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Qualitative, quantitative phytochemical analysis and in-vitro anti-mycobacterial evaluation of allspice

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Abstract---Phytochemical study of plant products has gained popularity in both plant biochemistry and organic chemistry in recent years. One of the current issues in phytochemistry is carrying out all of the above operations with little amounts of material. *Pimenta dioica* Linn, often called as "All-spice," contains a number of secondary metabolites with medicinal potential. This study examines the key phytochemicals found in the leaves of the medicinally important plant *Pimenta dioica* on a qualitative and quantitative level. The goal of this study is to evaluate phytochemicals by analysing diverse extracts quantitatively and qualitatively. Quantification and phytochemical screening revealed the presence of alkaloids, flavonoids, phenols, proteins, amino acids, saponins, tannins, terpenoids, and glycosides. In addition, the study found that an ethanolic extract of leaves extract was more effective against *Mycobacterium tuberculosis*. A microplate-based Alamar blue assay has been applied to search for novel antimicrobials to treat tuberculosis from phytochemicals in *Pimenta dioica*.

Keywords---*Pimenta dioica*, MABA, Alkaloids, flavonoids, phenols.

Introduction

Nature has provided everything for mankind's well-being over the millennia, including the instruments for the first therapeutic procedures. Plants have been used to heal a variety of diseases since ancient times. Many of these plants have been studied for novel drugs or used as templates for the development of new therapeutic agents, food additives, agrochemicals, and industrial chemicals, and many of these plants have been studied for novel drugs or used as templates for the development of new therapeutic agents, food additives, agrochemicals, and industrial chemicals, and many of these plants have been studied for novel drugs

or used as templates for the development of new therapeutic agents, food additives, agrochemicals, industrial chemicals and many of a photochemical is a bioactive substance found naturally in plants that functions as a disease defence mechanism. (Borris et al., 1996). Primary and secondary components are the two types of phytochemicals that act in plant metabolism. Only a small percentage of the world's 2,50,000 to 5,00,000 plant species have been studied for phytochemicals. (Habila et al., 2011).

In recent years, phytochemical research on plant products has grown in prominence in both plant biochemistry and organic chemistry. It examines the chemical structure, distribution, and biological function of plant components, as well as the variety of organic compounds accumulated by plants. As a result, developments in phytochemistry are closely tied to the effective use of existing techniques and the constant development of new approaches to unsolved problems as they occur. Completing all of the above duties with such a small amount of material is one of the difficulties in phytochemistry. To solve a biological problem, such as plant growth regulation, plant-animal interactions biochemistry, or the genesis of ancient plants, researchers must first find a spectrum of complex chemical compounds that may only be available in microgram quantities. As a result, comprehensive plant species screening with the goal of discovering new bioactive compounds can aid in the treatment of a variety of fungal and bacterial illnesses that affect economically important crops and animals, including humans. Plants are beneficial because they contain chemical components that have physiological effects on the human body. Alkaloids, tannins, flavonoids, and phenolic compounds are the most important bioactive components in plants. Because of their low mammalian toxicity, target selectivity, and biodegradability, these insecticides outperform synthetic pesticides. (Stevens et al., 192)

Pimenta dioica (L.) Merr., a member of the Myrtaceae family, is the botanical name for all spices. Jamaica pepper is a natural plant that grows in the Caribbean island of Jamaica. Maharashtra, Karnataka, Tamil Nadu, and Kerala are just a few of the Indian states where it grows. The classic evergreen tropical tree with light grey bark and dark green leaves (4-8 cm long) that develops to a height of 22-35 feet. The dried berries are about 13-14 grammes in weight and 6.5 to 9.5 millimetres in diameter. In the summer, the allspice tree that produces the berries blossoms with small white flowers. The berries are collected when they are still green and sun-dried. It has a stronger flavour because to the higher quantity of essential oils. It's also known as allspice because of its flavour and scent, which many people associate with cloves, cinnamon, ginger, and nutmeg. (Dhrubo et al., 2016) Phytocomponents such as terpenoids, glycosides, steroids, alkaloids, tannins, saponins, and polyphenols were discovered in the leaves, fruits, stem, and roots of the *Pimenta dioica* plant after a thorough investigation. These phytocomponents are useful in the treatment of a variety of infectious and non-infectious diseases. (Lorence et al., 1995, Rao et al., 2015 & Mukherjee et al., 2002). Plants and their ingredients, as well as the manner, in which they are administered, differ from person to person. As a result, the goal of this study was to find the best ways to use *Pimenta dioica* phytoconstituents. In the recent time, flavone and polyphenolics nucleus have gathered an immense attention among all

phytochemists as well as biologists as it is one of the key building elements for many naturally occurring compounds.

