Journal of Health Science 6 (2018) 296-303
doi: 10.17265/2328-7136/2018.04.008

In Vitro Anti-tuberculosis Activity of Total Crude Extract of *Echinops Amplexicaulis* against Multi-drug Resistant *Mycobacterium Tuberculosis*

Komakech Kevin[2], Kateregga John[1], Namaganda Carolyn[2], Semugenze Derrick[2] and Aloysius Lubega[3]

1. Department of Veterinary Pharmacology, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, P.O. Box 7062, Kampala, Uganda

2. Department of Microbiology, Mycobacteriology (BSL-3) Laboratory, College of Health Science, Makerere University, P.O. Box 7072, Kampala, Uganda

3. Department of Pharmacology and Therapeutics, College of Health Sciences, Makerere University, P.O. Box 7072, Kampala, Uganda

Abstract: Background: TB (Tuberculosis) is the second leading killer infectious disease after HIV (human immunodeficiency virus). Its incidence is worsened by development of multi-drug resistant and extensive drug resistant TB strains. Available treatment regimens are expensive, toxic and lengthy resulting to problems of non-adherence and inadequate response. Medicinal plants on the other hand may offer hope for developing alternative medicine for treatment of TB. This study evaluated the anti-tuberculosis activity of *Echinops amplexicaulis*. Materials and methods: Total crude extracts of *E. amplexicaulis* were tested for activity against a wild strain resistant to Rifampicin and Isoniazid (MDR), a fully susceptible laboratory strain (H37Rv) and *Mycobacterium bovis* (BCG strain) using disk diffusion method. MIC (minimum inhibitory concentration) was determined using Middlebrook 7H9 broth. The strains were sub-cultured on Middlebrook 7H10 medium and MBC (minimum bactericidal concentration) determined. Susceptibility was evaluated by measuring zones of inhibition; MIC was obtained as the lowest concentration with no significant growth as shown by clog formation of MTB (*Mycobacteria tuberculosis*) cells on the walls of the macro broth tube and MBC was obtained as the lowest concentration that inhibited growth of MTB colonies on Middlebrook 7H10 medium. Results: The extract showed a significant effect at a concentration of 50 mg/mL against all the three test strains F (2, 18) = 437.7, p = 0.00. It exhibited a MIC of 0.0488 mg/mL against MDR-TB and *M. bovis*. Its MBC was the same at 0.0977 mg/mL against both MDR TB and *M. bovis*. The MIC was much lower (0.0122 mg/mL) for the H37Rv strain. Terpenoids, alkaloids and tannins were present in large amount in the extract while saponins were present in small amounts. Flavonoids were not detected in the extract. Conclusion: *E. amplexicaulis* has the potential to be developed into new anti-TB drug and outcome of the study supports the folkloric claims of anti-tuberculosis activity of the plant.

Key words: *Echinops amplexicaulis*, anti-tuberculosis activity, multi-drug resistant tuberculosis.

1. Introduction

TB (Tuberculosis) is one of the major causes of ill-health and mortality worldwide; it had an incidence rate of 10.4 million cases with a mortality of more than 1.3 million in 2016 [1]. It affects about one third of world's population with severe burden implicated in the sub-Saharan Africa regions where 22 high burden countries account for 80% of the world's TB burden [2]. In Uganda, the situation is alarming with 43,858 new TB cases [3] and a mortality of 25,000 deaths [4]. In 2010, the Global WHO report ranked Uganda 16th among the twenty two high burden countries [5]. However, in the recent WHO report, it exits among the top 20 countries with the highest estimated number of TB cases among people living with HIV [1].

