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Research Article

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Phytochemical potential and *in vitro* antimicrobial activity of *Piper betle* Linn. leaf extracts

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ABSTRACT

The present investigation was undertaken to find out the phytochemical profile of aqueous, ethanolic, methanolic, butanolic and acetone extract from the leaves of Piper betle and to determine in vitro antimicrobial activity of Piper betle leaves against various microorganisms. The phytochemical screening reveals that, the aqueous, ethanolic and butanolic extracts contains valuable phytochemicals like steroids, diterpenes, tannin, flavonoids etc. and found rich in phytochemicals. Comparatively the aqueous extract yields more secondary metabolites qualitatively and quantitatively among all. The butanolic extracts shown higher inhibition zone against all the bacteria except the fungus and found more effective and efficient among the extracts prepared in various solvent systems although aqueous extract yielded more phytochemicals. All the extracts were fails to show zone of inhibition against A. niger. Thus, these results confirms the presence of active antibacterial compounds in the leaves of Piper betle, might be used as a source of antibiotics for the treatment of various respiratory diseases and the diseases caused by various bacteria.

Keywords: *Piper betle* Linn, Phytochemicals, *in vitro* Antimicrobial activity.

INTRODUCTION

Piper betle Linn is an evergreen, perennial, a Vedic plant with its Vedic name is Saptasira[1] and in sankrit it is known as Tambool, Nagvelleri, Nagani[2] and it belongs to the family Piperaceae; commonly known as “Paan”. It is extensively grown in India, Srilanka, Thailand, Taiwan and other Southeast Asian countries. The parts of *Piper betle* like leaves, roots, stems, stalks and fruits are utilized for various purposes. The plant has large number of bio-molecules which show various pharmacological activities. The leaves of *Piper betle* possess antitumor, antimutagenic and antihelminthic activities[3]. The study by Bhalerao *et al.*[4] reveals it to be a good for its antimicrobial activity, protective and healing activity, antidiabetic activity, gastro-protective activity, immunomodulatory activity, Antioxidant activity, platelet inhibition activity, antifertility activity, hepato-protective activity, antiphotosensitizer, cytotoxicity / Anticancer Potential , radio-protective activity etc. In Ayurveda its leaf extract is frequently used as an adjuvant; mixed with different medicines possibly for better effects beside its independent use as a medicine.

The current study has been carried to find out the phytochemical profile of aqueous, ethanolic, methanolic, butanolic and acetone extract of the leaves of *Piper betle* and to determine *in vitro* antimicrobial activity of *Piper betle* leaves against various microorganisms.

EXPERIMENTAL SECTION

Collection of the Plant material

The fresh leaves of *Piper betle* were used in the present study are brought from the local market of Gadhinglaj, Kolhapur district, Maharashtra, India during the month January 2015. Leaves were washed with distilled water to remove the dirt and other particles. Then the leaves were crushed and dried in shaded area at room temperature (25°C) for a period of a week. Then the dried leaves were grinded by using ordinary grinder and then sieved through sever.

Identification and source detail

The leaves of *Piper betle* Linn were authenticated by Associate Professor Mr. R. S. Sawant, Head, Department of Botany, Dr. Ghali College, Gadhinglaj, Kolhapur district, Maharashtra, India.

Preparation of Test extract

Various solvents like water, ethanol, methanol, butanol and acetone were used for extract preparation. The respective extracts of leaves of *Piper betle* were prepared by addition of 0.5 gm and 1.0 gm of powder into 10 ml of respective solvents to obtain the concentration 5 % and 10 % respectively and kept at room temperature for overnight. Sample further used after centrifugation at 5000 rpm for 10 minutes.

Phytochemical Screening

Phytochemical analysis of *Piper betle* Linn leaves were carried out for the aqueous, ethanolic, methanolic, butanolic and acetone extracts to evaluate the presence of secondary metabolites like steroids, saponins, flavonoids, phytosterols, phenolic compound, tannins, etc. (Table 1) by using various standard methods[5];6];[7] with slight modifications.

Test for Steroids

1 ml of test extract was added in 10 ml of chloroform and equal quantity of conc. H_2SO_4 was added in test tube from side. The upper layer turns in red colour while H_2SO_4 layer shows yellow colour with green fluorescence, which shows the presence of steroids.

