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Phytochemical Analysis and Determination of *In vitro* Antioxidant and Antimicrobial Activity of *Phyllanthus amarus* Leaves Extracts

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Abstract
Phyllanthus amarus Linn. (Family: Euphorbiaceae) is found in tropical and sub-tropical region of the world. It is being used for the treatment of severe microbially infected disorders. The present study quantifies the phytochemical constituents. The present study also evaluated the *in vitro* antioxidant and antimicrobial properties of aqueous and 70% ethanolic extracts of leaves of the plant. The total polyphenolic content of the water and 70% ethanolic extracts showed the amounts are 60.28 ± 0.05 and 59.58 ± 0.13 mg GAE/g DW and the total flavonoids content of aqueous and 70% ethanolic extracts showed the values 85.88 ± 1.77 and 95.15 ± 2.70 mg QE/g DW, and the total tannin content for aqueous and 70% ethanolic extracts 53.21 ± 0.6 and 40.91 ± 0.70 mg TAE/g DW, respectively. According to the result, aqueous extract showed more effective antioxidant and antimicrobial activity than ethanolic extract. The plant leaves possesses significant amount of phytochemicals which are effective in the treatment of many oxidative stress-related damages and microbial infections. Based on the present research investigation it may be concluded that *Phyllanthus amarus* can be used for the preparation of natural drugs for pharmaceutical and nutraceutical industries.

Keywords: *Phyllanthus amarus*, phytochemical analysis, Antioxidant, Antimicrobial.

Introduction

Phyllanthus amarus Linn. (Figure 1) is a small annual herbaceous weed that grows 40 cm in height and it is locally known as Bhui Amalaki, belonging to the family of Euphorbiaceae [1]. It is widely found throughout the tropical and sub-tropical countries of the world. It is mostly used in the Indian Ayurvedic System of Medicine. *Phyllanthus amarus* is a plant that was reported of having a broad spectrum of pharmacological properties [1, 2].

Fig 1: The Plant Phyllanthus amarus Linn.

The medicinal or pharmacological values of this ethnomedicinal plant lie in bioactive constituents that produce definite physiological action on the human body. The important phytomolecules are alkaloids, flavonoids, tannins, terpenoids, and phenolic compounds [3]. Due to their specialized biochemical capabilities, plants are able to synthesize a vast array of primary and secondary phytochemicals which are useful for humans [4].

Medicinal and aromatic plants are the vast source of potentially useful phytochemicals for the development of new therapeutic agents or pharmaceutical products [5]. Phytochemicals such as tannins and polyphenol from *Phyllanthus amarus* have been associated with some antimicrobial importance. Major Phytochemicals and mineral contents are also reported on the whole plants [6]. Many lignans were isolated from different parts of the plants such as phyllanthin and hypophyllanthin. The highest amount of phyllanthin, hypophyllanthin and flavonoids such as gallocatechin, quercetin-3-O-glucopyranoside, gluco pyranoside, kaempferol has been also reported in leaves [1, 2]. This small herbaceous weed also used in the folk medicine from ancient time [5]. This ethnomedicinal plant reported to have several biological properties such as antimicrobial and antiviral activities. It acts against hepatitis B and it is chemo-protective, anti-mutagenic and hypoglycaemic agents as well.

The plant parts also acts against cytotoxicity and improves the immune system of patients, too. It is also used as a tonic. It is used as diuretic and in the treatment of diabetes, dysentery, hepatitis, and skin disorders. It exhibited immuno-modulatory activity [7]. The plant also contains several pharmacological important phyto molecules whose efficacy is well established by several biochemical and pharmacological studies [8]. So from the previous studies it was found that this medicinal herb possesses huge ethnomedicinal potentiality and considering those reports it was decided to investigate some important parameters of the experimental plant extracts.

The current study investigated the proximate, phyto chemicals, nutritional as well as *in vitro* antioxidant and antimicrobial potentiality of the leaves of *Phyllanthus amarus*.

