Original Article

Phytochemical Analysis And Antitubercular Potential Of All Spice

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Abstract

Phytochemical analysis of plant products took a distinct place in plant biochemistry as well as organic chemistry in recent years. Now days it is one of the challenges of phytochemistry is to carry out all the above operations in small amounts of material. Pimenta dioica Linn also called as "All-spice" contain various secondary metabolites with great potentials. The present study investigates the qualitative and quantitative analysis of the major phytochemicals from fruits of medicinally important plant Pimenta dioica with their antitubercular potential. The findings from quantification and phytochemical screening showed the presence of alkaloids, flavonoids, Phenols, proteins, amino acids, saponins, tannins, terpenoids and glycosides. Further, the study findings revealed that ethanolic extract of fruits extract was found to more competent to isolate phytocomponents from plant. A Microplate based Alamar blue assay has been applied to search for novel antimicrobials to treat tuberculosis. The selective ethanolic phytochemicals from fruits of Pimenta dioica confirms the presence antitubercular potential.

Keywords: Pimenta dioica, antitubercular activity, Alkaloids, flavonoids

1. Introduction

Nature provides many things for the well-being of humankind over the years, including the tools for the first attempts at therapeutic intervention. In ancient times, people rely on plants for the treatment of various ailments. Today, plant derived materials remain an important resource for combating illnesses, including infectious diseases and many of these plants have been investigated for novel drugs or used as templates for the development of new therapeutic agents, food additives, agrochemicals and industrial chemicals.[1] The photochemical is natural bioactive compound(s) found in plants which as act as a defence system against diseases.

Based on the functions in plant metabolism, phytochemicals are two kinds viz., primary and secondary constituents. Among the 2, 50,000 - 5, 00,000 plant species in the world, only a small percentage of phytochemicals has been investigated.[2]

Phytochemical analysis of plant products took a distinct place in plant biochemistry as well as organic chemistry in recent years.

It is concerned not only the variety of organic substances accumulated by plants but also helps to determine chemical structure, distribution and biological function of plant substances. Thus, advances in our understanding of phytochemistry are directly related to the successful exploitation of known techniques, and the continuing development of new techniques to solve outstanding problems as they appear. One of the challenges of phytochemistry is to carry out all the above operations in small amounts of material. Frequently, the solution of a biological problem in, say, plant growth regulation, in the biochemistry of plant animal interactions, or in understanding the origin of fossil plants depends on identifying a range of complex chemical structures which may only be available for study in microgram amounts.

Therefore, the systematic screening of plant species with the purpose of discovering new bioactive compounds can help us to cure many fungal and bacterial diseases of economically important crops and animals including human being. The value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds. These are superior to synthetic pesticides in a number of ways like low mammalian toxicity, target specificity and biodegradability. [3]

Botanical name of all spice is Pimenta dioica (L.) Merr. and it belongs to family Myrtaceae. A native plant from the Caribbean island Jamaica, so it called as Jamaica pepper. In India, it grows in Maharashtra, Karnataka, Tamil Nadu and Kerala. The typical evergreen, tropical tree, grown height of 22-35 ft. with light grey bark and dark green leaves (4–8 cm long). The dried berries range in size (6.5-9.5 mm in diameter) and there is 13-14 gram. in weight. Small whitish flowers grow on the allspice

tree in the summer that produces the berries. The berries are picked while still green and dried in the sun. It contains a higher level of essential oils, which give it more flavours. Commonly it also known as Allspice due its taste and aroma remind many of us the mixture of cloves, cinnamon, ginger and nutmeg. [4] The various parts like leaves, fruits, stem and roots of Pimenta dioica plant discovered after systematic investigation containing phytocomponents like terpenoids, glycosides, steroids, alkaloids, tannins, saponins and polyphenols are useful in management of various infectious and non-infectious diseases. [5-7]

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. 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.

Selected plant parts with their application against disease manifestation vary from person to person. Hence, in the present study it was aimed to screen the potential phytoconstituents of Pimenta dioica fruit.

2. Materials And Methods

2.1 Plant Material Collections and Drying

Fresh matured fruits of plant identified and collected in month of September from Pune district (Maharashtra, India). Dried in the shade at normal room temperature. Crude drug powder stored in sealed bottles away from light and humidity until use for extract preparation.

