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In vitro anti-tuberculous study on the combination of extracts of stem-bark of *Erythrina abyssinica* Lam. ex DC and conventional drugs

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Abstract

Background: Tuberculosis (TB) caused by *Mycobacterium tuberculosis* remains an important public health issue worldwide and TB patients are reported to use conventional drugs concurrently with medicinal plants. This study investigated effect of co-administration of *Erythrina abyssinica* stem-bark extract with Rifampicin and Isoniazid on *Mycobacterium tuberculosis in vitro*.

Methods: The stem-bark of *E. abyssinica* was extracted using Methanol and Ethanol, separately. The extract, Rifampicin and Isoniazid drugs were separately reconstituted with distilled water for the experiment. Preserved strain of Tuberculosis H₃₇RV was used with 7H11 middle brook agar. The Minimum concentrations of each extract was mixed with Minimum Inhibitory Concentrations of Rifampicin and Isoniazid and tested on strain of Tuberculosis H₃₇RV to determine the inhibitory effect.

Results: The combination of 0.06µg/ml ethanol extract with 0.01µg/ml RI gave 99.2% inhibition and the combination of 0.06µg/ml methanol extract with 0.01µg/ml RI gave 99% inhibition while the combination of 0.01µg/ml RI gave 86.2% inhibition

Conclusion: The addition of *Erythrina abyssinica* extracts to the two conventional drugs produced antituberculous effect and thus corroborates the use of *E. abyssinica* with conventional drugs to treat tuberculosis patients. There is thus need to further investigate clinically the effects of the combination of the *E. abyssinica* extracts with the conventional anti tuberculosis drugs on patients.

Keywords: Tuberculosis, *Erythrina abyssinica*, *Mycobacterium tuberculosis*, conventional drugs

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is an important public health issue worldwide, accounting for eight million new cases per year (Garbuli, 2012). Globally, about 3 million mortalities caused by *M. tuberculosis* are recorded annually with Uganda ranked 18th out of the 22 TB endemic countries throughout the world in 2009. More than 50% of untreated cases of TB cause mortality worldwide as a single infectious agent (Pendland *et al.*, 2004) [9]. Moreover, the epidemiology of TB is affected by multidrug resistant (MDR) tuberculosis strains and of HIV/AIDS infections (Leite *et al.*, 2008) [8]. The increasing rate of TB cases and the resistance of the bacteria to antibiotics have prompted scientists to investigate medicinal plants with reputation of use in folk medicine as alternative therapies. Some literatures reported that medicinal plants and natural products used in folklore have anti-mycobacterial activity *in vitro* (Buzayan, M. M. & El-Garbuli, F. R., 2017) [3]. Medicinal plants reportedly in folklore for TB include *Eucalyptus spp.*, *Warbugia salutaris* Chiov., *Ocimum suave.*, *Zanthoxylum chalybum* Eng., *Momordica foetida*, *Persea americana*, *Acacia hockii*, *Erythrina abyssinica*, *Cryptolepis sanguinolenta* and *Solanum incanum* (Tabuti, Kukunda, & Waako, 2010; Bunalema, 2010) [12, 2]. Some of these plants have been established to have anti mycobacterium activity in which *E. abyssinica* was among (Bunalema, 2010) [2]. Communities use *E. abyssinica* concurrently with modern tuberculosis drugs, but there was no documentation on this neither was any science to back this proof. Therefore, this study investigated *in vitro* interaction of *E. abyssinica* extracts with Rifampicin plus Isoniazid which are commonly-used as anti-tuberculosis drugs to establish the claim.

Methods

This *in vitro* study was conducted in Makerere University Tuberculosis Laboratory, Uganda and required no human and animal participants.

Plant extract

The stem-bark of *Erythrina abyssinica* was collected from Natural Chemotherapeutic Research Institute medicinal garden, identified and authenticated at the Makerere University Herbarium.

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The stem-bark was shredded, washed, air dried, pulverized mechanically and separately extracted with ethanol and methanol of analytical grade using maceration method to obtain the crude extracts. The extracts were concentrated *in vacuo* at 40 °C and evaporated to dryness in a desiccator. Preserved strain of Tuberculosis H₃₇RV, a fully susceptible standard isolate, sensitive to all mycobacterium standard drugs and pure Rifampicin drug, sterilized bio discs were obtained from Makerere University Tuberculosis Laboratory, Mulago.

Cultures

A standard DIFco™ middle brook agar enriched with Oleic acid albumin catalase (OADC) was then prepared. The stability of the plant extract was carried out by sub culturing to ensure there was no growth of any microorganism. The Minimum Inhibitory Concentrations (MIC) of the plant extract, Rifampicin and the effect of the plant extract alone and in combination with Rifampicin were determined *in vitro*.