Tuberculosis (TB) is a lung infection caused mainly by *Mycobacterium tuberculosis* (M. tuberculosis [MTB]). From last 4-5 decades it is considered a major threat for public health due to most contagious and deadly diseases. (Brooks et al., 2009). Tuberculosis, also known as TB and 'white plaque', is caused by infection with members of the MTB complex, which includes *Mycobacterium tuberculosis* itself, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium pinnipedii* and *Mycobacterium canettii*. (Grange et al., 2009 & Dye et al., 1999). Although the existing method of curing is very effective against TB, the length of treatment, the toxicity and the potential for drug-drug interactions are factors that highlight the need for new anti-TB drugs. (Dye et al., 2010 & Schecter et al., 2010). Among pharmacologically important heterocyclic compounds, quinoline and its derivatives are significant because of their wide spectrum of biological activities and their presence in naturally occurring compounds.

Materials and Methods

Plant Material Collections and Drying

Fresh leaves of the plant were discovered and gathered in the Pune district in September (Maharashtra, India). Dried in the shade at ordinary room temperature. Store crude medicine powder in sealed bottles away from light and humidity until utilized for extract extraction.

Authentication

The plant specimen was authenticated by Dr. Priyanka A. Ingle, Scientist, Botanical Survey of India, Pune. The Voucher specimen number VVD 01 for Plant *Pimenta dioica* (L.) Merr., (Ref. No. BSI/WRC/IDEN.CER./2020/92, Date 01/10/2020)

Crude Extract preparation

In the Soxhlet system, 100 grammes of dry powdered leaf materials were subjected to 12 hours of successive organic solvent extraction. Graded polarity solvents such as hexane, chloroform, ethyl acetate, ethanol, and distilled water were utilised in the extraction operation. The solvent is kept at a boiling temperature throughout the extraction process, and all laboratory criteria are met. The pure dry extract was labelled and stored in the refrigerator until further testing. (Harborne et al., 1998, Gokhale et al., 2020 & Trease et al., 2002).

Phyto-chemical Screening

The physical properties of concentrated dried extracts of *Pimenta dioica* leaves, as well as their percent yield, were investigated. It was also used to do preliminary qualitative screening of phytochemicals using well-established methods. (Mukherjee et al., 2002, Harborne et al., 1998 & Khandelwal et al., 2005).

Tests for Acidic compounds

- Add sodium bicarbonate to the test solution; effervescence occurs when acidic chemicals are present.
- Warm water treatment and filtering of the test solution Litmus paper is used to test the filtrate. When acidic substances are present, litmus paper becomes blue.

Test for Carbohydrates

- **Molisch test (General test):**

In a test tube, two ml of extract solution was combined with a few drops of 15% ethanolic-naphthol solution, and 2 ml of concentrated sulphuric acid was gently applied along the side of the test tube. The presence of carbohydrates is shown by the creation of a reddish violet ring at the intersection of two layers.

- **Test for reducing sugar:**

- **Benedict's test:**

In a test tube, combine an equal volume of Benedict's reagent and extract solution. 5 minutes in a hot water bath. Depending on the quantity of reducing sugar present, the solution looks green, yellow, or red.

- **Fehling's test:**

Five ml of extract solution was mixed with 5 ml Fehling's solution (equal mixture of Fehling's solution A and B) and boiled. Development of brick red precipitate indicates the presence of reducing sugars.

- **Test for monosaccharides:**

- **Barford's test:**

Barford's reagent and extract solution should be mixed in equal parts. In a boiling water bath, heat for 1- 2 minutes and then cool. The presence of monosaccharides is shown by the formation of red precipitate.

- **Selivanoffs test (Test for Ketones):** Add resorcinol crystals and equal volumes of strong hydrochloric acid to the test extract solution and heat on a water bath. If ketone is present, a rose colour is generated.

- **Osazone formation test:** Heat the phenyl hydrazine hydrochloride, sodium acetate, and acetic acid solution in the test solution. Under a microscope, needle-shaped or yellow crystals produced during complex formation with carbohydrates (disaccharides).

Test for Proteins

- **Heat test:** Heat the test solution in boiling water bath. Proteins get coagulated.

- **Biuret test:**

The extract was heated after being treated with 1 ml of a 10% sodium hydroxide solution. To the aforesaid combination, a drop of 0.7 percent copper sulphate solution was added. The presence of proteins is shown by the production of purple violet blue.

- **Millon's test:**

The extract was treated with 2 ml of Millon's reagent. Formation of white precipitate indicates the presence of proteins and amino acids.

