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ASSESMENT OF PHYTOCONSTITUENTS AND IN-VITRO ANTIMICROBIAL SCREENING OF *PIPER BETLE (L)* EXTRACTS

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ABSTRACT

Plants contain numerous biologically active compounds, many of which have been shown to have antimicrobial properties. Plant-derived medicines have been part of traditional healthcare in most parts of the world for thousands of years and there is increasing interest in plants as sources of agents to fight microbial diseases. Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents. The present study investigated the presence of phyto constituents from *Piper betle* leaves available in the study area and also screened its antimicrobial properties. Antibacterial activity against various bacterial strains were examined by agar well diffusion method(inhibitory zone). The phytochemical analysis revealed the presence of Carbohydrate, Flavanoids, Saponins, Tannins, Cardiac glycosides etc. The crude aqueous extracts inhibited the growth of bacterial strains with high susceptibility. Activity indices were also determined and this study will support the local use of the leaf of the plant, *Piper betle* for prophylactic and therapeutic purposes against bacterial infection. The antibacterial properties and phytochemical profiling of betel leaves firmly support their application in the development of various products, especially in the food and pharmaceutical industries.

Key words : piper betle, antibacterial activity, activity indices etc.

INTRODUCTION

Piper betle (local name 'paan') belong to piperaceae, a dioecious, annul creeper, climbing by many small adventitious rootless, grows to a height of about one meter, generally grown in hotter and damper parts of the country. In India they are widely found in damp forests and also cultivated in several states. In Tamilnadu, three varieties of *piper betle* leaves, sirugamani,

karpoori and vetrilaikodi are accessible mostly.(Dwivedi and Tripathi, 2014). In India, 11,000-18,000 species of flowering plants are found of which 6000-7000 are estimated to have medicinal properties. The usage of these medicinal plants is found in many Indian culture and is documented in Indian systems of medicine such as Ayurveda, Unani, and Homeopathy.(In India over 3000 plants has been recognized officially for its medicinal value and it was also estimated that 6000 plants were traditionally used in herbal medicine(Reddy *et al*.,2022). The various parts of the plants like roots, stems, leaves, flowers, fruits were used in traditional therapy in India particularly in southern states.(Saxena *et al*., 2013).

Betel plant(*piper betle)* considered as a kind of conventional medicine were utilized by traditional healers from different remote communities in India claimed that their medicine acquired from these betel leaves was more effective and cheaper than the modern medicine.(Datta *et al* .,2011). Presence of various phyto constituents like alkaloids, flavonoids, saponins, tannins and polyphenols . eugenol, chavibetol, chabetol acetate, camphene, α-pinene, β- pinene, α- limonene, safrole and 1,8- cineol were documented in various literature survey and these phytochemicals were some of the secondary metabolites present in the piper betle leavers(Sushma *et al.*,2020).

Since from the ancient time fresh betel leaves has been wrapped along with areca nut, mineral slaked lime, catechu, flavoring substances, spices and were chewed because of its aromatic stimulo-carminative, astringent and aphrodisiac properties. A kind of phenol namely chavicol a type of aromatic volatile oil present in betel leaves has powerful antiseptic properties and the leaves also contains an alkaloid arakene which resembles cocaine in some aspects.

Pharmacological effects of betel chewing include abundant flow of saliva, temporary dulled of taste perception, stimulation of muscular and mental efficiency.(Balaji *et al.*,2011).

MATERIALS AND METHODS

Collection of plant material

Fresh leaves of Piper betle were collected from the local study area (Villianur, Puducherry, South India) in the month of February 2023 for the current study. Taxonomic characteristics and expert consultation were done to authenticate the plant leaves.

