

# ***Piper betle* L., (Betel quid) shows bacteriostatic, additive and synergistic antimicrobial action when combined with conventional antibiotics**

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## **Highlights**

- *P. betle* (PB) is commonly chewed as betel quid (paan) and is reputed as a famous medicinal spice.
- We studied the antibacterial activities of PB extracts and its antibiotic modulating activity.
- PB were evaluated *via* the broth microdilution assay against 6 ATCC bacterial strains.
- Antibiotics modulating effects was assessed using a modified Checkerboard method.
- Five extracts inhibited at least one of the six bacterial strains tested.
- Greatest synergy was observed against ATCC strain of *Pseudomonas aeruginosa*.
- Lower MICs occurred with increasing phenol and flavonoid content.

## **Abstract**

*Piper betle* L., commonly chewed as betel quid (paan) has been extensively acclaimed in a panoply of countries for both its nutritive and medicinal values. The present study was geared towards investigating the antibacterial activities of *P. betle* extracts and its antibiotic modulating activity. A decoction of *P. betle* (AQE) leaves as used traditionally, as well as ethanolic (EE), ethylacetate (EAE), acetone (ACE) and dichloromethane (DCME) extracts were evaluated using the broth microdilution assay against six bacterial ATCC strains. The minimum inhibitory concentration (MIC), bacteriostatic and bacteriocidal activities of the extracts were evaluated. Additionally, the ability of the extracts to modulate conventional antibiotics (synergistic, additive, indifference, antagonistic) was assessed using a modified Checkerboard method and the fractional inhibitory concentration index ( $\Sigma$ FIC) was calculated. The phytochemical profile of each extract was determined and the Pearson's correlation coefficient was used to establish any association between the MIC,  $\Sigma$ FIC and phytochemical content. All the five extracts inhibited at least one of the six bacterial strains tested with EAE and ACE exhibiting the most potent antibacterial activity. The lowest MIC (0.2500  $\mu$ g/ $\mu$ l) recorded was against *Staphylococcus aureus*. *Piper betle* has been further shown to exhibit only bacteriostatic effect. Results from the Checkerboard indicated additive and synergistic effects of *P. betle* extracts especially in the 50% EAE-50% antibiotic and 50% ACE-50% antibiotic combinations. The greatest synergy was observed against *Pseudomonas aeruginosa* ( $\Sigma$ FIC 0.09) in the 70% ACE-30% Chloramphenicol combination. Synergy was also observed against *S. aureus*, *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes*. Phytochemical screening revealed that ACE and EAE contain the highest amount of phenols while DCME contains the highest amount of flavonoids. Statistical analysis showed that lower MICs occurred with increasing phenol content ( $R = -0.392$ ,  $p \leq 0.05$ ) and increasing flavonoid content ( $R = -0.551$ ,  $p \leq 0.01$ ). However, no correlation was established between  $\Sigma$ FIC and phytochemical content which could indicate a different mechanism of action employed by the combinations. Data amassed

have provided insight into the antibacterial activity, antibiotic modulating activity and the phytochemical profile of *P. betle*.

**Keywords:** *Piper betle*; bacteriocidal; bacteriostatic; antibiotic; Checkerboard; phytochemical

## 1. Introduction

Curative herbs and medicinal food plants have been the foundation of traditional herbal medicine amongst different ethnic groups across the globe since time immemorial. For these reasons, medicinal food plants represent fundamental resources for the study of their traditional uses such as novel anti-infectious agents. Indeed, the famous saying “*Let food be thy medicine and medicine be thy food*” by Hippocrates- the father of medicines, (431 B.C.) has become a unique reference statement throughout history (Totelin, 2015). It has also been observed that several drugs originated from plant sources. For instance, *Galega officinalis* gave rise to the antidiabetic drug namely Metformin<sup>®</sup> while *Illicium verum* (star anise) was used in the development of Tamiflu<sup>®</sup> to manage AH1N1 (Grover et al., 2002; Khan, 2010; Pulipaka et al., 2012).

One famous culturally used medicinal food plant that has attracted much interest is *P. betle* L., also known as ‘betel leaf’ which is very popular in Asian countries (Satyal and Setzer, 2011; Periyannayagam et al., 2012). *Piper betle* belonging to the botanical family Piperaceae, is often referred to as ‘Golden heart of nature’ and has been acclaimed for both its nutritive value and traditional uses (Pradhan et al., 2013). Native to Central and Eastern Malaysia, this perennial evergreen vine has been mentioned in ancient texts like Sri Lanka Historical Book ‘Mahawamsa’ Mahawamsa” written in pali as well as in Sanskrit texts, including Charaka, Sushruta Samhita and Astanga Hridayam (Pradhan et al., 2013). There are reports of *P. betle* use dating back to 3000 BC in Timor in Indonesia and in the blacked teeth of a human skeleton in Palawan in the Philippines 2600 BC (Pradhan et al., 2013). This shade loving vine, with strong aromatic taste and smell, has other popular names including *paan* (in Hindi/ Nepali), *vetta/ vettila* (in Malayalam), *phlu* (in Thai), and *sirih* (in Bahama Indonesian) (Satyal and Setzer, 2011; Khan et al., 2013; Kumari and Babu Rao, 2014). In relevance to citations in the Indian scriptures, the significance of *P. betle* has been explained in relation to every sphere of human life including social, cultural, religious and even day-to-

day life, which is relevant even in the contemporary day (Guha, 2006). Even today, a well-prepared betel quid or paan (betel in combination with many other ingredients like sliced areca nut, slaked lime, coriander, aniseed, clove, cardamom, sweetener, coconut scrapings, jelly, pepper mint, flavouring agent, and fruit pulp) is still viewed as a brilliant mouth freshener and mild vitalizer, usually served on the social, cultural and religious occasions like weddings, Puja (religious festivals), Sraddha ceremony (religious function performed after cremation) and is further offered to visiting guests in view to show respect (Guha, 2006). Furthermore, *P. betle* remains one of the most famous spices in Nepal, India, and China mainly due to its strong sweet- smelling aroma (Satyal and Setzer, 2011; Periyayagam et al., 2012).