- **Xanthoproteic test:**

Take 1 ml test extract solution; add 1 ml of conc. nitric acid and boil yellow precipitate is formed. After cooling it, add 40 % sodium hydroxide solution. Orange colour formed in presence of proteins.

- **Test for starch:**

Add 1 ml of mild aqueous iodine solution to 1 mL of test extract solution. The presence of starch is indicated by the development of a blue colour, which vanishes when heated and returns when cooled.

Test for Amino acids

- **Millon's Test:**

1 ml test extract solution prepared with Million's reagent and heated in a water bath at the same time. The presence of amino acids is shown by the formation of white precipitate.

- **Ninhydrin test:**

The extract was treated with ninhydrin reagent at pH range of 4-8 and boiled. Formation of purple color indicates the presence of amino acids.

Test for Steroids

- **Salkowski test:**

To 10 mg of extract dissolved in 1 ml of chloroform, 1 ml of strong sulphuric acid was added. The presence of steroids is indicated by the chloroform layer's reddish brown blue and the acid layer's green fluorescence.

- **Liebermann-burchard test:**

Following the addition of 2 ml of concentrated sulphuric acid from the side of the test tube, 10 mg extract was dissolved in 1 ml chloroform and 1 ml acetic anhydride was added. The presence of steroids is shown by the formation of a reddish violet ring at the junction.

- **Liebermann's test:**

A few cc of acetic anhydride was added to 2 ml of the residual and gently heated. The contents of the test tube were chilled before adding 2 ml of concentrated sulphuric acid from the test tube's side. The presence of steroids was demonstrated by the development of a blue.

Test for Terpenoids

1 ml test extract mixed with 1 ml Vanillin sulfuric acid. Result showed violet blue colour in presence of terpenoids.

Test for Glycosides

- **General test:**

- **Test A:** Warm 5 ml concentrated test extract in 5 ml dilute sulphuric acid over a water bath, filter and neutralize the acid extract with a 5% sodium hydroxide solution. Heat on a water bath for 2 minutes after adding 0.1 ml of Fehling's solution A and B until it turns alkaline (test with pH paper). Presence of glycoside derivatives, a precipitate is generated.

- **Test B:**
Repeat above procedure by using 5 ml of water instead of dilute sulphuric acid. Note the quantity of red precipitate formed compared with precipitate of test A.
- **Chemical tests for specific glycosides:**
- **Anthraquinone glycosides:**
- **Borntrager's test:**
Boil and filter 3 mL test extract with dilute sulphuric acid. Add an equal amount of benzene or chloroform to the cooled filtrate. Shake vigorously. Distinguish the organic solvent. When ammonia is added, the ammonia layer turns pink or crimson.
- **Modified Borntrager's test:**
1ml concentrated test extract, 5ml of dilute HCl and 5 ml of 5% solution of ferric chloride were added and boiled for few minutes and then subsequently cooled and filtered part is shaken with benzene; the separated benzene layer and add equal volume of dilute solution of ammonia shows pink colour in presence of anthraquinone glycosides .
- **Cardiac glycoside**
- **Keller-Killiani test (Test for deoxysugars):**
Glacial acetic acid, one drop 5 percent Ferric chloride, and conc. Sulphuric acid were added to 2 ml of test extract. The creation of a reddish brown blue at the confluence of the two liquid layers indicates the presence of cardiac glycosides, whereas the top layer looked bluish green.
Kedde's test: To 2 ml of test extract with chloroform, evaporate to dryness. Add one drop of 90 % alcohol and 2 drops of 2 % sodium hydroxide solution. Purple colour is produced.
Baljet's Test: The test solution treated with sodium picrate or picric acid. Gives yellow to orange colour.
Legal's Test: Test solution treated with pyridine [made alkaline by adding sodium nitroprusside solution]. Gives blood red colour
- **Tests for coumarins glycosides:**
Fill a test tube with a tiny quantity of sample and cover it with filter paper soaked with weak sodium hydroxide solution. For many minutes, I placed the covered test tube in a water bath. Remove the paper off the package and expose it to UV light. Green fluorescence is seen on the paper.
- **Cynogenic glycosides:**
In a conical flask, place 1 ml concentrated test extract and a few drops of water. (The hydrogen cyanide created in the flask should be absolutely dry since it will dissolve in the water rather than escape as a gas and react with the paper.) Using a 5 percent aqueous sodium carbonate solution, wet a piece of picric acid paper and suspend it in the flask's neck. Warm to around 37°C. The reddish purple indicates the presence of glycosides.

Test for Saponins

Foam formation test

One ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. The development of stable foam indicates the presence of Saponins.