One of the major reasons contributing to the rise in global incidence of TB is the emergence of drug resistant *Mycobacterium tuberculosis* strains [6]. MDR-TB (multidrug-resistant tuberculosis) is a form of TB caused by TB strain that does not respond to at

Corresponding author: Komakech Kevin, bachelor of biomedical laboratory technology, laboratory technologist, research field: mycobacteria tuberculosis.

least isoniazid and rifampicin, the two most powerful first-line anti-TB drugs while extensive drug resistant tuberculosis (XDR-TB) is a form of TB that is resistant to first line drugs, fluoroquinolones and at least one of the three injectable second-line drugs [7]. Drug resistance is thus a major threat to global tuberculosis care and control; for example in 2016; WHO (World Health Organization) estimated 490,000 new cases of MDR-TB [1] and approximately 240,000 deaths from its worldwide [8]. In Uganda, 1,900 of all new cases of tuberculosis are multi drug resistant [4].

Treatment of MDR-TB requires use of second-line drugs that are more costly, less effective and more toxic then the first-line isoniazid and rifampin-based regimens [7]. On the other hand, co-infection with HIV among TB patients has worsened the situation making effort to control tuberculosis much more difficult [7]. Globally by the end of 2016, WHO estimated 10% of people having TB were co-infected with HIV [1]. Treating TB patients co-infected with HIV has been linked to relapses, treatment failure, acquiring drug resistance and finally drug interactions that increase risk of toxicity [10]. Alternative medicine especially those of natural origin are therefore highly recommended because they are safe and dependable compared to synthetic drugs that are expensive and have adverse effects [11].

The ability of plants to synthesize aromatic substances and secondary metabolites has been of great importance in development of new clinically effective drugs [12]. The major classes of phytochemicals with disease-preventing functions are dietary fibre, antioxidants, anticancer, detoxifying agents, immunity-potentiating agents and neuro-pharmacological agents [11]. Each class of these phytochemicals consists of a wide range of compounds, however; some of the most important bioactive phytochemical constituents with medicinal uses are alkaloids, essential oils, flavonoids, tannins, terpenoid, saponins and phenolic compounds [13]. These compounds are extracted from plants using different solvents ranging from alcohols, chloroform, ether, acetone, hexane and water. Alcohols, acetone and water solvents extract the polar components of the plant while chloroform, hexane and ether extract the non-polar compounds [14].

Medicinal plants or their semi synthetic derivatives offer hope for developing alternative medicines for treatment of TB [15]. Several plants are used locally to treat TB-related disease according to ethnobotanical survey done by [16, 17]. In an experimental study done by Gupta etal [18], they revealed that aqueous leaf extracts of *Acalypha indica*, *Adhatoda vasica*, bulbs of *Allium cepa*, cloves of *Allium sativum* and pure gel of *Aloe vera* leaves exhibited anti-tuberculosis activity in Lowenstein Jensen medium. Natural products have really provided new drugs especially for tuberculosis therapy, for examples streptomycin and kanamycin were developed from *Streptomyces griseus* and capreomycin from *Streptomyces capreolus* [19]. Rifampicin on the other hand, is a semi-synthetic drug that was derived from rifamycin, a product of *Amycolatopsis mediterranei* [20]. The plant kingdom thus continues to provide new and important leads against various pharmacological targets. For example, several drugs have been derived from medicinal plants such as quinine from cinchona tree, codeine and morphine from *Papaver somniferum* and artemether and artemisinin from *Artemisia annua* [21].

1.1 Echinops Amplexicaulis

The genus *Echinops* is composed of more than 120 species of perennials, annuals, and biennials [22]. It belongs to the family *Asteraceae* and its species are found in Eastern and Southern Europe, Tropical and North Africa and Asia [23]. Few health benefits of the plant have been documented based on clinical studies however, many documented ethnobotanical/veterinary studies have shown increasing use of the plant as traditional medicine but based on folkloric claims. For example a study done in Uganda by [16, 17], showed

the increasing use of the plant as anti-TB. Another study done by Lamorde etal [24] on medicinal plants used by traditional medicine practitioners for the treatment of HIV/AIDS and related conditions in Uganda also mentioned *E. amplexicaulis* as one of the plants used. A study done in Ethiopia by [25, 26] showed the use of the plant for treatment of ulcerative lymphagitis and hepatitis respectively. In all studies, whole root of the plant was the main part used.