2.2 Authentication

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

2.3 Crude Extract preparation

100 gram of dry powdered fruit materials were subjected to successive organic solvent extraction by refluxing in the Soxhlet apparatus each for 12hours. Solvents of graded polarities like Hexane, chloroform, ethyl acetate, ethanol and distilled water selected for extraction process.[8, 9] Boiling temperature of solvent and all optimum laboratory requirements are maintained during extraction process. The concentrated dried extract were labelled and stored in sterile containers in the refrigerator until further analysis.

2.4 Phyto-chemical Screening

The concentrated dried extracts of Pimenta dioica fruits were observed for their physical characristics and percent yield. Further, all extracts were evaluated for preliminary qualitative test for primary and secondary phytochemicals as per standard procedures. [9-12]

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

2.5 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 mg of the extract of the sample was weighed accurately and dissolved in 100 ml of distilled water. 1 ml of this solution was transferred to a test tube, then 0.5 ml 2N of the Folin-Ciocalteu reagent and 1.5 ml 20% of Na2CO3 solution was added and ultimately the volume was made up to 8 ml with distilled water followed by vigorous shaking and finally allowed to stand for 2 hours. The absorbance was taken at 765 nm. These data were used to estimate the total phenolic content using a standard calibration curve obtained from various diluted concentrations of gallic acid. The absorption of standard gallic acid solution (0.5 mg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates. [13]

Determination of total flavonoids

The method is based on the formation of the flavonoids - aluminium complex that has an absorptivity maximum at 415nm. 100µl of the sample extracts in methanol (10 mg/ml) was mixed with 100 µl of 20 % aluminium trichloride in methanol. A drop of acetic acid added and then diluted with methanol to 5ml. After 40 minutes the absorption was measured at 415 nm. Blank samples were prepared from 100 ml of sample extracts and a drop of acetic acid and then diluted to 5ml with methanol. The absorption of standard quercetin solution (0.5 mg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates. [14]

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. [14, 15]

Determination of total tannins

A 0.5 ml of test extract is added with 3.75 ml of distilled water and added 0.25 ml of Folin Phenol reagent, 0.5 ml of 35% sodium carbonate solution. The absorbance of above mixture was measured at 725nm. Tannic acid dilutions (0 to 0.5mg/ml) were used as standard solutions. The results of tannins are expressed in terms of tannic acid in mg/ml of extract. [15, 16]

Determination of total glycosides

The extract (1gm) was macerated with 50 ml of distilled water and filtered. To the filtrate (1 ml), 4 ml of alkaline pirate solution was added. The mixture was boiled for 5 min and allowed to cool. The absorbance was read at 490 nm. [17, 18]

Test for Terpenoids

The 1gm test extract was macerated with 50 ml of ethanol and filtered. 2.5ml filtrate mixed with 2.5 ml of 5% aqueous phosphomolybdic acid solution and 2.5 ml of concentrated H2SO4. Allow to stand for 30min. and makeup 12 ml volume with ethanol. The absorbance was taken at 700 nm. [19, 20]

Test for Steroids

The 1gm test extract was macerated with 20 ml of ethanol and filtered. To the filtrate (2 ml), 2 ml of chromagen solution was added and the solution left to stand for 30 min. The absorbance was taken at 550 nm. The difference between the intensity of colours or absorbance of the test and blank samples and is proportional to the concentration of the specific phytocomponents present in test extract observed. The all above quantitative data results are expressed as mg /gm. of dried sample. [21, 22]

2.6 In-vitro Anti-Tubercular activity: Microplate Alamar Blue Assay (MABA)

Microplate Alamar Blue Assay- The antitubercular activity of Pimenta dioica Linn. fruit 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 fruit showed presence of different phytocomponents which are fractionated by column chromatography in isocratic manner. [23] 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 ºC 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 colour 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. [24-27]

3. Results And Discussion

3.1 Phyto-chemical screening test

The details phytochemical analysis of Pimenta dioica fruits extracts have been analysed in this study, following observations was reported. Table No. 1 mentioned details of characteristics of all extract. The data reveals that chloroform, ethyl acetate and ethanol extract showed more percentage yield.