MIC of Rifampicin

Each concentration of 0.01, 0.1, 0.2, 0.3, 0.4, and 0.5 µg/mL of Rifampicin was prepared from standard concentration of 1.0 µg/mL. 50µl of middle brook agar 7H₁₁ was dispensed into the sections of the sterile bio-disc. Concentration of 0.01µg/mL of Rifampicin was pipetted and added into the first section of the sterile bio-disc and the second left blank. Two hundred (200) µL of the H₃₇ RV *M. tuberculosis* strain was then pipetted and added on the sterile impregnated bio disc. This was repeated for all the prepared concentrations of Rifampicin. The inoculated discs were then allowed to dry in a hood for 6hours. The discs were sealed and put in an incubator for 3 weeks. Colony of organism was then determined by counting the growth of the organism. The lowest concentration of Rifampicin that inhibits growth was then taken as the MIC of Rifampicin used.

MIC of ethanol and methanol extracts

Concentration of ethanol and plant extracts of 6.8.10 and 14µl/mL were firstly prepared and later scaled down to 4, 3, 2, 1, 0.5, 0.25 and 0.125 µl/mL when there was no observed growth. Four (4) µl/mL ethanol and methanol extract were separately pipetted and added in the first section of the sterile bio disc and the second left blank. Two hundred (200) µL of H₃₇ RV strain of *M. tuberculosis* was pipetted and added on both sections. The inoculated disc was then allowed to dry in a hood for 6 hrs., later sealed and put in an incubator at 37°C for three weeks. This was repeated for other prepared concentrates. The activity of the drug on *M. tuberculosis* was determined by counting the colony of growth of organism. The lowest concentration of the extracts that inhibits growth was then taken as the MIC of the extracts

Combination of the extracts with rifampicin

The culture media used was middle brook 7H₁₁ agar placed in a bio-disc. A pair of bio discs was used for each concentration of methanol and Rifampicin. In each section of the disc, 50µL of the culture media was added. First section was impregnated with 0.01µg/mL Rifampicin as a positive control and the second left blank as a negative control. In another, bio-disc was impregnated with 4µl/mL methanol extract and the second section with combination of 4µl/ml methanol extract and 0.01µg/ml Rifampicin. The sets of the bio disc were then left in the hood for 6 hours to allow diffusion of the drugs. The sets were then incubated at 37 °C for three weeks. The

synergistic effect of the combination of Rifampicin and methanol extract on *Mycobacterium tuberculosis* was then determined by counting the colony of growth of the organism. The experiment was later repeated for the other concentration of methanol extracts at 0.74, 0.49, 0.25, 0.12, 0.06 and 0.03µl/mL. This method was repeated for the synergistic combination of ethanol extract with Rifampicin at the same time and the same condition.

Combination of the extracts, rifampicin and isoniazid

The culture media used was middle brook 7H₁₁ agar placed in a bio disc with two sections. A pair of bio-discs was used for each concentration of Rifampicin, Isoniazid and extracts. In each section of the bio disc, 50uL of the culture media was added. Concentration of 0.01 µg/mL Rifampicin and Isoniazid impregnated disc was separately prepared and put in the first section as positive control and the second section left blank as negative control. In another bio-disc, 0.99 µg/mL methanol extract impregnated disc was placed in the first section and a combination of 0.01 µg/ml Rifampicin, 0.01 µg/ml Isoniazid and 0.09 µl/ml methanol impregnated disc was placed in the second section. The sets of the bio-disc were inoculated with 200 µL of H₃₇RV strain of *M. Tuberculosis* strain. The bio-discs were then left in the hood for 6hours to allow diffusion of the drugs and the sets were then incubated at 37°C for three weeks. The effect of the drugs and methanol extract on *M. tuberculosis* was determined by counting the colony of growth of the organism. The experiment was repeated for the concentrations of 0.74, 0.49, 0.25, 0.12, 0.06 and 0.03µg/ml of methanol extract and for ethanol extract at the same time and condition.

Qualitative phytochemical profile

Qualitative tests of the methanol and ethanol extracts for Terpenoids, Tannins, flavones and alkaloids was carried out using the methods described by Edeoga (2005) ^[5] (Edeoga *et al.*, 2005) ^[5]. The presence of terpenoids was tested by dissolving 1mL of the methanol extract in 1mL of chloroform and evaporating to dryness. 1mL of concentrated H₂SO₄ was then added and heated for 2 minutes with a bluish red coloration indicating the presence.

