
RESEARCH ARTICLE

Pharmacognostic Evaluation and *In-Vitro* Anti-Candidal Activity of Young Leaves of Common *Piper betle* Types in Sri Lanka

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Highlights

- Sri Lankan *Piper betle* cultivars exhibited only minor morphological and anatomical variations
 - All *Piper betle* cultivars were rich in secondary metabolites, indicating strong phytochemical potential
 - *Piper betle* leaf extracts demonstrated broad-spectrum anti-candidal activity
 - Extraction efficiency was solvent-dependent, with certain solvents yielding superior anti-candidal activity
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Pharmacognostic Evaluation and *In-Vitro* Anti-Candidal Activity of Young Leaves of Common *Piper betle* Types in Sri Lanka

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Abstract

Piper betle is an evergreen, semi-woody climber possessing various pharmacological properties. The current study evaluates the morphological, anatomical, and phytochemical characteristics, as well as the antifungal activity, of the young leaves of common betel types in Sri Lanka: 'Maneru', 'Ratadalu', 'Narammali', 'SuduNagawalli', and 'KahaNagawalli'. Plant samples were collected from the Intercropping and Betel Research Station at Narammala, Sri Lanka. Morphological and anatomical characteristics were compared using the second leaf from the apical bud. Phytochemical screening of the crude ethanolic extract was performed using thin-layer chromatography. *In-vitro* anti-candidal activity and Minimum Inhibitory Concentrations were investigated against standard cultures of *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis*. Ethanol, Chloroform, Ethyl acetate, Iso-propyl alcohol and Methanol were used for extraction of betel crude and to determine the anti-candidal activity. Variation among betel types in leaf shape, base, odor, apex, margin, and the length-to-width ratio was found to be marginal. Anomocytic stomatal arrangement, vein islet number, vein termination, and stomatal index were similar for all types. Oil bodies were observed in the lower surface of 'KahaNagawalli', while the other cultivars possessed oil bodies in both the lower and upper surfaces of the leaf. All betel types contained flavonoids, tannins, phenolic compounds, terpenoids, and alkaloids. Extracts of all betel types showed significant anti-candidal activity against *Candida* sp. at 6.4 mg/mL concentration. Ethyl acetate, Chloroform, and Methanol extracts of *P. betle* were more effective as anti-candidal agents compared to Isopropyl alcohol and ethanolic betel crude extracts.

keywords: *Candida* sp.; Extractions; *in-vitro* anti-candidal activity; Pharmacognostic evaluation: *Piper betle*

INTRODUCTION

Piper betle is an evergreen perennial vine with glossy, heart-shaped leaves that belongs to the family Piperaceae. Cultivation of this plant has vast potential in South Asian countries, especially in Sri Lanka, Thailand, India, and Taiwan (Dwivedi & Tripathi, 2014). In Sri Lanka, the plant is widely cultivated in the districts of Kurunegala, Gampaha, and Colombo for export purposes. The plant has a long-lasting history in traditional Ayurveda uses as *Deshiya Chikitsa* or *Sinhala Vedakama*, which, in the course of time, got abridged with the North Indian Ayurveda system of medicine. The leaves of this plant have

traditionally been used for the treatment of various diseases (Rai et al., 2011).

The plant has several pharmacognostic properties, including anti-microbial, anti-coagulant, anti-ulcerogenic, radioprotective, anti-inflammatory, hepato-protective, anti-allergic, antioxidant, antidiabetic, gastroprotective, immunomodulatory, anti-photosensitizer, and anti-cancer potential (Arawwawala et al., 2014). Therefore, *P. betle* has also been tested for the development of neuropharmacological as well as oral health care agents. Various biologically active compounds possessed by *P. betle* are responsible for its numerous pharmacological

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actions (Chauhan et al., 2016). It contains several primary and secondary metabolic compounds such as tannin (0.1-1.3%), starch and sugars (0.5-6.1%), proteins (3-3.5%), fat (0.4-1%), fibers (2.3%), vitamins, minerals and essential oils (0.08-0.2%) etc. in different plant parts (Guha, 2006). Leaves also possess a wide array of biologically active chemical compounds and enzymes in different plant parts, including diastases and an essential oil composed of terpinen-4-ol, saffrole, allyl-pyrocatechol monoacetate, eugenol, eugenyl acetate, hydroxyl chavicol, eugenol, piper betel. and betle oil, which contains cadinene, carvacrol, allyl catechol, chavicol, p-cymene, caryophyllene, chavibetol, cineole, quercetin, estragol, etc. as the key components (Umar et al., 2018). Therefore, the leaf has a significant antimicrobial activity against a broad spectrum of microorganisms (Hossain et al., 2017). The investigation has identified that hydroxichavicol, carvacrol, eugenol, and chavicol from *P. betel* are shown to exhibit antifungal activity as they increase the permeability of fungal membranes (Sharma, 2009). Researchers found that most organic extracts of *P. betel* leaf have potent activity against the yeasts of *Candida albicans* (Saini et al., 2016). Composition and concentrations of biologically active compounds depend on the variety of the plant, the season, and the climate (Umar et al., 2018). In addition, the relative amounts of secondary metabolic compounds differ according to the stage of maturity of the plant.

Anti-candidal activity has been reported in a previous study on ethanolic crude extracts of *P. betle* leaves without recording the *P. betle* cultivars in Sri Lanka (Nanayakkara et al., 2014). Existing *P. betle* cultivars in Sri Lanka include 'Narammali', 'SuduNagawalli', 'KahaNagawalli', 'Maneru', 'Ratadalu', 'Galdalu', and 'Mala-bulath'. 'Narammali' is a betel type that was developed from selecting the best type of 'Maneru' collected from different localities in Sri Lanka. Plant material authentication is essential before its use in a herbal formula. Thus, the present study aims to evaluate the morphological, anatomical, phytochemical characteristics, and the anti-candidal activity of young leaves of common betel types in Sri Lanka using standard isolates of the *Candida*; *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilopsis* and *C. tropicalis*.

MATERIALS AND METHOD

Collection of Plant Material - *Piper betle*

Young leaves (second leaf from the shoot apex) were collected from five of the betel cultivars, 'KahaNagawalli' (K), 'Maneru' (M), 'Narammali' (N), 'Ratadalu' (R), and 'SuduNagawalli' (S) available at the intercropping betel research station at Narammala (Kurunegala district), and one betel cultivar ('Koul bulath' type) was collected from Gelioya (Kandy district), Sri Lanka, which was previously studied by Nanayakkara et al. (2014). Leaves of all betel cultivars that were devoid of any pathogenic attacks were collected on the same day.

