

A Study on *Phyllanthus amarus*; Pharmacognostic, Mycobactericidal and Mutagenic Properties

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ABSTRACT

Background: *Phyllanthus amarus* is a medicinal plant used in the treatment of various ailments which include gonorrhoea, jaundice, diabetes, kidney diseases, bladder and intestinal infections, influenza, measles, viral infections, and tuberculosis. Tuberculosis treatment is faced with many challenges, resulting in a prolonged treatment regimen and potential treatment failure. There is a need to search for more favourable treatment options. **Objective:** This study aimed at investigating the pharmacognostic and mycobactericidal properties of *P. amarus*. Since toxicity could also be an issue, the mutagenic activity of this plant was also assessed. **Materials and Methods:** The macroscopic, microscopic, and physicochemical characteristics were assessed with reference to the Quality Control Methods for Herbal Material WHO (2011). The mycobactericidal activity was determined by the agar diffusion and broth dilution methods, while mutagenicity was investigated by the Ames test. **Results:** *P. amarus* contained tannins, flavonoids, glycosides, saponins and steroids. The 50% ethanol extract exhibited activity against *M. smegmatis* at 100 mg/mL with an inhibitory zone of 2.0 cm. *P. amarus* had a minimum inhibitory concentration of 50 mg/mL while that of rifampin was 0.1 µg/mL. *P. amarus* showed weak mutagenicity at a concentration of 10 µg/mL. **Conclusion:** The documented pharmacognostic characteristics can be used for quality control of the crude plant material. The mycobactericidal activity also affirmed its folkloric use in the treatment of tuberculosis. The mycobactericidal activity can be further exploited for drug development.

Key Words: *P. amarus*, Pharmacognostic, Mutagenic activity, Tuberculosis, Fluorescence.

INTRODUCTION

Phyllanthus amarus Schum. & Thonn is a plant belonging to the Euphorbiaceae family and is widely distributed across tropical and subtropical countries. This plant is known in English as black catnip, carry me seed, child pick-a-back, among many other names.¹ It is currently found in many countries including India, Philippines, Cuba, Brazil, Ghana, Ivory Coast, Kenya, and Nigeria Etc.²⁻⁴ *P. amarus* is an erect perennial herb not reaching one and a half foot tall having small leaves and yellow flowers.⁵ The appearance of dioecious cymes at the end of the branches are considered to be a distinct characteristic, in many respects it highly resembles its close relatives, *P. debilis* and *P. fraternus* of the same Swartziani sub-section.^{6,7} The genus *Phyllanthus* (Euphorbiaceae) consists of about 6500 species in 300 genera, of which 200 are American, 100 African, 70 from Madagascar and the remaining Asian and Australasian.⁸ The name 'Phyllanthus' means "leaf and flower" because the flower, as well as the fruit, seems to become one with the leaf.⁹ *P. amarus* has long been used worldwide in traditional folkloric medicine for treating a myriad of diseases including irregular menstruation, gonorrhoea, diabetes, urinary and intestinal infections, alleviation of kidney and gallbladder stones, influenza, measles, tuberculosis, liver diseases and disorders like hepatitis, jaundice and liver cancer.^{2,4,5,10} It is also used in treating

hepatotoxicity, boosting the immune system, and active against hepatitis A.¹¹ In Ayurveda medicine, *P. amarus* is also used for dropsy, arthritis, jaundice, asthma and bronchial infections.¹² The whole plant material is also used for menorrhagia, genital illnesses, gastropathy, diarrhoea, dysentery, intermittent fevers, ophthalmopathy, scabies, ulcers and wounds. It is also used as a good tonic.¹³