Preparation of plant extract

The extraction of the plant leaves were carried out using ethanol and distilled water as extracting solvents. The cold maceration extraction method was used. 50g of leaf powder was weighed and dissolved in 1000mL of the extracting solvent inside a 2L conical flask and covered with parafilm. The flasks were shaken vigorously at 30 minutes interval and left to stand for 24 hours at room temperature. The resultant mixture was then filtered with Whatman's No.4 filter paper and cotton wool to remove particles of plant sample. The clear solution obtained was distilled at 65°C under low pressure on a steam bath. The semi solid concentrations of the extracts were then collected in sterile pre-weighed screw capped bottles and labeled accordingly (Ogunjobi *et al*., 2007). The extracts were Stored at 4°C until when needed.

Phytochemical analysis

Phytochemical test were done to find the presence of the active chemical constituents such as Carbohydrates, Phenolics Compounds, Steroids, Alkaloids, Proteins, Terpenoids, Flavonoids Cardiac glycosides, Saponins, Tannis, Gums and mucilages, Cardiac glycosides by the following procedure (Siddique and Ali 1997).

Test for carbohydrate

Molisch's test

To 2mL of the extract, 2 drops of Molisch reagent was added and mixed. 2mL of concentrated Sulphuric acid was added to this solution. Formation of the violet ring at the junction of the solution and its disappearance on addition of excess alkaline solution indicates the presence of carbohydrate. (Singh and Kumar 2017).

Benedict's test

Few drops of Benedict's reagent was added to the test solution and boiled on water bath. Formation of reddish precipitate indicates the presence of sugars. Depending on the concentration of the reducing sugars, the amount and color of the precipitate produced varied. A positive Benedict's test appears green, yellow, orange, or red (Singh and Kumar2017).

Fehling's test

To 1mL of the extracts, 1mL of Fehling's A and 1mL of Fehling's B solution were added in a test tube and heated in a water bath for 10 minutes formation of red precipitate indicates the presence of a reducing sugar. The filtrate was treated with a 1mL of Fehling's A and B and heated in a boiling water bath 5- 10 minute. Appearance of reddish orange precipitate shows the presence of carbohydrates (Singh and Kumar 2017).

Test for phenolic compound

Ferric chloride test

A little extracts was dissolved in distilled water. To this, 1mL of 5% Ferric chloride solution was added. Formation of blue, green, or violet color indicates the presence of phenolic compounds. (Audu *et al*., 2007) Lead acetate test A little extracts was dissolved in distilled water. To this, a few drops of lead acetate solution was added. Formation of white precipitate indicates presence of phenolic compounds (Raaman *et al.,* 2006).

Lead acetate test

A little extracts was dissolved in distilled water. To this, a few drops of lead acetate solution was added. Formation of white precipitate indicates presence of phenolic compounds (Raaman *et al.,* 2006)

Dilute iodine solution test

To 2-3mL of extract, a few drops of dilute iodine solution were added. Formation of transient red color indicates the presence of phenolic compounds (Raaman *et al*., 2006)

Test for steroid

Liebermann burchard test

The extracts were dissolved in 2mL of chloroform to which ten drops of acetic acid and 5 drops of concentrated Sulphuric acid were added and mixed. The change of red color from blue to green indicates the presences of steroids (Ciulci, 1994).

Test for alkaloids

Wagner's test

To about 1-2mL of the filtrate, 2mL of Wagner's reagent was added. Reddish brown coloured precipitate indicates the presence of alkaloids (Wagner 1990).

Test for protein

Biuret test

To 3mL of extracts add equal volume of 4% sodium hydroxide and add a few drops of 1% solution CuSo4 to form violet or pink color.(Kumar *et al.*, 2018).

Millon's test

Add 5mL of million's reagent to 3mL extract warm the solution till it boils white precipitates formed where precipitate turns brick red color solution.(Kumar *et al*., 2018)

Test for terpenoids

Salkowski test

5mL of the extract was added to chloroform along with a few drops of concentrated sulphuric acid. The mixture was shaken well and kept aside for some time. Appearance of red color in the lower layer indicates the presence of steroids and the formation of yellow color in the lower layer indicates the presence of terpenoids.(Tiwari *et al*., 2011)

Test for flavonoid

Ammonia test

5mL of dilute ammonia solution was added to a portion of the leaf extracts followed by addition of concentrated sulphuric acid. Formation of a yellow coloration in the extract indicates the presence of flavonoids. The yellow coloration disappears after some time (Kumar *et al*., 2018).