Materials

Collection and Identification of plant sample

Fresh leaves of *Phyllanthus amarus* were collected from Salt Lake City, Kolkata and identified by Dr. Madhusudan Mondal, Former Additional Director, Botanical Survey of India, Howrah, and West Bengal (Figure 1).

Collection of Microorganisms

Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) strains were obtained from the Department of Microbiology, Calcutta University, West Bengal, India.

Chemicals and Reagents

All the used chemicals and reagents for the experiments were of analytical grade. Folin ciocalteu reagent was obtained from Merck Life Science Pvt. Ltd., Mumbai. Gallic acid was purchased from SD Fine-Chem Ltd., Mumbai. Quercetins, DPPH and tannic acid were obtained from Sisco Research Laboratories Pvt. Ltd., Maharashtra, Dextrose from Finar Limited, Ahmedabad. Bovine Serum Albumin (BSA), agar and broth were purchased from HiMedia, Mumbai.

Methods

Extraction of the Plant Sample

At first leaf samples were washed with distilled water and placed for sun drying for several days, at considerably lower temperature (40°C) for better grinding. Dried powder leaves extracted by using water and 70% ethanol (1g of powder dissolved in 25 ml solvent). Stock concentration was diluted according to the need for the assay.

Proximate Analysis

Determination of Ph

5g of the freshly collected leaves was homogenized in 10 ml deionized water and filtered. The pH of the leaf extract determined after calibrating pH meter with a buffer solution [9].

Determination of Conductivity

5g of the freshly collected leave sample was homogenized in 10 ml deionized water. This was filtered and the conductivity of a leaf extract determined after calibrating conductivity meter with a standard buffer solution.

Phytochemical Screening

To detect the presence of carbohydrate [10], protein [11], reducing sugars [12], polyphenols [13], flavonoids [14], tannin [15], cardiac glycosides [16], anthocyanins [17], quinones [18] and alkaloids [19] standard protocol were used.

Nutritional Analysis

Estimation of Total Polysaccharides Content

Total polysaccharides content was determined according to the standard method [20]. Dextrose was used as standard. The absorbance was read at 490 nm. The polysaccharides content was expressed as mg Dextrose Equivalent/g of Dry Weight (mg DE/g DW).

Estimation of Total Protein Content

Total protein content was measured by Lowry Method [21] by using BSA as standard reagent. The absorbance was read at 660 nm. The results were expressed as mg BSA Equivalent/g of Dry Weight (mg BSAE/g DW).

Phytochemical Analysis

Estimation of Total Polyphenols Content

The total polyphenols content was determined by using Folin-Ciocalteu method [22]. Gallic acid was used as standard reagent. The absorbance was read at 765 nm. The total polyphenols content was expressed as mg Gallic Acid Equivalent/g Dry Weight (mg GAE/ g DW).

Estimation of Total Flavonoids Content

Total flavonoids content was quantified by the aluminum chloride colorimetric assay [23]. The absorbance was read at 510 nm. Quercetin was used as standard reagent. The total flavonoids content was measured as mg Quercetin Equivalent/g Dry Weight (mg QE/g DW).

Estimation of Total Tannin Content

Total tannin content was measured by using Broadhurst and Jones method [24]. Tannic acid was used as a standard reagent. The absorbance was read at 500 nm after 20 min of incubation at room temperature. Total tannin content was measured in terms of mg Tannic Acid Equivalent/g Dry Weight (mg TAE/g DW).

Determination of *In vitro* Antioxidant Activity

DPPH Free Radical Scavenging Assay

The free scavenging activity was evaluated by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) following the standard protocol [25]. Ascorbic acid was used as standard reagent. Absorbance was read at 517 nm. DPPH free radical scavenging activity was expressed in terms of Ascorbic Acid Equivalent, as a percentage inhibition was calculated by the following formula.

