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RESEARCH ARTICLE

Determination of Anti-tuberculosis activity of *Psychotria sarmentosa*, *Aponogeton crispus* and two species of *Pleurotus* mushrooms

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ABSTRACT:

Tuberculosis (TB) is a chronic disease caused by *Mycobacterium tuberculosis* (Mtb) complex. The global TB epidemic has been aggravated by the emergence of disease outbreaks caused by multi-drug resistant and extensively drug-resistant strains. The aim of the present study was to evaluate the *in-vitro*, anti-TB activity of leaves of *Psychotria sarmentosa*, *Aponogeton crispus* and the mushrooms *Pleurotus ostreatus* and *Pleurotus cystidiosus* found in Sri Lanka. Leaves of *Psychotria sarmentosa*, *Aponogeton crispus* and the mushrooms*; Pleurotus ostreatus* and *P. cystidiosus* were dried until a constant weight and 120 g each were taken to prepare crude extracts with distilled water (1.9 L) by heating at a moderate temperature and the final volume was reduced to 240 ml. Freeze dried aqueous extracts were incorporated in Middle Brook 7H11 medium (1mg/ml) using pour plate method.Two ten-fold dilutions (10^{-2} and 10^{-4}) of standard H_{37}Rv Mtb suspensions were inoculated on Middle Brook 7H11 media with the crude extracts. The plates were incubated at 37 ^{0}C for 4 weeks until visible appearance of Mtb colonies. The inhibitory effect of each extract was calculated by the mean reduction of number of colonies on extract containing medium compared to extract-free control medium. Accordingly, the highest mean percentage inhibition was shown by *P. sarmentosa* (71.0 %). The mean percentage inhibition exerted by *A. crispus*, *P. ostreatus* and *P. cystidiosus* were 46.0 %, 43.4 % and 39.5 % respectively. Therefore, freeze-dried aqueous extract from leaves of *P. sarmentosa* has certain activity against the tested standard mycobacterial strain and has a potential to be used as an anti-TB drug component.

KEYWORDS: *Psychotria sarmentosa*, *Aponogeton crispus*, *Pleurotus ostreatus*, *Pleurotus cystidiosus*, *Mycobacterium tuberculosis*.

INTRODUCTION:

Tuberculosis (TB) is one of the deadliest and chronic infectious, disease resulting from infection with the pathogen *Mycobacterium tuberculosis* complex (MTC).

Tuberculosis remains one of the top 10 causes of death worldwide and one-third of the world's population infected with it. In 2019, 1.4 million deaths occurred due to this global burden disease and currently around 10 million people are suffering from TB[1]. (World Health Organization (WHO), 2020). This disease affects both developed and developing countries including Sri Lanka[2].

In 1882, Robert Koch has discovered *Mycobacterium tuberculosis* (Mtb) which is responsible for TB, thus identifying TB as an infectious disease[3]. TB is

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effectively cured by a combination of WHO recommended first-line oral antibiotics including isoniazid (INH), rifampin (RIF), pyrazinamide (PZA) and ethambutol (EMB)[4]. In addition, emergence of drug resistance has led to the use of second line drugs for treatment of TB in certain situations. Four agents of Nitroimidazole class including CGI-17341, PA-824, TBA-354 and OPC-67683, have shown the potential anti-TB property[5].

Medicinal plants are considered as one of the best sources to obtain different types of medicines[6]. Many plants are used in traditional medicine and Ayurveda to treat respiratory diseases including TB[7]. Therapeutic information of some plants is only folkloric based and hence there is need to undertake their scientific rationalization through *in vitro* and *in vivo* testing[8].

Psychotria sarmentosa (Family: Rubiaceae), commonly known as Gonica in Sinhala, is a climbing shrub which is native to tropical Asia and has been used in folk medicine in Sri Lanka. Extracts of different species of the Genus Psychotria have been evaluated for antimycobacterial activity[9]. The literature survey revealed that published scientific information regarding this plant is rare.

Aponogeton crispus (Family: Aponogetonaceae) is an aquatic plant and widely used in Ayrvedic medicine. The plant has medicinal properties like anti-pyretic, anti-inflammatory, hypoglycaemic and anti-nociceptive activities[10]. In Ayurveda, *A. undulatus* is claimed to be effective against tuberculosis[11].