Test for Alkaloids

- **Dragendroff's test:**
2-3 ml test solution and 0.1 ml Dragendroff's reagent was added in test tube. Formation of orange brown precipitate indicates the presence of alkaloids.
- **Mayer's test:**
2-3 ml test solution and 0.1 ml of Mayer's reagent were added. Formation of yellowish buff precipitate indicates the presence of alkaloids.
- **Hager's test:**
2-3 ml test solution and 0.1 ml of Hager's reagent. Formation of yellowish precipitate indicates the presence of alkaloids.
- **Wagner's test:**
2-3 ml test solution and 0.1 ml of Wagner's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.
- **Tannic acid test:**
Test extract solution treated with Tannic acid solution. Gives buff coloured precipitate
- **Picrolonic acid test:**
Test solution treated with Picrolonic acid. Gives yellow coloured precipitate

Test for Tannins and Phenolic compounds

- **5 %Ferric chloride:**
Five ml of extract solution was allowed to react with 1 ml of 5 percent ferric chloride solution. Deep blue-black coloration indicates the presence of tannins.
- **Dilute nitric acid test:**
Two ml of extract solution was allowed to react with few drops of dilute HNO_3 solution. Formation of reddish to yellow color indicates the presence of tannins.
- **Bromine water test:**
Two ml of extract solution mix with 2 ml of bromine water. Discoloration of bromine water indicates presence of tannins.
- **Potassium dichromate test:**
2-3 ml of extract solution and mix with 2 ml of Potassium dichromate. The formation of red precipitate indicates presence of tannins.
- **Gelatin test:** To the test solution add 1 % gelatin solution containing 10 % sodium chloride. In presence of tannins precipitate formed.

Test for Flavonoids

- **Shinoda test:**
To the test extract 5 ml (95%) ethanol and few drops of con. HCl and 0.5 g of magnesium turnings was added gives pink color.
- **Lead acetate test:**
Few drops of 10 percent lead acetate are added to the test extract. Development of yellow colored precipitate confirms the presence of flavonoids.

- **Sodium hydroxide test:**

Increasing amount of sodium hydroxide add in an extract solution which shows yellow coloration, which disappears after addition of acid.

- **Zinc-Hydrochloride test:**

Treat test extract solution with zinc dust and few drops of hydrochloric acid, in presence of flavone derivatives shows red colour after few minutes.

Determination of Minerals Elements

The minerals were determined by the dry ash extraction method using atomic spectrometry.

Quantification of phyto-chemicals in crude extract of *Pimenta dioica*

- **Quantitative phytochemical analysis:** The phytochemicals which are extracted may show response in qualitative analysis. The phytochemicals present in the all extracts was determined and quantified by standard procedures as follows.
- **Determination of total phenolic compounds:** 100 milligrams of the sample extract was properly weighed and diluted in 100 mL of distilled water. 1 ml of this solution was transferred to a test tube, followed by the addition of 0.5 ml 2N Folin-Ciocalteu reagent and 1.5 ml 20 percent Na₂CO₃ solution, and lastly the volume was filled up to 8 ml with distilled water, vigorous shaking, and let to stand for 2 hours. At 765 nm, the absorbance was measured. Using a standard calibration curve derived from varying diluted quantities of gallic acid, these data were utilised to determine the total phenolic content. Under the same circumstances, the absorption of a standard gallic acid solution (0.5 mg/ml) in methanol was determined. All of the tests were performed in duplicate. (Wagner et al., 1996 & Ekwueme et al., 2015).
- **Determination of total flavonoids:** The approach relies on the production of a flavonoids-aluminium combination with a maximum absorptivity of 415nm. A mixture of 100 l of sample extracts in methanol (10 mg/ml) and 100 l of 20 percent aluminium trichloride in methanol was used. A drop of acetic acid was added, followed by a 5ml methanol dilution. The absorbance was measured at 415 nm after 40 minutes. 100 mL of sample extracts and a drop of acetic acid were used to make blank samples, which were then diluted to 5 mL with methanol. Under the same circumstances, the absorption of a standard quercetin solution (0.5 mg/ml) in methanol was determined. All of the tests were performed in duplicate. (Ekwueme et al., 2015).
- **Determination of total alkaloids:**
The 1gm test extract was macerated with 20 ml of ethanol and 20% H₂SO₄ (1:1 v/v). The filtrate (1 ml) was added to 5 ml of 60% H₂SO₄. After 5 min, 5 ml of 0.5% formaldehyde in 60% H₂SO₄ was mixed with the above mixture and allowed to stand for 3 hr. The absorbance was read at 565 nm. (Wagner et al., 1996 & Ekwueme et al., 2015).
- **Determination of total tannins:** 0.25 ml Folin Phenol reagent, 0.5 ml 35 percent sodium carbonate solution, 3.75 ml distilled water, 0.5 ml test extract, 3.75 ml distilled water, 0.25 ml Folin Phenol reagent, 0.25 ml Folin

Phenol reagent, 0.25 ml Folin Phenol reagent, 0.25 ml Folin Phenol reagent at 725nm, the absorbance of the aforementioned combination was measured. Standard solutions were tannic acid dilutions (0 to 0.5 mg/ml). Tannic acid content is measured in milligrams per millilitre of extract. (Chukwuma et al., 2016 & Gul et al., 2017).