% Inhibition of DPPH = [(OD of control - OD of sample) / OD of control] *100

H_2O_2 Free Radical Scavenging Assay

Hydrogen peroxide (H_2O_2) scavenging ability was measured by using a standard method [26]. The optical density was read at 230 nm. Gallic acid was used as a standard. H_2O_2 free radical scavenging activity was expressed in terms of Gallic Acid Equivalent, as a percentage inhibition was calculated by the following formula.

% Inhibition of H_2O_2 = [(OD of control - OD of sample) / OD of control] *100

Evaluation of *In vitro* Antimicrobial Activity

Antimicrobial activity of *Phyllanthus amarus* was performed by using Agar Plate Disc Diffusion Method [8]. Aqueous and 70% ethanolic extracts were screened against gram-positive *Staphylococcus aureus*, denoted by (J) and gram-negative *Escherichia coli*, denoted by (H) compared with standard antibiotic Streptomycin as positive control and two solvents as negative control. Incubate the plate aerobically for 24 hrs at 37ºC. After that zone of inhibition was taken by using ruler, in triplicates.

Statistical Analysis

All the experimental measurements were performed in triplicates and expressed as the average ± standard deviations. The magnitude of the correlation coefficient

Between two variables, means, standard errors, standard deviations, and one-way ANOVA were calculated by using MS Excel 2007 Software. p>0.05 was categorized as non-significant data.

Results and Discussions

Proximate Analysis

From the results of proximate analysis, it was observed that the analytical potential of the experimental plant extracts showed acidic pH property (Table 1) that could enhance the antimicrobial activity as curative measures for some diseases. In this pH range, plant extracts may exhibit sensitivity against some organisms and could contain antimicrobial property. It also indicates that the plant contains useful nutrients for that in future it may be used as functional foods and as nutraceuticals. Electrical conductivity measures the ionic exchange of plant sample. Conductivity is necessary for plant growth, reproduction, development and for seed germination [27, 28].

Table 1: Value of pH and Conductivity

Ph	Conductivity
4.98	1.6

Phytochemical Screening

Results obtained from phytochemical screening of leaf extracts are represented in Table 2.

Total 10 tests were performed for the detection of various phytomolecules. Among them, 6 were present in both the extracts.

These were polyphenol, flavonoids, carbohydrate, protein, reducing sugar and tannin.

On the other hand, 4 phytochemicals were absent in both the extracts.

The results indicated that the leaf sample has significant prospects as a source of therapeutically and pharma ceutically important bioactive compounds [28, 29].

Table 2: Results of Phytochemical Screening

Sl No.	Test Name	Solvent Name	
		Aqueous	Ethanol
1.	Carbohydrate	+	+
2.	Protein	+	+
3.	Reducing Sugar	+	+
4.	Polyphenol	+	+
5.	Flavonoid	+	+
6.	Tannin	+	+
7.	Cardiac Glycoside	-	-
8.	Anthocyanin	-	-
9.	Quinones	-	-
10.	Alkaloid	-	-

