# Characterization of leaf and vine morphological diversity, phytochemical composition and antibacterial activity in the leaf extracts of six *Piper betle* L. cultivars in Sri Lanka

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## Abstract

Betel (*P. betle*) is an important cash crop. Two types of leaves namely matured-leaf (*Peedunu kola*) and stem-leaf (*Kanda kola*) are found in betel vines. The matured-leaves are the economically important part of the plant and consumed as an exhilarant-quid. These leaves contain many phytochemicals possessing numerous medicinal properties. Sri Lanka exports betel leaves however, industrial applications such as manufacturing of betel based herbal formulations cannot be seen. Therefore, this study was conducted to assess the morphological diversity of vines and matured-leaves, phytochemical composition and antibacterial activity of six betel cultivars within the county. The samples were collected from Intercropping and Betel Research Station at *Narammala*, Sri Lanka. The morphology of vines and matured-leaves were assessed. The flavonoids, tannins, terpenoids, reducing sugars, phlobatannins, saponins and ascorbic acid were assessed using standard tests. The antibacterial activity of the water extracts of matured-leaves were tested against three model pathogenic species *Escherichia coli* (JM109), Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus aureus* (NCTC4838). The highest petiole length and inter-nodal length were observed in *Nagawalli-yellow* and *Malabulath* respectively ($P < 0.05$). The highest concentration of flavonoids were detected in *Malabulath* and all cultivars contained variable levels of these phytochemicals. All the betel cultivars exhibited significant antibacterial activity however, Gram negative *E. coli* was less affected. Although *Malabulath* is not used for consumption, it contains largest leaves, phytochemical and antibacterial properties similar to the other cultivars. These information could be used in planning betel breeding programs and to select cultivars for industrial applications such as phytochemical extractions and herbal formulations.

**Keywords:** *Maneru, Nagawalli, phytochemicals in betel, flavonoid, tannin, betel cultivars*

## 1. Introduction

Betel (*Piper betle* L.), an evergreen perennial vine, is predominantly cultivated in India, Sri Lanka, Malay Peninsula and Philippines, and consumed in many Asian and African countries[1,2]. There are two types of leaves, matured-leaves (i.e. *peedunu kola*) and stem leaves (*kanda kola* which is more hard, crunchy, non-edible and always symmetric and monomorphic except size) in betel vine. Matured-leaves of betel, the economically important part of the plant, are mainly consumed as a quid, an exhilarant which prevents halitosis, with variable mixtures of other components such as nut pieces of *Areca catechu* and sometimes spices like cardamom and nutmeg with or without tobacco and mild formulations of lime[3,4]. In addition, betel has a significant place in religious and cultural activities in societies where Hindus and Buddhists are living. It is believed that betel is a blessed plant to be evergreen and perpetual with the shape of God's heart[5]. It has been documented in ancient scripture for social, domestic, cultural and religious activities. It is a momentous symbol of paying respect in social events and an icon of prosperity and blessing in marriages and religious festivals[6]. Betel has been tremendously used in traditional medicine for centuries in South Asia especially in Ayurvedic Medicine in India and Sri Lanka[5,7]. It has been a promising remedy for adenopathy, bruises, cancer, catarrh, colic, congestion, diphtheria, edema, gastroenteritis, inflammations and wounds[8]. The leaves are also supposed to harden the gum, conserve the teeth and prevent indigestion, bronchitis and constipation[3]. Betel leaves are warmed with oil and applied on chest in breathing difficulties, cough, asthma and bronchitis[8].

The leaf aqueous extracts of betel contain high concentrations of alkaloids, flavonoids, glycosides and tannins, while the leaf methanolic extracts contain alkaloids, sterols, phenols, tannins and flavonoids, with moderate levels of saponins and terpenoids. These leaf extracts possess antibacterial, antifungal, anti-fertility, anti-estrogenic, anti-oxidative, anti-hemolytic and anti-carcinogenic activities[9-13]. Numerous studies conducted using betel leaves isolated variety of important phytochemicals such as steroids, flavonoids, terpenoids, saponins, polysaccharides, essential oils, vitamins, minerals, alkaloids, amides, polyphenols, kawapyrones, piperolides, chalcones, phenylpropenes and allylpyrocatechols[14-16]. The concentrations of these constituents may vary depending on the type of cultivar, maturity level and environmental conditions[6,17].

Betel leaves contain phenolic compounds such as hydroxychavicol which is a promising antimicrobial agent against pathogens in the oral cavity[18]. Therefore, betel leaves have a great potential to be used in manufacturing mouth-washing formula[19,20]. The leaf extracts of betel inhibit human pathogenic bacteria such as *Bacillus cereus*, *Staphylococcus aureus*,

*Pseudomonas aeruginosa*, *Proteus vulgaris*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Streptococcus mutans* and *Diplococcus pneumoniae*[21-24] and clinically significant fungal species *Aspergillus* spp., Candida[25], *Corynespora cassiicola*, *Colletotrichum* spp. and *Rigidoporus* spp.[8].

Betel is a popular intercrop in wet and intermediate zones of Sri Lanka. Betel grows in well-drained and fertile lateritic soils[26] which are mainly present in *Kurunegala* and *Gampaha*, two major Districts of betel cultivation. *Colombo*, *Kalutara*, *Kegalle*, *Ratnapura*, *Matale* and *Galle* are the other betel cultivating Districts in the country[17]. There are improved betel cultivars (i.e. commercial betel) available for growers in Sri Lanka and other cultivars like *Malabulath* (i.e. wild germplasm) are available as potential parental genotypes to characterize for important agronomic and phytochemical traits which could be used in betel breeding. The annual betel leaf production in Sri Lanka is 30, 000 MT mainly exported to Pakistan[27].

There are numerous problems can be identified in betel industry of Sri Lanka although it is a very important cash crop for the livelihood of many people. The pest and diseases, low yield, sensitivity to drought and problems in post-harvest practices can be considered as the key limiting factors in betel industry. Sri Lanka exports betel as leaves and very limited attention has been given to produce value added products such as betel based herbal formulations. Novel studies on chemical constitutes and bioactivities on betel would surely help to develop value added products such as toothpaste, mouthwash and body lotions[28,29] to augment the value of betel as a cash crop to expand the future prospects of the industry. The present study was conducted to assess the matured-leaf and vine morphological diversity, phytochemical composition and antibacterial activity of six betel cultivars to lay a platform to characterize them to be used in breeding and industrial applications.