Pleurotus ostreatus (American oyster) belongs to the family *Pleurotaceae* and is considered as one of the most popular edible mushrooms in all over the world. It exhibits antimicrobial, anti-cancer, anti-inflammatory, anti-diabetic, anti-hypercholesterolemia, anti-hypertensive, and hepato-protective activities[12,13]. In addition, antimycobacterial activity of ethanolic extract of *P.ostreatus* has also been recorded[14]. *Pleurotus cystidiosus* (Abalone) belongs to the family *Pleurotaceae* and has exhibited antimicrobial, antidiabetic, anti-cancer antihypertensive activities and anti-platelet aggregation properties[12]. Dichloromethane extract of *Pleurotus tuber-regium* has exhibited significant anti-TB activity against clinical isolated Mtb[15].

Anti-TB allopathic medications which are prescribed for this disease are associated with numerous side effects. The global TB epidemic has been aggravated by the emergence of disease outbreaks caused by multi drug resistant (MDR)-TB and extensively drug-resistant (XDR)-TB[16]. Therefore, there is an urgent need to develop newer anti-TB drugs with unique drug targets.

Even though various biological activities of *P. sarmentosa*, *A. crispus*, *P. ostreatus* and *P. cystidiosus* have been reported, there were no studies carried out to test anti-TB activity of aqueous extracts of said plants and mushrooms. Hence, this study was aimed to assess the anti-tuberculosis activity of *P. sarmentosa*, *A. crispus* and two species of *Pleurotus* mushrooms.

MATERIAL AND METHODS:

Collection of plant materials:

The leaves of *P. sarmentosa* were purchased from a supermarket in Rajagiriya, Sri Lanka. *A. crispus* leaves were collected from a lake located at Kurunegala area in North Western province, Sri Lanka. Fresh *P. ostreatus* and *P. cystidiosus* grown using the spawn provided by the Mushroom Cultivation Centre, Export Research Board (Ratmalana, Sri Lanka), were collected from a local farm.

Identification and authentication of plants and mushrooms:

Dried plant specimens of all interested plants were submitted to the National Herbarium located in the premises of the Royal Botanical Gardens, Peradeniya for identification and authentication process. The identification and authentication of *P.ostreatus* and *P. cystidiosus* was done by studying the spore print and the shape of the cap (fan-shaped) and the stipe (eccentric).

Preparation of aqueous extracts:

Freshly collected leaves of *P. sarmentosa*, *A. crispus* and the whole mushrooms of *P. ostreatus* and *P. cystidiosus* were washed under slow running tap water to remove humus and other debris on the plant part surfaces and then were rinsed 3-4 times with distilled water followed by washing in hydrogen peroxide (6%) for 15 minutes. The leaves and mushrooms were dried until a constant weight under the ambient condition. The dried materials were pulverized into particles. A weight of 120g of the powdered plant materials was allowed to heat at a moderate temperature in 1.9 L of distilled water and the final volume was reduced to 240ml according to Ayurvedic protocol. Filtrate was obtained by filtering through a muslin cloth. Each filtrate was transferred to a sterile glass container and stored in a refrigerator at 4ºC until subjected to freeze-drying process. The freeze-dried extracts were collected into a clean, dry, well-closed container and were stored in a desiccator.

Preparation of Middlebrook 7H11 media:

A weight of 10.25g of Middlebrook 7H11 agar base was dissolved in 450ml distilled water containing 2.5ml glycerol. The mixture was heated to boiling to dissolve the medium completely. Prepared media were sterilized

by autoclaving at 15 lbs pressure, 121^0C for 10 minutes. Media were cooled to 50^0C and aseptically added contents of 1 vial (50ml) of Middlebrook OADC (Oleic acid, bovine albumin, sodium chloride, dextrose and catalase) growth supplement and mixed thoroughly without letting bubbles to form.

Preparation of serial dilutions of inoculum suspension:

Fresh growth of the standard strain of Mtb H_{37}Rv grown on L-J medium was used as the source of inoculum. A loopful (3mm disposable loop) of the inoculum was scraped from the L-J medium and the culture was emulsified on the wall of a sterile McFarland bottle with 1 ml sterile distilled water and six 5mm glass beads. The suspension was vortexed for 20-30 seconds until homogenized. A volume of 4ml of sterile distilled water was added and mixed thoroughly. Then the suspension was rested for 5 minutes. The supernatant was decanted to another sterile McFarland bottle. Turbidity of Mtb H_{37}Rv suspension was visually adjusted by comparing it to the reference McFarland standard 1.0 which contained approximately 10^8 colony forming units (CFU)/ml (NEAT), by addition of sterile distilled water. Serial dilutions of 10^{-2} (10^6 CFU/ml) and 10^{-4} (10^4 CFU/ml) of the inoculum suspensions were prepared by diluting sequentially 100.0µl of the standard suspension in a sterile bijou bottle containing 900.0µl of sterile distilled water.