- **Determination of total glycosides:**

The extract (1 gram) was macerated in 50 ml distilled water before being filtered. 4 ml alkaline pirate solution was added to the filtrate (1 ml). After boiling for 5 minutes, the mixture was allowed to cool. At 490 nm, the absorbance was measured. (Edem et al., 2016 & Roghini et al., 2018).

- **Test for Terpenoids:**

The 1gm test extract was macerated in 50 ml ethanol before being filtered. 2.5 ml filtrate, 2.5 ml aqueous phosphomolybdic acid solution (5%), and 2.5 ml concentrated H₂SO₄ Allow 30 minutes for the mixture to settle before adding 12 mL of ethanol. At 700 nm, the absorbance was measured. (Ajiboye et al., 2013, Hagerman et al., 2000).

- **Test for Steroids:**

The 1gm test extract was macerated in ethanol for 20 minutes before being filtered. 2 ml chromagen solution was added to the filtrate (2 ml), and the solution was allowed to stand for 30 minutes. At 550 nm, the absorbance was measured. The difference in colour intensity or absorbance between the test and blank samples is proportional to the concentration of the particular phytocomponents found in the test extract. All of the above quantitative data is presented in milligrams per gm of dried sample. (Van-Burden et al., 1981, Durai et al., 2016, Tsuzuki et al., 2007, Zablotowicz et al., 1996 & Mir et al., 2013).

***In-vitro* Anti-Mycobacterial activity: Microplate Alamar Blue Assay (MABA)**

Microplate Alamar Blue Assay- The antitubercular activity of *Pimenta dioica* Linn. Leaves extract are planned to perform Screening against Mycobacterial Pathogens (*Mycobacterial tuberculosis*) (H37Rv) (ATCC No-27294) using 96 well plate in MABA.

Requirement

Microbial strain *M. Tuberculosis* (H37Rv) (ATCC No-27294), Middlebrook 7H9 culture medium, 96 well plate, sterile deionized water, parafilm, incubator, Almar Blue reagent, Tween 80,

Test sample

The Ethanol extract of *Pimenta dioica* leaves presence of different phytocomponents which are fractionated by column chromatography in isocratic manner. (Majumder et al., 2016). All collected fractions were concentrated and evaluated for Microplate Alamar Blue Assay to determine potential of active fractions and separated phytocomponents. Required test concentrations 0.8 to 100µg/ml were prepared.

Microplate Alamar Blue Assay

The antitubercular activity of fractions was assessed against microbial strain *M. Tuberculosis* (H37Rv) (ATCC No-27294) using MABA. This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method. The *M. Tuberculosis* (MTB) were cultured in 7H9 medium in the presence of the plant extracts in a 96 well plate was tested at concentration 0.8, 1.6, 3.12, 6.25, 12.5, 25, 50 and 100 µg/ml. 200µl of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100µl of the Middlebrook 7H9 broth, and serial dilution of compounds was made directly on the plate. The final drug concentrations tested were 0.8 to 100µg/ml. Plates were covered and sealed with parafilm and incubated at 37 °C for five days. After this 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% Tween 80 was added to the plate and incubated for 24 h. A blue color in the well was interpreted as no bacterial growth and pink color was scored as growth. Interpretations were based on the percent reduction of the dye which is directly proportional to the bacterial growth. The extracts were considered active if the percent reduction value of Alamar blue dye was less than that observed for the standard. Triplicate wells were maintained for each variable in assay. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink. (Franzblau et al., 1998, Collins et al., 1997, Maria et al., 2007 & Mandewale et al., 2015).

Results and Discussion

Phyto-chemical screening test

In this work, the qualitative analyses of *Pimenta dioica* leaves crude extracts were examined, and the findings were presented. Table 1 shows the physical properties and % yield of all extracts. The data shows that the percentage yield of chloroform, ethyl acetate, and ethanol extracts were higher.