## 2. Materials and methods

### 2.1 Sampling

The *Piper betle* samples were collected from Intercropping and Betel Research Station, *Narammala* (7°26'4"N, 80°13'17"E) which is coming under Department of Export Agriculture, Sri Lanka. The matured-leaves which are generally harvested for consumption and marketing purposes were collected from six *P. betle* cultivars namely *Malabulath*, *Maneru*, *Maneru-yellow*, *Nagawalli-white*, *Nagawalli-yellow* and *Ratadalu*. The samples were taken from the vines grown under uniform growth conditions and they were at equal maturity stage.

### 2.2 Assessment of the morphology of the matured-leaves and vines

The leaves were photographed using a high resolution camera (Canon EOS 60D, Canon USA, Inc.) and the mean RGB (Red, Green, Blue Color Spectrum) values of the leaf color were obtained using Adobe® Photoshop® Software (Adobe Systems Incorporated, CA, USA). The color pattern, shape, symmetricity of shape, base and apex, and venation pattern of leaves were observed. In addition, the texture of vines and rooting at nodes were also observed. These data were recorded in multiple replicates and reported according to the botanical key available in Suwanphakdee and Chantaranothai,[30] and Chaveerach *et al*. [4]. The width, length, number of veins and petiole length of the leaves and the length of the internodes were also measured.

### 2.3 Phytochemical analysis

The leaves were harvested and washed under running tap-water. The leaf samples were then air dried and cut into small pieces. A total of 20 g of leaf pieces were measured and crushed followed by the addition of 350 ml of distilled water. The mixtures were sonicated three times for 30 mins and filtered in each time using Whatman filter paper no. 2 (WHA1002125). A portion of final filtrate (50 ml) was collected from each sample and stored in 4 $^{0}$C for phytochemical analysis. The key phytochemicals namely flavonoids, tannins, terpenoids, reducing sugars, phlobatannins and saponins present in the matured-leaves of betel cultivars were tested according to the methods given in Table 1. The ascorbic acid content was measured as follows. A total volume of 5 ml leaf water extract was pipetted into a 50 ml conical flask and three drops of starch indicator solution was added. The sample was titrated with 0.005 M iodine solution until first permanent trace of a dark bluish black color was observed (i.e. formation of starch-iodine complex) as the end point. The titration was repeated three times for each sample. The ascorbic acid concentration was calculated according to the stoichiometry of following reaction.

$$\text{Ascorbic acid} + I_2 \rightarrow 2I^- + \text{Dehydroascorbic acid}$$

**Table 1** Qualitative tests to detect the presence of phytochemicals

| Phytochemical | Test | Reference |
|---|---|---|
| Flavonoids | 5 ml diluted $NH_3$ was added to 1 ml of leaf extract. This resulted in an intense yellow color indicating the presence of flavonoids. Then concentrated $H_2SO_4$ was added until the disappearance of yellow color and the amount of $H_2SO_4$ added to disappear the yellow color was proportional to the level of flavonoids present. | Modified from Hossain *et al*.[31] |
| Tannins | Few drops of 10 % ferric chloride was added to 1 ml of leaf extract. Formation of green or a bluish black color confirmed the presence of tannins. | Modified from Auwal *et al*.[32] |
| Terpenoids | ***Salkowski test:*** 1 ml of leaf extract was mixed with 2 ml of chloroform in a test tube and 3 ml of concentrated $H_2SO_4$ was added along the sides of the tube. The occurrence of reddish brown color at the interface confirmed the presence of terpenoids. | Sing *et al*.[33] |
| Reducing sugars | 1 ml of leaf extract was boiled with few drops of Benedict's solution for few minutes in a water bath. An orange red precipitate was observed as the indication of the presence of reducing sugars. | Benedict,[34] |
| Phlobatannins | 1 ml of leaf extract was boiled with 2 % HCl solution and observed for the development of red precipitate to confirm the presence of phlobatanins | Auwal *et al*.[32] |
| Saponins | The extract was shaken vigorously and observed for the formation of stable and persistent froth. | Sing *et al*.[33] |

## 2.4 Antibacterial activity

A total of 20 g of fresh leaf samples were cleaned, cut into small pieces and dissolved in 350 ml of water. The solutions were sonicated (Branson® Ultrasonic Bath, 230Vac, 50Hz, Z245100) to obtain leaf extracts and filtered through Whatman filter paper no. 2 (WHA1002125) and the filtrates were kept in freeze-dryer for 48 hrs. The obtained freeze-dried samples were crushed into fine powder and 50 mg of the powder was dissolved in 300 µl of distilled water to achieve the final concentration of 1.6 x $10^5$ ppm. The antibacterial activity of the extracts were tested using *Kirby-Bauer* agar disc-diffusion assay[35] against three model pathogenic bacterial strains, *Escherichia coli* (JM109), Methicillin resistant *S. aureus* (MRSA) and *Staphylococcus aureus* (NCTC4838). A total volume of 20 ml of autoclaved *Mueller Hinton Agar* (MHA) medium was poured into sterile petri dish and allowed to solidify. Then, 200 µl of bacterial cell culture was spread evenly on the MHA plate. After that, the autoclaved Whatman filter paper discs with 6 mm diameter were soaked with leaf extracts of the cultivars and placed on the spread plate ensuring equal distances among them.

Distilled water was used as the control. The plates were incubated at 37 ºC for 24 hrs and the diameter of the zone of bacterial inhibition (DZBI) around each filter paper disc was measured. This experiment was conducted in triplicate.

### 2.5 Data analysis

The qualitative parameters of the leaves and vine morphology were descriptively analyzed and reported. The morphometric data of matured-leaves and vines were subjected to General Linear Model (GLM) Procedure, correlation analysis through the calculation of Pearson's Correlation Coefficient (PCC) in CORR Procedure, regression analysis, cluster procedure and dendrogram construction using the statistical package SAS® 9.1 (SAS Institute, Cary, NC, USA). The phytochemical data except ascorbic acid concentration were descriptively interpreted with reference to the intended color changes, formation of precipitates and froths. The ascorbic acid concentrations and DZBI data of antibacterial activity were subjected to GLM Procedure.