Owing to the plant kingdom's enormous chemical diversity, it can therefore be looked at as an important source of new anti-TB agent [15, 18]. This study therefore aimed at testing the efficacy of *E. amplexicaulis* against multi-drug resistant *M. tuberculosis.*

2. Materials and Methods

2.1 Plant Collection and Identification

Roots of *E. amplexicaulis* were collected from Padibe west sub-county in Lamwo district, Northern Uganda, approximately at latitude 3°28′49.7″ N (3.4804800°) and longitude 32°48′40.8″ E (32.8113300°). Only plants judged as mature and healthy were collected; after which, a new root tuber of the plant was planted to maintain diversity. Its shoot with leaves and flowers was used for identification and a voucher specimen (38,766) was kept at the Makerere University herbarium.

2.2 Extract Preparation

The total crude plant extract was serially extracted by soaking the course plant material (200 g) in ether (500 mL) then in methanol (500 mL) in the order of increasing polarity of the solvents for three days each with occasional shaking. Whatman's filter paper No. 1 was used for filtering in order to obtain the crude solution. This solution was concentrated to a minimum volume by a rotary evaporator at 40 °C and at reduced pressure. The minimum concentrated volume left was further concentrated in an oven at 25 °C in order to obtain the solid crude extracts at constant weight.

2.3 Mycobacterial Strains/Isolates

Three *Mycobacterial* strains were used that is to say rifampicin-isoniazid resistant strain as an indicator of MDR-TB, a fully susceptible laboratory strain (H37Rv) as a control and *M. bovis* (BCG strain). The MDR-TB strain was obtained from National Tuberculosis and Leprosy Referral Laboratory, Wandegeya while the remaining two were obtained from Mycobacteriology (BSL-3) laboratory, College of Health Science, Makerere University.

2.4 Preparation of the Drugs/Extracts

Each of the dried crude extracts (1 g) was dissolved in DMSO (dimethyl sulfoxide) (10 mL) to give a concentration of 100 mg/mL. The extract was later sterilized using 0.2 µm single use filters before use. Rifampicin and isoniazid stock solution of 1,000 µg/mL was serially diluted to obtain a working solution of 125 µg/mL.

2.5 Preparation of Bio Discs

Bio discs of 6 mm diameter were punched from Whatman's filter paper No. 1. They were sterilized at 121 °C for 15 min before impregnating them with 20 µL of extract/drug prepared earlier. The discs were left to dry in a hood for 12 h before use.

2.6 Preparation of Inoculum

Preserved strains of *Mycobacteria* were revived on Middlebrook 7H10 agar prior to susceptibility testing. Cells were scraped from freshly growing colonies (three weeks old) and introduced into normal saline (10 mL). A bacterial suspension equivalent to 0.5 McFarland standards (10^8 CFU) was prepared by adding more cells or diluting with more normal saline.

2.7 Susceptibility Test

Middlebrooks 7H10 agar was used as the culture medium. Then 20 mL of this medium was poured in 90 mm diameter petri dishes with quadrants such that each quadrant contained 5 mL of the medium. Then

100 µL of MTB strains inoculum at 0.5 McFarland concentrations was inoculated using a sterile single use plastic loop on the solidified medium such that each quadrant contained 25 µL. An extract impregnated disc with a concentration of 50 mg/mL was placed in the first quadrant. An isoniazid and rifampicin impregnated disc containing 125 µg/mL was placed in the second and third quadrants and finally a blank disc impregnated with DMSO was placed in the fourth quadrant as a negative control.

All tests for the three strains of mycobacterium were done in triplicate. The Petri dishes were left in the hood for 12 h to allow diffusion of the extracts and drug before sealing them with a carbon dioxide-permeable tape. They were then incubated at 37 °C in a carbon dioxide incubator for three weeks. The sensitivity of MTB strains to the extracts and the drug was determined by measuring the zones of inhibition (mm) surrounding the disc using a ruler.