Organoleptic, morphological, microscopic, and phytochemical evaluation of young *Piper betle* leaves

Various sensory parameters of fresh leaf samples, including color, odor, shape, size, and taste, were studied by organoleptic evaluation (Pandya et al., 2010). Various macroscopic characteristics of fresh leaves of the species, *P. betle*, were recorded, such as the type of leaf base and characteristics of the lamina. Lamina consists of characteristic features such as composition, incision, shape, venation, margin, apex, base, surface, and texture.

Microscopic studies were conducted on both qualitative and quantitative grounds. The qualitative microscopy was evaluated by studying various microscopic characteristics with or without staining and micrographs. Transverse and longitudinal sections of betel leaf cultivars were studied under a photomicrograph. Staining reagents (such as safranin, lignified cell walls, and methyl orange; oil bodies) were used as per standard procedures (Ali, 2008; Biradar, 2013; Kokate, 2005; Pandya et al., 2010; Wallis, 1985).

Quantitative microscopy of the *P. betle* leaves was evaluated by vein islet number, vein termination number, stomatal index, and stomatal arrangement. Stomatal number is the average number of stomata per square millimeter of epidermis. The percentage proportion of stomata per total stomatal and epidermal cells per square millimeter of epidermis is termed the stomatal index. Stomatal index can be calculated by using the following equation: $I = S / E + S \times 100$, Where, I = stomatal index, S = number of stomata per mm² and E = number of ordinary epidermal cells per mm².

A cleaned leaf piece was peeled, mounted, and observed under a camera lucida. Epidermal cells and stomata were traced, and stomatal number and index were calculated. The number of stomata was counted as the stomatal number, and the stomatal index was calculated separately for upper and lower surfaces, using the above formula.

Vein islets are small photosynthetic areas enclosed by conducting strands, while the vein termination number refers to veinlet terminations per mm² of leaf surface. A leaf piece was cleared in 10% KOH overnight, then observed under a camera lucida. A 1 mm square was drawn, and vein islets and terminations were traced, counted, and averaged from four adjoining squares. Illustrations and photomicrographs were captured using an Olympus CX 31 microscope.

Preparation of ethanolic extracts of betel cultivars

Well-cleaned and air-dried immature (young) leaves of *P. betle* cultivars were cut into pieces (0.5 cm×0.5 cm), and 20 g of each sample was measured. The sample was soaked in 200 ml of 99% (absolute) ethanol at room temperature (25±1°C), separately. Using the vacuum infiltration technique, the plant parts were shaken with the same aliquot of alcohol in a magnetic stirrer at a speed of 3 revolutions per second, at room temperature for 1 hour. Subsequently, the obtained extract was filtered through a Whatman No.1 filter paper, and the filtrate was collected. To remove ethanol, the extract was concentrated by evaporating the

ethanol in the sample at 40 °C overnight until a constant weight. The crude extract was stored at 4 °C until further use. The above procedure was repeated for all the betel cultivars studied.

Candida isolates

Five standard cultures of *Candida* species, namely *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019), and *Candida tropicalis* (ATCC 13803) were obtained from the Faculty of Dental Sciences, University of Peradeniya. All the *Candida* species were sub-cultured on sterile Mueller-Hinton Agar (MHA) plates separately at 37 °C for 24 hours. The resultant colonies were kept at 4 °C for short-term storage.

Agar well diffusion bioassay for determining the anti-candidal activity of the crude extracts of young leaves of common *P. betle* cultivars in Sri Lanka

For the well diffusion bioassay, fresh *Candida* suspension were prepared by suspending overnight colonies in sterile saline, adjusting turbidity to McFarland 0.5 standard ($\sim 10^7$ – 10^8 CFU/ml). Sterile Mueller-Hinton Agar (MHA) plates were prepared, and 2 ml of each suspension was spread evenly before drying at 44 °C. Using an 8 mm cork borer, wells (5 mm diameter, 4 mm depth) were created and labeled for different betel leaf extracts, controls (Fluconazole, DMSO), and previously tested cultivars. Ethanolic extracts (600 mg in 6 ml DMSO) and water extracts (200 μ l each) were loaded into wells. Plates were incubated at 37°C for 24 hours, and anti-candidal activity was assessed by measuring inhibition zones.

Determination of the Minimum Inhibitory Concentration (MIC) of the ethanolic extract of young leaves of common *P. betle* cultivars in Sri Lanka

All the tested ethanolic extracts of betel cultivars were subjected to the determination of the minimum inhibitory concentration MIC, as all of them showed significant anti-candidal activity. It was determined using the British Society for Antimicrobial Chemotherapy (BSAC) method, modified by Andrews (2005), which was followed by Nanayakkara et al. (2014).

Determination of the inhibition activity of other crude extracts

In addition to ethanol, several other solvent types, such as chloroform, ethyl acetate, isopropyl alcohol, and methanol, were also used for the extraction and preparation of betel crude extracts of betel leaves following the method. The 'Ratadalu' betel type was used for extraction and crude preparation. Finally, crude extracts from different solvents were used to assess the growth of the *Candida* sp. tested under the obtained MIC value and the lower value.

Statistical Analysis

Data obtained from the well diffusion bioassay were subjected to statistical analysis of variance using the Mini Tab software version 16.0. Mean separation test was performed with General Linear Model in ANOVA and Turkey test to determine whether there is any significant difference between the anti-candidal activity of different betel types. All statistical analyses were carried out at 95 % confidence level.