Apart from its cultural and culinary use, *P. betle* has also acquired an esteemed position in traditional medicine. Several ethnopharmacological surveys have revealed that betel leaf juice is taken as a sex stimulant, applied on wounds to stop bleeding, and taken orally with a mixture of seed powder of *Tamarindus indica* and *Piper nigrum* together with salt at a dose of 3 teaspoons twice daily for the treatment of fever until alleviation of symptoms (Rahmatullah et al., 2011; Uddin et al., 2013). Further therapeutic use of *P. betle* includes the treatment of urinary complicacy, sore throat, cough, pulmonary infection, boils, constipation and during breast-feeding as lactogogues (Mula et al., 2008; Satyal and Setzer, 2011; Pradhan et al., 2013; Dwivedi and Tripathi, 2014). Scientific evaluation has further confirmed the biological activities of *P. betle* ascribed to its reservoir of panoply of phytochemicals (Satyal and Setzer, 2011). The leaves of *P. betle* have been shown to possess significant free radicals scavenging power mainly due to the presence of catechol and allylpyrocatechol (Parillon and Edward, 2006; Pradhan et al., 2013). The essential oil of *P. betle* has also been demonstrated to exhibit larvicidal, insecticidal, and anti-filarial activities (Singh et al., 2009; Nagori et al., 2011; Rekha et al., 2014). Moreover, it has been shown that *P. betle* exhibits anti- allergic (Wirotsangthong et al., 2007), anti-cancer (Banerjee and Shah, 2014), anti-hypercholesterolemic (Rekha et al., 2014), anti-diabetic (Arefin et al., 2012), anti-platelet (Nagori et al., 2011), and anti-nociceptive (Arefin et al., 2012) effects. Additionally, *P. betle* has been demonstrated *in vitro* to have significant antimicrobial potential against a broad spectrum of microorganisms including bacteria and fungi and has been further described as an effective alternative to reduce biofilm formation in membrane bioreactors caused by both *Pseudomonas aeruginosa* and *Candida albicans* (Ali et al., 2010; Siddiqui et al., 2012; Pradhan et al., 2013; Rekha et al., 2014). The antimicrobial potential is most likely due to hydroxichavicol, stearic acid, and palmitic acid. The antimicrobial activity explains the use of

*P. betle* as an oral care agent in Asian countries (Niranjan et al., 2002; Rekha et al., 2014). *P. betle* has also displayed the greatest potential value, among 12 Philippine medicinal plants, against both Gram-negative and Gram-positive multidrug-resistant bacteria (methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, extended spectrum  $\beta$ -lactamase-producing, carbapenem-resistant *Enterobacteriaceae* and metallo- $\beta$ -lactamase-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) which are responsible for nosocomial infections (Valle et al., 2015).

Nosocomial infections represent one of the main causes of premature deaths worldwide (Davis, 1994; Robin et al., 1998; Udgirkar et al., 2012). Previously, antibiotics represented a major option for treating bacterial infections. However, it was soon discovered that bacteria eventually adapted themselves to become resistant to these antimicrobial drugs, leading to complications in managing infections. Therefore, there is a critical need to investigate alternative options, namely products from plant origin in combination with conventional antimicrobial drugs, to tackle the emerging issue of multi-drug resistant pathogens. With the reported pharmacological traits of *P. betle*, this plant product can be exploited in order to find an efficient treatment and/or management strategy against nosocomial infections. Additionally, the antibiotic (Gentamicin) potentiating effect of essential oil of *P. betle* has been evidenced recently making it an ideal candidate for further investigation (Aumeeruddy-Elalfi et al., 2015).

A number of *in vitro* and *in vivo* studies have shown the efficacy of *P. betle* in the treatment of numerous conditions like sore throats, allergies, hypercholesterolemia, diabetes mellitus, pain, and oral health, amongst others. Despite the availability of panoply of information on *P. betle*, there is limited information on the efficacy *P. betle* and its antibiotic potentiating effect. Therefore, the main aim of this *in vitro* study was to investigate the antibacterial and antibiotic potentiating activities of different extracts of the local cultivar of *P. betle*. It is anticipated that data gathered will open new avenues for the development of pharmacological bioproducts from local resources that can be used to treat and/or manage infectious diseases.

## **2. Methodology**

### **2.1. Collection of plant sample**

*P. betle* leaves were collected from Triolet (District Pamplemousses), Mauritius in October 2014. They were authenticated by the curator of the National Herbarium, Mauritius

Sugar Industry Research Institute (MSIRI), Réduit. The leaves were washed with water to remove all unwanted materials, air-dried for a minimum of 3 days, pulverized in a mill and stored in a sterile air-tight container until further use (Hübsch et al., 2014).

## **2.2. Materials**

All reagents were obtained from Sigma Aldrich® (Germany). ATCC strains (*Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Propionibacterium acnes* (ATCC 6919), *Staphylococcus epidermidis* (ATCC 12228) and *Streptococcus pyogenes* (ATCC 19615) were purchased from Sigma Aldrich®.

The Clinical Laboratory Standards Institute (CLSI), formerly known as the National Committee for Clinical Laboratory Standards (NCCLS) (CLSI 2003), guidelines were used to ensure that accurate microbiological assay and transfer techniques were followed (de Rapper et al., 2012).

## **2.3. Preparation of plant extracts**

For the aqueous extract preparation, 250 ml of boiling water was poured over 50 g of powdered leaves placed in a 500 ml conical flask (Arawwawala et al., 2011). The mixture was shaken with the help of a magnetic hot plate stirrer for over 4h. Extraction with other solvents was carried out based on a modified method of Ishnava et al., (2013) where 50 g of powdered leaves were macerated in 250 ml solvent for 24 h using the following solvents with decreasing polarity: ethanol, ethyl acetate, acetone, and dichloromethane. The macerates were, then, filtered through filter paper (Whatman). The residual materials from the funnel are used for re-extraction with a same volume (250 ml) of solvent. The filtered solution was evaporated in a rotary evaporator under vacuum at 45 °C. The dry extract and stock solutions were kept at +4 °C until further analysis.

## **2.4. Antibacterial activity**

### *2.4.1. Inoculum preparation*

Fresh inoculums were prepared by streaking a loopful of bacterial suspension into autoclaved peptone water and incubated at 37 °C in aerobic conditions in order to maintain an approximately uniform growth rate. The bacterial cultures were compared with 0.5

McFarland turbidity standard, which is equivalent to approximately  $1 \times 10^8$  bacterial cell count per ml (Perilla, 2003; Ishnava et al., 2013), which was maintained throughout the experimentation. Purity of the culture and isolation of pure colonies for sub-culturing were ensured by preparing streak plates (Hübsch et al., 2014).