## 3. Results

### 3.1 Leaf morphology

The color of the matured-leaves of the six betel cultivars was measured as RGB values to show the variation. The leaves of the two cultivars *Nagawalli-white* and *Nagawalli-yellow* got predominant and variably sized, white and yellow patches respectively around the mid rib and other major veins (Table 2) (Figure 1). In addition, redness was observed at the base of *Malabulath* leaves. The cultivars *Malabulath* and *Nagawalli-yellow* got ovate and broad shaped leaves whereas *Maneru*, *Maneru-yellow*, *Nagawalli-white* and *Ratadalu* got ovate and elongate shaped leaves. The cultivars *Maneru*, *Maneru-yellow*, *Nagawalli-white* and *Ratadalu* got asymmetric leaves with asymmetric leaf base and apex, and other two cultivars got perfectly symmetric leaves. There were two basic venation patterns observed among the six cultivars. The cultivar *Malabulath* got flexuous vines whereas all the others got stout and smooth vines. The cultivar *Nagawalli-yellow* exhibited rooting at nodes whereas other cultivars did not show this trait (Table 2).

**Table 2** Morphological variation of matured-leaves and vines of *P. betle* cultivars

| Cultivar | Leaf Color (R, G, B)* | Leaf color pattern[$] | Leaf Shape | Leaf-shape symmetricity | Leaf-base symmetricity | Leaf-apex symmetricity | Venation pattern | Vine texture | Rooting at nodes |
|---|---|---|---|---|---|---|---|---|---|
| *Malabulath* | 0, 84, 0 | Redness at the base | Ovate and broad | Symmetric | Symmetric | Symmetric | One pair starts from mid rib and other pairs start from the base or One pair starts from mid rib, some pairs start in-between and other pairs start from the base | Flexuous | No |
| *Maneru* | 36, 110, 36 | Plain | Ovate and elongated | Asymmetric | Asymmetric | Asymmetric | | Stout and smooth | |
| *Maneru-yellow* | 88, 198, 6 | | | | | | | | |
| *Nagawalli-white* | 36, 110, 36 / 200, 210, 131 | Whitish patch starting from the base | | | | | One pair starts from mid rib and other pairs start from the base | | |
| *Nagawalli-yellow* | 36, 110, 36 / 193, 192, 70 | Yellowish patch starting from the base | Ovate and broad | Symmetric | Symmetric | Symmetric | | | Yes |
| *Ratadalu* | 83, 136, 44 | Plain | Ovate and elongated | Asymmetric | Asymmetric | Asymmetric | | | No |

*The Red, Green and Blue (R, G, B) values are indicated for the color of the leaf as given by Adobe® Photoshop® Software. The color represented by RGB values are given as the shades of the respective cells. *Nagawalli-white* and *Nagawalli-yellow* got variegated leaves.

[$]The specific features in addition to the prominent green color are indicated.

The mean leaf width was significantly highest in *Malabulath* (15.76 cm) and other five *P. betle* cultivars got significantly similar mean leaf widths (8.48 cm to - 10.57 cm). Similarly mean leaf length was also highest in *Malabulath* (19.01 cm) and other five cultivars got significantly similar mean leaf lengths (14.40 cm to 15.75 cm). Number of veins were significantly higher in the cultivars *Maneru*, *Malabulath*, *Nagawalli-white* and *Ratadalu* (mean number of veins = 9) and lower in *Maneru-yellow* and *Nagawalli-yellow* (7.44 and 8.22 respectively). The mean highest petiole length was observed in *Nagawalli-yellow* (3.93 cm) and lowest in *Maneru-yellow* (2.05 cm). The mean inter-nodal length was significantly highest in *Malabulath, Nagawalli-white* and *Maneru* (9.39 cm, 7.08 cm and 6.92 cm respectively) and significantly lowest in *Ratadalu* (3.90 cm) ($P < 0.05$) (Table 3). There was a significant and highest correlation observed among length and width of leaves (PCC = 88 %). Number of veins was also significantly and positively correlated with leaf width (PCC = 37 %). Inter-nodal length was also significantly correlated to the leaf width (PCC = 35 %) and to the number of veins (35 %). However, number of veins and petiole length were not significantly correlated with other parameters measured (Table 4) ($P < 0.05$). The images of the matured-leaves of the six cultivars are shown in Figure 1.

**Table 3** Morphological variation of matured-leaves and inter-nodal length among *P. betle* cultivars

| Cultivar | Leaf width (cm) | Leaf length (cm) | Number of veins | Petiole length (cm) | Inter-nodal length (cm) |
|---|---|---|---|---|---|
| *Malabulath* | 15.76$^{a}$ | 19.01$^{a}$ | 9.00$^{a}$ | 2.44$^{b}$ | 9.39$^{a}$ |
| *Maneru* | 9.96$^{b}$ | 14.40$^{b}$ | 9.00$^{a}$ | 2.70$^{b}$ | 6.92$^{b}$ |
| *Maneru-yellow* | 8.48$^{b}$ | 14.55$^{b}$ | 7.44$^{b}$ | 2.05$^{b}$ | 4.71$^{c}$ |
| *Nagawalli-white* | 10.44$^{b}$ | 15.44$^{b}$ | 9.00$^{a}$ | 2.84$^{b}$ | 7.08$^{b}$ |
| *Nagawalli-yellow* | 10.57$^{b}$ | 15.67$^{b}$ | 8.22$^{b}$ | 3.93$^{a}$ | 4.73$^{c}$ |
| *Ratadalu* | 10.00$^{b}$ | 15.75$^{b}$ | 9.00$^{a}$ | 2.20$^{b}$ | 3.90$^{c}$ |

Means denoted by the same letters within the column are not significantly different at $P < 0.05$.