Test for Diterpenes: Copper acetate test

Test extract was dissolved in distilled water and treated with 8-10 drops of copper acetate solution. Formation of emerald green colour indicates presence of the diterpenes.

Test for Phlobatannins

Aqueous extract of sample is boiled with 1% aqueous HCl, the deposition of red ppt taken as evidence for presence of Phlobatannins.

Test for Tannin:-

Lead acetate test: 2 ml test extract was added to 1% lead acetate and observed for yellowish precipitate which indicates the presence of tannin.

$FeCl_3$ test: 4 ml test extract was treated with 4 ml of $FeCl_3$. Formation of green colour indicates presence of condensed tannin.

Test for Cardial Glycosides: Keller-Killani Test

2 ml glacial acetic acid with a drop of $FeCl_3$ used to treat the extract. The formation of brown colour ring indicates the presence of cardial glycosides.

Test for Flavonoid:-

Alkaline reagent test: Test extract was treated with 10 % NaOH solution; formation of the intense yellow colour evidence for presence of flavonoid.

NH_4OH test: 10 % NH_4OH was used to treat the 3 ml of test extract, development of yellow fluorescence indicates positive test.

Mg turning test: Test extract was treated with Mg followed by few drops of conc. HCl and finally 5 ml of 95 % ethanol was added. Formation of crimson red colour indicates presence of flavonoid.

Zinc dust test: 2 ml test extract were treated with Zn dust followed by few drops of conc. HCl, the development of red colour indicates positive test.

Test for Anthocyanin
2 ml of aqueous test extract was added to 2 ml of 2 N HCl and NH_3, the appearance of pink red colour turns to blue violet taken as an evidence for presence of anthocyanin.

Test for Phytosterol: Salkowskis test
Test extract was treated with chloroform and then filtered. The filtrate was treated with few drops of conc. H_2SO_4, shacked well, allowed for stand, the appearance of golden red colour indicates positive test.

Test for Alkaloids:
Wagner's reagent test: Filtrate (1 ml HCl was added into a test tube containing 3 ml conc. test extract. The mixture was heated gently for 20 minutes and filtered after cooling) was treated with Wagner's reagent, the formation of reddish precipitate shows presence of alkaloids.

Hager's test: The filtrate was treated with Hager's reagent; formation of yellow precipitate shows presence of alkaloids.
Test for Phenol: Ferric Chloride test
Test extract treated with 4-5 drops of Alcoholic $FeCl_3$ solution; formation of the bluish black colour indicates positive test for Phenol.

Test for Emodins
2 ml of NH_4OH and 3 ml of benzene was added to the test extract. Appearance of the red colour indicates emodins.

Test for Coumarin
3 ml of 10% NaOH was added to 2 ml of aqueous test extract, the formation of yellow colour shows presence of coumarin.

Test for Leucoanthocyanin
5 ml of iso-amyl alcohol was added in 5 ml of aqueous test extract. The upper layer appears red in colour which indicates presence of Leuanthocyanin.

Test for Saponin: Foam test
5 ml test extract was mixed with 20 ml of distilled water and agitated in graduated cylinder for 15 minutes. The formation of foam taken as an evidence for saponin.

Test organisms
The standard microorganisms were used for this study is, *Bacillus subtilis* NCIM 2635, *Salmonella typhimurium* NCIM 2501, *Proteus vulgaris* NCIM 2813, *Staphylococcus aureus* NCIM 2654, *Aspergillus niger* NCIM 503.

Preparation of bacterial suspension
Standard loop full suspension of the test organisms were aseptically streaked onto nutrient agar slants and were incubated at 37°C for 24 hours. Then bacterial growth was harvested from the respective slant and suspension was prepared using sterile 1 ml normal saline (0.85 gm NaCl in 100 ml of distilled water). The suspensions were stored in the refrigerator at 4°C until used[7].

Preparation of fungal suspension
The fungal culture was maintained on Potato dextrose agar (PDA), incubated at room temperature for 4-5 days. Then the fungal growth was harvested from the agar medium and suspension was prepared by using sterile 1 ml normal saline, finally stored in the refrigerator until used[7].

***In vitro* Antimicrobial activity**
In vitro testing of extracts for antimicrobial activity in the aqueous, ethanolic, methanolic, butanolic and acetone extracts of leaves of *Piper betle* Linn against various microorganisms (Table 2) was determined by using agar well diffusion method, using Nutrient agar medium for antibacterial activity while Potato dextrose agar (PDA) for antifungal activity[5].