#### 2.4.2. Antibacterial assay

The antibacterial ability of the *P. betle* extracts was assessed using modified antimicrobial assay using 96 well microtitre-plate as described by Drummond and Waigh, (2000). Briefly, using aseptic techniques a single colony of microbe was transferred into a 100 ml bottle of peptone water broth, capped and placed in incubator overnight at 35 °C. After 12–18 h of incubation, using aseptic preparation and the aid of a centrifuge, a clean sample of microbe was prepared. To the sterile microtitre plates, 100 µl of peptone water broth was first added to all wells, followed by 100 µl of extract or control. Then, 100 µl of microbial culture was further added. The resulting mixtures were then left to incubate for 24 h at ambient temperature. After incubation, 40 µl INT (0.2 mg/ml) was added to all the wells and left to incubate for a further 20 minutes. The microplates were then assessed visually to determine the minimum inhibitory concentration (MIC). Controls used are Chloramphenicol and Streptomycin, prepared in sterile distilled water at a concentration of 0.5 µg/µl. The extracts which showed antimicrobial activity were then further assessed for their bacteriostatic, bacteriocidal and antibiotic potentiating effect.

#### 2.5. Bacteriostatic and bacteriocidal activity

The bacteriostatic and bacteriocidal activity was determined using a modified assay by Aumeeruddy-Elalfi et al., (2015). Briefly, 10 µl of broth from uncoloured wells (where no bacterial growth was observed in the previous MIC assay), corresponding to the MIC value was inoculated on sterile Mueller-Hinton agar plates, and incubated for 18- 24 h. Growth of bacteria indicates bacteriostasis while no growth indicates bacteriocidal effect.

#### 2.6. Antibiotic potentiating assay

The antibiotic potentiating effects of the different extracts of *P. betle* were determined using an adapted Checkerboard method (Orhan et al., 2005). According to the ratio of plant extract: antibiotic being tested, appropriate amount of peptone water was distributed into 56 wells (8 x 8) so that the final volume of the combination is 100 µl. The antibiotic was serially diluted along the vertical axis while the plant extract was serially diluted along the horizontal

axis so that each well represents a unique concentration of the combination being tested. 100  $\mu$ l of previously prepared bacterial culture was then added to each well and the plates were left to incubate overnight in aerobic conditions and ambient temperature. After the incubation period, 40  $\mu$ l of iodinitrotetrazolium chloride (INT) (0.2 mg/ml) was added to the wells and left to incubate for an additional 20 minutes. The plates were assessed visually. All the tests were carried out in triplicates.

### 2.6.1. Fractional inhibitory concentration index and interpretation

The wells at the growth- no growth interface were determined and the individual fractional inhibitory concentration index ( $\Sigma$ FIC) of the combination was calculated according to the equation below. The final  $\Sigma$ FIC was obtained by averaging the individual  $\Sigma$ FICs (Michael et al., 1993). The selection of wells to calculate the final  $\Sigma$ FIC is highly debatable.

$$\Sigma\text{FIC} = \frac{\text{MIC of plant extract in combination}}{\text{MIC of plant extract alone}} + \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

Formerly,  $\Sigma$ FIC was interpreted as synergistic when  $\Sigma$ FIC was less than 1.00 and antagonistic if  $\Sigma$ FIC was more than 1.00. This study has used a more recent conservative and adapted approach where synergy was interpreted if  $\Sigma$ FIC was less or equal than 0.5 (Odds, 2003). Additive effects were considered for  $\Sigma$ FIC more than 0.5 but less than 1.00, indifference was indicated by  $\Sigma$ FIC more than 1.00 but less than 4.00 and antagonism was shown by  $\Sigma$ FIC more than 4.00 (de Rapper et al., 2012).

## 2.7. Determination of total phenolic, flavonoid, tannin, and anthocyanin content

The total phenolic content (TPC), was evaluated using the modified Folin-Ciocalteu assay described by Nickavar and Esbati, (2012). The plant extract (500  $\mu$ l) was added to a test-tube containing a 10-fold diluted Folin-Ciocalteu reagent solution (2500  $\mu$ l), and sodium carbonate (2000  $\mu$ l, 7.5%). The mixture was allowed to react for 30 minutes at room temperature. The total phenolic content was then spectrophotometrically determined at 760 nm. All determinations were carried out in triplicates and results obtained were expressed as  $\mu$ g gallic acid equivalent (GAE)/mg.

The total flavonoid content (TFC) was evaluated according to the aluminium chloride colometric method (Amaeze et al., 2011). The plant extract (2 ml) was added to 2%

aluminium chloride solution (2 ml). The mixture was allowed to react for 30 minutes at room temperature and the absorbance was read at 420 nm. All determinations were performed in triplicates and results obtained were expressed as  $\mu\text{g}$  rutin equivalent (RE) / mg crude extract.

Quantitative estimation of tannin (TTC), as catechin equivalent, was evaluated using the vanillin- HCl method with slight modifications. 1 ml of extract was added in 5 ml of reagent mix containing 4% vanillin (in methanol) and 8% concentrated hydrochloric acid (in methanol). The resulting reaction mixture was vortexed and kept in the dark for 20 minutes. The absorbance was then determined at 500 nm using a spectrophotometer, using catechin (400  $\mu\text{g}/\text{ml}$ ) as standard.

The total anthocyanin content (TAC) was calculated using the pH differential method (Sutharut and Sudarat, 2012). Briefly, 1 ml (1 mg/ml) of plant extract was transferred into 10 ml volumetric flask and the volume was adjusted with buffer pH 1.0 and pH 4.5. Mixtures were allowed to equilibrate for 15 minutes. Absorbance of each dilution was spectrophotometrically determined at 510 and 700 nm. Absorbance of diluted samples was evaluated using the following equation:  $A = (A_{510} - A_{700})_{\text{pH}1.0} - (A_{510} - A_{700})_{\text{pH}4.5}$ . The monomeric anthocyanin pigment concentration in the original sample was calculated according to the following equation: Anthocyanin content (mg/ ml) =  $A * \text{MW} * \text{DF} * 1000 / (\epsilon * l)$ ; Where MW is the molecular weight of cyaniding-3-glucoside (484.5), DF is the dilution factor and  $\epsilon$  the molar extinction coefficient ( $\epsilon=26,900$ ).