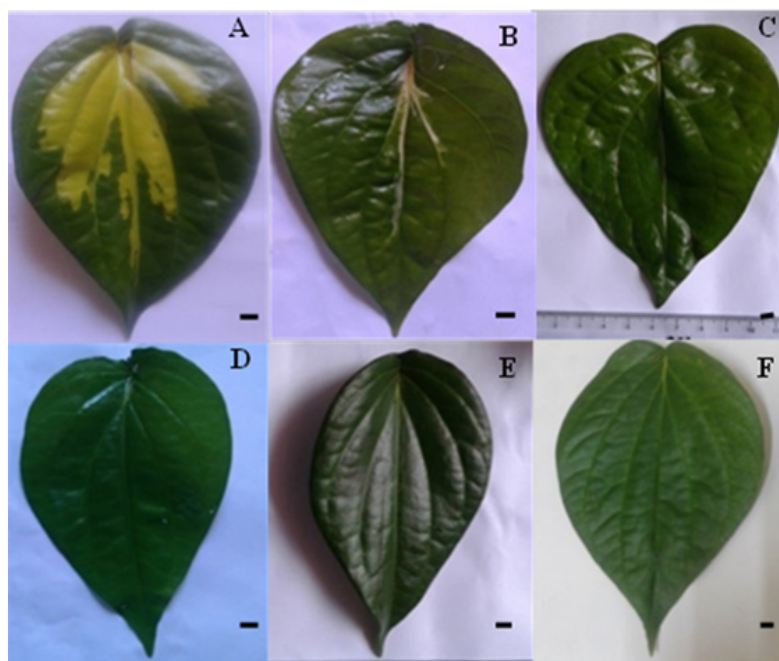


Figure 1: Available betel cultivars in Sri Lanka. A) 'KahaNagawalli' B) 'SuduNagawalli' C) 'Maneru' D) 'Narammali' E) 'Ratadalu', and F) Previously tested betel type (not distinguished as a cultivar) which was collected from the Gelioya region in Kandy District. Scale bar represents 1cm. (All cultivars were collected from Intercropping and Betel Research Station at Narammala, Kurunegala)

The macroscopic evaluation involved the study of morphological characteristics of the betel cultivars studied, along with the previously tested betel-type young leaves. Table 1 illustrates some organoleptic characteristics and morphology. The composition of lamina was found to be simple, pinnate reticulate venation, and a symmetric petiole for all studied cultivars and the previously tested

betel type. Both the leaf apex and leaf base varied from acute to acuminate. Leaf surfaces were shiny for all leaf types, but in different amounts. The leaf margin varied from undulate to entire. The length-to-width ratio of each leaf type slightly varies from one to another. Therefore, the morphology of each cultivar was slightly different from that of the others (Table 1).

Table 1: Organoleptic characteristics and morphology of young *Piper betle* leaves of common cultivars in Sri Lanka

Morphology	Betel variety					
	‘Kaha Nagawalli’	‘Maneru’	‘Narammali’	‘Ratadalu’	‘Sudu Nagawalli’	Previously tested betel
Color	Dark green with a yellow color line through the mid-vein	Light green	Green	Dark green	Dark green with a white color line through the mid vein	Deep green
Shape	Abcordate	Cordate	Ovate	Ovate	Abcordate	Cordate to ovate
Base	Rounded	Cordate	Rounded	Rounded	Rounded	Asymmetric
Apex	Acuminate	Acute	Acute	Acuminate	Acuminate	Caudate
Upper surface	Shining	Shining	Shining	Shining	Shining	Less shining
Odor	Pungent	Fragrant	Fragrant	Fragrant	Pungent	Less odor
Margin	Undulate	Undulate	Entire	Entire	Undulate	Entire
Length to width ratio	1.47	1.83	1.62	1.65	1.37	1.50

For microscopic analysis, qualitative characteristics were studied by observing a transverse section of the leaf (Figure 2). The upper epidermis consisted of small polygonal parenchymatous cells, which appeared wavy in outline after the epidermis. The chlorenchyma cells were seen as upper palisade and lower spongy tissue. The cortex consisted of 4 to 5 layers of parenchymatous cells in the midrib region. The vascular bundle was ovoid in shape, and a zone of sclerenchymatous tissues was present below it. In between the upper epidermis and the vascular bundle, 5 to 6 layers of irregularly shaped collenchyma cells were present. Oil glands were also present between them and were detected by red color when treated with methyl orange. ‘SuduNagawalli’ has oil bodies at the lower surface, whereas ‘Narammali’, ‘Maneru’, ‘Ratadalu’, and ‘KahaNagawalli’ have oil bodies in both lower and upper leaf surfaces. Other than the leaf epidermis, the midrib region contained oil bodies. Lignified Xylem cells were seen on the upper side, whereas phloem was seen towards the lower side of the epidermis. The uniseriate covering and multicellular trichomes were present on both surfaces of the leaf, more along the midrib region and less along the lamina. The covering trichomes were simple, uniseriate, unbranched, and unicellular with a blunt apex and smooth walls (Figure 2). ‘KahaNagawalli’ had a higher number

of epidermal hairs on both upper and lower leaf surface respectively.

In quantitative microscopy, Anomocytic types of stomata were present on the lower surface of leaves. Vein islet number, vein termination number, and stomatal index were similar for all types. The stomata were not observed in the upper surface of the leaves in all betel cultivars. The stomatal index of the lower surface was found to range from 8 -12, and the vein islet and vein termination were calculated to be 2-5 and 3-6, respectively, for all the tested cultivars (Table 2).

Thin Layer Chromatography (TLC) profiling of plant extracts in different solvent systems confirmed the presence of diverse groups of phytochemicals. According to the phytochemical screening of ethanolic crude extracts, two classes of compounds, flavonoids and tannins, were observed; a phenolic compound and three alkaloid compounds were detected, respectively, according to the R_f values. Two terpenoids were observed in all betel types, except ‘Ratadalu’, which gave three R_f values indicating the presence of three compounds (Table 3 and Figure 3).