Zinc-hydrochloride test

To the extract, a pinch of zinc dust was added followed by addition of concentrated hydrochloric acid long the sides of the test tubes. Appearance of magenta color indicates the presence of flavonoids.

Alkaline reagent test

The extract was treated with a few drops of sodium hydroxide. Formation of intense yellow color, which becomes color less on addition of few drops of dilute acid, indicates the presence of flavonoids (Gul *et al*.,2017).

Test for amino acid

Ninhydrin test

To two mL of protein solution one mL of 40% NaOH solution and one to two drops of 1% CuSO4 solution was added. A violet color indicates the presence of peptide linkage of the molecule. (Kumar *et al*., 2018)

Cardiac glycosides

Test for cardiac glycosides

To the solution of the extract glacial acetic acid, few drops of 5% ferric chloride and concentrated Sulphuric acid are added, and observed for a reddish brown coloration at the junction of two layers and the bluish green color in the upper layer.

Saponins

Test for foam

To 1mL of extract 2mL of distilled water is added and shaken well. Persistent foam formation indicates presence of Saponins (Kokate 1999)

Tannins

Test for braymer's test

Take 1mL of extract and treat it with 1ml of 10% alcoholic ferric chloride solution and observe for the formation of blue or greenish color (Ciulci, 1994)

Quinones

Test for Quinones

Take 1 mL of extract and add 5 mL distilled water and observe for the turbidity.

Coumarins.

Test for Coumarins

Take 1 mL of extract and add 1.5ml of 10% NaOH then observe for the formation of yellow color which indicates the presence of coumarins.

Anthroquinones

Test for Anthroquinones

About 0.5 g of extract was taken in a dry test tube and 5 mL of chloroform was added and shaken for 5 min. The extract was filtered and the filtrate shaken with equal volume of 10% of ammonia solution. A pink violet or red color in the ammonical layer indicates the presence of anthroquinones.

Gums and mucilages

Test for gums and mucilages

The extract is dissolved in 10mL of distilled water and to this 2mL of absolute alcohol is added with constant stirring. White or cloudy precipitate indicates the presence of Gums and Mucilage (Whistler and BeMiller 1993).

Antimicrobial activity

Selection of Test organisms

In vitro antibacterial activity was evaluated against five bacterial pathogens of clinical origin. Gram positive *Staphylococcus aureus*, Gram negative *Pseudomonas* sp, *Proteus* sp *Escherichia Coli* and *Shigella* sp. were used in this study. All the clinical strains were procured from Microbiology laboratory of Divine Mother College, Puducherry, India.

Inoculum preparation

Bacterial inoculum was prepared by inoculating a loopful of test organisms in 5 mL of Muller Hinton Broth and incubated at 37°C for 3-5 hours till a moderate turbidity was developed. The turbidity was matched with 0.5 Mc Farland standards.

Screening of antibacterial activity of Aqueous Extracts using agar well diffusion method

Immediately after standardization, a sterile cotton swab was immersed in the bacterial suspension and swabbed aseptically on the surface of sterile Mueller-Hinton agar (Hi-media, Mumbai) plates. Wells of 8mm diameter were punched in to the agar medium and filled with 10µL, 50 µL and 100 µL of plant extracts (100mg/ml. water). Well loaded with 100 µL of Levofloxacin (5µg) served as positive control. The plates were incubated at37°C for overnight in a bacteriological incubator. Antimicrobial activity was measured by measuring the zone of inhibition in mm around each well, negotiating the diameter of well and finally activity indices, designated as AI, were calculated as the division of zone of inhibition of the extract by that of the standard drug (Singh *et al.*, 2002).