## 2.8. Statistical analyses

All calculations and graphical representations were carried out using Microsoft Excel 2007. Correlations were established using Pearson's correlation coefficient through the aid of statistical software SPSS Version 21.

## 3. Results

### 3.1. Antibacterial activities

The antibacterial effectiveness of each extract was investigated by determining their MICs using the micro-dilution assay. Efficacy of *P. betle* towards several microorganisms was defined by determining the MIC relative to standards (Streptomycin and Chloramphenicol). The results are summarised in Table 1.



**Table 1:** Antibacterial activities of *P. betle* extracts

Bacteria	Type of strain	BC/BS	MICs obtained by different extracts/ ( $\mu\text{g}/\mu\text{l}$ )						
			Aqueous	Ethanol	Ethyl acetate	Acetone	Dichloromethane	Streptomycin	Chloramphenicol
<i>E. coli</i>	G-	BS	-	-	4.0000	4.0000	-	0.0078	0.0078
<i>P. aeruginosa</i>	G-	BS	-	-	4.0000	4.0000	-	-	0.1250
<i>S. aureus</i>	G+	BS	1.0000	1.0000	0.5000	0.2500	1.0000	0.0019	0.0156
<i>P. acnes</i>	G+	BS	-	4.0000	2.0000	2.0000	4.0000	0.1250	0.0625
<i>S. epidermidis</i>	G+	BS	-	-	4.0000	4.0000	4.0000	0.1250	0.0625
<i>S. pyogenes</i>	G+	BS	-	-	2.0000	4.0000	4.0000	-	0.0625

Note: - refers to no inhibition; G+ refers to Gram-positive; G- refers to Gram-negative; BS refers to bacteriostatic effect.

All the extracts showed antibacterial activity, with the greatest activity observed by ethyl acetate and acetone extracts. The lowest MIC, i.e., greatest antibacterial activity was observed against *S. aureus*. Upon re-plating, all agar plates displayed growth of bacteria, indicating bacteriostatic activity only. This was observed against all the bacterial strains tested.

### **3.2. Antibiotic potentiating effect**

The antibiotic potentiating activity of the plant extracts were determined using an adapted Checkerboard method and the  $\Sigma$ FICs were determined.

#### *3.2.1. Streptomycin potentiating activity*

Different combinations of plant extracts and Streptomycin were tested and the results are summarised in Table 2.

The ethanolic extract-Streptomycin (EE-Strep) combinations, synergy was observed against *S. aureus* ( $\Sigma$ FIC 0.40) in the 70% EE- 30% Strep combination. It is to be noted that the combinations did not inhibit the growth of *P. acnes* at any concentration tested, and therefore no FIC was determined.

The ethyl acetate extract-Streptomycin (EAE-Strep) combinations exhibited synergistic or additive properties against the highest number of bacteria species. It was observed that the 30% EAE- 70% Strep combination displayed additive effects against *P. acnes* ( $\Sigma$ FIC 0.85) and *S. epidermidis* ( $\Sigma$ FIC 0.85). The 50% EAE- 50% Strep combination displayed interesting synergistic activity against multiple bacterial strains namely *E. coli* ( $\Sigma$ FIC 0.42), *P. acnes* ( $\Sigma$ FIC 0.29), and *S. epidermidis* ( $\Sigma$ FIC 0.29). Furthermore, the same combination displayed additive effects against *S. aureus* ( $\Sigma$ FIC 0.55). The 70% EAE- 30% Strep combination displayed synergistic activity against *P. acnes* ( $\Sigma$ FIC 0.37), and *S. epidermidis* ( $\Sigma$ FIC 0.37). Additionally, additive properties were indicated against *E. coli* ( $\Sigma$ FIC 0.52).

The acetone extract- Streptomycin (ACE- Strep) combinations were the second most active and exhibited synergistic or additive effects against the tested bacterial strains. The 30% ACE- 70% Strep combination demonstrated addition properties against *E. coli* ( $\Sigma$ FIC 0.66), and *P. acnes* ( $\Sigma$ FIC 0.85). The 50% ACE- 50% Strep combination revealed synergistic activity against *P. acnes* ( $\Sigma$ FIC 0.29), and *S. epidermidis* ( $\Sigma$ FIC 0.29) while additive effects were displayed against *E. coli* ( $\Sigma$ FIC 0.62). The 70% ACE- 30% Strep combination indicated

**Table 2:** Streptomycin potentiating effect of *P. betle*

Bacteria	ΣFIC of plant extract: streptomycin														
	Aqueous			Ethanol			Ethyl acetate			Acetone			Dichloromethane		
	30:70	50:50	70:30	30:70	50:50	70:30	30:70	50:50	70:30	30:70	50:50	70:30	30:70	50:50	70:30
<i>E. coli</i>	-	-	-	-	-	-	1.46	<u>0.42</u>	<b>0.52</b>	<b>0.66</b>	<b>0.62</b>	<b>0.52/</b> 2.41	-	-	-
<i>P. aeruginosa</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. aureus</i>	4.59	1.58	1.43	1.87	1.87	<u>0.40</u>	2.53	<b>0.55</b>	2.58	3.67	2.89	2.57	> 4.00	1.85	2.26
<i>P. acnes</i>	-	-	-	N.I	N.I	N.I	<b>0.85</b>	<u>0.29</u>	<u>0.37</u>	<b>0.85</b>	<u>0.29</u>	<u>0.37</u>	N.I	<b>0.75</b>	<u>0.42</u>
<i>S. epidermidis</i>	-	-	-	-	-	-	<b>0.85</b>	<u>0.29</u>	<u>0.37</u>	> 4.00	<u>0.29</u>	<u>0.37</u>	N.I	<b>0.75</b>	1.50
<i>S. pyogenes</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: No antimicrobial activity is indicated by -; Additive effects are indicated with figures in bold; Synergy is indicated with figures underlined; / refers to dose dependent ΣFIC; N.I refers to no inhibition by the plant-antibiotic combination

synergy against *P. acnes* ( $\Sigma$ FIC 0.37) and *S. epidermidis* ( $\Sigma$ FIC 0.37). Moreover, the same combination showed additive effects against *E. coli* ( $\Sigma$ FIC 0.52) for ACE concentration greater or equal to 0.175  $\mu\text{g}/\mu\text{l}$ .