**Table 4** Pearson's Correlation Coefficients (PCC) among leaf traits and inter-nodal length of *P. betle* cultivars

| | Leaf length | Number of veins | Petiole length | Inter-nodal length |
|---|---|---|---|---|
| **Leaf width** | 88%* | 37%* | 12% | 45%* |
| **Leaf length** | | 31% | 8% | 27% |
| **Number of veins** | | | 9% | 35%* |
| **Petiole length** | | | | 21% |

*Pearson's correlation coefficient is significant at $P < 0.05$.

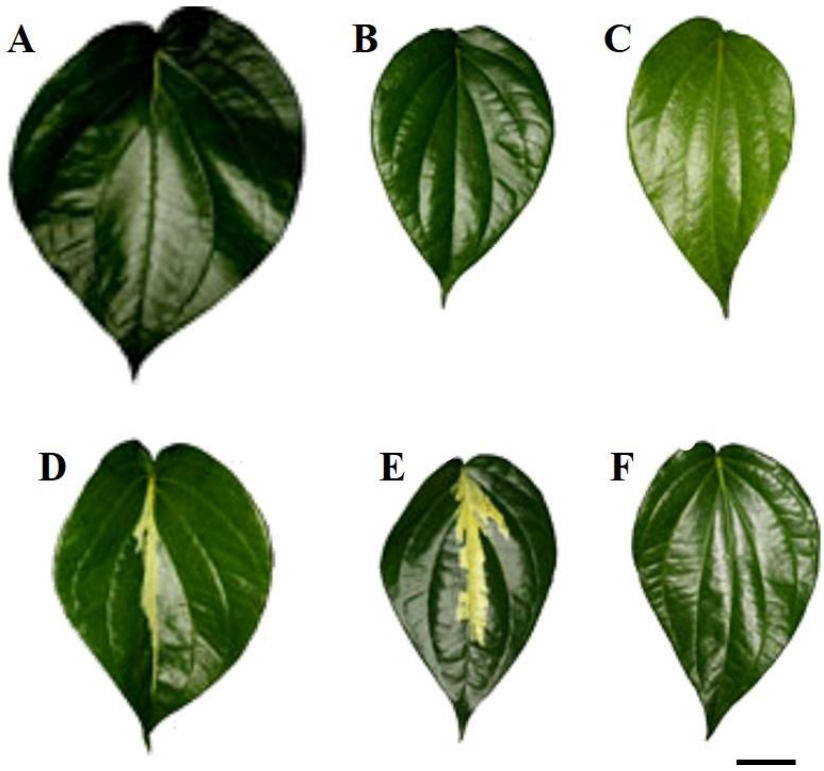


**Figure 1** Representative images of the matured-leaves of six *P. betle* cultivars. A: *Malabulath*, B: *Maneru*, C: *Maneru-yellow*, D: *Nagawalli-white*, E: *Nagawalli-yellow*, F: *Ratadalu*. Scale bar represents 3 cm.

The regression analysis between leaf length and width clearly placed *Malabulath* separately compared to the leaves of other cultivars indicating that *Malabulath* has the largest matured-leaves (Figure 2). The dendrogram constructed based on the leaf length and width values also proved that the leaf size of the *Malabulath* was different from the rest of the cultivars. A total of three clusters (C1, C2 and C3) were observed based on the leaf size similarities. C1 contains *Ratadalu*, *Nagawalli-yellow* and *Nagawalli-white*, C2 contains *Maneru-yellow* and *Maneru* whereas C3 contains *Malabulath* only (Figure 3). The regression analysis between the inter-nodal length and leaf petiole length indicated that the cultivar *Ratadalu* got shortest nodes and petiole length whereas *Malabulath* got longest internodes and shorter petiole lengths. *Nagawalli-yellow* got the longest petiole length (Figure 4).

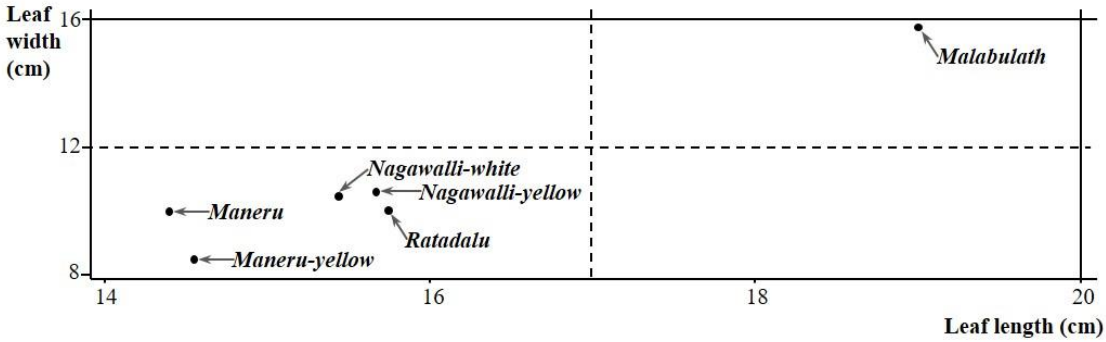


**Figure 2** The scatter plot showing the matured-leaf length and width differences of six betel cultivars. Arrows are used to clearly label the cultivar position with names.