RESULT AND DISCUSSION

The present phytochemical findings of *piper betle* leaf extracts using Aqueous solvents confirmed the presence of various phytoconstituents(**Table.1**). Carbohydrates were present in the extract and when the *piper betle* leaf consumed after a meal that can influences glucose metabolism \beneficially. Earlier reports also demonstrated leaf suspension of *piper* has significant antioxidant effects in STZ diabetic rats. **(Fig. 1)**. The presence of flavonoids in plants regulates plant development, pigmentation and also protects from UV rays. It also enhances defence and signaling between plants and microorganisms. The presence of flavonoids in human diet may control inflammation and play a significant role in cancer prevention(Ulrik Mathesius.,2018). Foam test showed positive reaction which indicated the presence of sapononins.**(Fig.3).** They are eco-friendly because of their natural origin, biodegradable and non-toxic which is of utmost importance from environmental and health perspectives.. The physiochemical and biological characteristics of Saponin rich plants were the natural sources of surfactants and were utilized for research and commercial purposes. The molecular and physicochemical properties of plant-based natural surfactants, saponins, emphasizing surfactant properties similar to those of conventional surfactants along with their potential applications.(Summi Rai *et al*.,2021).

Presence of tannins in the extracts were confirmed by Braymer's test. **(Fig.4)** Tannins are basically polyphenolic compound which gets attached to proteins, basic compounds, pigments, large-molecular compounds and metallic ions with antioxidant activities, etc. These characteristic

features of tannins lead to qualitative and quantitative analytical distinction between tannins and other polyphenolic compounds.(Takuo and Hideyuki *et al*.,2011).

Prevalence of Cardiac glycosides were noticed during the investigation **(Fig.5)** which are organic compounds comprise of steroid molecule bound to a carbohydrate unit. These glycosides also interferes and influence in heart functioning by acting on cellular sodium-potassium ATPase pump by an increased rate of contractions.(Nidhi Mishra *et al*., 2020).

None of the phytoconstituents like phenolic compounds, steroids, alkaloids, Terpenoids, Coumarins, anthroquiones gums and mucilages, proteins and amino acids were found present in the extract during the investigation

. **Table 1. Phytochemical Constituents Of *Piper Betle***

Sl.No	Phytochemical analysis	Aqueous extracts
1.	**Carbohydrate**	
	Molisch's test	+
	Benedict's test	+
	Fehling's test	+
2.	**Protein**	
	Biuret test	-
	Millon's test	-
3.	**Phenolic compounds**	
	Ferric chloride test	-
	Lead acetate teat	-
	Dilute iodine test	-
4.	**Steroid**	
	Libemann burchard	-
5.	**Alkaloids**	
	Wagner's test	-
6.	**Flavonoids**	
	Ammonia test	+
	Zinc hydrochloride test	+
	Alkaline regent test	+
7.	**Terpenoids**	
	Salkawski test	-
8.	**Amino acid**	
	Nihydrin test	-
9.	**Saponins**	
	Foam's test	+
10.	**Tannis**	
	Broymer's test	+