Tannins was tested by stirring 2mL of the methanol extract in 2 mL of the distilled water and addition of drops of 0.1M FeCl₃ with blue black coloration indicating the presence.

Flavone was detected by adding 5mL of ammonium solution to 1mL of methanol extract followed by the addition of 2mL H₂SO₄. A yellow coloration indicated presence of flavones.

Presence of Alkaloid was tested by mixing 50g of the powder with 250mL of 1% H₂SO₄. It was allowed to stand, filtered and Meyer's reagents was added to 10mL of the filtrate and then shaken. Formation of a white precipitate indicated presence of alkaloids.

Data analysis

The results were expressed as mean±SEM. The variation in a set of data was analyzed through one-way analysis of variance and the difference among the means was considered at 95% confidence level using post-hoc test of Dunnett.

Results

The methanol extract of *Erythrina abyssinica* showed activity against *Mycobacterium tuberculosis* with colony of growth ranging from 119 colonies to innumerable colonies(IC) at concentration of 0.49µg/mL to 0.125µg/mL (Table 1). The ethanol extract showed activity against *M. tuberculosis* with colony of growth ranging from 1 to innumerable colonies (IC) at 0.49 µg/mL to 0.03µg/mL (Table 1).

Table 1: Effect of extracts with Rifampicin plus Isoniazid on *M. tuberculosis* growth in vitro

Variable	No. of Observations	CFU	95% I.C	P-values
Negative control (blank)	10	500.00±0.00	-	-
RI (0.01 µg/mL)	10	135.80±1.12	130 to 142	<0.001
Ethanol ext (0.99 µg/mL)+RI (0.01 µg/mL)	10	4.60±1.96	0 to 15	<0.001
Methanol ext (0.99 µg/mL)+RI (0.01 µg/mL)	10	7.10±5.66	0 to 16	<0.001

CFU- Colony Forming Units, RI- Rifampicin and Isoniazid

Data are expressed as mean±SEM, *P<0.001 compared with negative control

Table 2: Phytochemical groups identified in the active extracts

Group	Methanol extract	Ethanol extract
Terpenoids	+	+
Alkaloids	+	+
Tannins	+	+
Flavonoids	+	+

+ denotes positive

Discussion

The results of phytochemical screening showed the presence of some secondary metabolites like terpenoids, alkaloids, tannins and flavonoids. The presence of these phytochemicals was linked to anti-mycobacterium (Bunalema, 2010) [2]. A vast array of alkaloids, flavanones and tannins in the plant extracts have been found in the genus *Erythrina* in the previous studies (Copp, 2003; Yenesew *et al.*, 2004; Bunalema, 2010) [2, 14, 4] and it is in agreement with this study (Table 2).

The study on the combination of Rifampicin with *Erythrina abyssinica* methanol and ethanol extracts were tested on *Mycobacterium tuberculosis* and results showed a synergistic activity. The results of the anti-mycobacterium activity of the plant extracts and the MIC show that the plant extracts were active against the bacteria.

The presence of some of the secondary metabolites like terpenoids lead to the increasing number of natural products with reported anti-mycobacterial activity, this is due to the lipophilic nature of terpenoid and their ability to penetrate the mycobacterial cell wall (Bunalema, 2010) [2].

Novel drug leads in tuberculosis treatment have in recent years been provided by natural products or their semi synthetic derivatives (Shu, 1998) [11]. Streptomycin and kanamycin from *Streptomyces griseus*, Capreomycin isolated from *S. capreolus* are examples of such natural compounds in clinical use against TB (Shu, 1998; Tribuddharat & Fennewald, 1999; Copp, 2003) [11, 13, 4].

Rifampicin, a semi synthetic drug derived from Rifamycin isolated from *Amycolatopsis mediterranei* (Tribuddharat & Fennewald, 1999) [13] had an MIC at 0.3 µg/ml (Table 1) in this study. A pure isolated compound often exhibited a better MIC with much higher activity. However, with increased dilution of Rifampicin, its activity against *M. tuberculosis* decreased which showed that it is concentration independent.