Table 1
Characteristics of Plant extracts

Extract particulars	Percent Yield (%W/W)	Characteristics	
		Colour	Appearance/ Consistency
Hexane extract (PLH)	06.21%	Green	Solid
Chloroform extract (PLC)	14.20%	Brown	Solid
Ethyl acetate extract (PLEA)	11.01%	Dark brown	Semisolid
Ethanol extract (PLET)	20.15%	Dark yellowish brown	Semisolid
Aqueous (Water) extract (PLA)	04.91%	Faint Brown/ Black	Liquid

The extract variety yielded positive results for a variety of phytochemicals reported in table 2. In *Pimenta dioica* leaves, polarity gradient solvent selection resulted in greater separation of distinct complex metabolites.

Table 2
Qualitative analysis of *Pimenta dioica* Linn. Leaves extract

Sr.N o.	Tests	PLH	PLC	PLEA	PLET	PLA
1.	Tests for Acidic compounds	-	-	+	+	-
	Test for carbohydrate					
	Molish's test	-	-	-	+	-
	Fehling test	-	+	+	+	-
2	Benedicts test	-	-	+	+	-
	Barfoed test	-	+	-	+	-
	Selivanoffs test	-	+	+	-	-
	Osazone formation test	-	-	+	-	-
	Test for Proteins					
3	Biuret Test	-	-	-	+	+
	Millons Test	-	-	-	-	-
4	Test for amino acids					
	Ninhydrine test	-	+	-	+	+
	Test for Steroids					
5	Salkowski test	-	+	-	-	-
	Libermann test	-	-	-	+	-
	Libermann-Burchard reaction	-	+	-	-	-
	Test for Glycosides					
6	Anthraquinone glycoside test	-	+	+	+	-
	Cardiac glycoside test	-	+	-	-	-
	Cynogentic glycosides test	-	-	-	+	-
7	Test for Terpenoids:	-	+	-	+	-
8	Test for Saponin					
	Foam test	-	+	-	-	+
	Test for Alkaloids					
	Dragondorff's test	-	-	-	+	-
9	Mayer's test	-	-	-	-	-
	Hager's test	-	-	+	+	-
	Wagner's test	-	-	-	+	-
	Test for Tannins and Phenolic compounds					
10	5% FeCl ₃ test	-	+	-	+	-
	Lead acetate solution	-	+	+	+	-
	Test for Flavonoids					
11	Shinoda test	-	+	+	+	-
	Sulphuric acid test	-	-	+	+	-

Abbreviation: PLH- Pimenta leaves Hexane extract; PLC- Pimenta leaves Chloroform extract; PLEA- Pimenta leaves Ethyl Acetate extract; PLET- Pimenta leaves Ethanol Extract, PLA- Pimenta leaves Aqueous Extract., (-):Absent, (+): Presence.As per above observation all solvents showed their efficiency to separate variety of phytocomponents based on affinity. As it observed phytochemical complexity need to isolate and quantify them for better characterization.

The elemental analysis of the plant revealed the findings reported in Table 3.

Table 3
Elemental Composition

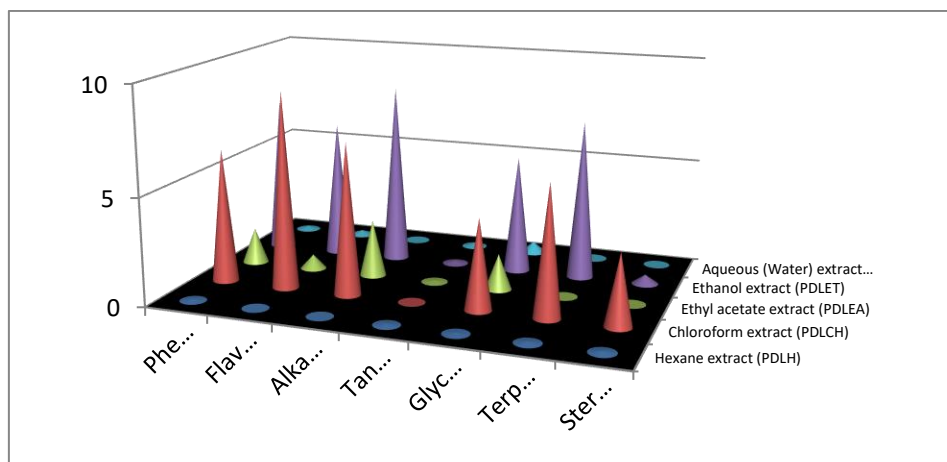
Sr. no.	Minerals	Composition (mg/100g)
1	Magnesium	4.56 mg
2	Zinc	9.11 mg
3	Selenium	1.06 mg
4	Iron	4.12 mg
5	Manganese	5.15 mg

Quantification of phyto-chemicals in crude extract of *Pimenta dioica*

In quantitative examination of all assays, extracts of *Pimenta dioica* leaves indicated good findings for seven phytochemicals, including phenol, flavonoids, alkaloids, tannin, glycosides, terpenoids, and steroids. The findings were depicted in Table 4 and Graph 1. Although alkaloids, phenolic chemicals, flavonoids, and glycosides are important secondary metabolites that contribute to the plant's therapeutic properties, they are also important primary metabolites. Additional analytical methods were used to determine the phytochemical contents of the extract.