11.	Quinones	-
12.	Coumarins	-
13.	Anthraquinones	-
14.	Gums and mucilages	-
15.	Cardiac gtycosides	+

Test for Carbohydrates

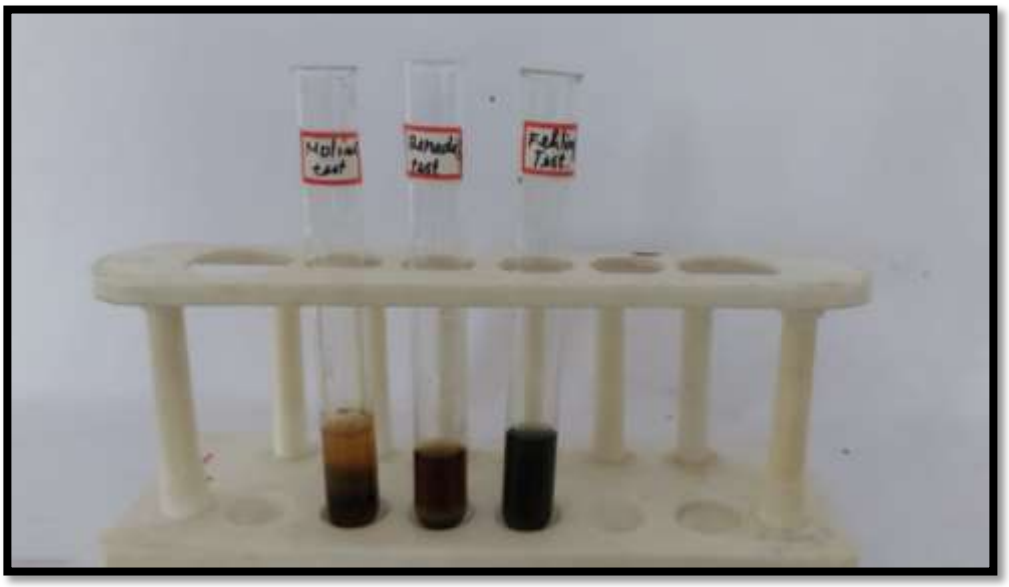


Fig.1. Shows the test for Molisch's, Benedicts and Fehling's test

Test for Flavanoids

Fig.2 Shows the Test for Flavanoids

Test for Saponins

Fig.3 Shows the Test for Saponins

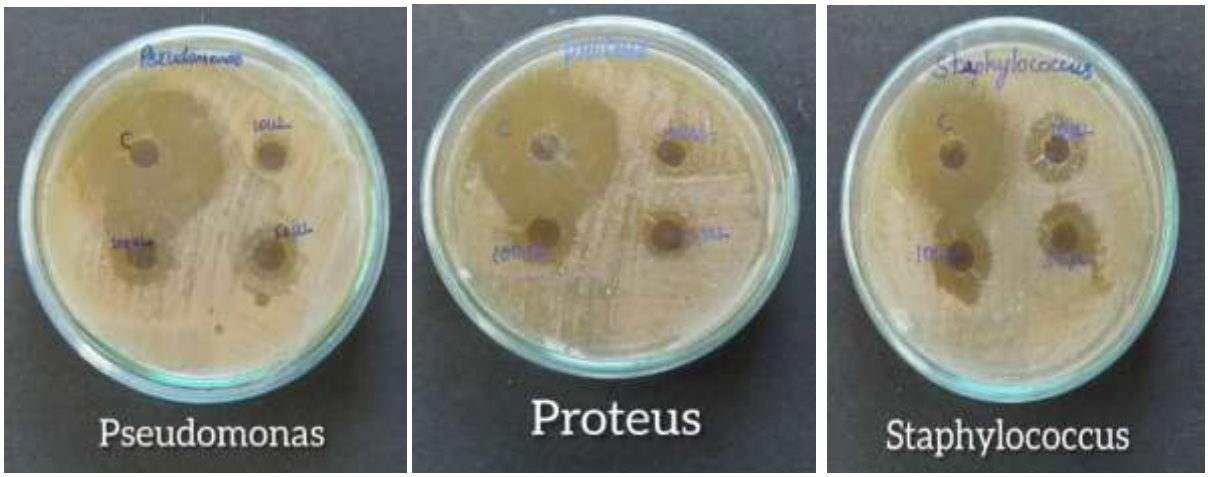
Test for Tannins

Fig.4 Shows the Test for Tannins

Test for Cardiac glycosides

Fig.5 Shows the test for Cardiac glycosides

Agar Well Diffusion Method



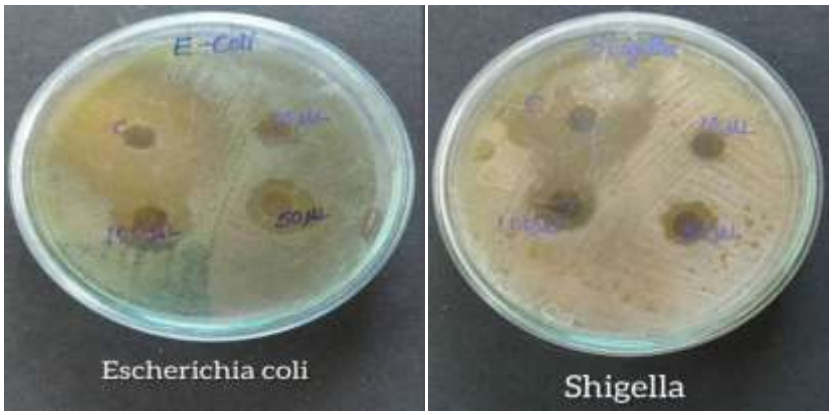


Fig.6. Zone of inhibition

An antimicrobial agent is a substance that kills or inhibits the growth of microorganisms.Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. Plant extracts are being expected towards the target sites than routine antibiotics will be effective to combat drug resistant pathogenic microorganisms(Valle *et al* .,2016). The phytoconstituents which are the biologically active compounds are accountable and are very potent in action against microbial pathogens(Jayarajan *et al*.,2023).

In the present investigation we used aqueous solvent for the extraction of active components from the leaves of *Piper betel* plant. The antibacterial activity of the leaf extracts was assessed using agar well diffusion method by measuring the diameter of growth inhibition zones with 10µL 50µL and 100µL of aqueous extracts (**Table. 