"+" Present; "-" Absent

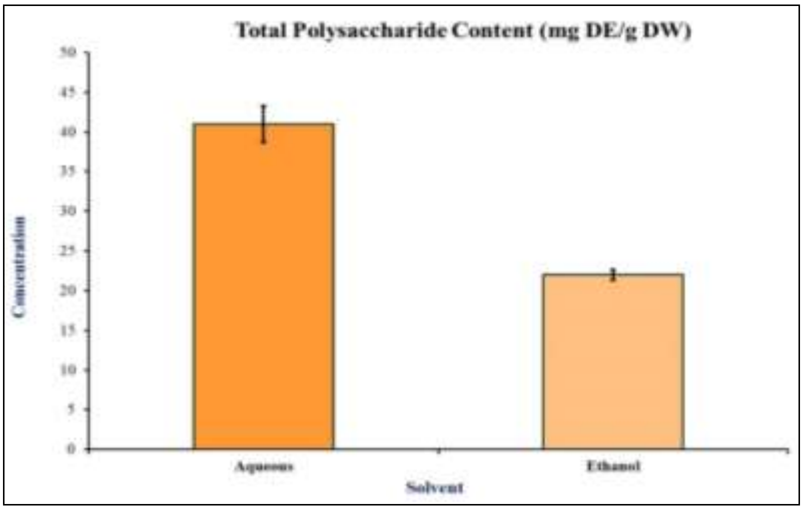
Nutritional Analysis

Total Polysaccharides Content

The total polysaccharides content was quantified to be 41.14 ± 2.29 mg DE/g DW for aqueous extract and 22.22 ± 0.63 mg DE/g DW for 70% ethanolic extract. The total polysaccharides content of the leaf sample was showed in Figure 2a. So, it was clearly observed that aqueous extract exhibited higher amount of polysaccharide content than ethanolic extracts. The p-value<0.05 showed the presence of significant amount of polysaccharides in this plant extract. Polysaccharide exhibits binding, suspending, emulsifying, stabilizing and water-holding capacities and it can be used for the preparation of pharmaceutical and nutraceutical products in the form of tablets and capsules [20, 28, 30].

Total Protein Content

The total protein content was quantified to be 40.12 ± 0.06 mg BSAE/g DW for aqueous extract and 39.36 ±0.07 mg BSAE/g DW for 70% ethanolic extract. The total protein content was showed in Figure 2b. From the results it was showed that the acceptable amount of protein was present in aqueous extracts than ethanolic extracts. The p-value<0.05 showed the presence of significant amount of protein in the extracts [21, 28, 31].



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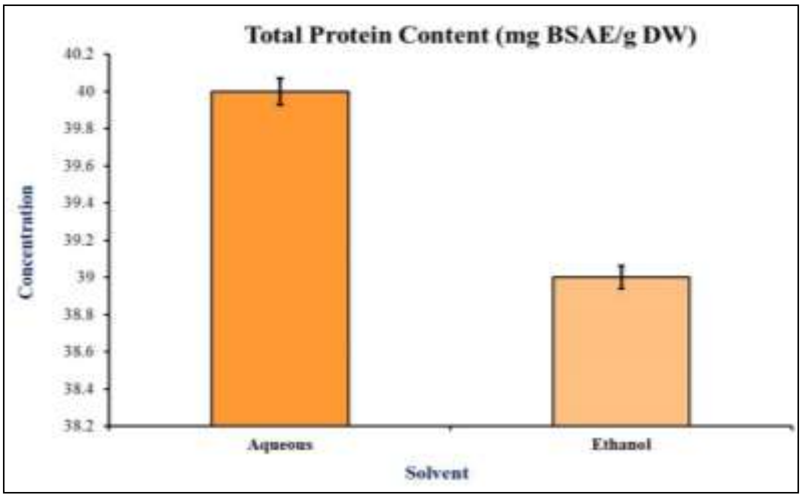

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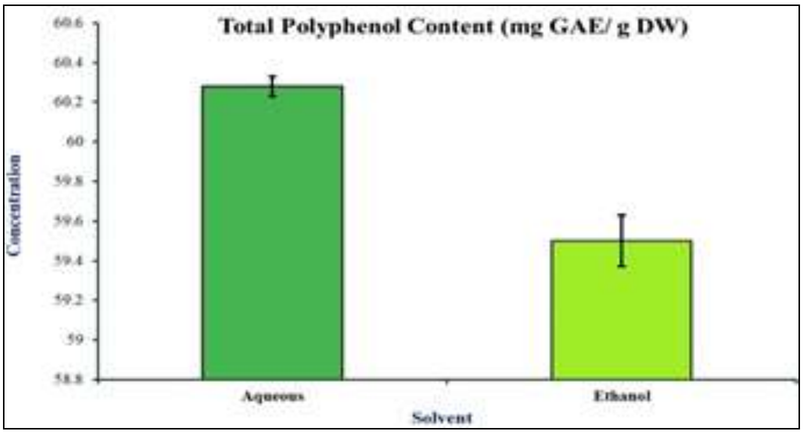
Fig 2: Nutritional Analysis (a) Total Polysaccharides Content (mg DE/g Dry Weight) (b) Total Protein Content (mg BSA/g Dry Weight)