The Variety of extract showed positive result for different phytochemicals. The polarity gradient solvent selection showed better separation in different complex metabolites in Pimenta dioica fruits reported in Table No. 2.

In Pimenta dioica Linn. Fruits hexane extracts – steroids, chloroform extracts – Saponins, Steroids, carbohydrates, glycosides, saponins, ethyl acetate extract – acidic compounds, glycosides, alkaloids, Phenolic compounds as well as flavonoids., Ethanol extract - glycosides, alkaloids, Phenoilic compounds as well as flavonoids, Aqueous extracts- proteins and saponins were positively present. Table no.3 mentioned details of elemental analysis observed in fruit.

Table 1: Characteristics of Plant extracts

Extract particulars	*Percent Yield (%W/W)*	*Characteristics*	
		Colour	*Appearance/ Consistency*
Hexane extract (PFH)	05.71%	Green	Solid
Chloroform extract (PFC)	12.11%	Brown	Solid
Ethyl acetate extract (PFEA)	18.28%	Dark brown	Semisolid
Ethanol extract (PFET)	18.65%	Yellowish brown	Solid
Aqueous (Water) extract (PFA)	10.66%	Black	Liquid

Table 2: Qualitative analysis of Pimenta dioica Linn. Fruits extract

Sr.No.	*Tests*	*PFH*	*PFC*	*PFEA*	*PFE*	*PFA*
1.	Tests for Acidic compounds	-	-	-	+	-
2	Test for carbohydrate					
	Molish's test	-	-	-	+	-
	Fehling test	+	-	+	-	-
	Benedicts test	-	-	+	+	-
	Barfoed test	-	+	-	+	-
	Selivanoffs test	-	+	+	-	-
	Osazone formation test	-	-	+	-	-
3	Test for Proteins					

Sr.No.	*Tests*	*PFH*	*PFC*	*PFEA*	*PFE*	*PFA*
	Biuret Test	-	-	-	+	+
	Millons Test	-	-	-	-	+
4	Test for amino acids					
	Ninhydrine test	-	+	-	+	+
	Test for Steroids					
	Salkowski test	-	+	-	-	-
5	Libermann test	-	-	-	+	-
	Libermann-Burchard reaction	-	+	-	-	-
	Test for Glycosides					
	Anthraquinone glycoside test	-	+	+	+	-
6	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% FeCl3 test	-	-	+	+	-
	Lead acetate solution	-	-	+	+	-
	Test for Flavonoids					
11	Shinoda test	-	-	+	+	-
	Sulphuric acid test	-	-	+	+	-

Abbreviations: HE- Hexane; CH- Chloroform; EA-Ethyl Acetate; EO; Ethanol; AQ; Aqueous

Note: (-):Absent, (+): Presence

Table 3: Elemental Composition

Sr. no.	*Minerals*	*Composition (mg/100g)*
1	Magnesium	2.51 mg
2	Zinc	6.23 mg
3	Selenium	1.01 mg
4	Iron	815 mg
5	Manganese	4.22 mg

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

In quantitative analysis of all tests extracts of Pimenta dioica fruits exhibited positive results for seven phytochemicals, such as phenol, flavonoids, alkaloids, tannin, glycosides, terpenoids and steroids were screened in the extract by quantitative means as per methods reported in literature and the results have been reported in Table 4. and Figure 1. Although, among these compounds alkaloids, phenolic compounds, flavonoids and glycosides are important secondary metabolites and are responsible principles for medicinal values of the respective plant. Furthermore, the extract was subjected to further analytical tests for the

quantification of phytochemical compounds.

The highest concentration of phenols, alkaloids and flavonoids observed in ethanol extract. The highest concentration of terpenoids and steroids observed in chloroform extract. It reveals that specified plant metabolites are separated in selected solvents.