P. amarus is known to biosynthesize many groups of naturally occurring compounds of medicinal importance. Its phytoconstituents include alkaloids, flavonoids, hydrolysable tannins, triterpenes, lignans, polyphenols, alkaloids, sterols, and oils.⁷⁻¹⁴ Many lignans have been isolated from this plant; phyllanthin and hypophyllanthin. Other lignans isolated from *P. amarus* are niranthin, phyltetralin, nirtetralin, isonirtetralin, hinokinin, lintetralin, isolintetralin, demethylenedioxy-niranthin and 5-demethoxyniranthin. Flavonoids such as rutin, gallocatechin, kaempferol and various flavonoid glycosides have been isolated from this plant. The present in this plant material sellagitannins include geraniinic acid B, geraniin, amariin, amarulone furosin, amariinic acid, phyllanthusiin A, B, C, D and melatonin. The securiniga-type alkaloids isolated include epibubbialine and isobubbialine. The sterols also present in this plant include amarosterol A and amarosterol B.⁷⁻¹⁴ The extracts and compounds isolated from *P. amarus* have been shown to possess antibacterial, antiviral, antiplasmodial, antimalarial,

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antimicrobial, anti-inflammatory, anticancer, hypolipidemic, antidiabetic, antioxidant, nephroprotective, hepatoprotective, diuretic, anti-diarrheal, antiulcer, antifungal, analgesic, anti-allodynic, anti-oedematogenic, antinociceptive, antiplasmodial, and antiviral activities.^{13,14}

MATERIALS AND METHODS

Plant collection

The leaves of *P. amarus* were collected from the campus of the Kwame Nkrumah University of Science and Technology in January 2017 and authenticated by Mr. Asare of the Department of Pharmacognosy. A herbarium voucher with specimen number of PSM64/1/17 has been deposited at the institution's herbarium. The leaves were air-dried for two weeks and milled into a coarse powder.

Macroscopic and microscopic analysis

The leaves of *P. amarus* were examined macroscopically for characteristics such as colour, shape, texture, leaf venations and leaf margin.¹⁵ The microscopic characteristics of the leaves were studied following the standard protocols of the WHO guidelines on Quality Control Methods for Herbal Materials, 2011.¹⁵ Four-millimetre square (4 mm²) sizes of the mature lamina were cut. These were then boiled for about two hours in a solution of chloral hydrate. The sections were in glycerin and covered with a glass slip. The slides were observed with a LEICAD light microscope under low power and high power magnifications of $\times 10$ and $\times 40$. The mounts were observed for the presence of calcium oxalate crystals, stomata and trichomes were observed. Quantitative parameters such as vein islet number, veinlet termination numbers, stomatal number and stomatal index were determined. This was done by counting each determination under ten different fields of view and calculating the number per millimetre square of each leaf surface.

Physico-chemical analysis

Air-dried samples of *P. amarus* were analyzed for their physicochemical parameters. The loss on drying method estimated the moisture content. Total ash, acid insoluble ash and water-soluble ash were also determined using the WHO, 2011 guidelines.¹⁵

Extractive values determination

Twenty grams (20 g) of coarsely powdered air-dried leaves of plant material was weighed and 200 mL of water, 50% ethanol or petroleum ether was separately added to each sample. The plant materials were also sonicated for 45 minutes. The filtrates were obtained and concentrated *in vacuo* at 40 °C. The concentrates were then freeze-dried to obtain dry crude extracts and stored at -20 °C prior to use. The percentage yields of petroleum ether, water and 50% ethanol were calculated with reference to the air-dried material.

Phytochemical screening

The crude plant material and extracts of *P. amarus* were screened phytochemically for the presence of tannins, glycosides, saponins, alkaloids and flavonoids using standard methods.^{16,17}

Fluorescence studies

Fluorescence analysis of the powdered crude drug was carried out to determine the characteristic fluorescence. The pulverized leaf sample was dissolved in specific solvents according to methods described by Ranjith, 2018.¹⁸ Observations were also carried out under visible daylight, UV light of short wavelength (254 nm) and UV light of long wavelength (365 nm) for resultant characteristic colour.¹⁸ The solvents employed were distilled water, 1N HCl, 1N NaOH, 50% H₂SO₄,

methanol, glacial acetic acid, nitric acid, chloroform, 50% FeCl₃ and 96% ethanol.

Determination of mycobactericidal activity

Test organism

Mycobacterium smegmatis (MC2 155) was obtained from the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana. Middlebrook 7H9 powder (VWR, U.S.A.) was used for cultivation. A loopful of *M. smegmatis* culture was introduced into 20 mL of 7H9 Middlebrook broth and incubated for 24 hours at 37 °C. The bacterial culture was then standardized to contain 1 x 10⁶ cell/mL by the help of a previously calibrated bacterial suspension curve at 680 nm using a calorimeter.