The 50% DCME- 50% Strep combination pointed towards additive properties when used against *P. acnes* ( $\Sigma$ FIC 0.75) and *S. epidermidis* ( $\Sigma$ FIC 0.75). The 70% DCME- 30% Strep combination exhibited synergy against *P. acnes* ( $\Sigma$ FIC 0.42).

### 3.2.2. Chloramphenicol potentiating activity

Different combinations of the plant extract were analysed further for their ability to potentiate Chloramphenicol. The results are summarised in Table 3. The 30% aqueous extract (AQE)- 70% Chloramphenicol (Chlo) combination showed neither additive nor synergistic action against any of the tested bacterial strains. However, the 50% AQE- 50% Chlo combination exhibited synergistic action against *S. aureus* ( $\Sigma$ FIC 0.30). The 70% AQE- 30% Chlo combination displayed synergy against *S. aureus* ( $\Sigma$ FIC 0.42).

The ethanolic extract-Chloramphenicol (EE-Chlo) indicated additive properties against *P. acnes* ( $\Sigma$ FIC 0.73) when used in 30%-70% ratio. Additive effects were observed against the EE concentration greater or equal to 1.4  $\mu\text{g}/\mu\text{l}$  against *P. acnes*.

The ethyl acetate extract-Chloramphenicol (EAE-Chlo) combination displayed synergy against *P. aeruginosa* ( $\Sigma$ FIC 0.23) when used in the 30%- 70% ratio. The 50% EAE- 50% Chlo combination exhibited dose dependent synergy against *E. coli* ( $\Sigma$ FIC 0.45) and *S. epidermidis* ( $\Sigma$ FIC 0.39) for EAE concentration greater or equal to 1 $\mu\text{g}/\mu\text{l}$ . Synergy was also observed by the same combination against *S. aureus* ( $\Sigma$ FIC 0.33) and *S. pyogenes* ( $\Sigma$ FIC 0.50). Additionally, additive effects were noted against *P. acnes* ( $\Sigma$ FIC 0.81). The 70% EAE- 30% Chlo combination displayed synergy against *S. aureus* ( $\Sigma$ FIC 0.31) and *S. epidermidis* ( $\Sigma$ FIC 0.44). Additive effect was observed against *S. pyogenes* ( $\Sigma$ FIC 0.53) and, in a dose dependent behaviour, against *E. coli* ( $\Sigma$ FIC 0.74) for EAE concentration greater or equal to 1.4  $\mu\text{g}/\mu\text{l}$ .

The acetone extract- Chloramphenicol (ACE-Chlo) combination, when tested in 30%- 70% ratio, exhibited synergy against *P. aeruginosa* ( $\Sigma$ FIC 0.27) and was dose dependent, against *S. epidermidis* ( $\Sigma$ FIC 0.40) for ACE concentration greater or equal to 0.075  $\mu\text{g}/\mu\text{l}$ . The same combination also displayed additive properties against *P. acnes* ( $\Sigma$ FIC 0.53) for ACE concentration greater or equal to 0.6  $\mu\text{g}/\mu\text{l}$ . The 50% ACE- 50% Chlo combination displayed synergy against *P. aeruginosa* ( $\Sigma$ FIC 0.15) and *S. pyogenes* ( $\Sigma$ FIC 0.41). Synergy

**Table 3:** Chloramphenicol potentiating activity of *P. betle*

Bacteria	ΣFIC of plant extract: chloramphenicol														
	Aqueous			Ethanol			Ethyl acetate			Acetone			Dichloromethane		
	30:70	50:50	70:30	30:70	50:50	70:30	30:70	50:50	70:30	30:70	50:50	70:30	30:70	50:50	70:30
<i>E. coli</i>	-	-	-	-	-	-	3.94	<u>0.45/</u> >4.00	<b>0.74/</b> 4.51	3.95	0.42/ 3.33	<b>0.51/</b> 3.24	-	-	-
<i>P. aeruginosa</i>	-	-	-	-	-	-	<u>0.23</u>	2.32	1.89	<u>0.27</u>	<u>0.15</u>	<u>0.09</u>	-	-	-
<i>S. aureus</i>	2.44	<u>0.30</u>	<u>0.42</u>	2.77	2.06	2.52	2.36	<u>0.33</u>	<u>0.31</u>	2.87	2.18	3.38	<u>0.22</u>	<u>0.30</u>	<u>0.42</u>
<i>P. acnes</i>	-	-	-	<b>0.73</b>	1.06	0.95	1.25	<b>0.81</b>	<b>0.80</b>	<b>0.53/</b> 1.41	<u>0.37/</u> 1.02	<u>0.50</u>	1.06	1.06	<b>0.95</b>
<i>S. epidermidis</i>	-	-	-	-	-	-	1.35	<u>0.39/</u> 1.04	<u>0.44</u>	<u>0.40/</u> 1.41	<u>0.39/</u> 1.03	<u>0.48</u>	1.06	1.06	<b>0.95</b>
<i>S. pyogenes</i>	-	-	-	-	-	-	<u>0.39/</u> <b>0.71</b>	<u>0.50</u>	<b>0.53</b>	<b>1.22</b>	<u>0.41</u>	<u>0.46</u>	<b>0.56</b>	1.00	<b>0.65</b>