Indirect agar proportion method for drug susceptibility test:

Drug susceptibility test was conducted using the standard indirect agar proportion method. Anti-TB drugs; INH, RIF, streptomycin and EMB were tested against standard strain of H_{37}Rv Mtb. Stock solutions of standard drugs were prepared. Each standard drug solution was filter sterilized using 0.2 µm filter and stored at -20^0C. Stock solutions were thawed and mixed with Middlebrook 7H11 medium with OADC supplement under sterile conditions in order to get the required drug concentration in the final agar mixture as represented in Table 1.

Media with standard drugs and the drug- free control media were then transferred separately into the sterile culture plates (20ml) under sterile conditions. Inoculation and incubation were carried out in a bio-safety level 3 (BSL3) laboratory. The culture plates were inoculated with 0.01ml of freshly prepared two ten-fold dilutions (10^{-2} and 10^{-4}) of McFarland 1 suspension of standard Mtb strain H_{37}Rv suspensions. Plates were sealed with parafilm to avoid contamination and incubated at 37°C in the presence of 5-10% CO_2 incubator. Results were recorded after four weeks. The assay was done in duplicate. The numbers of colony forming units, growing on the medium containing the drug were compared with the numbers on the control plate. Inhibition percentage of each test sample was calculated using following formula.

$$\text{Percentage of inhibition} = \frac{[\text{No. of colonies on control medium} - \text{No. colonies on test medium}]}{\text{No. of colonies on control medium}} \times 100$$

Table 1. Stock concentrations and final concentrations of standard anti-TB drugs

Standard anti-TB drugs	Concentration of stock solution (µg/ml)	Concentration of working solution (µg/ml)
Isoniazid	1000	0.2
Rifampicin	100	1
Ethambutol	100	5
Streptomycin	1000	2

Determination of anti-tuberculosis activity of *P. sarmentosa*, *A. crispus*, *P. ostreatus* and *P. cystidiosus*:

A weight of 20.00mg of freeze-dried aqueous extract of each plant and mushroom were dissolved in sterile distilled water (4.00ml) separately. The solutions were filter sterilized using 0.2µm filter and the sterile filtrates were obtained at a concentration of 5.00mg/ml. Sterile crude extracts were in cooperated in to freshly prepared Middlebrook 7H11 agar medium containing the OADC enrichment separately and the concentrations of final agar media were 1mg/ml. The media with each crude extract were then transferred in 20ml amounts into sterile culture plates separately under sterile conditions using the pour plate method. A volume of 0.01ml of freshly prepared dilution (10^{-2} and 10^{-4}) of the inoculum was placed in plates containing crude extracts. This assay was done in triplicate and inoculation and incubation were carried out in a bio-safety level 3 (BSL3) laboratory.

After inoculation, the plates were sealed with parafilm and incubated at 37°C in the presence of 5-10% CO_2 incubator. Results were recorded after 4 weeks. The numbers of CFU, growing on the medium containing each crude extract were compared with the number on the control plate. Inhibition percentage of each test sample was calculated using the following formula.

$$\text{Percentage of inhibition} = \frac{[\text{No. of colonies on control medium} - \text{No. colonies on test medium}]}{\text{No. of colonies on control medium}} \times 100$$

RESULTS:

Indirect agar proportion method for drug susceptibility test:

The inhibition percentages of anti-TB drugs at 10^{-2} dilution inoculum of H_{37}Rv Mtb strain and the inhibition percentages of anti-TB drugs at 10^{-4} dilution inoculum of H_{37}Rv Mtb strain are shown in Table 2 and Table 3 respectively. Mean inhibition percentages of standard

anti-TB drugs inoculated with two ten-fold dilutions (10^{-2} and 10^{-4}) of $H_{37}Rv$ Mtb strain are shown in Table 4.