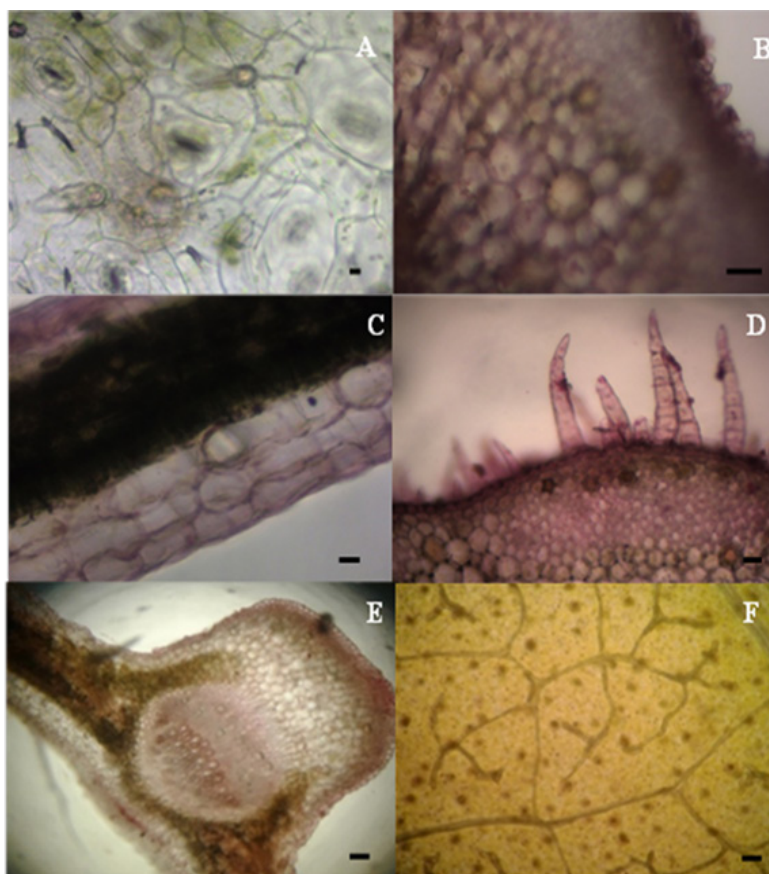


Figure 2: Prominent microscopic characteristics of *P. betle* cultivars. Transverse section of *P. betle* leaf A) Oil bodies at the lower leaf epidermis of 'KahaNagawalli' B) Oil bodies at the leaf petiole in 'Ratadalu' C) Oil bodies below the spongy mesophyll cells in young betel leaf L.S. D) Epidermal outer growth "Trichoms"—uniseriate, multicellular, unbranched in the petiole of 'SuduNagawalli' stained with safranin. E) Transverse section of *P. betle* leaf petiole. F) Vein islet and vein terminations representing overnight dipping in 10% Potassium Hydroxide (KOH) solution at room temperature. Scale bar represents 1µm.

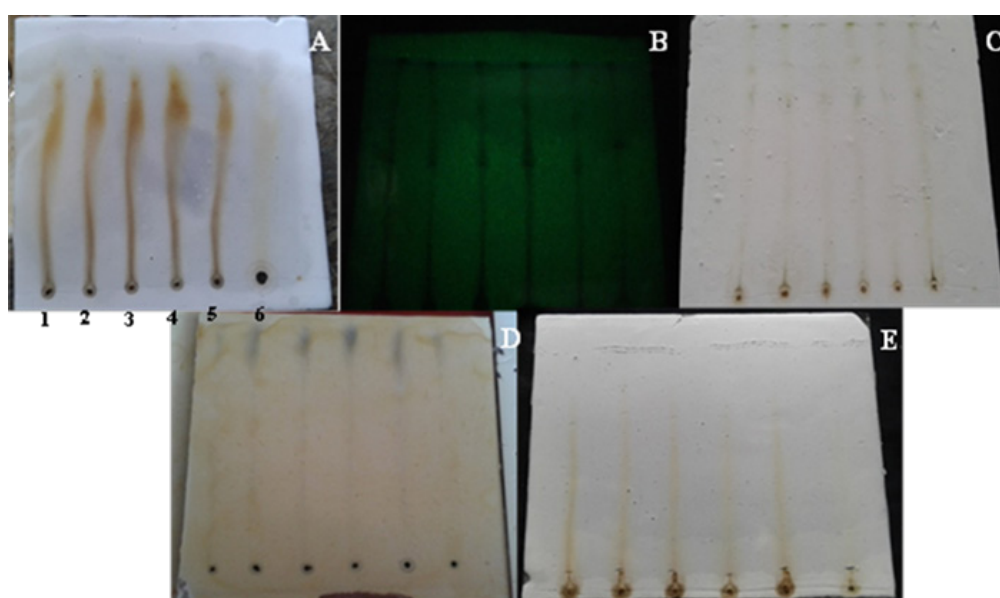


Figure 3: Photoplates of phytochemical screening of different *Piper betle* crude extracts. (A) Alkaloids after spraying with Mayer's reagent; (B) Flavonoids observed under UV light at 254 nm; (C) Terpenoids after spraying with 10% H₂SO₄; (D) Phenols after spraying with FeCl₃; and (E) Tannins after spraying with FeCl₃. Lanes represent: (1) 'KahaNagawalli' (2) 'Maneru' (3) 'Narammali' (4) 'Ratadalu' (5) 'SuduNagawalli' and (6) previously tested betel-type ethanolic crude extract dissolved in DMSO at room temperature.

Table 2: Comparison of anatomical characteristics of the upper and lower epidermis among six Sri Lankan *Piper betle* cultivars, highlighting variations in vein islet number, vein termination number, oil bodies, stomatal features, epidermal cell density, stomatal index, and trichome occurrence

Betel type	'KahaNagawalli'		'Maneru'		'Narammali'		'Ratadalu'		'SuduNagawalli'		'Koul bulath'	
Character	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Vein islet No.	4.07(5)	2.73(3)	2.9(3)	2.97(3)	1.4(2)	1.67(2)	1.77(2)	2.3(3)	1.9(2)	2.4(3)	3.97(4)	4.4(5)
Vein termination No.	2.63(3)	3.5(4)	4.07(5)	4.17(5)	2.33(3)	3.23(4)	2.93(3)	3.53(4)	3.37(4)	2.6(3)	5.23(6)	3.8(4)
Oil bodies	5.17(6)	29.17(30)	14.17(15)	20.83(21)	28.97(29)	34.07(35)	61.97(62)	39.4(40)	79.27(80)	87	33.87(34)	16.37(17)
Stomata No.	0	105.83	0	99.17	0	120.83	0	114.17	0	110.83	0	175.83
Epidermal cells	180	1209.17	615.83	1155	636.67	960	652	953.33	810	1051	1276.67	1261.67
Stomatal index	0	11.43(12)	0	11.65(12)	0	7.95(8)	0	8.35(9)	0	9.48(10)	0	7.18(8)
Trichomes	present (more)		Absent		Present (few)		Absent		Present (more)		Present	

Table 3: Different phytochemical compounds detected in ethanolic crude extracts of young betel leaves

Class of chemical compounds	Solvent system	Confirmatory test	Betel cultivar	Rf Values in crude (after the confirmatory test)	No. compounds in the Crude
Flavonoids	Chloroform: Methanol (18:2)	UV light (254 nm)	1 to 6	0.58, 0.98	2
Terpenoids	Benzene: Ethyl acetate (1:1)	10% H ₂ SO ₄	1,2,3,5,6 4	0.76, 0.95 0.71, 0.77, 0.98	2 3
Phenols	Methanol: Water (6:3)	FeCl ₃ spray	1 to 6	0.9	1
Tannins	Chloroform: Water (6:4)	FeCl ₃ spray	1 to 6	0.13, 0.69	2
Alkaloids	Methanol: NH ₄ OH (17:3)	Mayer's reagent spray	1 to 6	0.47, 0.72, 0.85	3

Determination of the anti-candidal activity of the young leaves of common *Piper betle* cultivars in Sri Lanka using Agar well diffusion bioassay

The anti-candidal activity of the ethanolic extracts of young leaves of *P. betle* cultivars and previously tested betel types against five *Candida* sp. is summarized in Figure 04. The ethanolic extract of all betel cultivars, including

the previously tested betel type, showed a significant anti-candidal activity against all the *Candida* species used in the current study. No significant difference could be observed among the anti-candidal activity of young *P. betle* leaves collected from five betel cultivars and previously tested betel type ($p > 0.05$; Figure 04). 'Ratadalu' cultivar showed the highest inhibition zone compared to the other tested samples.