Agar well diffusion method

A concentration of 100 mg/mL of each of the water, 50% ethanol and petroleum ether extracts were prepared in sterile distilled water and sterile filtered through a 0.22 µm membrane filter.

A hundred microliters (100 µL) of 1x 10⁶ cell/mL culture of *M. smegmatis* was added to 20 mL of nutrient agar, stabilized at 45°C, mixed, poured into a sterile petri dish and the agar allowed to set. Five holes were bored equidistant from each other using a sterile cork borer of diameter 13 mm (Number 6). A volume of 150 µL of each extract was introduced into a separate well. Sterile water of 150 µL was introduced into the fourth well as the untreated control, and 150 µL of rifampicin (10 µg/mL) was introduced into the fifth well as the positive control. The extracts were allowed to diffuse for 15 minutes at room temperature, and thereafter incubated at 37 °C for 24 hours. The diameter of the zones of inhibition was measured. The experiment was performed in triplicates.

Determination of Minimum inhibitory concentration

The active extracts (aqueous and 50% ethanol), were assessed for their MIC. Two hundred milligram per millilitres (200 mg/mL) concentration of the active 50% ethanol leaf extract of *P. amarus* was prepared. A volume of 500 µL of double strength nutrient broth, 250 µL of plant extract, 50 µL of sterile water and 200 µL of bacterial culture were added to a 24 well plate to produce a concentration of 100 mg/mL. Another 500 µL of double strength (D/S) nutrient broth, 125 µL of extract, 175 µL of sterile water and 200 µL of culture were added to the next well to make a concentration of 50 mg/mL. Double strength nutrient broth (500 µL), 62.50 µL plant extract, 237.5 µL of sterile water and 200 µL culture was added to another well to make a concentration of 25 mg/mL. A growth control was set up with 500 µL of D/S nutrient broth, 300 µL sterile water and 200 µL culture added together in a well. The negative control contained 500 µL D/S nutrient broth, 250 µL plant extract and 250 µL sterile water. The well plates were incubated at 37 °C for 24 hours and observed. A concentration of 0.004 µg/mL of rifampin was used as a positive control. To 500 µL of D/S nutrient broth was added 250 µL of rifampin, 50 µL sterile water and 200 µL culture were added in one well to make 10 µg/mL. Also, 500 µL D/S nutrient broth, 25 µL rifampin, 275 µL sterile water and 200 µL were added to the next well to make 1 µg/mL. Finally, 500 µL DS nutrient broth, 2.5 µL rifampicin, 297.5 µL sterile water and 200 µL were added to the next well. It was incubated at the same conditions. Each experiment was carried out in triplicates.

Determination of kill kinetics

Using the method described by Gbedemah *et al.*, 2010¹⁹, mid-logarithmic phase cultures of *M. smegmatis* were transferred into 50 mL portions of pre-warmed nutrient broth containing twice the MIC of the ethanol extract (100 mg/mL), to yield final concentrations of

1×10^6 cell/mL. The broths were maintained in a stabilized water bath at intermittent shaking. Aliquots (1 ml) were drawn after 0, 1, 2, 3, 4, 5, 6, 12 and 24 h and diluted in another 10 mL nutrient broth to neutralize the effect of the extract. Viable counts were then determined by the pour plate method. One millilitre (1 mL) of the diluted culture suspensions was added to a sterile petri dish, and 20 mL of molten nutrient agar, stabilized at 45°C was poured over it and shaken. Growth controls were also set up in the nutrient broth. Same was done for 0.4 µg/mL rifampicin as the positive control. All plates were then incubated inverted at 37 °C for 24 hours, after which the number of viable cells was counted. All experiments were performed in triplicates. Survivors were estimated by counting the number of colonies. Survivor-time curves were drawn for the test organisms, exposed to the extract and controls. A graph of log of the number of viable cells against time was plotted and analyzed using 2 - way ANNOVA, Bonferroni post-test.