Table 2: Inhibition percentages of anti-tuberculosis drugs at 10^{-2} dilution inoculum of $H_{37}Rv$ Mtb

Test sample	Concentration of drug (µg/ml)	Mean CFU	Standard error of mean (SEM)	Inhibition percentage %
Control	-	>300	0	-
Isoniazid	0.2	0	0	100
Rifampicin	1	0	0	100
Ethambutol	5	0	0	100
Streptomycin	2	0	0	100

Table 3: Inhibition percentages of anti-tuberculosis drugs at 10^{-4} dilution inoculum of $H_{37}Rv$ Mtb

Test sample	Concentration of drug (µg/ml)	Mean CFU	SEM	Inhibition percentage %
Control	-	>300	0	-
Isoniazid	0.2	0	0	100
Rifampicin	1	0	0	100
Ethambutol	5	0	0	100
Streptomycin	2	0	0	100

Table 4: Mean inhibition percentages of standard anti-Tuberculosis drugs

Test sample	Concentration of drug (µg/ml)	Mean inhibition percentage %	SEM
Isoniazid	0.2	100	0
Rifampicin	1	100	0
Ethambutol	5	100	0
Streptomycin	2	100	0

The sensitivity to detect susceptibility to INH, RIF, streptomycin and EMB were 100%. As per the results, there was no growth of standard strain colonies observed at both ten-fold dilutions of the inoculum in the presence of standard drugs.

Determination of anti-tuberculosis activity of *P. sarmentosa, A. crispus, P. ostreatus and P. cystidiosus:*

In the present study, we examined the *in vitro* antimycobacterial activity of *P. sarmentosa*, *A. crispus*, *P. cystidiosus* and *P. ostreatus* against a slow growing Mtb strain of $H_{37}Rv$ which was susceptible to the first-line TB drugs. Inhibition percentages of *P. sarmentosa*, *A. crispus*, *P. cystidiosus* and *P. ostreatus* at 10^{-2} and 10^{-4} dilution inoculum of $H_{37}Rv$ Mtb are indicated in Table 5 and Table 6 respectively. The inhibitory effect of each extract was calculated by the mean reduction of the number of colonies on the extract containing medium compared to control based on measures of central tendency as the standard strain of $H_{37}Rv$ Mtb was tested in triplicate.

Table 5: Inhibition percentage of *P. sarmentosa, A. crispus, P. cystidiosus* and *P. ostreatus* extracts at 10^{-2} dilution inoculum of $H_{37}Rv$ Mtb

Test sample	Mean CFU	SEM	Percentage inhibition %
Control	>300	0	-
P. sarmentosa (Gonika)	110.33	67.9	66.6
A. crispus (Kekatiya)	152	120.8	49.3
P. cystidiosus (Abalone)	>300	0	0
P. ostreatus (Oyster)	>300	0	0

Table 6: Inhibition percentage of *P. sarmentosa, A. crispus, P. cystidiosus* and *P. ostreatus* extracts at 10^{-4} dilution inoculum of $H_{37}Rv$ Mtb

Test sample	Mean CFU	SEM	Percentage inhibition %
Control	41.8	28.5	-
P.sarmentosa (Gonika)	10.33	3.4	75.4
A.crispus (Kekatiya)	24	17.1	42.6
P.cystidiosus (Abalone)	8.75	0.86	79.1
P. ostreatus (Oyster)	5.5	1.2	86.8

The inhibitory effect of each extract was calculated by the number of colonies on the extract containing medium compared to extract-free control medium. The mean percentage inhibition exerted by *P. sarmentosa*, *A. crispus*, *P. ostreatus* and *P. cystidiosus* were 71.0 %, 46.0 %, 43.4 % and 39.5 % respectively. Accordingly, the highest mean percentage inhibition was exhibited by *P. sarmentosa* (71.0 %).

Furthermore, figure 1 shows the appearance of Mtb colonies on *P. sarmentosa* extract containing plate and the control plate after inoculating 10^{-2} dilution inoculum of $H_{37}Rv$ Mtb. This Figure further supports the effectiveness of *P. sarmentosa* on the inhibition of $H_{37}Rv$ Mtb.