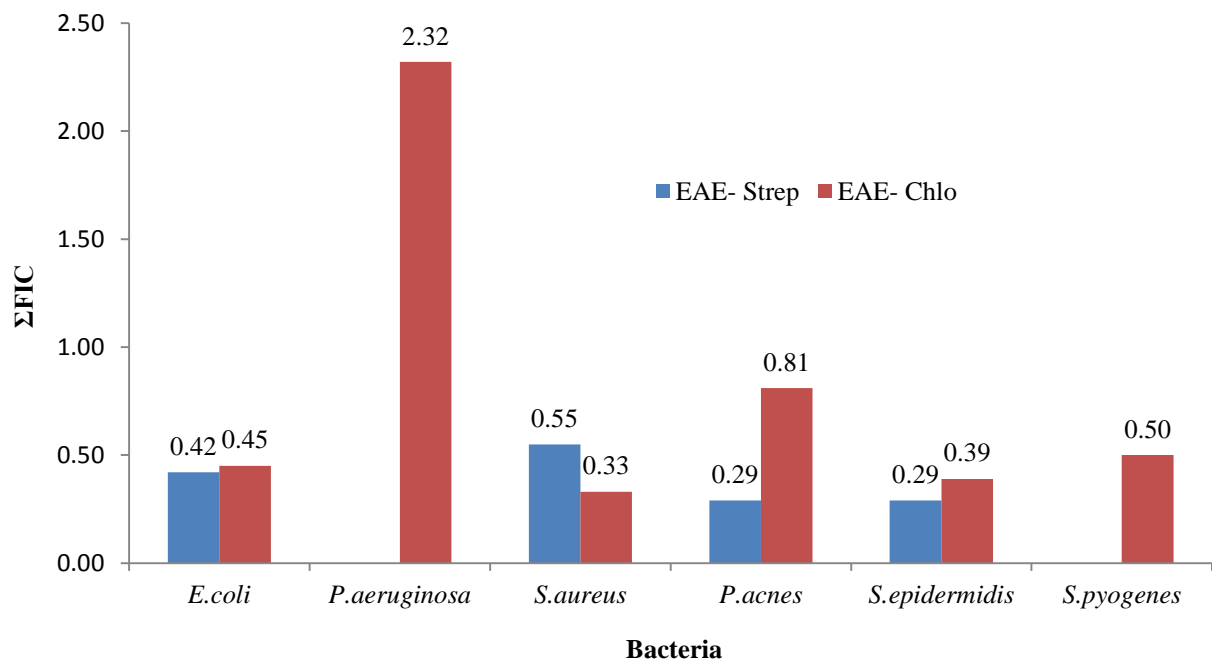
Note: No antimicrobial activity is indicated by -; Additive effects are indicated with figures in bold; Synergy is indicated with figures underlined; / refers to dose dependent ΣFIC; N.I refers to no inhibition by the plant-antibiotic combination.

was also observed in a dose dependent manner against *E. coli* ( $\Sigma$ FIC 0.42; ACE concentration  $\geq 0.5 \mu\text{g}/\mu\text{l}$ ) and *S. epidermidis* ( $\Sigma$ FIC 0.39; ACE concentration  $\geq 0.5 \mu\text{g}/\mu\text{l}$ ). The 70% ACE-30% Chlo combination showed synergistic activity against *P. aeruginosa* ( $\Sigma$ FIC 0.09), *P. acnes* ( $\Sigma$ FIC 0.50), *S. epidermidis* ( $\Sigma$ FIC 0.48) and *S. pyogenes* ( $\Sigma$ FIC 0.46). Additive effect, in a dose dependent behavior, was also noted against *E. coli* ( $\Sigma$ FIC 0.51; ACE concentration  $\geq 0.7 \mu\text{g}/\mu\text{l}$ ).

The dichloromethane extract- Chloramphenicol (DCME- Chlo) showed synergy against *S. aureus* in all 3 combinations tested. The 30% DCME- 70% Chlo combination displayed additive effects against *S. pyogenes* ( $\Sigma$ FIC 0.56) while the 70% DCME- 30% Chlo combination demonstrated additive effects against *P. acnes* ( $\Sigma$ FIC 0.95), *S. epidermidis* ( $\Sigma$ FIC 0.95) and *S. pyogenes* ( $\Sigma$ FIC 0.65).

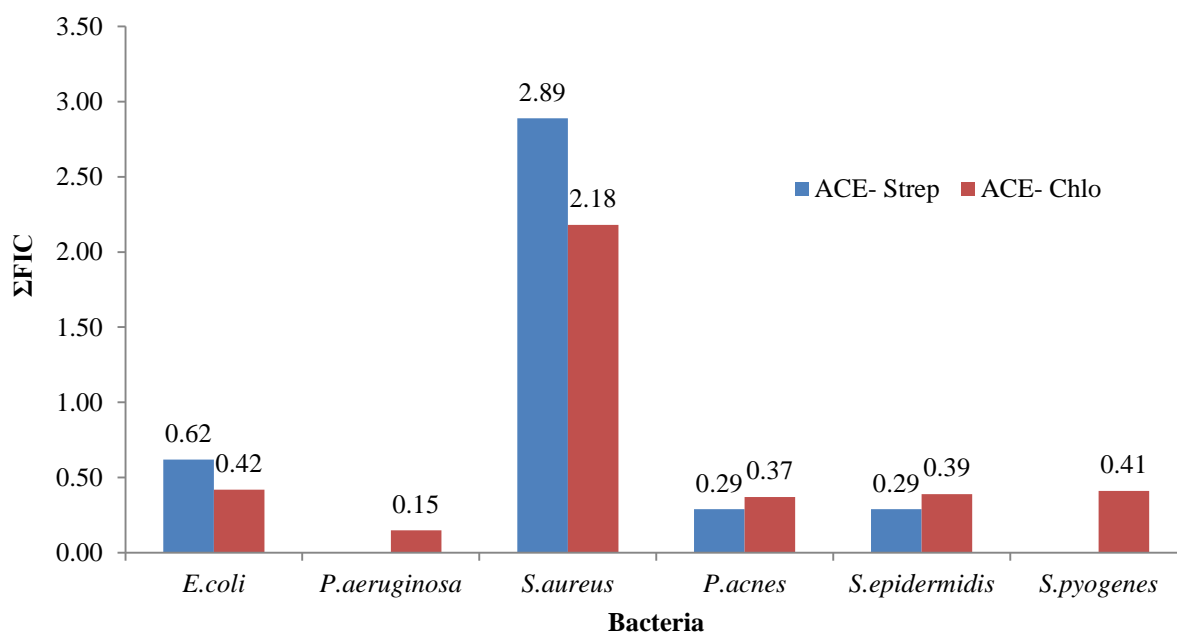
### 3.3. Comparison between effects of *P. betle* on Streptomycin and Chloramphenicol

EAE and ACE have been observed to display the greatest activity, especially in the 50%-50% combinations. Thus, they were further compared and it was found that most combination with Streptomycin tend to show lower  $\Sigma$ FIC values (Figures 1 and 2). It is interesting to note that



**Figure 1.** Comparison between effects of EAE on Streptomycin and Chloramphenicol in 50%- 50% ratio

while EAE- Strep showed additive effects against *S. aureus*, EAE- Chlo showed synergistic effects. Furthermore, EAE- Strep showed synergistic effects against *P. acnes* while EAE- Chlo showed additive effects. Also, ACE- Strep displayed additive effects against *E. coli* whereas ACE- Chlo exhibited synergistic effects.