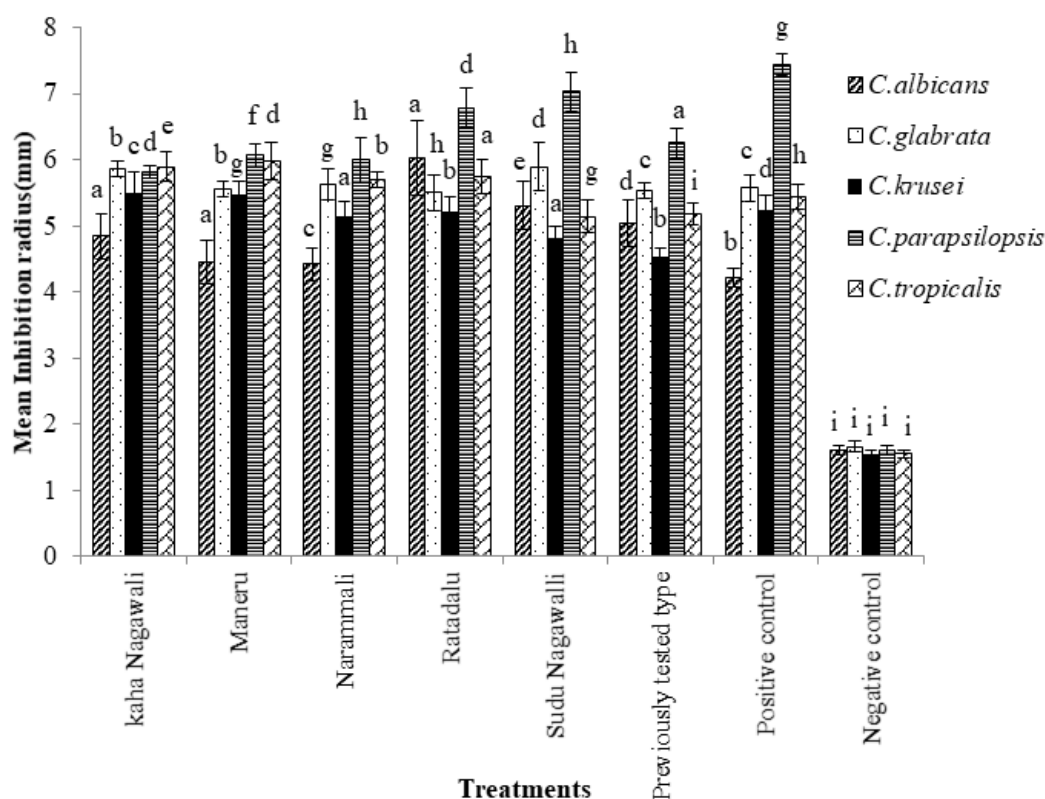


Figure 4: Effect of the ethanolic extracts of young leaves in five betel cultivars collected from the betel research station and the previously tested type on five different *Candida* species after incubation at 37 °C overnight. Within each *P. betle* cultivar, columns with the same letter are not significantly different ($p > 0.05$)

Determination of the Minimum Inhibitory Concentration (MIC) of the ethanolic extract of young leaves of common *Piper betle* cultivars in Sri Lanka

Figure 5 illustrates the MIC values and the mean zones of inhibition produced by the ethanolic extracts of five betel cultivars, along with previously tested young leaves of a betel type of *P. betle*, against each tested *Candida* species, in comparison with fluconazole used as the positive control. A negative correlation was observed between the MIC values obtained and the average radius of the zone of inhibition, viz; when the MIC value was 3.2 mg/ml *C. albicans* were inhibited by all betel samples. *C. tropicalis* and *C. krusei* strains were slightly resistant to young betel leaves extracts,

while *C. parapsilosis* and *C. glabrata* were less resistant. The minimum inhibition concentration for the *C. tropicalis* and *C. krusei* was 3.2 mg/ml. *Candida parapsilosis* showed inhibition below 0.8 mg/ml, while *C. glabrata* showed the inhibition at 1.6 mg/ml concentration for all the tested betel samples. However, results of the minimum inhibitory concentration of ethanolic crude extracts from each betel cultivar show that 'SuduNagawalli', 'KahaNagawalli', 'Maneru', 'Narammali' crude extracts inhibit the growth of *C. albicans*, *C. glabrata*, *C. parapsilopsis*, and *C. krusei* at a concentration of 6.4 mg/ml. Obviously, at this crude concentration 'Ratadalu' type inhibited the growth of all tested *Candida* species (Figure 6).

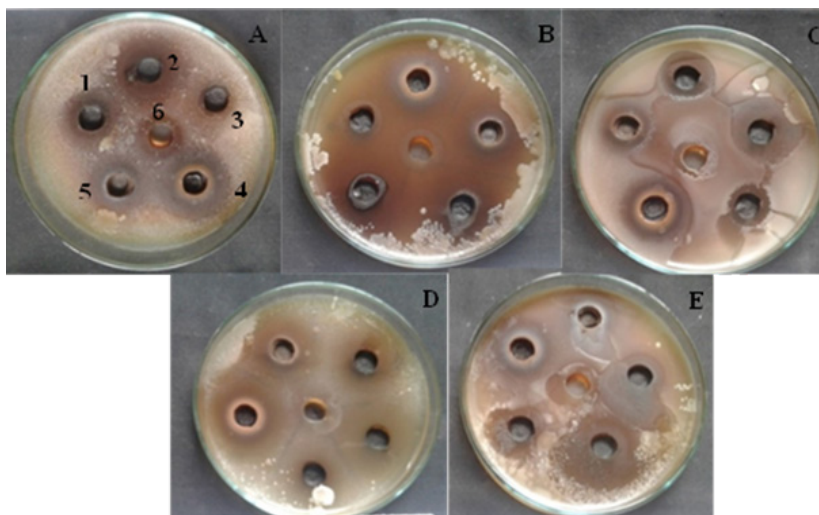


Figure 5: Zones of inhibition produced by crude extracts of young leaves of the 'Ratadalu' cultivar prepared using five different solvents: (1) ethanol, (2) chloroform, (3) ethyl acetate, (4) isopropyl alcohol, (5) methanol, and (6) DMSO (negative control), against *Candida albicans* after overnight incubation at 37 °C.