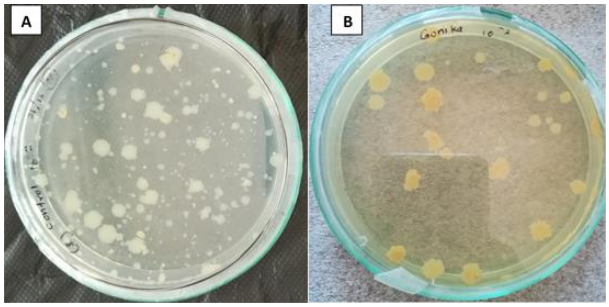

Figure 1: Number of colonies recorded after 4 weeks at 10^{-2} dilution inoculum of H37Rv Mtb. A: Control plate; B: *P. sarmentosa* extract incorporated plate.

DISCUSSION:

According to the results of the present study, the highest anti-mycobacterial activity was shown by *P. sarmentosa*, followed by *A.crispus*, *P. ostreatus* and *P. cystidiosus*. Thus, by having an inhibitory activity greater than 50%, *P. sarmentosa* has a potential to be used in the treatment of TB. To the best of our knowledge, the *in vitro* anti-mycobacterial activity of *P.*

sarmentosa, *A. crispus* and *P. cystidiosus.*is reported for the first time in the current study.

In the present study, the highest anti-mycobacterial activity was shown by *P. sarmentosa.* Acetone extract of *Psychotria zombamontana* and *P. capensis* had been evaluated against different mycobacterial species including *M. tuberculosis* $H_{37}Rv$ in a previous study. The minimum inhibitory concentration (MIC) values of the above extracts have demonstrated reasonable antimycobacterial activity against all tested strains and comparable with the positive control; RIF[17]. Ethanolic extract of *P. vellosiana* had exhibited anti-mycobacterial activity against *M. tuberculosis* $H_{37}Rv$[18]. In the present study, aqueous extract of *P. sarmentosa* was evaluated against *M. tuberculosis* $H_{37}Rv$. Anti-micobacterial activity of ten *Psychotria* species including *P. pubigera, P. ruelliifolia, P. suterela, P. stachyoides, P. capitata, P. glaziovii, P. leiocarpa, P. nuda, P. pubigera, P. racemosa, P. vellosiana* have been evaluated in a previous study[19]. All ten species have demonstrated anti-mycobacterial activity against *Mycobacterium bovis*[19]. In the present study, anti-micobacterial activity of *P. sarmentosa* was evaluated against *M. tuberculosis* $H_{37}Rv$.

Anti-mycobacterial activity of *P. vellosiana* is related to the presence of alkaloids[18]. Further, Junior et al., 2019 has demonstrated the anti-TB activity of a mixture of triterpenes and two alkaloids which were isolated from methanol extract of *P. nuda* against *Mycobacterium tuberculosis* $H_{37}Rv$[20]. Presence of various bioactive phytochemicals such as tannins, polyphenols and flavonoids has been observed in *P. sarmentosa*[21]. Therefore, the antimycobacterial activity of *P. sarmentosa* observed in this study could be attributed to the presence of various bioactive principles which were investigated in *P. sarmentosa*. However, in the present study, the active compounds present in the crude extract of *P. sarmentosa* have not been investigated. Therefore, further studies are recommended to isolate and characterize compounds from *P. sarmentosa* aiming at developing a new class of drugs against tuberculosis.

Aponogeton crispus has emerged as the next candidate of interest with an inhibition percentage of 46.0 % among the other extracts. Published scientific information regarding this plant is rare and effectiveness as an anti-mycobacterial agent is also not reported. In Ayurveda, *Aponogeton undulatus* is claimed to be effective against tuberculosis[11]. However, this is the first report of anti-TB activity of *A. crispus.*

Dichloromethane extract of *Pleurotus tuber-regium* has exhibited significant anti-TB activity against clinical isolated *M. tuberculosis*[15]. Ethanolic extract of *Pleurotus ostreatus* had demonstrated significant activity against $H_{37}Ra$ Mtb, 2 clinical isolates and one Mycobacterium other than tuberculosis[14]. In the present study, anti-mycobacterial activity of aqueous extract of *P. ostreatus* was evaluated against *M. tuberculosis* $H_{37}Rv$.