**Figure 2.** Comparison between effects of ACE on Streptomycin and Chloramphenicol in 50%- 50% ratio

### 3.4. Phytochemical screening

Total phenolic content is reported as gallic acid equivalents by reference to standard curve ( $y = 0.0099x$  and  $R^2 = 0.9881$ ), the total flavonoid content was determined using the reference standard curve ( $y = 0.0152x$  and  $R^2 = 0.9981$  while the tannin content was calculated using standard curve  $y=0.0012x$  and  $R^2= 0.9971$ . ACE and EAE showed significantly higher ( $p < 0.05$ ) amount of phenolic compounds with  $186.26 \pm 0.73(\text{GAE})/\text{mg}$  and  $183.24 \pm 0.76 (\text{GAE})/\text{mg}$  respectively, when compared with the other extracts. The phenolic content of AQE and EE was not significantly different ( $p > 0.05$ ). The total flavonoid content increased with decreasing polarity, with DCME displayed significantly higher ( $p < 0.05$ ) flavonoid content ( $128.44 \pm 0.53 \text{ RE}/\text{mg}$ ) than the other extracts. AQE showed significantly lower ( $p < 0.05$ ) amount of flavonoids ( $8.9 \pm 0.14 \text{ RE}/\text{mg}$ ) compared to

the other extracts. The highest amount of tannins was found in the EE followed by EAE. Anthocyanin were not detected in most cases. The results have been summarised in Table 4.

**Table 4:** Phytochemical content of different extracts of *P. betle*

Extract	Assays			
	Total phenol <sup>a</sup>	Total flavonoid <sup>b</sup>	Total tannin <sup>c</sup>	Total anthocyanin
Aqueous	128.89 ± 0.36	8.90 ± 0.14	7.22 ± 0.48	N.D
Ethanol	129.43 ± 0.50	42.54 ± 0.77	17.78 ± 0.48	Trace
Ethyl acetate	183.24 ± 0.76	111.73 ± 0.89	14.16 ± 0.83	N.D
Acetone	186.26 ± 0.73	123.46 ± 0.17	0.83 ± 0.00	N.D
DCM	95.76 ± 0.53	128.44 ± 0.53	2.22 ± 0.48	N.D

<sup>a</sup>: µg gallic acid equivalent (GAE)/mg, <sup>b</sup>:µg rutin equivalent (RE) / mg, <sup>c</sup>: µg catechin equivalent(CE), N.D: Not detected

### 3.5. Correlations between antibacterial activity and phytochemical content

The relationship between MIC, ΣFIC and the phytochemical content was analysed. Only MICs of bacteria which have been inhibited by all 5 extracts have been used in this analysis. It was found that when total phenolic content increases, MIC decreases ( $R = -0.392$ ,  $p \leq 0.05$ ). That is, lower concentration of plant extract results in inhibitory action against bacteria. Furthermore, when total flavonoid content increases, MIC decreases ( $R = -0.551$ ,  $p \leq 0.01$ ). However, no significant relationship was found between total tannin content and MIC. Additionally, no significant association was found between ΣFIC and phytochemical content.

## 4. Discussion

The present study investigated the antibacterial potential of the different extracts of *P. betle* against six ATCC bacterial strains. Interestingly, we found *P. betle* to possess promising antibacterial potential with inhibitory activity against at least one out of the six strains tested. The EAE and ACE showed the highest antimicrobial potential by inhibiting the growth of all strains. As indicated by the correlation analysis, the highest activity can be attributed to the highest total phenolic content of both EAE and ACE as well as their high flavonoids content. The DCME followed by ACE and EAE in antimicrobial activity, inhibiting four out six



bacterial strains tested. Despite having lowest level of phenolic compounds, DCME showed the highest amount of flavonoids explaining its high activity. Therefore, DCME might contain compounds not present in EAE and ACE which may have acted against *P. aeruginosa*. The difference in phenolic and flavonoid content of the extracts can be explained by the use of solvents varying in polarity which have been reported to influence the amount of phytochemicals. Polar solvents extract polar compounds while non-polar solvents extract non-polar ones. In the current study, the polar solvents include water and ethanol while medium-polar solvents include ethyl acetate, acetone and dichloromethane. In numerous studies, the phenol content was highest in the acetone extract of plant (Tatiya et al., 2011) which is in accordance to the highest phenol yield of ACE in the present study. Interestingly, ACE, EAE and DCME inhibited the growth of *P. aeruginosa* and *S. pyogenes* in contrast to Streptomycin which may indicate the presence of undiscovered bioactive constituents having antibacterial attributes in *P. betle*.

In support of the present study, fresh and processed leaves of *P. betle* have been formerly reported as an effective antibacterial agent (Tan and Chan, 2014). Additionally, the essential oil of *P. betle* has been demonstrated to exhibit significant antimicrobial activity against a broad spectrum of microorganisms among which feature *E. coli*, *P. aeruginosa*, *S. aureus* and *Acinetobacter* amongst others (Aumeeruddy- Elalfi et al., 2015). The presence of tannins, anthraquinones, flavonoids, alkaloids, terpenoids, saponins, cardiac glycosides, glycosides, phlobatanins, hydroxychavicol acetate, allylpyrocatechol piperbetol, isoeugenol, anethole, stearic acid, methyl eugenol, carvacrol, chavicol, and allylpyrocatechol amongst others have found in *P. betle* (Kumari and Babu Rao, 2014; Rekha et al., 2014) which could contribute to its therapeutic potential. However, the current study revealed that *P. betle* exhibit only bacteriostatic effect contradicting the findings of Datta et al., (2011) where the antibacterial activity of ethanolic extracts of *P. betle* via disc diffusion method and time- kill kinetics was analysed and it was concluded that the relative efficiency of the bactericidal activity of ethanolic extract of *P. betle* to Ceftriaxone (antibiotic) represents the possibility of a more cost-effective and harmless antimicrobial agent. This disparity can be explained by different environmental and geographical factors including climate, time of harvest, storage conditions, and altitude which have an impact on the chemical composition and hence therapeutic potential of medicinal plants. Nonetheless, there are numerous benefits in using plant derived antimicrobials namely because of fewer side effects, better tolerance by patients, reduced cost, consumer acceptance due to history of long use, as well as their representation as green alternatives (Gur et al., 2006; Chanda and Rakholiya, 2011).