Table 4: Quantitative Analysis of Pimenta dioica Linn. Fruits extracts

Extracts/ Test	*Phytochemical Mean ± STD*						
	Phenols	*Flavonoids*	*Alkaloids*	*Tannins*	*Glycosides*	*Terpenoids*	*Steroids*
Pimenta dioica Linn. Fruits extracts							
Hexane extract (PFH)	0.06 ± 0.020	-	0.16 ± 0.021	0.06 ± 0.003	0.80 ± 0.020	0.16 ± 0.033	0.44 ± 0.003
Chloroform extract (PFCH)	0.94 ± 0.020	0.31 ± 0.012	0.82 ± 0.020	1.34 ± 0.012	4.31 ± 0.052	6.44 ± 0.038	8.41 ± 0.062
Ethyl acetate extract (PFEA)	2.08 ± 0.003	4.68 ± 0.011	3.77 ± 0.071	0.53 ± 0.013	4.48 ± 0.043	-	0.18 ± 0.001
Ethanol extract (PFET)	5.44 ± 0.055	7.45 ± 0.075	6.45 ± 0.075	2.87 ± 0.085	2.43 ± 0.035	3.47 ± 0.055	-
Aqueous (Water) extract (PFAQ)	1.81 ± 0.083	0.06 ± 0.063	-	0.01 ± 0.003	0.55 ± 0.003	0.46 ± 0.014	-

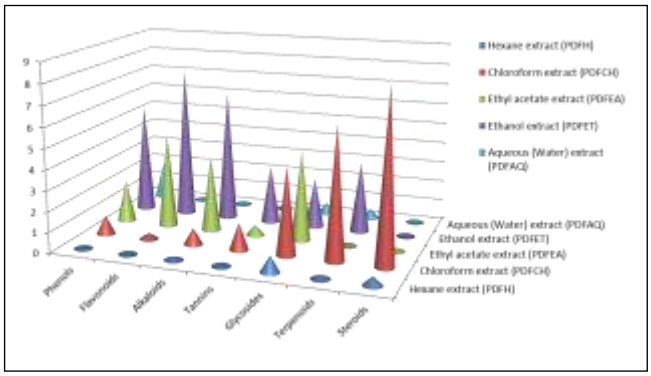

Figure 1: Quantitative Analysis of Pimenta dioica Fruits extract (Concentration vs Test compound in extract)

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

The test fractions of ethanol extracts of Pimenta dioica were fruits shown better potential and prevent mycobacterial growth. Observation in figure 2. Test fraction 6 and 7 showed better inhibition and MIC at 3.2 and 6.25 µg/ml respectively.

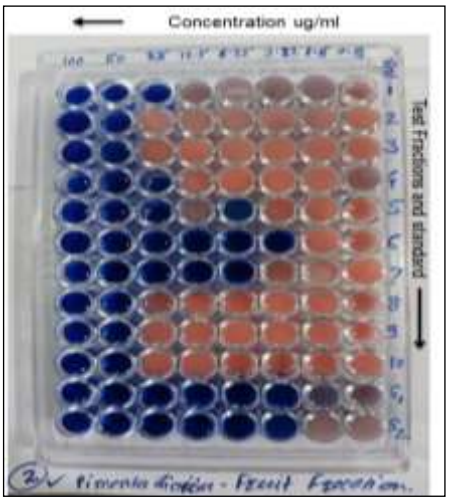

Figure 2: Observation for Microplate Alamar Blue Assay

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 6 and 7 showed inhibition 3.2 and 6.25 µg/ml respectively mentioned in table 6 and figure 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 µg/ml*							
		100	*50*	*25*	*12.5*	*6.25*	*3.12*	*1.60*	*0.8*
	Fraction 1	S	S	S	R	R	R	R	R
	Fraction 2	S	S	R	R	R	R	R	R
	Fraction 3	S	S	R	R	R	R	R	R
	Fraction 4	S	S	S	R	R	R	R	R
	Fraction 5	S	S	S	R	R	R	R	R
	Fraction 6	S	S	S	S	S	S	R	R
	Fraction 7	S	S	S	S	S	R	R	R
	Fraction 8	S	S	R	R	R	R	R	R
	Fraction 9	S	S	R	R	R	R	R	R
	Fraction 10	S	S	R	R	R	R	R	R
	Std. 1: Isoniazid	S	S	S	S	S	S	R	R
	Std. 2: Rifampicin	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 µg/ml*	*Sr. No.*	*Samples*	*MIC µg/ml*
	Fraction 1	25	7	Fraction 7	6.25
	Fraction 2	50	8	Fraction 8	50
	Fraction 3	50	9	Fraction 9	50
	Fraction 4	25	10	Fraction 10	50
	Fraction 5	25	11	Std. 1: Isoniazid	3.12
	Fraction 6	3.12	12	Std. 2: Rifampicin	3.12