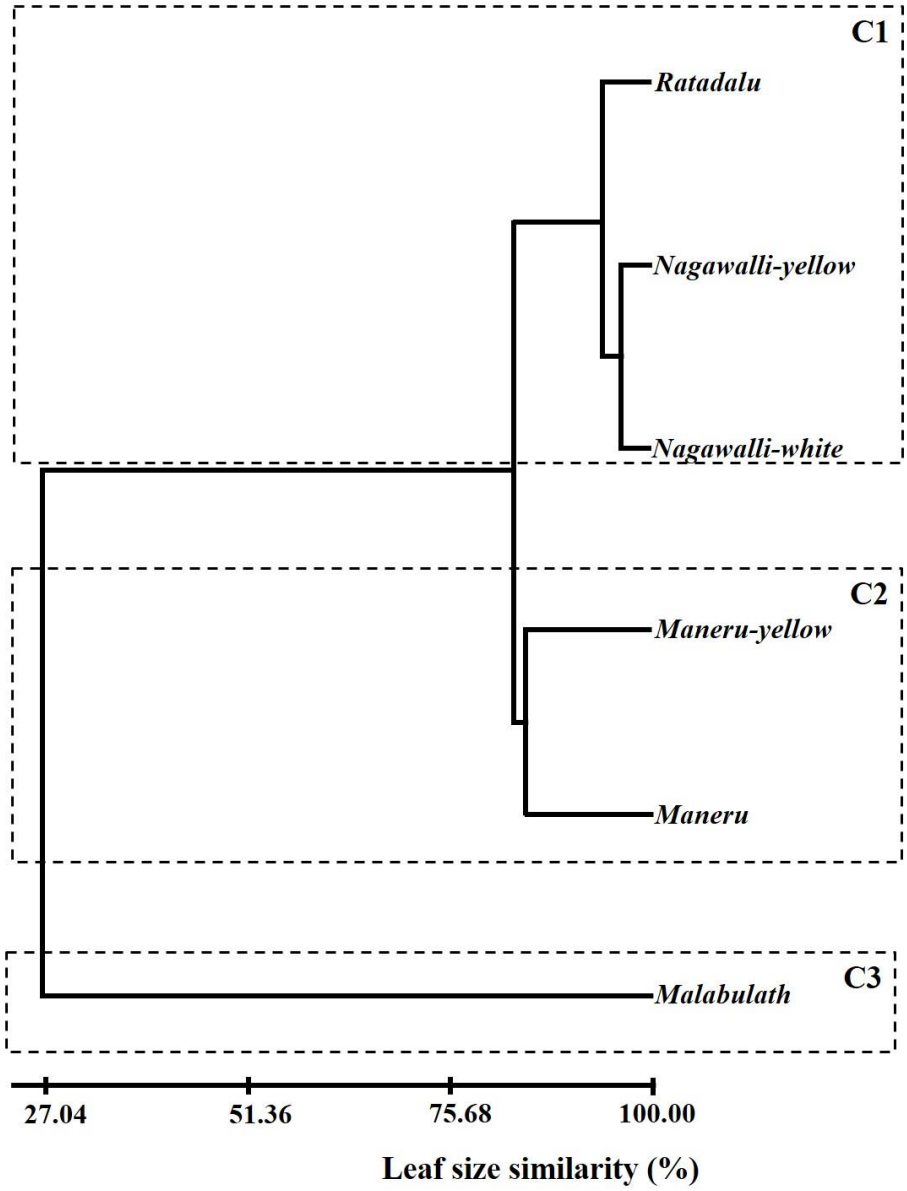


**Figure 3** Dendrogram showing the differences/similarity of matured-leaf size among six *P. betle* cultivars. The dendrogram was constructed based on the leaf length and width parameters. C1, C2 and C3 are the three major clusters obtained.

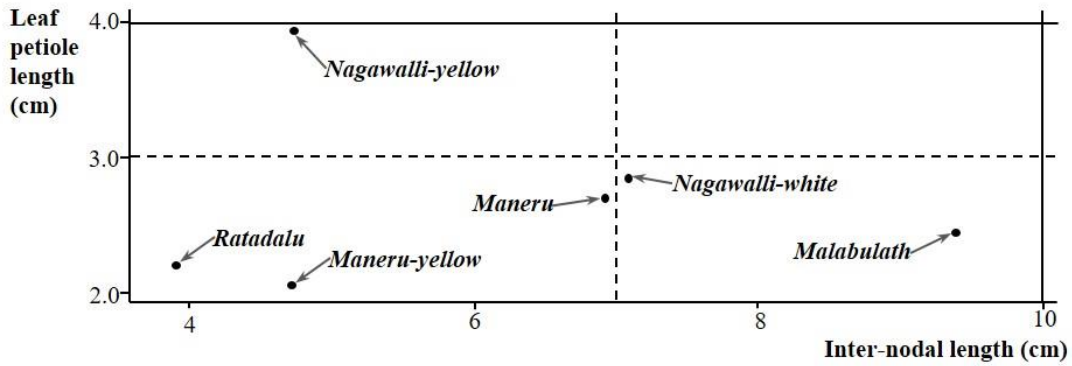


**Figure 4** The scatter plot showing the inter-nodal length and petiole length of matured-leaves of six betel cultivars. Arrows are used to clearly label the cultivar position with names.

### 3.2 Phytochemical analysis

The relative flavonoid content was different among the matured-leaves of six betel cultivars. According to the diluted ammonia test, the cultivars *Malabulath*, *Maneru-yellow* and *Nagawalli-yellow* got the higher flavonoid content whereas according to the concentrated sulfuric test, the cultivar *Malabulath*, *Maneru* and *Nagawalli-yellow* got the higher flavonoid content. If the two tests are combined, the cultivars *Malabulath* and *Nagawalli-yellow* got the higher flavonoid content whereas the cultivar *Nagawalli-white* and *Ratadalu* got lower flavonoid content. The highest level of tannin was observed in *Maneru-yellow* and *Nagawalli-white* whereas lowest level was observed in *Ratadalu*. The highest terpenoid content was observed in *Maneru* and least in *Maneru-yellow*. The content of reducing sugars was highest in *Maneru-yellow* and least in *Malabulath*. The phlobatannin content was highest in *Malabulath* and *Ratadalu* and least in *Maneru-yellow*. The froth forming test indicated that all cultivars got saponins (Figure 5 and Table 5). The mean ascorbic acid concentration was significantly highest in *Nagawalli-white* (0.00167 M) and least in *Malabulath*, *Nagawalli-yellow* and *Ratadalu* ($P < 0.05$) (Table 5).