However, bacterial resistance can occur against plant molecules as well thereby increasing the need to develop newer and more powerful therapies to combat resistant microorganisms.

Therefore, a novel concept which has been recently been explored is the synergism between known antimicrobial drugs and bioactive plant components. Reported synergism between conventional antimicrobial drugs and medicinal plant products has motivated researchers to test such possible interactions geared towards novel antimicrobial therapeutics. This therapy can be employed to expand the antimicrobial spectrum, to prevent the surfacing of bacterial mutants and to minimize toxicity, thereby exhibiting antimicrobial action greater than what would be normally expected from each individual antibiotic (Chanda and Rakholiya, 2011). Synergy between plant derived products and antimicrobial drugs has been frequently reported. Subramaniam et al., (2014) showed that sitosterol-D-glucopyranoside isolated from *Desmostachya bipinnata* leaves combined with antibiotics showed antibacterial potential against numerous common human pathogens. Another study showed that the methanolic extract of *Punica granatum* (pomegranate) drastically enhanced the activity of all the tested antibiotics in order to combat MRSA (Braga et al., 2005). Clove, guava and lemongrass extracts showed high synergism rate with antimicrobial drugs when tested against *S. aureus* strains (Btoni et al., 2006). Additionally, the essential oil of *Lippia sidoides* Cham. (Verbenaceae) was shown to influence the activity of aminoglycosides and thus may be used as an adjuvant in antibiotic therapy against respiratory tract bacterial pathogens (Veras et al., 2012).

The present study revealed the synergistic effect of the different extracts of *P. betle* in combination with Streptomycin or Chloramphenicol against a wide range of bacterial strains. The highest activity was observed by the EAE- Strep combination against *E. coli*, *P. acnes*, and *S. epidermidis*, especially in the 50%- 50% ratio. With Chloramphenicol, synergistic effect was shown against *E. coli*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *S. pyogenes*. The EAE- Chlo and ACE- Chlo combinations showed the highest level of activity attributed to their high phytochemical content. It is to be noted that remarkable synergistic action was observed against *P. acnes* and *S. epidermidis* by both EAE- Strep and ACE- Strep combinations. *P. acnes* has long been linked with skin inflammatory condition acne vulgaris (McDowell et al., 2013) and recently associated with implants infections such as prosthetic joints, cardiac devices, breast implants, intraocular lenses, neurosurgical devices, and spine implants (Portillo et al., 2013). *S. epidermidis* has been associated with infections of un-dwelling medical devices like prosthetic heart valves (Monk et al., 2008). Therefore, the

combination therapy involving Streptomycin and *P. betle* extract can represent a novel approach in the management of the several infections.

Apart from *S. epidermidis*, Chloramphenicol combination with *P. betle* exhibited synergistic action against *S. pyogenes* primarily by 50%ACE- 50% Chlo and 70% ACE- 30% Chlo combinations. Moreover, synergy was observed against *P. aeruginosa*. *S. pyogenes* has been linked to numerous diseases in immune-compromised individuals and have also been associated with deep tissue infections whereas *P. aeruginosa* has been implicated as the most prevalent pathogen in cystic fibrosis lung disease (Sousa and Pereira, 2014). Therefore, combination therapy involving *P. betle* and Chloramphenicol tend to open new avenues and a viable treatment option. Furthermore, the present study revealed synergistic effects against *S. aureus* by the DCME- Chlo combination. *S. aureus* are implicated in a plethora of diseases ranging from mild skin diseases to more serious bloodstream infections (Rondevaldova et al., 2015) and treatment options could include combined use of *P. betle* and Chloramphenicol. In support of the present study's findings, another study demonstrated the antibiotic (Gentamicin) potentiating effect of the essential oil of *P. betle* against *E. coli* and *S. epidermidis* (Aumeeruddy-Elalfi et al., 2015).

It has been pointed out that plants contain several components to protect themselves from aggressor agents (Silva et al., 2010). Some of these components exert antimicrobial properties through different lines of actions namely disintegration of cytoplasmic membrane, destabilization of the proton motive force, electron flow, active transport and coagulation of the cell content (Burt, 2004; Silva et al., 2010, 2013). While some plant molecules can distort lipid- protein interaction, others (e.g. essential oils) can stimulate the growth of pseudo-mycelia which may act on enzymes involved in the synthesis of bacterium structural components (Silva et al., 2010). Eugenol, present in *P. betle*, interferes with the production of amylase and protease by *Bacillus cereus* and also contributes to cell wall degradation or lysis (Silva et al., 2010). The combination therapy, i.e., plant extract and antibiotics, tends to employ alternative mechanisms of action to combat pathogens and may represent a successful option to treat resistant bacteria (Chanda and Rakholiya, 2011).

## 5. Conclusion

In this study, the decoction of *P. betle* leaves as used traditionally, as well as ethanolic, ethylacetate, acetone and dichloromethane extracts were evaluated *via* the broth microdilution assay against six bacterial ATCC strains. In addition, the antibiotic modulating

activity of the extracts was studied. Interesting antibacterial and antibiotic activity of the extracts were observed which tend to validate its traditional use in the treatment and management of infectious diseases. The antibacterial activity was shown to correlate with the high amount of phenols and flavonoids present in the leaves. Additionally, synergistic or additive action was observed against numerous bacteria when *P. betle* was combined with conventional antibiotics. While *P. betle* is a potential candidate for further studies, it must be emphasized that interactions between plant natural components and synthetic drugs depend on several factors including pharmacokinetics and employed doses and therefore, combinations established *in vitro* may not have the same effect *in vivo* or in clinical trials (Szalek et al., 2006; Silva et al., 2010). Further research should be undertaken to ensure the safety and efficacy of *P. betle* extracts.

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