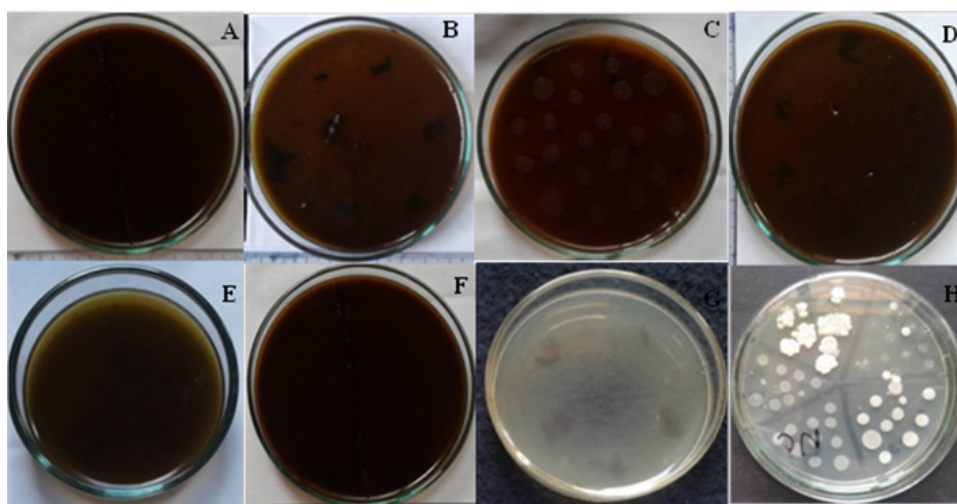


Figure 6: Suppression of growth of *Candida albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* at a concentration of 6.4 mg mL⁻¹. Treatments include crude extracts of (A) 'Kaha Nagawalli' (B) 'Maneru' (C) 'Narammali' (D) 'Ratadalu' (E) 'SuduNagawalli', (F) previously tested betel type, (G) fluconazole (positive control), and (H) DMSO (negative control), following overnight incubation at 37 °C. Except for *C. tropicalis*, the growth of other *Candida* species was suppressed by the tested cultivar crude extracts.

Determination of the inhibition activity of *Piper betle* crude extracts from different solvents using agar well diffusion bioassay

Anti-candidal activity of different solvent extracts (ethanol, chloroform, ethyl acetate, iso-propyl alcohol and methanol) of young leaves of 'Ratadalu' cultivar against *Candida albicans* is shown in Figure 07. Ethyl acetate 'Ratadalu' crude extract exhibited the highest inhibition diameter for tested five *Candida* sp., while the crude extract of iso-propyl alcohol also showed significant anti-candidal activity. No significant difference could be observed for the anti-candidal activity of different solvent betel crude extracts ($p > 0.05$; Figure 08). According to

the results obtained from agar well diffusion bioassay on crude extracts of different solvent's, all the tested solvent types showed inhibition against *Candida* species compared to the positive fluconazole control and negative DMSO control. However, all the tested crude extracts of different solvents at a MIC of 6.4 mg/ml inhibited all the tested *Candida* species. When the solvent crude extracts were treated with MIC below 3.2 mg/ml, all the tested *Candida* species inhibited their growth in the crude extracts obtained from solvents of Chloroform, Ethyl acetate and Methanol. Therefore, the solvent can be changed for the extraction according to the industrial perspective.

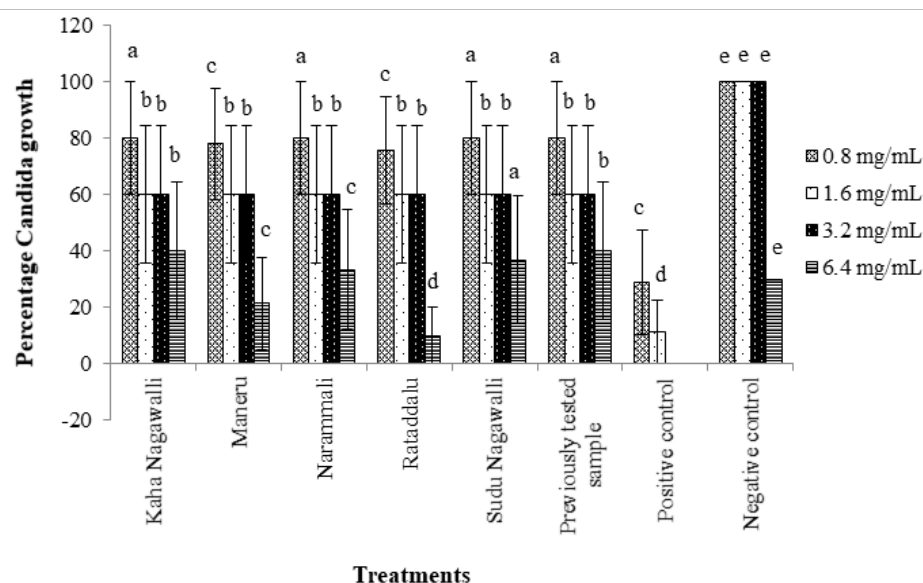


Figure 7: Determination of the growth of five *Candida* sp. in crude extracts of different solvents under previously obtained minimum inhibitory concentration after incubation at 37 °C overnight. Within each solvent extract, columns with the same letter are not significantly different ($p > 0.05$)

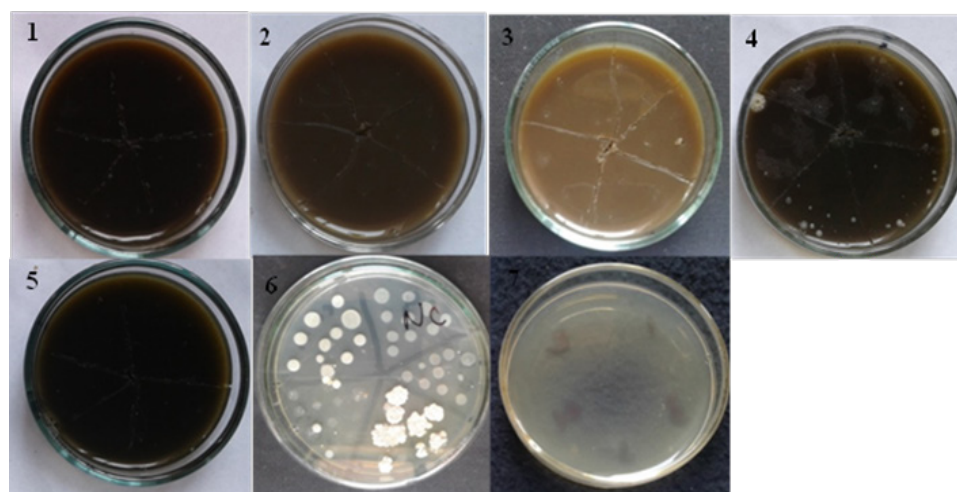


Figure 8: Suppression of the growth of five different *Candida* sp.; *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilopsis*, *C. tropicalis* in crude extracts of different solvents at 6.4 mg/ml concentration after incubation at 37 °C overnight 1) Ethyl alcohol, 2) Chloroform 3) Ethyl acetate 4) Iso-propyl alcohol 5) Methanol 6) Negative control (DMSO) and 7) Positive control (Fluconazole)