Determination of mutagenicity

The aqueous extract of *P. amarus* was dissolved in sterile water on the day of the assay, to give a concentration of 10 µg/mL and sterile filtered using a 0.22 µM membrane filter. The Muta-ChromoPlate™ two strain kit (Environmental Bio-Detection Products Inc, Ontario, Canada) which works on the principle of the Ames test was employed to determine the mutagenicity potential of the extracts.²⁰ The experiment was carried out under the protocol provided by the manufacturer using *Salmonella typhimurium* TA98 strain. The experiment was performed similarly to methods already published in the literature.²¹ The number of positive wells scored in the 96 well plates determined the significance of mutation in the fluctuation test.²² The statistical table provided in the kit was used to compare the natural background rate of reverse mutation to the rate of reverse mutation within a sample assay. Based on these samples, p values of 0.001 were classified as highly mutagenic, p values of 0.01 moderately mutagenic and p values of 0.05 weakly mutagenic.

RESULTS

Macroscopic characteristics

Organoleptic examination of the leaves of *P. amarus* showed that it was deep green and had non-specific odour and tastes. A summary of these features is given in Table 1. *P. amarus* consists of pinnate compound leaves which are alternately arranged on the stem (Figure 1). The leaflets were oblong to elliptical, had entire margins reticulate venation, and glabrous surfaces. On the lower side of each rachis, small, ovoid fruitlets arranged in a row were observed.

Microscopic characteristics

The microscopic analysis involved the qualitative observations and the quantitative investigations, also known as leaf constants. The vein islet number was calculated to be 10 per mm². Veinlet terminations were calculated as 6 per mm². Details of the results are shown in Table 2. Internally, *P. amarus* leaves showed the presence of wavy epidermal cells, paracytic stomata, prismatic calcium oxalate crystals which were lined up along the veins. The reticulate veins can be categorised as dichotomizing with poorly developed areoles (Figure 2).

Physicochemical characteristics

The physico-chemical parameters of the leaves of *P. amarus* are provided in Table 3. The water-soluble ash content was of 13.5% w/w, followed by the total ash content of 6.17 % w/w, and acid insoluble ash value of 5.67 % w/w. The average moisture content was approximately 14% w/w. *P. amarus* had the highest extract yield in the 50 % ethanol solvent and the least amount in petroleum ether (Table 4).

Fluorescence analysis

Analysis for characteristic fluorescence of the samples at short and long wavelengths showed varying colours for all the samples. These results

Table 1: Macroscopic characteristics of *P. amarus* leaf.

Morphology	<i>P. amarus</i>
Shape	Oblong
Margin	Entire
Venation	Pinnately reticulate
Texture	Smooth
Type	Compound leaf

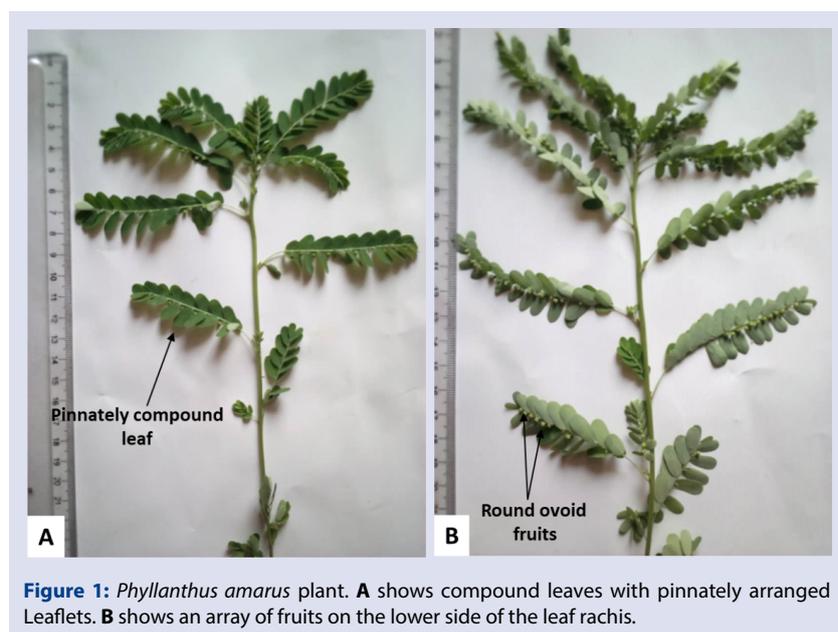


Figure 1: *Phyllanthus amarus* plant. **A** shows compound leaves with pinnately arranged leaflets. **B** shows an array of fruits on the lower side of the leaf rachis.