RESULTS AND DISCUSSION

The Phytohemical tests of various extracts from *Piper betle* leaves were performed and the results were presented in Table 1. In the phytochemical screening, aqueous extract yielded steroids, diterpenes, tannin, cardial glycosides, flavonoids, saponin, phenols, coumarin and alkaloids. Ethanolic extract contains various phytochemicals like steroids, diterpenes, tannin, flavonoids, saponin and coumarin. The methanolic extract of *Piper betle* leaves was shown presence of steroids, diterpenes, tannin, and saponin. Butanolic extract contains steroids, diterpenes, tannin, flavonoids, emodins and alkaloids while the acetone extract shown steroids, diterpenes, tannin, flavonoids, saponin and coumarin. Comparatively the aqueous extract yielded more secondary metabolites qualitatively and quantitatively among all. Flavonoids shows anti-inflamatory, vascular activities, antioxidant, antimicrobial as well as other medicinal properties[8]. Several reports are available in the literature on the antimicrobial activity of flavonoids[9];[10];[11]. According to Harborne[12] tannin may be toxic to bacteria, yeast and filamentous fungi. Tannin also shows potential antiviral[13] as well as antibacterial activity[14];[15].

The results of *in vitro* antimicrobial activity of *Piper betle* leaves were represented in Table 2. The results (Table 2) revealed that the butanol extracted *Piper betle* leaves with 5 % of 50 µl (Fig 1) was found effective concentration which inhibited the growth of *Salmonella typhimurium, Staphylococcus aureus,* and *Bacillus cereus* rather 10 % of 50 µl concentration (Fig 3) was found effective against *Proteus vulgaris.* The butanolic extracts shown higher inhibition zone against all the bacteria except the fungus and found more effective and efficient among the extracts prepared in various solvent systems (Fig 1, Fig 2, Fig 3 and Fig 4). Even though aqueous extract yields more phytochemicals, the efficiency of antimicrobial activity was not noted up to mark. The aqueous and acetone extract of *Piper betle* leaves might be not effective against *Proteus vulgaris.* All the extracts were fails to shown zone of inhibition against *A. niger* (Fig 1 to 4). *Piper betle* leaves might be used as a repellent. The results obtained may support the use of *Piper betle* leaves in the traditional medicine for treatment of various respiratory diseases, the diseases like Pneumonia, Carbuncles, Boils, etc.; May be used in healing of wounds.

Table 1: Phytochemicals of *Piper betle* Linn leaf extracts

Sr. No.	Phytochemical		Results				
			Aqueous extract	Ethanolic extract	Methanolic extract	Butanolic extract	Acetone extract
1	Steroids		+++	+	++	+++	+
2	Diterpenes: Copper acetate test		++	+	+	+++	+
3	Phlobatannins		-	-	-	-	-
4	Tannin:	Lead acetate test	+++	+	++	-	++
		$FeCl_3$	-	+	++	+	++
5	Cardial Glycosides: Keller-Killani test		+	-	-	-	-
6	Flavonoid:	Alkaline Reagent Test	++	+	-	+	++
		NH_4OH	++	+	-	+	-
		Mg turning test	-	-	-	-	-
		Zn dust test	+	-	-	-	-
7	Anthocyanin		-	-	-	-	-
8	Phytosterol: Salkowski's test		-	-	-	-	-
9	Alkaloids	Wagner's reagent	+	-	-	++	-
		Hager's reagent	++	-	-	+++	-
10	Phenols: $FeCl_3$ test		+	-	-	-	-
11	Emodins		-	-	-	+	-
12	Coumarin		+++	+	-	-	++
13	Leucoanthocyanin		-	-	-	-	-
14	Saponin: Foam test		+++	+	+	-	+

Key: (+) Positive test, (-) Negative test, '+' low; '++' moderate; '+++' high

Table 2: *In vitro* Antimicrobial activity of *Piper betle* Linn leaf extracts

Organism used	Zone of inhibition (mm)							
	Aqueous extract				Ethanolic extract			
	5 %		10 %		5 %		10 %	
	50µl	100µl	50µl	100µl	50µl	100µl	50µl	100µl
Bacillus subtilis NCIM 2635	-	14±1.00	-	-	-	17.6±0.57	12±1.00	15.6±0.57
Salmonella typhimurium NCIM 2501	14.6±0.57	-	-	-	-	-	-	-
Proteus vulgaris NCIM 2813	-	-	-	-	-	16.0±1.00	-	19±1.00
Staphylococcus aureus NCIM 2654	18.3±1.52	20.3±0.57	17±1.00	19.3±0.57	12.6±0.57	18±1.00	19.6±0.57	20±1.00
Aspergillus niger NCIM 503	-	-	-	-	-	-	-	-