DISCUSSION

Pharmacognostic evaluation

Natural products are in great demand at present for their extensive biological properties and bioactive components, which have been proven to be useful against a large number of causative agents of diseases (Sivareddy et al., 2019). Standardization of an active part of a plant and distinguishing the pharmacognostic properties is an integral part of making herbal drugs in order to establish their purity, identity, safety, and quality. Before any crude drug can be included in an herbal pharmacopoeia, pharmacognostic parameters and standards must be established (Abere et al., 2007). In order to standardize an herbal drug, various organoleptic, macroscopic, microscopic, and phytochemical analyses need to be carried out. The microscopic method is one of the cheapest and simplest methods to start establishing the correct identification of the source material (Kumar et al., 2012). The quantitative determination of some pharmacognostic parameters is useful for setting standards for crude drugs. Stomatal number, stomatal index value, and palisade ratio, vein islet, and vein termination value determination are equally important for the evaluation of crude drugs. These values help in the evaluation of the purity of drugs (Kumar et al., 2011). The information obtained from phytochemical screening will be useful in finding out the quality of the drug (Kumar et al., 2012). 9). Standardization of plant materials, including the assessment of pharmacognostic properties, is essential to ensure the identity, purity, quality, and safety of herbal drugs (Abere et al., 2007). This involves organoleptic, macroscopic, microscopic, and phytochemical analyses, with microscopic methods providing a simple and cost-effective means of verifying source material (Kumar et al., 2012). Quantitative pharmacognostic parameters, such as stomatal number, stomatal index, palisade ratio, and vein islet and termination values, are crucial for evaluating crude drug quality and purity. Phytochemical screening further aids in assessing the drug's overall quality (Kumar et al., 2012). *Piper betle* is a well-known worldwide growing herbal plant and is consumed frequently in medical herbalism for a wide array of diseases (Venkateswarlu et al., 2014). This plant has been botanically identified and authenticated in India (Kumar et al., 2012), but such authentication has not yet been reported in Sri Lanka. Since *P. betle* is useful as a traditional medicine for the treatment of some ailments, it is important to standardize before it is used as a drug (Musa et al., 2006). Leaves of *P. betle* are rich in nutrients, antioxidants, phytochemicals, and nutraceuticals. The leaf juice is given systemically to treat cough and indigestion in children (Chauhan et al., 2016). The leaf maturity stages, along with various other features, are important to distinguish the active plant part and its confirmation. Microscopic, organoleptic, macroscopic, and phytochemical screening have distinguished the related plant part since the plant species has several cultivars and possess varying anti-microbial properties in each plant part (Nanayakkara et al., 2014).

The present study focused on pharmacognostic evaluation of distinguished organoleptic, macroscopic, microscopic,

and phytochemical properties in order to differentiate authentic young leaves of common *P. betle* cultivars in Sri Lanka. According to the results of the study, these characteristics vary slightly due to the differences in variety in the young leaves of common *P. betle* cultivars. Arambewela et al. (2005) reported that the stomatal index and the length to width ratio of 'Ratadalu' and 'Nagawalli' were quite similar, with differed appearance of leaves from other cultivars. The current study referred to 'Nagawalli' as two cultivars, namely 'SuduNagawalli' and 'KahaNagawalli'. Taxonomic studies are required to prove that 'SuduNagawalli' and 'KahaNagawalli' have derived from the same *P. betle* cultivar. However, the stomatal index and the ratio are similar in 'Ratadalu', 'SuduNagawalli', and 'KahaNagawalli' in this study. The 'Mala-bulath' cultivar, studied previously, was not included in this study due to its extreme rarity in Sri Lanka. The current study considered 'Maneru' as a single betel cultivar and did not distinguish it as two separate cultivars of 'KudaManeru' and 'MahaManeru', as suggested by Arambewela et al. (2005). The 'Ratadalu' cultivar could be obtained in the current study, which was collected in the previous study as well. 'Narammali' cultivar is a new betel cultivar that was obtained by the best type of 'Maneru' cultivar, which was not recorded and tested in the previous studies. Characteristics such as color and shape affect the amount and the content of secondary metabolites among betel cultivars due to the phylogenetic phenotypical relationships between betel cultivars.

According to the data collected from the Export Agriculture Department in Sri Lanka, no more than twelve *P. betle* cultivars, such as 'Ratadalu', 'Maneru', 'SuduNagawalli', 'KahaNagawalli', 'Galdalu', 'Mala-bulath', have been recorded in Sri Lanka. There are two commercially available betel cultivars 'Ratadalu', 'Maneru', that are extensively grown by farmers in Sri Lanka for export to several countries, including Malaysia, Thailand, Japan, etc. The current study is only focused on the betel cultivars that are extensively grown in Sri Lanka.

Khan et al. (2013) studied the pharmacognostics of eight *P. betle* cultivars from the eastern region of India, including *Desi*, *Bangla*, *Kali*, *Haldia*, *Sanci*, *Meetha*, *Kirkoli*, and *Magahi* and found to have moderate variations among the studied betel cultivars in India. *Desi*, *Bangla*, and *Magahi* betel cultivars tested by Khan et al. (2013) are slightly similar morphologically to the common betel types of Sri Lanka.

Preliminary phytochemical screening

Preliminary phytochemical screening of a plant provides information regarding the chemical nature of the plant, such as the presence and absence of various phytochemicals. Thin Layer Chromatography is the most widely used analytical method, which could detect the different chemical constituents present in the plant extract, due to its simplicity, rapidness and cost effectiveness. The variation in Rf values of the phytochemicals provides a very important clue in understanding the polarity of constituents present in the plant extract. Preliminary phytochemical screening

reveals the presence or absence of major chemical constituents in plants. Thin-layer chromatography, being simple, rapid, and cost-effective, is widely used to detect these compounds. Differences in R_f values provide insights into the polarity and diversity of phytochemicals within the extract (Kumar et al., 2012).