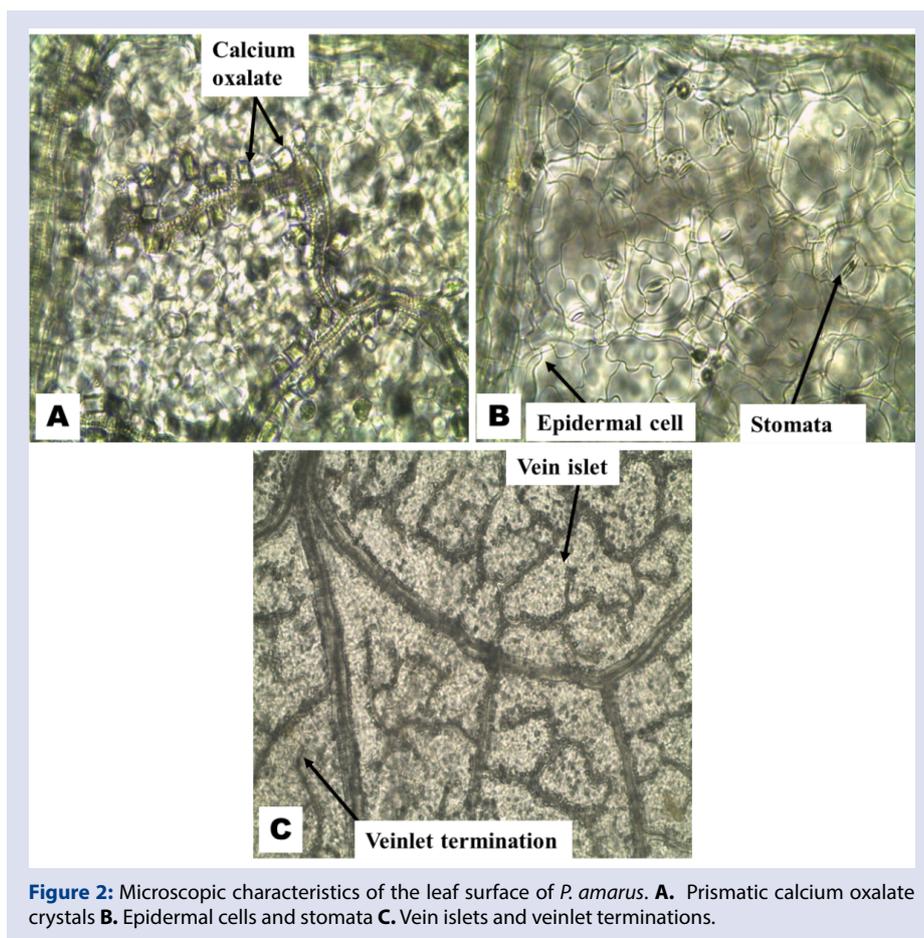


Table 2: Microscopic characteristics of *P. amarus*.

Parameter	<i>P. amarus</i>
Stomatal Number	5 ± 2.8
Epidermal Cell Number	35 ± 2.0
Vein islet Number	10 ± 1.7
Veinlet termination number	6 ± 1.9
The stomatal index [stomata per square mm of epidermis]	12.5 ± 2.1

Table 3: Physicochemical characteristics of *P. amarus*.

Parameters	<i>P. amarus</i>
Total ash	6.17 ± 1.2
Acid insoluble ash	5.67 ± 1.5
Water soluble ash	13.50 ± 1.4
Moisture content	13.67 ± 2.1
Foreign organic matter	-
Foaming index	<100
Swelling index [mL]	7.33 ± 2.2

Table 4: Extractive values.

Solvent	<i>P. amarus</i> (% W/W)
Water	11.29 ± 2.2
Ethanol 50%	20.76 ± 1.5
Petroleum Ether	7.00 ± 1.8

are useful for both identity and quality determination of the samples. These are presented in Tables 6.