2**). The results illustrated that extracts possess antibacterial activity against the tested gram positive (*Staphylococcus aureus*) and gram negative (*Escherichia coli*, *Pseudomonas* sp, *Proteus* sp and *Shigella*sp) bacteria. At 100µL concentration, the extract expressed significant level of inhibition against all the tested organisms, the maximum inhibition zone was observed on *Escherichia coli* (20 mm; A.I=0.58), *Staphylococcus* sp (18mm; A.I=0.75) and moderate inhibition was observed on *Pseudomonas* sp (12 mm; A.I=0.30) *Proteus* sp(10mm; A.I=0.4) and *Shigella* sp (10mm; A.I=0.41) **(Fig.6 &7).** The results from the current study were in agreement with our previous studies in *Amaranthus blitum* (Jayarajan *et al*.,2022) and the same also demonstrated in reports of Adegoke *et al*.,2009 in *Lasienthera africanum* and *Rakshit et al*.,2011 in *Scindapsus officinalis* (Roxb.) Schott. It has been described in various reports that the *Piper betel* plant is biologically very active against various bacterial species such as *Bacillus cereus, Enterococcus faecalis, Listeria monocytogenes, Micrococcus luteus, Staphylococcus aureus, Aeromonas hydrophila, Escherichia coli, Pseudomonas aeruginosa, Salmonella Enteritidis, Streptococcus mutans* (Sharma and Khan,2010).

The leaves of *Piper betel* contains a wide range of secondary metabolites including phenolic compounds (chavicol, hydroxyl chavicol), volatile oils (safrole, eugenol, isoeugenol,

eugenol methyl ester), fatty acids (stearic and palmitic) and hydroxyl fatty acids (stearic, palmitic, myristic) which were proved that it has antibacterial properties and could be preferred as inexpensive and safe antibacterial agent for the treatment of microbial infections.