Phytochemical Analysis

Total Polyphenols Content

The total polyphenols content was quantified to be 60.28 ± 0.05 mg GAE/g DW for aqueous extract and 59.58 ± 0.13 mg GAE/g DW for 70% ethanolic extract. The total polyphenols content showed in Figure 3a. So, it was clearly observed that aqueous extract exhibited higher amount of polyphenols content than ethanolic extract. The p-value<0.05 showed the significant amount of polyphenols presence in the extracts. Phenolic compounds are reactive elements towards oxidative activity. The activity of polyphenols against oxidative stress-related processes can have therapeutic application in the pharmaceutical industry. Plants having more polyphenol content showed significant antioxidant activities [25, 32-36].



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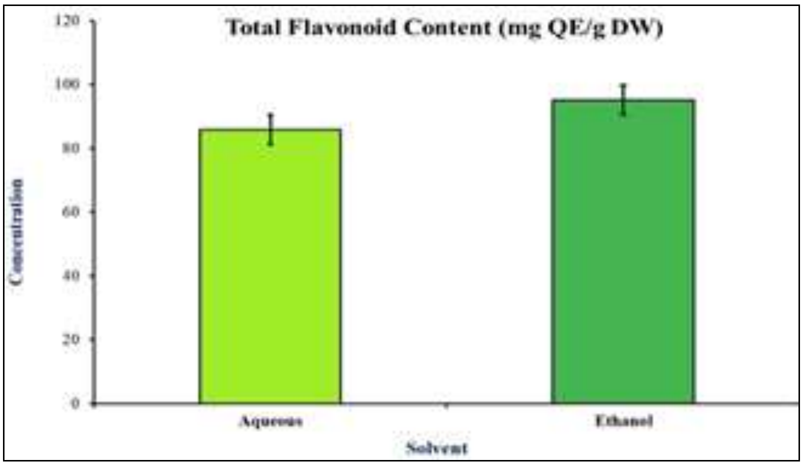

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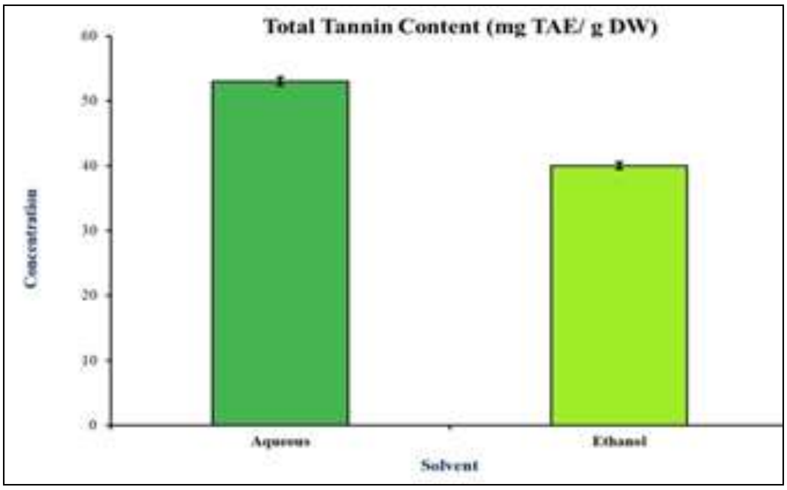

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Fig 3: Phytochemical Analysis (a) Total Polyphenols Content (mg GAE/g Dry Weight) (b) Total Flavonoids Content (mg QE/g Dry Weight) (c) Total Tannin Content (mg TAE/g Dry Weight)

Total Flavonoids Content

The amount of total flavonoids was estimated to be 85.88 ±1.77 mg QE/g DW for aqueous extract and 95.15 ± 2.70 mg QE/g DW for 70% ethanolic extract. The total flavonoids content was shown in Figure 3b. So, it was clearly observed that ethanolic extract exhibited higher amount of flavonoids content than aqueous extract. The p-value<0.05 showed the significant amount of flavonoids presence in the extract. Flavonoids are the most important class of plant secondary metabolites which are strongly related with antioxidant and chelating activities. The antioxidative activities of flavonoids are due to the various mechanisms, like scavenging of free radicals and chelation of metal ions. Flavonoids categories of substances can inhibit the Reactive Oxygen Species (ROS). Plants having more flavonoids content showed significant antioxidant activities [23, 33-37].