Mycobactericidal activity

When 100 mg/mL concentrations of the water, 50% ethanol and petroleum ether extracts of *P. amarus* was screened for preliminary mycobactericidal activity, the water and 50% ethanol extracts showed activity against *M. smegmatis* with a zone of inhibition of 1.5 ± 0.2 and $2 \text{ cm} \pm 0.5$ respectively. *P. amarus* had a MIC of 50 mg/mL and 100 mg/mL for the 50%, ethanol and aqueous extracts respectively, while rifampin had an MIC of 0.1 µg/mL. Kill kinetics studies of the 100 mg/mL 50% ethanol extract showed a significant reduction in the number of cells over 24-hour period. This was also observed for rifampicin as shown in Figure 3. The area under each curve was also significantly reduced when compared to the untreated control.

Mutagenic activity

The Ames test showed the aqueous extract of *P. amarus* to be weakly mutagenic.

DISCUSSION/CONCLUSION

The discovery and development of new antimicrobials is a significant health challenge in dealing with infections caused by especially multidrug-resistant bacteria such as the mycobacteria.²³ It is, therefore, imperative that new sources of drugs are developed. Plant sources have made significant contributions to the development of new drugs since the beginning of modern medicine.²⁴

The studied pharmacognostic characteristics of *P. amarus* are a prerequisite for standardization.²⁵ These characteristics can be used as a good indication of identity, purity and quality²⁶ and provide a means of detecting adulteration and substitution of this plant material. *P. amarus* is used in the treatment of numerous ailments,^{2,4,5,10,26} including tuberculosis hence these the pharmacognostic and mycobactericidal results will go a long way to assure the safe and efficacious use of this plant material.

Table 5: Preliminary phytochemical screening of secondary metabolites of *P. amarus*.

Constituent	Results
Saponins	+
Alkaloids	+
Flavonoids	+
Tannins	+
Steroids	+
Glycosides	+

+: Present; -: Absent

Table 6: Fluorescence studies of powdered *P. amarus* leaves in various solvents.

	Daylight	254 nm	365 nm
Powdered Sample only	Greenish brown	Dark brown	Brown
Distilled water	Brown	Purple	Purple
1N HCl	Green	Purple	Pale purple
1N NaOH	Red	Purple	Pale purple
10N H ₂ SO ₄	Deep green	Blue	Blue
Methanol	Pale brown	Red	Pale blue
Glacial acetic acid	Pale brown	Red	Pale blue
Nitric acid	Yellow	Blue	Pale blue
Chloroform	Brown	Red	Paler red
50 % FeCl ₃	Black	Deep blue	Pale blue
96 % Ethanol	Light green	Red	Pale blue