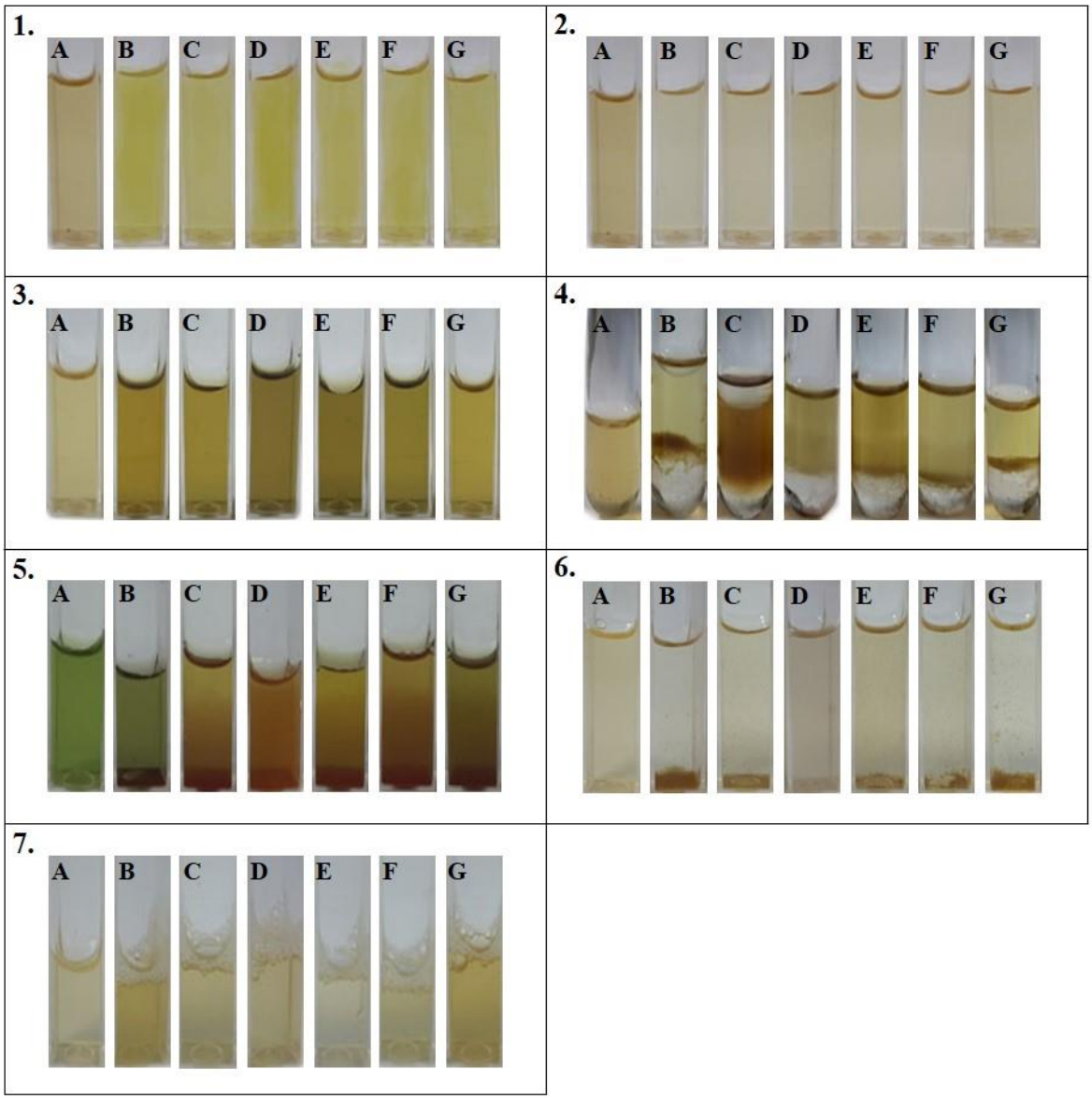


**Figure 5** The qualitative results obtained in phytochemical analysis. 1: Diluted $NH_3$ test for flavonoids 2: Concentrated $H_2SO_4$ test for flavonoids 3: $FeCl_3$ test for tannins 4: Salkowski test for terpenoids 5: Benedict's test for reducing Sugars 6: Diluted HCl test for Phlobatannins 7: Froth forming test for Saponins. In test 1, 2, 3, 5, 6 and 7 cuvettes were used to make the solutions whereas in test 4, larger glass tubes were used. Only portions of cuvettes and glass tubes are shown. A: Positive control (Standard sample) B: *Malabulath* C: *Maneru* D: *Maneru-yellow* E: *Nagawalli-white* F: *Nagawalli-yellow* G: *Ratadalu*

**Table 5** Detection of key phytochemicals in matured-leaves of betel cultivars

| Cultivar | Flavonoids | | Tannins | Terpenoids | Reducing Sugars | Phlobatannins | Saponins | Ascorbic acid conc. (M) |
|---|---|---|---|---|---|---|---|---|
| | (Dil. $NH_3$ test) | (Conc. $H_2SO_4$ test) | ($FeCl_3$ Test) | (Salkowski test) | (Benedict's test) | (Dil. HCl test) | (Froth forming test) | (Iodine test) |
| *Malabulath* | 2 | 2 | 2 | 4 | 2 | 4 | + | $0.00070^{d}$ |
| *Maneru* | 1 | 2 | 2 | 5 | 3 | 2 | + | $0.00103^{c}$ |
| *Maneru-yellow* | 2 | 1 | 3 | 1 | 5 | 1 | + | $0.00143^{b}$ |
| *Nagawalli-white* | 1 | 1 | 3 | 2 | 3 | 2 | + | $0.00167^{a}$ |
| *Nagawalli-yellow* | 2 | 2 | 2 | 2 | 3 | 3 | + | $0.00077^{d}$ |
| *Ratadalu* | 1 | 1 | 1 | 3 | 4 | 4 | + | $0.00057^{d}$ |

For flavonoids, tannins, terpenoids and reducing sugars, the relative color development (i.e. concentration) was identified as 1→5 rank scores according to the color intensities of the solutions indicated in the Figure 5 (1: least and 5: highest). For phlobatannins, the size of the precipitate was observed and ranked from 1 to 4 (1: smallest and 4: largest). For saponins, formation of froth or otherwise were ranked as positive and negative respectively. The ascorbic acid concentration was measured using iodine titrimetric method.

In the last column, means denoted by the same letters within the column are not significantly different at $P < 0.05$.

### 3.3 Antibacterial activity

The assessment of antibacterial activity of the water extracts of matured-leaves of betel demonstrated that each betel cultivar exhibited significant bactericidal effect on three model pathogenic bacteria. However, *E. coli* was significantly less affected (mean DZBI of 0.49 cm) compared to MR *S. aureus* and *S. aureus* (mean DZBI of 0.64) ($P < 0.05$) (Table 6). All six betel cultivars exhibited significantly similar activities on three model pathogens collectively (Table 7). The detailed DZBI values and their variances for each betel cultivar on three bacterial species are given in Figure 6 and bacterial culture plates that were undergone disc diffusion assay are displayed in Figure 7.