Total Tannin Content

The amount of total tannin was estimated to be 53.21 ± 0.60 mg TAE/g DW for aqueous extract and 40.91 ± 0.70 mg TAE/g DW for 70% ethanolic extract. Total tannin content of the extracts was shown in Figure 3c. So, it was clearly observed that aqueous extract exhibited higher amount of tannin content than ethanolic extracts. The p-value<0.05 showed the significant amount of tannin presence in plant extracts. Tannins are astringent and bitter plant polymeric compounds mainly found in the stem of many plants rather than leaves. The higher tannins content reflects the presence of antioxidant and antimicrobial properties. The tannin-protein complex can provide persistent antioxidant and antimicrobial activity [33-40].

***In vitro* Antioxidant Activity**

DPPH Free Radical Scavenging Assay

The maximum inhibition percentages of DPPH free radical scavenging assay is found maximum 69.29 ± 0.90% for aqueous extract and minimum is 58.53 ± 0.80% for 70% ethanolic extract.

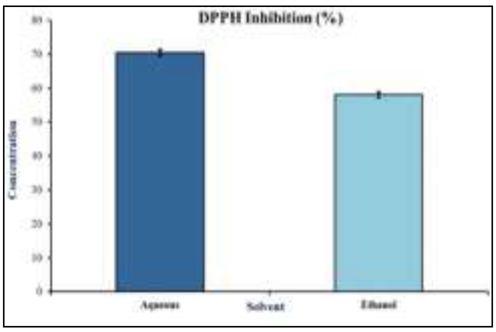
The aqueous extract showed (Figure 4a) higher inhibition percentages and scavenged maximum number of radicals as compared to 70% ethanolic extract.

The p-value<0.05 showed the significant level of antioxidant presence in the extracts. DPPH is a kind of free radical which is widely used to investigate the free radical scavenging capacity of the natural antioxidants. DPPH assay is basically functions on the aspects of reduction of stable free radicals in methanol solution in presence of hydrogen-donating antioxidants due to the formation of the non-radical shape of DPPH [25, 33-36].

H_2O_2 Free Radical Scavenging Assay

The highest inhibition percentage of H_2O_2 free radical scavenging assay was found to be highest for aqueous extract i.e. 70.29±0.29% and lowest is 60.53±0.25% for 70% ethanolic extract.

The aqueous extract showed (Figure 4b) higher inhibition percentage and scavenged maximum number of free radicals as compared to 70% ethanolic extract. The p-value<0.05 showed the significance amount of antioxidant presence in the extract. H_2O_2 becomes toxic to the cell because it is rapidly decomposed into oxygen and water and gives rise to the hydroxyl radical. The study investigates the presence of significant level of natural antioxidants presence in the leaf extracts [33-36, 41].



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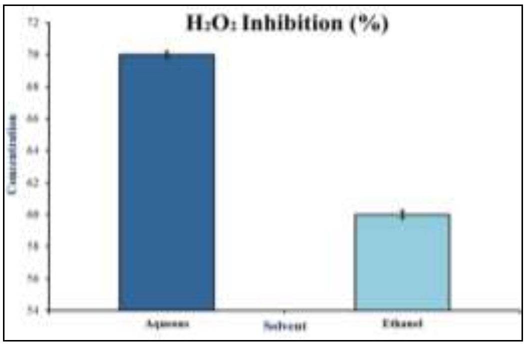

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Fig 4: Antioxidant Activity (a) DPPH Inhibition (%) (b) H_2O_2 Inhibition (%)

In vitro Antimicrobial Activity

From the results of the antimicrobial activity study it is indicated that the aqueous and 70% ethanolic extracts showed significant zone of inhibition against two bacterial strains (Table 3).