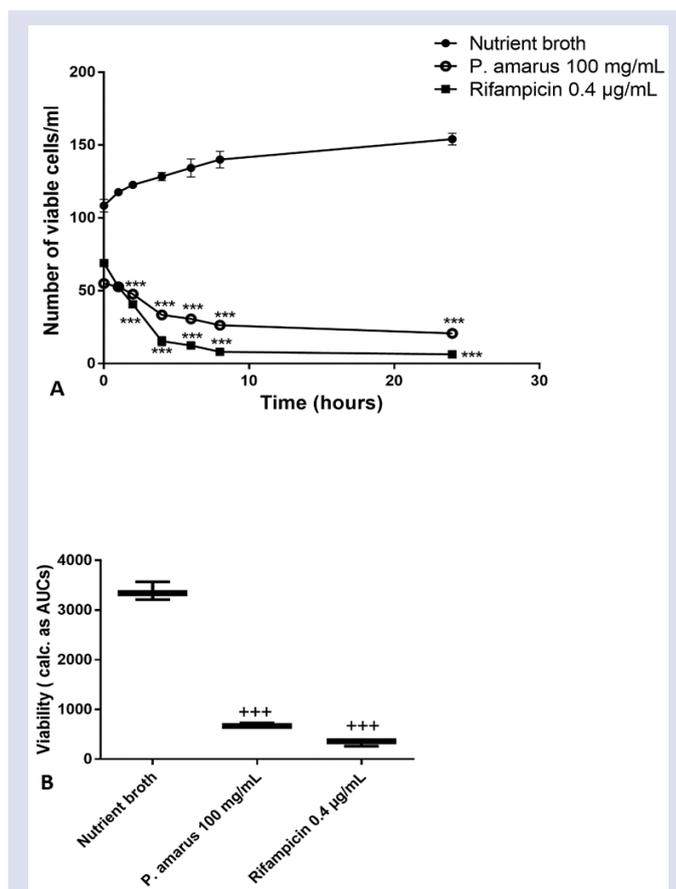


Figure 3: Time-kill kinetics of 50% ethanol extract of *P. amarus* leave on *M. smegmatis*. **A:** time-kill kinetics curve, **B:** Area under curve of time-kill kinetics. Values are mean \pm SEM, n=3. ***p<0.001 compared to the growth control (One-way ANOVA followed by Dunnett's post hoc test).

The macroscopic and microscopic examinations of the medicinal plant have served as tools for distinguishing between closely related species. They also enable the discovery of essential characteristics of species, thus preventing adulteration.²⁷ In the present study, the morphological features observed, were consistent with the literature.²⁸ Also, microscopic observations such as anisocytic stomata, wavy epidermal cells and prismatic calcium oxalate crystal are similar to finding made by Sharma and Sheela (2011).²⁹ Measuring the ash values of the plant can give an idea of the presence of inorganic constituents and other impurities that may be present in the plant material. Acid insoluble ash gives a measure of the amount of silica present, especially in the form of sand and siliceous earth.³⁰ The water-soluble ash content was 13.5% w/w, followed by the total ash content of 6.17% w/w, and acid insoluble ash value of 5.67 % w/w. These extractive values are similar to that already published.³¹ In which the total ash, water-soluble ash and acid-insoluble were reported to be 7.5, 9-12, 4-9% w/w respectively.³¹ The slight variations may likely be due to environmental factors.³²

The total ash corresponds to the total quantity of physiological ash results from mineral components of the plant tissues as well as and non-physiological ash due to extraneous content such as sand and soil that could have been present in the collected plant sample.³³ The total ash value aids in the detection of low-grade products and the establishment of purity characteristics.^{33,34} The water-soluble ash, which was 13.5%, on the other hand, corresponds to the ash produced from the water extract of the plant material, this value could be used to detect previously extracted plant materials. The acid insoluble ash, which was determined to be 5.7% also gives the amount of inorganic material such as silica or metals that could be present. A significant increase or

decrease in this value could indicate that plant material that could have been contaminated or adulterated. The average moisture content was approximately 14% w/w, which is significantly lower than that reported by Dhongade and Chandewar, 2013.³¹ A higher moisture content could indicate the presence of excess moisture and hence degradation. Low moisture content is indicative of better stability against degradation of the herbal material.³⁰ Extractive values indicate weights of the extractable chemical constituents of the crude drug under different solvents environment.³⁰ Per the results obtained, the leaves of *P. amarus* contained a high proportion of middle polar constituents.

The presence of alkaloids, flavonoids, tannins, steroids, saponins, glycosides as seen in *P. amarus* is already in accordance with the published literature.³¹

When herbal materials contain appreciable amounts of saponins, there is a persistent foam when an aqueous decoction is shaken.¹⁵ The foaming index was determined to be less than a 100 for all the aqueous decoction, indicating possibly the presence of a low concentration of saponins.¹⁵

The pulverised leaf gives a characteristic red and purple fluorescence in several solvents and reagents under UV light of both short and long wavelength (Table 6). These fluorescent behaviour can be a useful tool in further confirming the purity of crude plant materials from this plant. The pulverised leaf suspended in ethanol is shown to be light green, red and pale blue under daylight, 254 and 365 UV light respectively. The ethanol extract is, however, reported to be yellow, brown and light green, respectively.³¹

