because Chloroform extracts could contain lipid soluble phytochemicals like terpenes that would easily penetrate the highly lipophilic wall of *Mycobacterium*.

The pan sensitive strain showed the highest susceptibility to the extracts as compared to the resistant strains; this is expected as the strain is susceptible on all the first line drugs however the potency of the extracts on H37Rv as compared to the pure drugs was still very low. This could be attributed to the fact that crude extracts were used in the study and if purified compounds are tested they could confer comparable efficacy.

Many plants have been evaluated for their antimycobacterial activity according to previous reviews done [22-25]. Most of these studies have worked on the fast growing *Mycobacteria* however a few of them have evaluated activity on the resistant strains of the slow growing Mycobacteria and the results are promising.

## CONCLUSION

This study shows that *C. citrinus* and *P. africanum* are active not only on the pan sensitive strains of TB but also on resistant strains. This study also supports the fact that selecting plants by ethnobotanical criteria enhances the probability of finding species with activity.

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## COMPETING INTERESTS

There are no competing interests

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