Aqueous extract showed maximum zone of inhibition against *Staphylococcus aureus* compared with *Escherichia coli* which showed minimum zone of inhibition. On the other hand, in 70% ethanolic extracts *Staphylococcus aureus* exhibited highest zone of inhibition and *Escherichia coli* showed lowest zone of inhibition.

This zone of inhibition compared with positive control streptomycin, a standard antibiotic which showed largest zone of inhibition for *Staphylococcus aureus* but negative control did not show any zone of inhibition against both the organisms (Figure 5). These indicated that leaf extracts had a significant antibacterial property in aqueous extracts than 70% ethanolic extracts [29, 33-36, 42, 43].

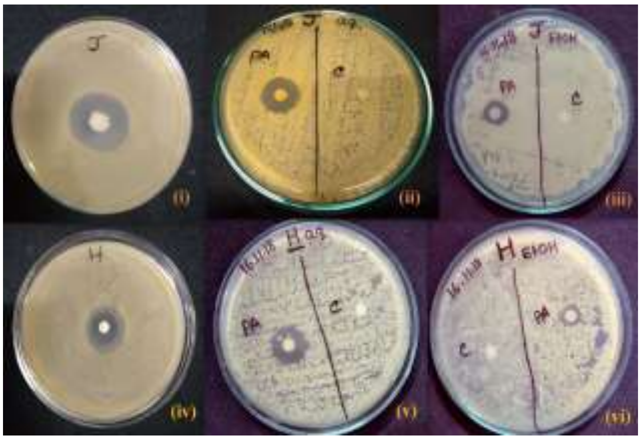

Fig 5: Zone of Inhibition against *S. aureus* and *E. coli*

1. Zone of Inhibition of positive control against *S. aureus*
2. Zone of Inhibition produced by aqueous extract against *S. aureus*
3. Zone of Inhibition produced by 70% ethanolic extract against *S. aureus*
4. Zone of Inhibition of positive control against *E. coli*
5. Zone of Inhibition produced by aqueous extract against *E. coli*
6. Zone of Inhibition produced by 70% ethanolic extract against *E. coli*

Table 3: Zone of inhibition

Organisms Name	Zone of Inhibition (mm)			
	Positive Control (Streptomycin)	Negative Control (Water, Ethanol)	Aqueous Extract	70% Ethanolic Extract
Staphylococcus aureus	30 mm	Nil	16.7 mm	12.33 mm
Escherichia coli	20 mm	Nil	10.66 mm	11.70 mm

Conclusions

Based on the current research study it can be concluded that the phytochemical components of the leaf extracts are highly responsible for its pharmacological properties. The extracts of the *Phyllanthus amarus* were found to be rich with plant primary and secondary metabolites and it showed the higher *in vitro* antioxidant and antimicrobial activities, too.

The aqueous extract showed the higher bioactive compounds presence as well as *in vitro* antioxidant and antimicrobial activities than the 70% ethanolic extract. The study showed that the aqueous extract is more potent than the 70% ethanolic extracts.

The natural antioxidants which are present in the leaf extracts may be attributed to their free radical scavenging capacity. Polyphenolic class of compounds and other important phytomolecules are generally responsible for antioxidant and antimicrobial properties of the leaf extracts [33-36]. The study also concluded that leaf aqueous extracts showed higher antimicrobial property than 70% ethanolic extracts. Based on the present research study findings, it is concluded that the zone of inhibition may be due to the presence of higher bioactive contents, which supports previous research investigations as well [33-36].

The study result which was obtained from the investigation highlighted that the *Phyllanthus amarus* leaves are the vast source of phytochemicals, natural antioxidants and it could be used to prepare pharmaceutical or nutraceutical products against many diseases related with microbial infections and for the oxidative stresses.

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Conflict of Interest

The authors declare that there was no conflict of interest in this research study.

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