In this study, dried plant materials and mushrooms were pulverized into particles. When the particle size is reduced, the surface area in contact with the solvent is increased. This in turn leads to efficient extraction. Moreover, moderate temperature was supplied by heating during the extraction process. Moderate temperature aids in the optimal extraction process while high temperature could reduce the activity of the compounds extracted into the solvent. Bioactive compounds such as phenolic compounds, enzymes etc. are thermal sensitive. Some compounds degrade in the presence of high temperature[22]. Therefore, it is recommended to evaluate the anti-TB activity of suspensions of freeze-dried and powdered *Pleurotus* mushrooms which were not exposed to high temperature. Jayasuriya et al., 2015 and 2020, have reported the hypoglycemic activity of *P. ostreatus* and *P cystidiosus* and anti-inflammatory activity of *P. ostreatus* using suspensions of freeze-dried and powdered mushrooms[12,13].

Freeze-dried aqueous extracts of *P. sarmentosa, A. crispus* and *Pleurotus* mushrooms were used to prepare the final known concentrations. By freeze-drying, the plant extracts properly preserve the medicinal qualities of the extracts and therefore using freeze-dried extracts is better when compared with other methods of drying. The principle involved in freeze-drying is called sublimation[23].

Anti-mycobacterial activity exhibited by aqueous extracts of *Pleurotus* mushrooms were less when compared with the anti-mycobacterial activity exhibited by *P. sarmentosa* in the present study. The compounds present in the *Pleurotus* mushroom extracts, which are responsible for anti-mycobacterial activity. may be extracted in less amount to the aqueous solvent. Hence, low polar compounds which are more likely to dissolve in organic solvents (less polar solvent than water) may not have been extracted into the aqueous extract. Those compounds could exert anti-mycobacterial activity. Anti-inflammatory activity of *P. ostreatus* was exhibited by the acetone extract of *P. ostraetus*[13]. Furthermore, an oxidized ergosterol active against anthracnose causing *Colletotrichum gloeosporioides* have been isolated from the acetone extract of *P. cystidiosus* and characterized[24]. Moreover, dichloromethane and ethanolic extracts of other *Pleurotus* species have exhibited significant anti-micobacterial activity[14,15]. Therefore, it is recommended to evaluate the anti-TB activity of different solvent

extracts of two *Pleurotus* species extracted with organic solvents with different polarities. The concentrations of the extracts of *Pleurotus* used in the agar plate may be insufficient to show the inhibitory effect on mycobacteria. In addition, the soil composition and other environmental factors could affect the concentration of active compounds present in a plant extract.

The H_{37}Rv Mtb was inhibited effectively by WHO recommended first-line oral therapeutic drugs including INH, RIF, EMB and by streptomycin which is usually administrated as a parental drug for TB treatment. However, sometimes, these drugs are ineffective in the presence of drug resistance strains[25]. Drug susceptibility testing were carried out to identify whether any particular drug is resistant to the tested TB strains. The anti TB activity of standard drugs were compared with anti TB activity of *P. sarmentosa* and *A. crispus* plants and *Pleurotus* mushrooms in the present study. Therefore, the susceptibility tests for standard drugs were carried out to determine the sensitivity of the H_{37}Rv strain to standard drugs. The major disadvantage of the agar based drug susceptibility test is that it is time- consuming since the results can be observed only after the visible appearance of the growth of the slow-growing colonies.

Recent studies have shown that there is a possibility of using plant extracts in combination of two or more as anti-mycobacterial agents and effective against different strains of Mtb[26]. Similarly, some studies have been carried out to evaluate the synergism between antimicrobial drugs and plant extracts[27]. It is recommended to conduct studies with different plant materials together in search of synergistic anti-TB activity.

This study provides novel information about the anti-TB activity of *P. sarmentosa*, *A crispus*, *P. ostreatus* and *P.cystidiosus*. Further studies to investigate anti-TB bioactive constituents from these plants and mushrooms are important to prove the possible application in TB therapy.

CONCLUSION:

In the current study, *P. sarmentosa A. crispus* and *P. ostreatus* and *P.cystidiosus* were screened for their anti-tuberculosis activity. Accordingly, the highest mean percentage inhibition was shown by *P. sarmentosa* (71.0 %). The mean percentage inhibition exerted by *A. crispus*, *P. ostreatus* and *P. cystidiosus* were 46.0%, 43.4% and 39.5% respectively against H_{37}Rv Mtb. Freeze-dried aqueous extract from leaves of *P. sarmentosa* has exhibited higher activity against the tested standard mycobacterial strain H37Rv and has a potential to be used as an anti-TB drug component.

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CONFLICT OF INTEREST:

Authors declare no conflict of interest.

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