**Table 6** Overall antibacterial activity of betel cultivars on three model bacterial pathogens

| Species | DZBI (cm) |
|---|---|
| *E. coli* | $0.49^{b}$ |
| MR *S. aureus* | $0.64^{a}$ |
| *S. aureus* | $0.64^{a}$ |

Means denoted by the same letters within the column are not significantly different at $P < 0.05$.

**Table 7** Antibacterial activity of six betel cultivars collectively

| Cultivar | DZBI (cm) |
|---|---|
| *Malabulath* | $0.70^{a}$ |
| *Maneru* | $0.66^{a}$ |
| *Maneru-yellow* | $0.68^{a}$ |
| *Nagawalli-white* | $0.71^{a}$ |
| *Nagawalli-yellow* | $0.70^{a}$ |
| *Ratadalu* | $0.66^{a}$ |

Means denoted by the same letters within the column are not significantly different at $P < 0.05$.

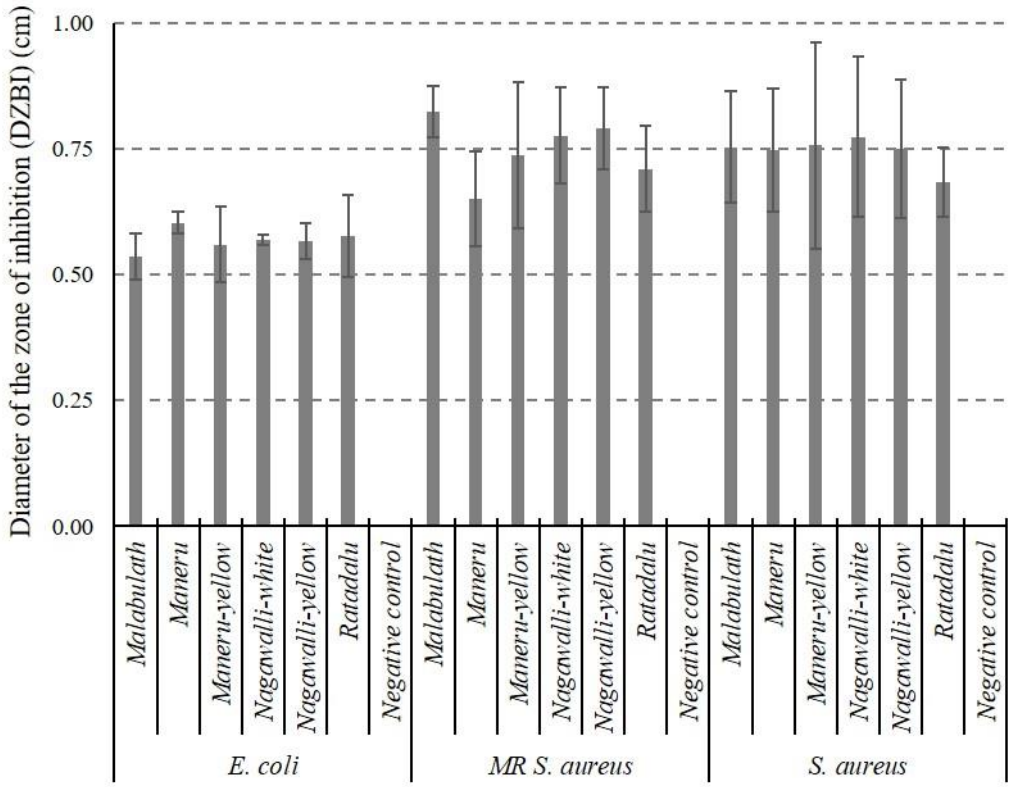


**Figure 6** Antibacterial activity exhibited by water extracts of matured-leaves of betel cultivars in disc diffusion assay against three model pathogenic bacteria. The error bars indicate the ± standard deviation.

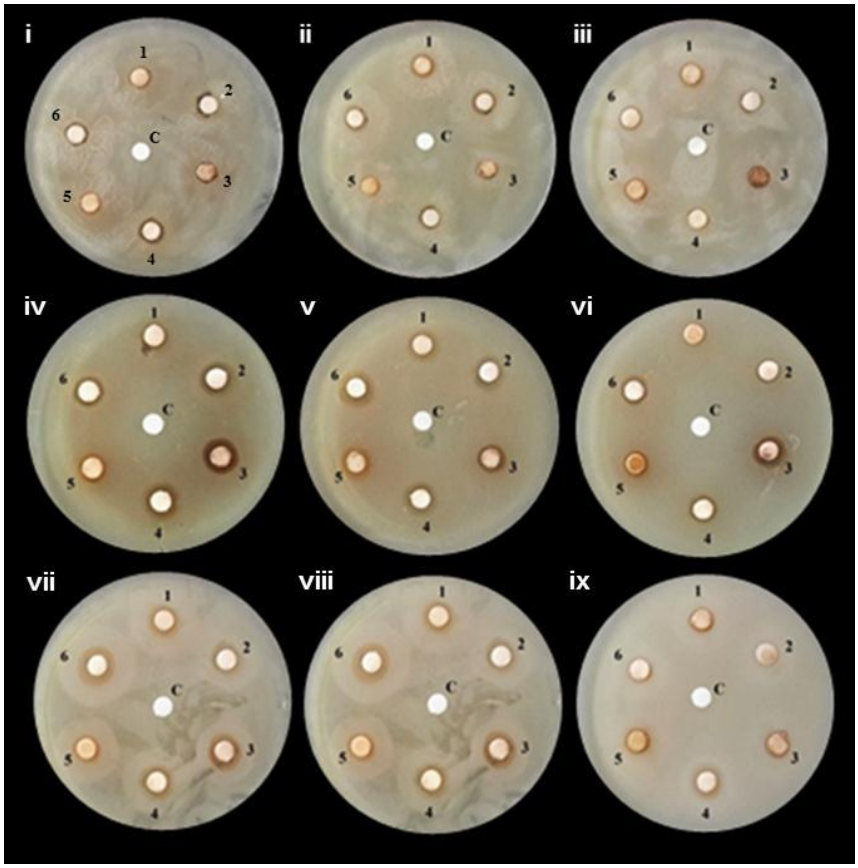


**Figure 7** Antibacterial activity of the water extracts of matured-leaves of betel. The top views of the petri dishes are shown. i, ii and iii: *E. coli*. iv, v and vi: Methicillin resistant (MR) *S. aureus*. vii, viii and ix: *S. aureus*. 1 to 6 are paper discs moistened with water extracts of betel leaves. 1: *Maneru*, 2: *Maneru-yellow*, 3: *Malabulath*, 4: *Nagawalli-white*, 5: *Ratadalu*, 6: *Nagawalli-yellow*. C: a paper disc moistened with distilled water (i.e.negative control).