2.8 Determination of MIC (Minimum Inhibitory Concentration)

MIC was determined using macro broth dilution method as described by [25] by serial diluting the extracts in Middle brook 7H9 medium. Five (5) mL of the medium was dispensed into 15 mL falcon tubes before adding an equal volume of the plant extract at concentration of 25 mg/mL. A twenty fold dilution was made by mixing the extract with the medium 6 times before pipetting off 5 mL to the next tube containing 5 mL of 7H9 medium until a final concentration of 1 µg/mL was obtained. The above procedure was done for each of the test strains (MDR, H37Rv and *M. bovis*) before inoculating them with 0.5 mL of 0.5 McFarland of each strain respectively. The tubes were left in the incubator shaker at 37 °C for 14 days. MIC was obtained as the lowest concentration with no visible growth as shown by clog formation of MTB cells on the walls of the macro broth tube. The tests were done in duplicates and Isoniazid (INH) & Rifampicin (RIF) and DMSO were used as positive and negative controls respectively.

2.9 Determination of MBC (Minimum Bactericidal Concentration)

All MIC tubes with no visible growth as shown by clog formation of MTB cells on the tube walls were plated on Middle brook 7H10 medium and incubated at 37 °C in a carbon dioxide incubator for three weeks. MBC was obtained as the lowest concentration that inhibited growth of MTB colonies on medium [25].

2.10 Qualitative Phytochemical Testing

Qualitative phytochemical testing for the most active phytochemicals such as tannins, alkaloids, flavonoids, saponins, and terpenoids was carried out as described by Refs. [11, 26, 27].

2.11 Terpenoids

It was tested by adding 0.5 mL of acetic acid followed by unequal volume of chloroform to 1 mL of diluted extract. The solution was transferred to a dry test tube before adding concentrated sulphuric acid to the bottom by means of a dropping pipette. Formation of brownish or violet ring at the point where the two layers met indicated the presence of terpenoids.

2.12 Tannins

It was tested by adding 2-3 drops of iron (III) chloride to 2 mL of diluted extract. Formation of a blackish-blue color or green-blackish color indicated the presence of gallic tannins or catechol tannins respectively.

2.13 Flavonoids

It was tested by dissolving the extract in methanol (2 mL, 50%) then magnesium ribbon was added followed by 5-6 drops of concentrated hydrochloric acid. A red solution or an orange solution indicated the presence of flavonols or flavonones respectively.

2.14 Alkaloids

When testing for alkaloids, the plant extract was

dissolved in 3 mL of 2% hydrochloric acid before dividing the resultant solution into three portions. To each portions, 0.5 mL of dilute hydrochloric acid, 2-3 drops of Bertrand's reagent and 2-3 drops of Mayer's reagent was added respectively. Formation of a yellowish white precipitate with either of the two reagents indicated presence of alkaloids while the portion where dilute hydrochloric acid was added acted as a reference solution.

2.15 Saponins

Saponins were tested by dissolving 0.5 g of the extract in 5 mL of distilled water before shaking it vigorously. Formation of a stable persistent froth indicated presence of saponins.

2.16 Statistical Analysis

Data were entered in Microsoft excel and exported to SPSS version 21 for statistical analysis. ANOVA was performed to compare mean zones of inhibition produced by the different strains after treating them with the extract. MIC and MBC were summarized in form of means and standard deviation.

3. Results

3.1 Susceptibility Test

The study showed that there was a significant difference in zones of inhibition obtained from the different stains $F(2, 18) = 437.7$, $p = 0.00$. The extract produced a large zone of inhibition (> 40 mm) for each of the test strains at a concentration of 50 mg/mL as shown in Table 1. Rifampicin and isoniazid produced a zone of 41.0 mm for both H37Rv and *M. bovis* at a concentration of 125 μg/mL except for MDR-TB wild strain.