For studies on the mycobactericidal activities of *P. amarus* leaves, *M. smegmatis*, a soil-dwelling saprophyte related to *Mycobacterium tuberculosis* was employed. It is a mycobacterium model that is used to understand the pathogenesis of *M. tuberculosis* because of the disadvantages in the direct use of the disease-causing tubercle bacillus.³⁵ Both the aqueous and 50% ethanol extracts of *P. amarus* showed activity. However, the ethanol extract had higher activity evident by a larger zone of inhibition. The zones of inhibition is indicative of the presence of antimicrobial constituents. The minimum inhibitory concentration was determined as 50 mg/mL and 100 mg/mL for the 50% ethanol and aqueous extract, respectively. This high MIC suggests the need for further optimization to determine active fractions and compounds. Further analysis of the active 50% ethanol extracts at 100 mg/mL in kill kinetic studies, showed a significant reduction in the number of viable cells as compared to that of the untreated control at all time points over a 24-hour period. Antimicrobial plants are known to act alone, synergistically or as potentiators to already existing drugs.³⁶ When antimicrobial plant extracts are used in therapy, they could reduce the emergence of resistant strains.³⁷ Hence the identified mycobactericidal activity of *P. amarus* can be further optimized for the treatment of tuberculosis. This preliminary activity also authenticates the folkloric use of this plant in the treatment of tuberculosis.¹⁰

In the mutagenic studies, *P. amarus* was weakly mutagenic. Mutation in the *S. typhimurium* TA 98 strain used in this study is indicative of a frameshift mutation; this could, therefore, infer that this plant could contain components that can intercalate between the normal bases to create mistakes during DNA synthesis.³⁸ However, this needs to be further investigated.

In conclusion, the determined pharmacognostic indices can be employed to determine the authenticity, purity and quality of *P. amarus*. The mycobactericidal activity of *P. amarus* against *M. smegmatis* is promising; hence this plant can be further exploited for the development of alternative treatments for mycobacterial infections. The mutagenic results for *P. amarus* suggest that this plant may need to be used with caution and further screened for toxicity.

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CONFLICTS OF INTEREST

None.

ABBREVIATIONS

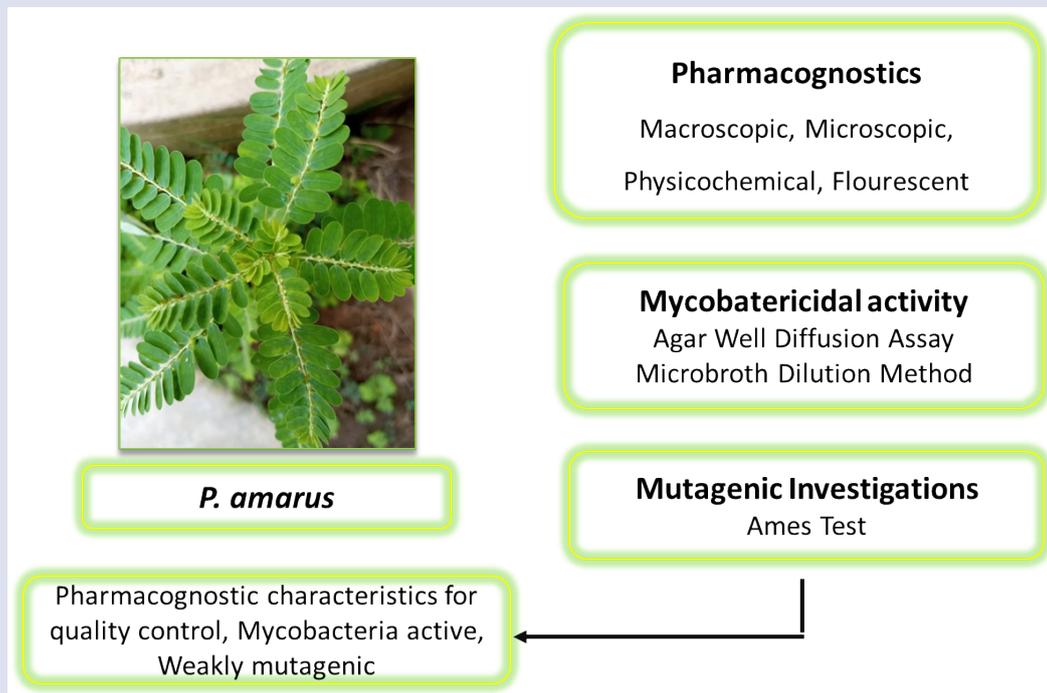
MIC: Minimum inhibitory concentration.

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GRAPHICAL ABSTRACT



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