The plant extracts of *E. abyssinica* when applied alone showed little activity against *M. tuberculosis* with 125 colonies of growth for methanol and 10 colonies of growth for ethanol extracts at 0.49 µg/mL respectively, as shown in Table 1

The combination of plant extract with Rifampicin had no colonies of growth at 0.49 µg/mL for both methanol and ethanol extracts, innumerable column of growth for Rifampicin alone, 10 and 125 colonies of growth for ethanol and methanol extracts respectively showing activity higher than for both plant extracts and Rifampicin alone. At plant extracts concentration of 0.12 µg/mL, there was 220 and 200 colonies of growth for combination of Rifampicin with methanol and with ethanol extracts respectively, innumerable

column of growth for Rifampicin alone, 125 and 300+ colonies of growth for ethanol and methanol extracts alone respectively showing a reduction on the plant extracts activity at reduced concentration

The combination of 0.06 µg/mL methanol extract with Rifampicin and Isoniazid had 10 colonies of growth, innumerable colonies of growth for methanol extract alone and 136 colonies of growth for Rifampicin and Isoniazid combination. there were 8 colonies of growth for the combination of ethanol extract with Rifampicin and Isoniazid, 300+ colonies of growth for ethanol extract alone and then 138 colonies of growth for Rifampicin and Isoniazid combination. The combination of the first line drugs with the plant extracts showed much greater activity at reduced concentration of the plant extracts

These results confirmed that the interaction of the plant extracts and Rifampicin alone exhibit synergistic anti-mycobacterium activity. The combination of the plant extracts and the two conventional drugs exhibited higher synergistic anti-mycobacterium activity.

The therapeutic indications of medicinal plant-based extracts are in most cases empirical and practitioners of phytotherapy intuitively believe that a total extract acts better than an equivalent dose of isolated substances (Kursar & Nelson, 1999) [7]. Substances that constitute a total extract may be academically divided into; active substances, co-effectors, matrix formers and the interaction between them can protect the active substances from decomposition (Biavatti, 2009) [1]. Combinational therapy is an important treatment modality in many disease settings including cancer, cardio-vascular disease and infectious diseases. A combination has substantial activity and provides greater than additive activity or more durable response compared to individual agents alone (source). Drug combination is most widely used in treating dreadful diseases such as malaria, cancer, HIV/AIDS and TB, and the main aims are to achieve improved therapeutic effect, dose and toxicity reduction and to minimize or delay the induction of drug resistance (source)

Two types of synergy are observed in the case of the pharmacological or clinical effects of herbal material; in one case, the activity of an active compound or extract which on its own has no effect and the other common situation is when all compounds concerned have activity (Houghton, 2009) [6] but in combination it is much greater than expected. In some traditional medicine systems, mixtures of plants are used rather than one species probably for the above reasons.

Pure drugs industrially produced or isolated from plants may be chosen for their high activity against a human disease however they rarely have the same degree of activity as the unrefined extract and this is attributed to the absence of interacting substances present in the extract (Rasoanaivo *et al.*, 2011) [10]. Many medicinal plants are known to contain substances that inhibit MDR, and probably, they could exist in many more plants than are currently recognized. These compounds often have little or no direct effect so would be discarded in the conventional process of screening and bio-

assay guided fractionation. However, when combined with compounds which in isolation have only moderate activity and already purified, they may reveal a much higher level of activity and this is in agreement with this study.

Conclusion

In the present study, the effect of the ethanol extract of *E. abyssinica* against *M. tuberculosis* was significantly better than the activity of the methanol extract. The effect of the ethanol extract alone was significantly lower than that of Rifampicin-Isoniazid combination. The ethanol extract offered better synergy with RI than did Methanol extract. The results of this study have shown that there is potential to develop new compounds against tuberculosis from ethanol extracts of *Erythrina abyssinica*. The findings from this study therefore corroborates their use by herbalists in the treatment of Tuberculosis especially in combination with anti TB drugs. Studies are already ongoing in our laboratories to test the combination on the resistant strains, evaluate the clinical potency and to isolate active constituent (s) from ethanol extract.

Declarations

Ethical considerations

Ethical approval was sought from the School of Health Sciences, Makerere University. Study registration number SHSREC REF 2013-016

Protection of the investigators was ensured by carrying out the work in collaboration with, and under the guidance of the Mycobacteriology laboratory technical staffs at the Makerere University TB laboratory, Mulago. Additionally, the necessary protective wears including respirators and gloves as well as safety cabinets were used, to minimize the risk of exposure to *M. tuberculosis*

Safe disposal of infectious waste

All infected materials, including closed specimen containers, were placed in the BSC in autoclavable bags.

All cultures and related materials were autoclaved, placing the infected materials inside autoclavable bags and following procedures for adequate decontamination.

Availability of data and materials

The data that support the findings of this study are available from [third party name] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [third party name].

Conflict of interest

All authors declare no conflict of interest

Authors Contribution

This work was carried out in collaboration between all authors. Author Jacqueline Aber managed the literature searches, carried out the bench-work and wrote the first draft of the manuscript as a postgraduate student under the supervision of author Patrick Engeu Ogwang and was co-supervised by author Norbert Anyama. Author Clement Olusoji Ajayi co-drafted the manuscript.

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