Organism used	Zone of inhibition (mm)							
	Methanolic extract				Butanolic extract			
	5 %		10 %		5 %		10 %	
	50µl	100µl	50µl	100µl	50µl	100µl	50µl	100µl
Bacillus subtilis NCIM 2635	-	14.0±1.00	-	-	25.0±1.00	32.6±0.57	16.0±1.00	25.6±0.57
Salmonella typhimurium NCIM 2501	13.6±0.57	15.3±1.52	-	15.6±0.57	28.6±0.57	31.0±1.15	20.0±1.00	30.0±0.00
Proteus vulgaris NCIM 2813	14.0±1.00	19.0±1.00	14.0±0.00	18.3±0.57	-	-	12.0±1.00	12.0±1.00
Staphylococcus aureus NCIM 2654	15.3±1.52	16.3±0.57	13.0±1.00	20.3±0.57	18.6±0.57	30.0±1.00	22.6±0.57	25.0±1.00
Aspergillus niger NCIM 503	-	-	-	-	-	-	-	-

Organism used	Zone of inhibition (mm)			
	Acetone extract			
	5 %		10 %	
	50µl	100µl	50µl	100µl
Bacillus subtilis NCIM 2635	-	12.6±0.57	11.6±0.57	15.0±0.57
Salmonella typhimurium NCIM 2501	-	-	-	18.0±0.00
Proteus vulgaris NCIM 2813	-	-	-	-
Staphylococcus aureus NCIM 2654	15.3±0.57	15.0±1.00	-	18.0±1.00
Aspergillus niger NCIM 503	-	-	-	-

Note: Each value is the mean of three readings ±SD.

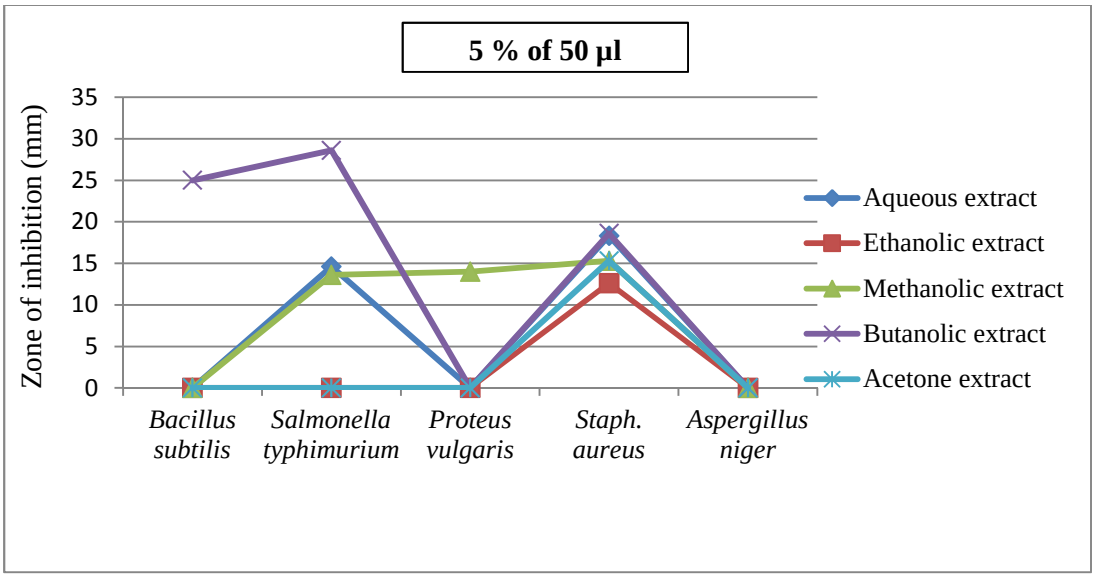

Figure 1: Effect of 5% (50 µl) *Piper betle* Linn leaf extracts against test organisms