Table 4
Quantitative Analysis of *Pimenta dioica* Linn. Leaves extracts

Extracts/ Test	Phytochemical Mean \pm STD						
	Phenols	Flavonoids	Alkaloids	Tannins	Glycosides	Terpenoids	Steroids
<i>Pimenta dioica</i> Linn. Leaves extracts							
Hexane extract (PLH)	0.06 \pm 0.023	-	-	0.07 \pm 0.012	0.06 \pm 0.020	0.08 \pm 0.016	0.09 \pm 0.011
Chloroform extract (PLCH)	6.30 \pm 0.032	9.21 \pm 0.030	7.11 \pm 0.021	-	4.23 \pm 0.042	6.11 \pm 0.030	3.34 \pm 0.082
Ethyl acetate extract (PLEA)	1.68 \pm 0.013	0.68 \pm 0.043	2.68 \pm 0.015	0.08 \pm 0.003	1.68 \pm 0.023	-	-
Ethanol extract (PLET)	7.41 \pm 0.052	6.45 \pm 0.078	8.45 \pm 0.065	0.14 \pm 0.044	5.51 \pm 0.012	7.44 \pm 0.055	0.44 \pm 0.051
Aqueous (Water) extract (PLAQ)	0.16 \pm 0.020	0.22 \pm 0.043	-	0.16 \pm 0.003	0.54 \pm 0.020	-	-



Graph 1. Quantitative Analysis of *Pimenta dioica* Leaf extract

The highest concentration of phenols (7.41 mg /g), alkaloids (8.45 mg /g), glycosides (5.51 mg /g) and terpenoids (7.44 mg /g) observed in ethanol extract. The highest concentration of flavonoids (9.21 mg /g), alkaloids (7.11 mg /g) and steroids (3.34 mg /g) observed in chloroform extract. The aqueous extract observed high tannins contents (0.16 mg /g).

***In-vitro* Anti-Mycobacterial activity: Microplate Alamar Blue Assay (MABA)**

The test fractions of ethanol extracts of *Pimenta dioica* were shown better potential and prevent mycobacterial growth. Observation in figure 1. Test fraction 1, 4, 6 and 7 showed better inhibition and MIC at 1.6, 6.25, 3.2 and 6.25 μ g/ml respectively.

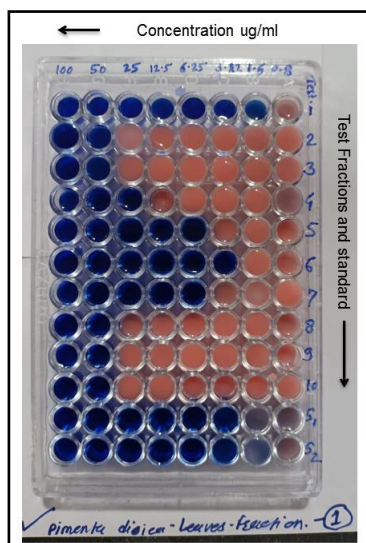


Figure 1. Observation for Microplate Alamar Blue Assay

The result of the *in-vitro* anti-tubercular activity table 5 reported sensitivity towards mycobacterium growth at different concentration. The MIC of the crude extract fraction number 1, 4, 6 and 7 showed inhibition 1.6, 6.25, 3.2 and 6.25 $\mu\text{g/ml}$ respectively mentioned in table 6 and graph. 2. So the isolated phytochemicals in fraction showed significant potential as compare with other fractions and standard drugs showed promising activity against *M. tuberculosis*.