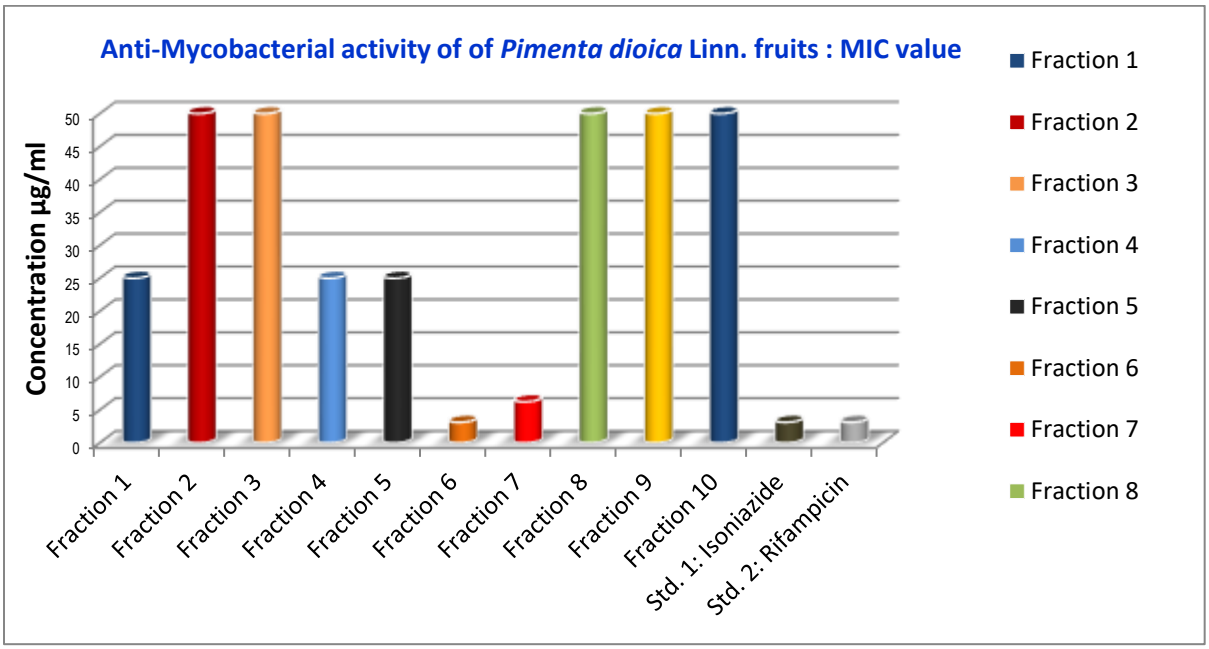

Figure 2: Graphical Representation of Minimum Inhibitory concentration of all fractions.

4. Conclusion

All-successive solvent extracts of Pimenta dioica fruits screened for different varieties of phytochemicals. In qualitative analysis primary metabolites like carbohydrates, proteins, fats and secondary metabolites like alkaloids, flavonoids, phenols, alkaloids, tannins, terpenoids and glycosides were showed positive test results. As the plant fruits have, variety of components may involve in biosynthesis which result formation of higher derivatives. In quantitative estimation phytocomponents was found to be in abundant such as alkaloids, flavonoids, phenols, alkaloids, tannins, terpenoids and glycosides. Especially it was found in high amount in chloroform and ethanol extract than other three extracts of Pimenta dioica fruits. Alkaloids are the most significant compounds play a metabolic role in the living systems and are involved in the protective function in animals. Flavonoids have been used against the cancer causing tumors and it inhibits the promotion of growth and progression of tumors.28 Phenolic and flavonoid proven their antioxidant potential in many more disease manifestation. Phenols when mixed with the flavonoid compounds in plants are reported to show multiple activities like antioxidant, anticarcinogenic, anti-inflammatory, etc.22 Tannins inhibit the pathogenic fungi and antimicrobial activity of extracts showed better activity by the presence of tannins.29

According to the findings of the study ethanol extracts fraction number 6 and 7 of Pimenta dioica fruit 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 phytocomponents of Pimenta dioica serve as potential antimycobacterial agents in the field of pharmaceutical as well as LED developments.

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