Table. 2 Antibacterial Activity of *Piper Betle*

Test Organism	Zone of inhibition				
	Control (Levofloxacin) (10 µg)	Aqueous extract			
		10(µL)	50(µL)	100(µL)	Activity index
Staphylococcus sp	24	10	14	18	0.75
Escherichia coli	34	2	16	20	0.58
Pseudomonas sp	33	2	6	10	0.30
Proteus sp	30	4	8	12	0.4
Shigella sp	24	2	8	10	0.41

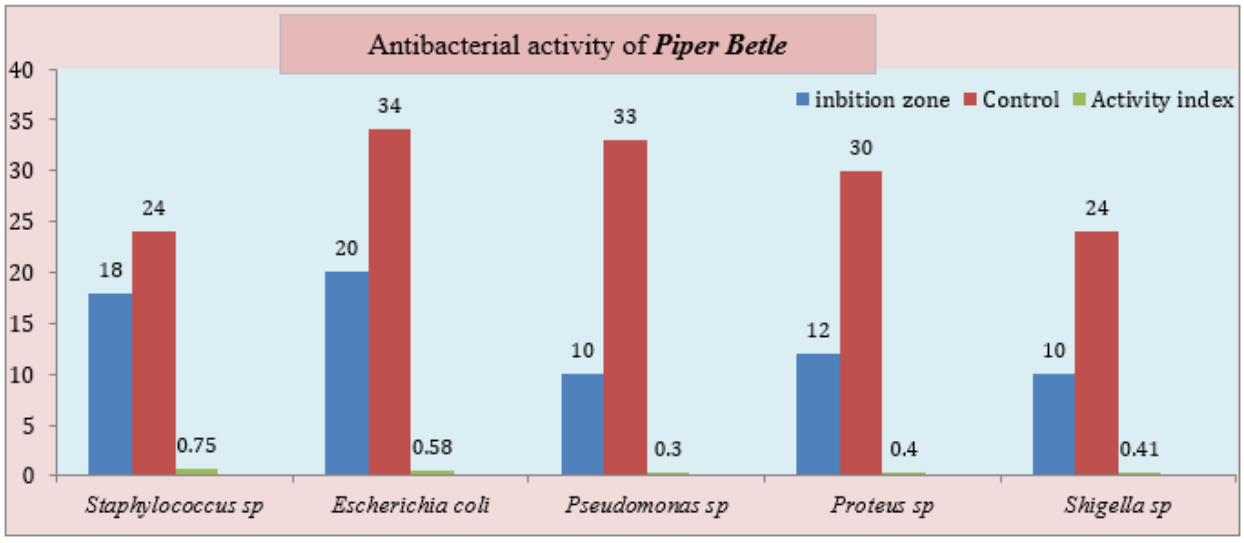


Fig.7. Antibacterial activity and Activity index of *Piper betle* on bacterial species

Piper betel has been conventionally used as compost, carminative, antiseptic agents, antifungal and antibacterial. It has also been reported for the cure of stomach problems, worms and as a general tonic. It is often chewed in combination with the betel nut (Areca catechu), as a stimulatory. Some evidence suggests that betel leaves have immune boosting properties as well as anti-cancer properties (Fathilah *et al.*, 2010).Traditionally *Piper betel* has been utilized as carminative, compost, antiseptic, antibacterial and antifungal agent and also been reported in curing of stomach problems, worms and chewed along with betel nut as a stimulatory as well used as a general tonic. Earlier studies suggests that the betel leaves

Conclusion

The *in vitro* antimicrobial potential established by *Piper betle* leaves extracts against the microbial test isolates designates that the plant has potential antimicrobial strength. Thus, validating the local use of *Piper betle* for medicinal purposes in treating infectious diseases such as gastrointestinal disorders, diarrhea, and other infections in which the tested pathogens may be concerned. The presence of phytochemicals and the ability of the leaves extracts to inhibit the growth of numerous microbial species is an indication of the broad-spectrum antimicrobial potential of *Piper betle*, which makes it a potential candidate for a prospective antimicrobial drug. Thus, this plant is effective and comprises natural compounds that could be used in the treatment of infections and helps promote alternative medicine as better substitutes to synthetic antimicrobials.

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CONFLICT OF INTEREST:

The author(s) declare(s) that they have no conflict of interest

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