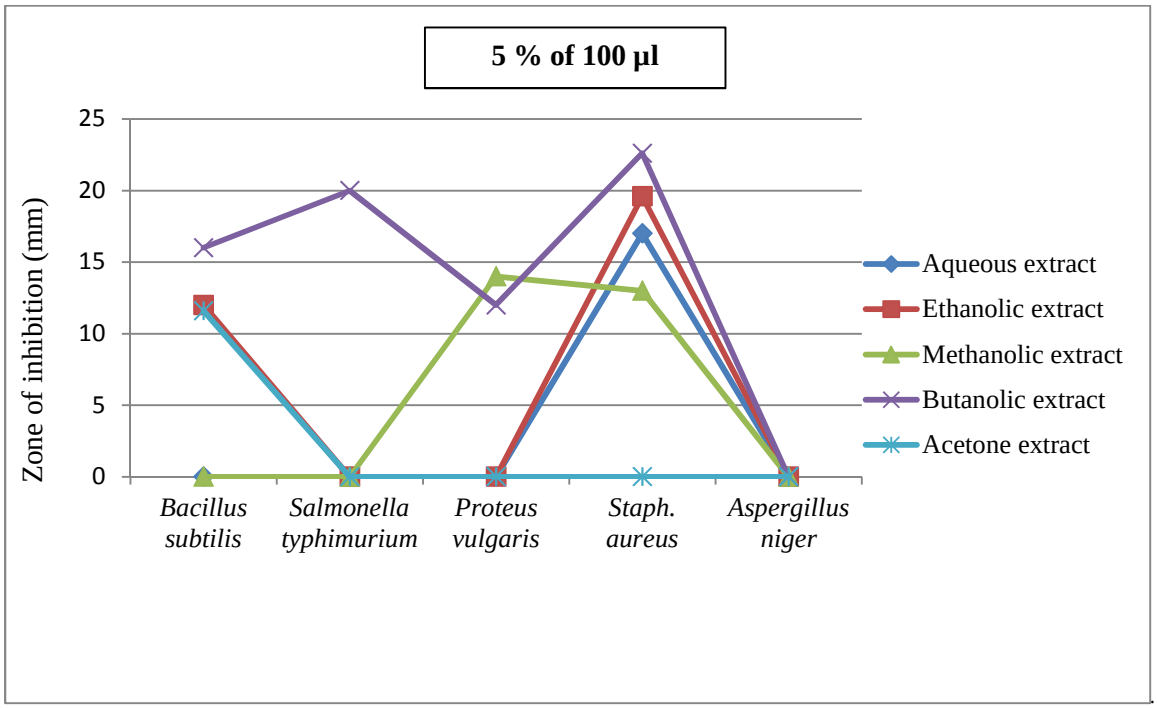

Figure 2: Effect of 5% (100 µl) *Piper betle* Linn leaf extracts against test organisms