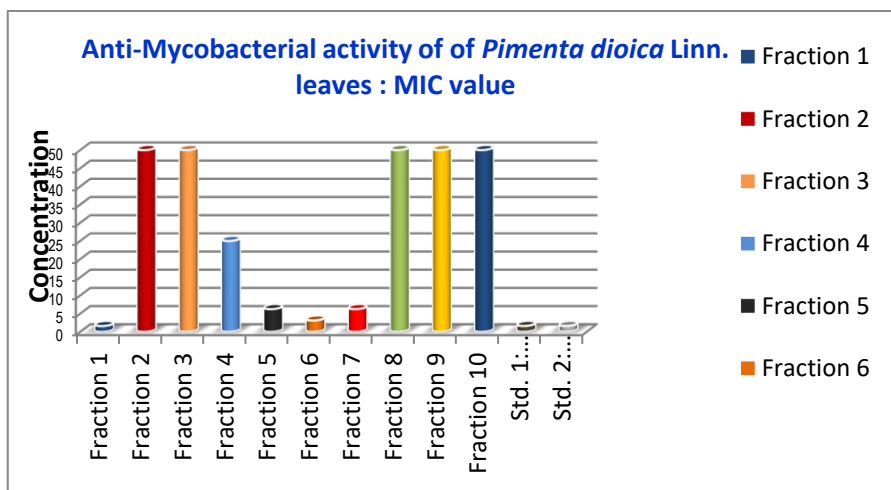
Table 5
Observation for *In-vitro* Anti-Mycobacterial activity of plant fractions by
Microplate Alamar Blue Assay (MABA)

Sr. No.	Samples	Concentration $\mu\text{g/ml}$							
		100	50	25	12.5	6.25	3.12	1.60	0.8
1.	Fraction 1	S	S	S	S	S	S	S	R
2.	Fraction 2	S	S	R	R	R	R	R	R
3.	Fraction 3	S	S	R	R	R	R	R	R
4.	Fraction 4	S	S	S	R	R	R	R	R
5.	Fraction 5	S	S	S	S	S	R	R	R
6.	Fraction 6	S	S	S	S	S	S	R	R
7.	Fraction 7	S	S	S	S	S	R	R	R
8.	Fraction 8	S	S	R	R	R	R	R	R
9.	Fraction 9	S	S	R	R	R	R	R	R
10.	Fraction 10	S	S	R	R	R	R	R	R
11.	Std. Isoniazide	1: S	S	S	S	S	S	R	R
12.	Std. Rifampicin	2: S	S	S	S	S	S	R	R

S-Sensitive R- Resistant

Table 6
Observation for *In-vitro* Anti-Mycobacterial activity of plant fractions
Minimum Inhibitory concentration

Sr. No.	Samples	MIC $\mu\text{g/ml}$	Sr. No.	Samples	MIC $\mu\text{g/ml}$
1.	Fraction 1	1.60	7	Fraction 7	6.25
2.	Fraction 2	50	8	Fraction 8	50
3.	Fraction 3	50	9	Fraction 9	50
4.	Fraction 4	25	10	Fraction 10	50
5.	Fraction 5	6.25	11	Std. Isoniazide	1: 3.12
6.	Fraction 6	3.12	12	Std. Rifampicin	2: 3.12



Graph 2. Graphical Representation of Minimum Inhibitory concentration of all fractions.

Conclusion

Several phytochemicals were evaluated in all subsequent solvent extracts of *Pimenta dioica* leaf. In qualitative examination, many primary metabolites such as carbohydrates, proteins, and lipids, as well as secondary metabolites such as alkaloids, flavonoids, phenols, alkaloids, tannins, terpenoids, and glycosides, were found to be positive. Plant leaves may be engaged in biosynthesis, culminating in the creation of higher derivatives, because they contain a diverse spectrum of components. Quantitative study revealed that phytochemicals such as alkaloids, flavonoids, phenols, alkaloids, tannins, terpenoids, and glycosides were abundant. The chloroform and ethanol extracts of *Pimenta dioica* leaves had higher quantities than the other three extracts. Alkaloids are the most significant compounds, as they play a metabolic role in biological systems and provide protection to animals. Flavonoids have been used to treat cancerous tumours and have been demonstrated to reduce the growth and progression of cancerous tumours. Antioxidant capacity of phenolic and flavonoid compounds has been proven in a range of disease presentations. When phenols are coupled with flavonoid molecules in plants, they have been found to have antioxidant, anticarcinogenic, and anti-inflammatory activities. Tannins inhibit harmful fungi, and adding tannins to extracts boosted their antibacterial activity. Plant-based compounds have a more effective dosage response and fewer side effects than manufactured chemicals. Furthermore, the plant examined for phytochemical elements appeared to have the potential to serve as a source of useful pharmaceuticals as well as to improve the health status of consumers, owing to the presence of several compounds that are important for good health.

According to the findings of the study ethanol extracts fraction number 1, 4, 6 and 7 of *Pimenta dioica* leaves can be used as a source of phytochemicals. They may become a revolutionary lead medicine source for the prevention of *tuberculosis* due to its promising activity against *M. tuberculosis* (H37Rv) strain. So it can be concluded that isolated bioactive fractions and phytochemicals of

Pimenta dioica serve as potential antimycobacterial agents in the field of pharmaceutical as well as LED developments.

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