The results showed that the extract had an MIC of 0.0488 mg/mL against MDR TB and *M. bovis*. It gave a much lower MIC of 0.0122 mg/mL against H37Rv strain (Table 2).

Just like for MIC, MBC of the extract was the same at 0.0977 mg/mL against MDR TB and *M. bovis*. The MBC for H37Rv strain was also much lower at 0.0244 mg/mL (Table 3).

Phytochemical screening detected large quantities of terpenoids, alkaloids and tannins. Saponins were detected in small amounts while flavonoids were not detected in the plant's crude extract (Table 4).

Table 1 Mean zones of inhibition of *E. amplexicaulis* extract against the three test strains

	Zones of inhibition (mm) ± standard deviation		
Extract	MDR wild strain	H37Rv	*M. bovis*
Echinops amplexicaulis total crude	41.0 ± 1.00	40.3 ± 0.58	40.7 ± 1.15
Positive control, RIF & INH	6.3 ± 0.58	41.0 ± 0.58	41.0 ± 1.73
Negative control, DMSO	6.3 ± 0.58	6.3 ± 0.58	6.3 ± 0.58
p-Value	0.000		

Table 2 MIC of total crude extract of *E. amplexicaulis.*

Strains	Concentration ± SD (mg/mL)
MDR TB	0.0488 ± 0.00
H37Rv	0.0122 ± 0.00
M. bovis	0.0488 ± 0.00

Table 3 MBC of total crude extract of *E. amplexicaulis.*

Strains	Concentration ± SD (mg/mL)
MDR TB	0.0977 ± 0.00
H37Rv	0.0244 ± 0.00
M. bovis	0.0977 ± 0.00

Table 4 Major phytochemicals in total crude extract of *E. amplexicaulis.*

Compounds	Amount detected
Terpenoids	++
Alkaloids	++
Tannins	++
Flavonoids	-
Saponins	+

++ much; + medium; - not detected.

4. Discussion

The study showed that *E. amplexicaulis* has anti-mycobacterial activity. During an ethnobotanical survey, this plant was reported to possess anti-mycobacterial activity [16, 17]. In the present study, the total crude extract of the plants showed promising anti-mycobacterial activity against a wild strain of MDR-TB, H37Rv and *M. bovis*. This could be due to the presence of bioactive constituents such as terpenoids, alkaloids, saponins and tannins which were found to have anti-mycobacterial activity [30, 34].

Earlier studies have reported various plants with anti-mycobacterial activity [15, 18, 31]. Studies had mostly reported activity in the plant families of *Asteraceae*, *Lamiaceae*, *Fabaceae*, *Apiaceae* and many others [15]. The plant that showed activity in this study belongs to the family of *Asteraceae*. Despite the fact that several ethnobotanical studies [24, 26] have reported the therapeutic benefit of the plant, no earlier studies had reported the clinical benefit of the plant except in a study done by [32] who reported the hepatoprotective effect of *Echinops echinatus.* Hence this investigation could be the first documented report on the anti-mycobacterial activity of *E. amplexicaulis*.

Phytochemical tests detected presence of alkaloids, terpenoids, tannins and saponins (Table 4). According to [33] , secondary metabolites of terpenoids lead the number of natural products with reported anti-mycobacterial activity due to their lipophilic nature and hence have ability to penetrate the mycobacterial cell wall. This could provide an explanation for the high anti-mycobacterial activity observed in the plant extracts. Earlier studies performed by [30] also reported the ant-mycobacterial activity of phytochemicals such as terpenoids, alkaloids, saponins and tannins. Although hepatoprotective studies of some *Echinops* spp. (*E. echinatus*) have been reported by [30], this study demonstrates the anti-TB potential of *E. amplexicaulis*.

Acknowledgments

The authors gratefully acknowledge Department of Microbiology, Mycobacteriology (BSL-3) Laboratory and Department of Pharmacology and Therapeutics, College of Health Science, Makerere University, for providing a platform from which the study was done.

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