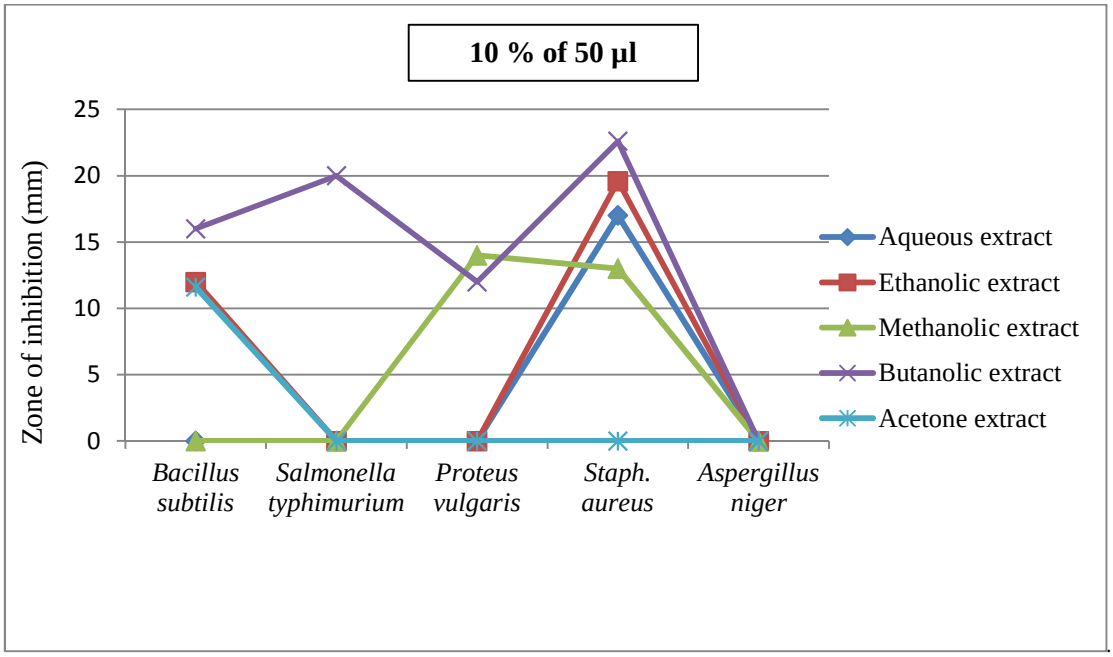

Figure 3: Effect of 10% (50 µl) *Piper betle* Linn leaf extracts against test organisms

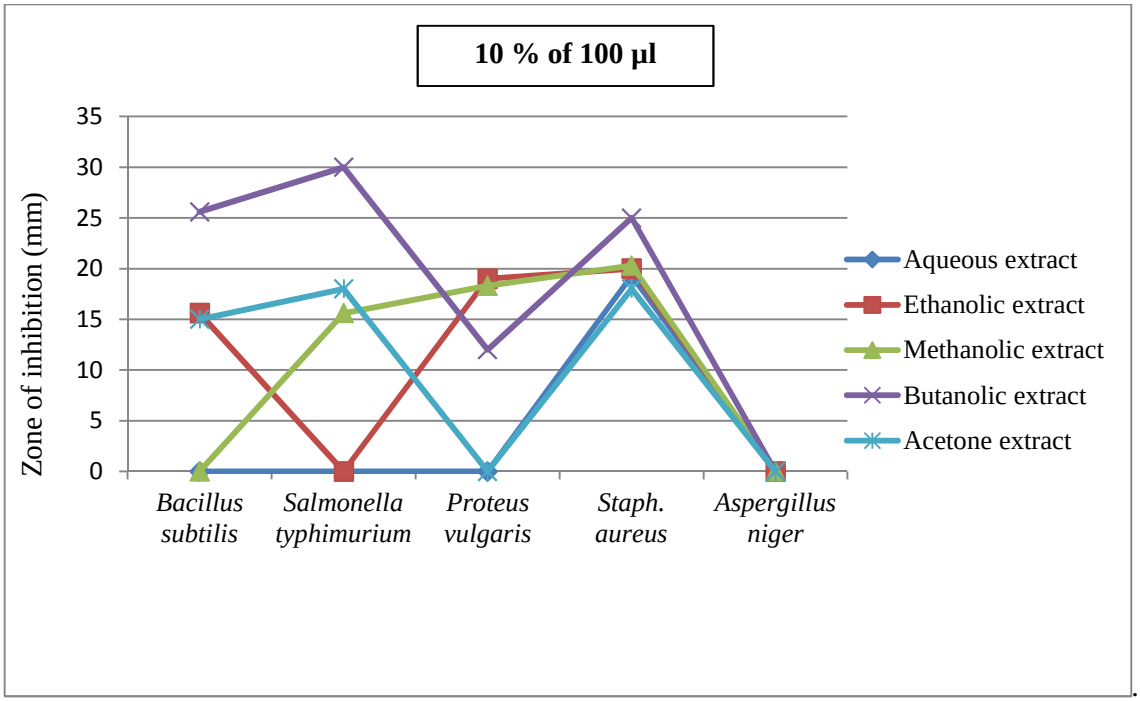

Figure 4: Effect of 10% (100 µl) *Piper betle* Linn leaf extracts against test organisms

CONCLUSION

Present investigation concludes that leaves of *Piper betle* are the rich source of various phytochemicals. Qualitatively and quantitatively aqueous extract yields more phytochemicals than the other extracts. All the extracts were shown antibacterial properties but comparatively the butanolic, acetone and methanolic extract were found more effective. The leaves extract of *Piper betle* from all the solvent system may be used as a source of antibiotics, more specifically butanolic. The study may contribute its use for the best oral hygiene to oral cavity and to help in proving it to be a promising source in pharmaceutical as well as neutraceutical industry.

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