## 4. Discussion

Betel is one of the most important medicinal plants extensively grown and used in Asian and South Asian countries[36]. The morphological diversity of matured-leaves and vines of six betel cultivars were assessed in the present study. The plant material was collected from Intercropping and Betel Research Station, Sri Lanka because it is hard to collect authenticated and reliable cultivars from the farming fields and often growers do not know exactly the name or type of cultivar that they grow. There are 12 betel cultivars reported to be grown in the country[26] however the selected six cultivars in the present study were available for accurate sampling within the Intercropping and Betel Research Station. The color of the matured-leaves was different among the cultivars indicating the possible carotene pigmentation within the leaves. However, yellowish green color is not a desirable trait in the betel export market. The dark green coloration was observed in *Malabulath* compared to the other non-variegated cultivars *Maneru* and *Ratadalu* (Table 2). However, the presence of red coloration at the base of *Malabulath* leaves is also an undesirable trait in leaf export market as customers look for plain dark green colored leaves for consumption, religious and cultural purposes. The far-mers, merchants and consumers identify the matured leaves by looking at the presence of asymmetric leaf base and if the leaf base is symmetric they just identify such leaves as stem-leaves. Therefore, *Maneru-yellow*, *Nagawalli-white* and *Ratadalu* are better cultivars because their leaf base is always asymmetrical. The vine texture of *Malabulath* is flexuous making it less useful in vine management and harvesting. However, all other five cultivated betel cultivars have stout and smooth texture in vines making the vine management and harvesting easier. The rooting at nodes was observed in *Nagawalli-yellow* which would be a desirable trait in generating planting material. However, excessive rooting at nodes would cause difficulties in pest and disease management. The largest leaves were observed in *Malabulath* and longest petiole length was observed in *Nagawalli-yellow* (Table 3) ($P < 0.05$). Shorter petiole lengths were observed in all the other betel cultivars. Higher petiole length is a desirable trait in harvesting in which farmers use their nails of thumb and index fingers to pluck the leaves. The longest inter-nodal length was observed in *Malabulath* compared to others and the inter-nodal length was positively and significantly correlated with leaf width (Table 4). This implies that higher inter-nodal lengths cause larger leaves and shorter internodes cause narrower leaves which is an adaptation to avoid mutual shading and an adaptive geometry to maintain balanced structure of the vines[37]. However, under intensive management systems, shorter internodes are preferred as they yield many leaves compared

to that of longer internodes. Overall *Malabulath*, a cultivar that is not used for chewing, has significantly largest leaves which is a highly desirable trait in betel industry (Table 3) (Figures 1, 2 and 3).

The present study attempted to qualitatively characterize the presence of flavonoids, tannins, terpenoids, reducing sugars, phlobatannins, and saponins. The highest concentration of terpenoids was observed in *Maneru* followed by *Malabulath* and phlobatannins in *Malabulath* and *Ratadalu*. The saponins were present in all the cultivars according to the froth forming test. The highest ascorbic acid concentration was observed in *Nagawalli-white* which is a highly desirable trait in food and drug formulations. These qualitative assessment details are important to select the cultivars for industrial and breeding purposes but the quantification of the concentration of each phytochemical is required under particular agro-ecological conditions before they are used in practical purposes. Because, the relative concentrations of the phytochemicals are varied based on the soil, agronomic and microclimatic conditions[6,17]. In the present study, the relative presence of these phytochemicals was reported under the conditions available at Intercropping and Betel Research Station, Sri Lanka.

A significant antibacterial activity was reported against Gram negative *E. coli* and Gram positive Methicillin resistant *S. aureus* and *S. aureus*. It is obvious that gram positive *E. coli* was less affected compared to other two because of the differences in the structure of the cell membrane. However, it is interesting to note that both MR *S. aureus* and *S. aureus* were equally inhibited by betel cultivars (Table 6) indicating that molecular mechanism of bacterial inhibition conferred by leaf water extracts of betel is different from methicillin. If the leaves of betel cultivars are to be used in antibacterial formulations such as mouth washes, body lotions and herbal drugs, further studies are required using target pathogens and the betel leaves grown under different agro-ecological conditions. The broad spectrum antibacterial activity of betel leaf extracts has been extensively reported against many pathogenic bacteria[21-23] in addition to the three species studied in this research. The characterization of antibacterial activity against model pathogens is a prerequisite to specifically assess the inhibitory activity against specific pathogens because if unique and locally available pathogens are used straightaway for characterization, the results cannot be compared with published parallel literature.

## 5. Conclusion

The assessment of the morphological diversity of matured-leaves and vines of six betel cultivars revealed that the wild cultivar *Malabulath* has largest leaves and longest internodes whereas, shortest petiole length was observed in *Maneru-yellow*. The inter-nodal length and the leaf width were found to be positively and significantly correlated. The qualitative phytochemical analysis revealed that all tested cultivars contain flavonoids, tannins, terpenoids, reducing sugars, phlobatannins and saponins. The ascorbic acid concentration was highest in *Nagawalli-white*. The leaf water extracts of all the cultivars exhibited significantly higher antibacterial activity against model pathogens *E. coli*, MR *S. aureus* and *S. aureus*. The information generated in the present study would provide a strong platform to select betel cultivars for breeding and industrial applications in the future.

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