In the present study, TLC fingerprints of the ethanolic crude extracts of young betel leaves of five betel cultivars and a previously tested type were compared. TLC profiling of ethanolic crude extracts confirmed the presence of a diverse group of phytochemicals in different betel cultivars and previously tested types. According to the results, two compounds of flavonoids and tannins were observed; a phenolic compound and three alkaloid compounds were detected with respect to the R_f values in ethanolic crude extracts of the studied plant cultivars. Two terpenoids were observed in all betel types, except for 'Ratadalu', which gave three spots having R_f values indicating the presence of three compounds. The present findings align with earlier studies reporting saponins, alkaloids, tannins, flavonoids, and steroid glycosides in *P. betle*. Young leaves, in particular, are rich in flavonoids and phenolic compounds (Abere et al., 2007). Phytochemical evaluation revealed the presence of tannins, which have been claimed to be responsible for its antimicrobial activity (Parekh & Chanda 2007)

Natural antioxidants from plants mainly come in the form of phenolic compounds such as flavonoids, phenolic acids (Ali et al., 2008). Several studies have described the antioxidant properties of medicinal plants rich in phenolic compounds. Natural antioxidants from plants are predominantly phenolic compounds, including flavonoids and phenolic acids, and studies have reported strong antioxidant properties in medicinal plants rich in these constituents (Ali et al., 2008). The results of the present study suggest that the identified phytochemical compounds may represent the bioactive constituents, indicating that these plants constitute a valuable reservoir of bioactive compounds with significant medicinal potential.

Piper betle leaves are associated with phenolic constituents such as hydroxychavicol (HC), hydroxylchavicol acetate, allypyrocatechol (APC), piper betel methyl, piper betel piperol A, and piperol B (Singburadom, 2015). Plants produce various secondary metabolites in response to plant maturation and pathogenic attack. These include chemical constituents belonging to phenolic compounds, flavonoids, alkaloids, tannins, and terpenoids, etc., which could be detected from the TLC technique from plant extracts using different solvent systems. The ethanolic betel extracts of the tested cultivars also gave a positive response for the presence of phenolic compounds in young *P. betle* leaves by the TLC technique. However, the number of phenolic compounds could not be separated, possibly due to their relative R_f values for each compound. The phenolic compounds are attributed to several types of anti-biological activity of human diseases (Kumar et al., 2012).

Flavonoids are hydroxylated phenolic substances known to be synthesized by plants and are found to exhibit antimicrobial properties against a wide array of

microorganisms *in-vitro* (Yadav & Agarwala, 2011). Flavonoids were detected under UV light due to some constituents showing fluorescence in the visible range in daylight. The ultraviolet light produces fluorescence in many natural products that do not visibly fluoresce in daylight. If the substance itself is not fluorescent, it may often be converted into fluorescent derivatives or decomposition products by applying different reagents (Zhao et al., 2011). It is well demonstrated that flavonoids are the polyphenolic compounds which showed potential beneficial effects on human health and possess antiviral, anti-inflammatory, anti-tumor, anti-hemolytic, and anti-oxidative activity (Chakraborty & Shah 2011).

The alkaloids contained in plants are used in medicine as anesthetic agents (Yadav & Agarwala 2011). Alkaloids have been associated with medicinal uses for centuries, and one of their common biological properties is their cytotoxicity.

Tannins bind to proline-rich proteins and interfere with protein synthesis (Yadav & Agarwala 2011) The presence of tannins and other phenolic compounds having antiseptic properties could explain the use of this plant for the treatment of sores and its use as an anti-helminthic (Musa et al., 2006). The pharmacognostic parameters for the leaves of this plant, the diagnostic microscopic features, and the numerical standards reported in this work could be useful for the compilation of a suitable monograph for its proper identification.

The antimicrobial effects of the plants studied are attributed to the presence of higher terpenoids that have carboxylic acid groups responsible for the antimicrobial activity of the organic extracts. Studies have reported on the analgesic properties of alkaloids as well as the anti-inflammatory and anti-bacterial properties of tannins (Njoku et al., 2011). The presence of saponins in plants has been reported to be responsible for the tonic and stimulating activities observed in Chinese and Japanese medical herbs (Zhao et al., 2011).

Saini et al. (2016) reported that the *P. betle* Linn. leaves of *Bangla* variety contains a higher amount of carbohydrates, proteins, phytosterols, flavanoids, alkaloids, tannins, and phenols in the ethanolic extract with higher concentrations of secondary metabolites (Saini et al., 2016). Flavonoids, alkaloids, tannins, terpenoids, and phenolic compounds could be detected from the ethanolic extract of betel cultivars in the current study. Different chemical compounds, such as alkaloids, tannins, and flavonoids, among others, were detected in different betel cultivars. If could make the plant useful for treating different ailments and having a potential of providing useful drugs for human use.

The results of the current investigation could, therefore, serve as a basis for proper identification, collection, and investigation of the plant. The macro and micro morphological features of the leaf described can distinguish it from other members of the same species in different cultivars. Chemomicroscopy, numerical data, and quantitative leaf microscopy are parameters that are unique to the plant and are required in its standardization. The identified phytochemical compounds may be the bioactive constituents responsible for the efficacy of the leaves of the plants studied. The presence of some of these compounds

has been confirmed to have antimicrobial activity, could be inferred that the plant extracts could be a source for the industrial manufacture of drugs useful in the chemotherapy of some microbial infections. Studies on the natural products of plant origin of *P. betle* proved that the released secondary metabolites have strong antimicrobial activity (Sivareddy 2019).

Agar well diffusion Assay and determination of MIC

In conventional allopathic medicine, widely spreading diseases and microbial infections in the world are controlled by synthetic drugs. However, with the development of antimicrobial drug resistance by certain pathogens, herbal remedies are tested widely as alternative novel herbal drugs. As *Candida* spp. evolve resistance towards available antimycotic agents, some studies have focused on the investigation of novel, potent antimicrobial compounds from natural sources, including plants (Rai & Mares 2003). The current study investigated the anti-candidal activity of the extracts of young *P. betle* leaves of locally available five betel cultivars against five standard isolates of *Candida* species, and the activities were compared with the previously tested betel type by Nanayakkara et al. (2014). The anti-candidal activity of the ethanolic betel crude extract has been previously investigated by Nanayakkara et al. (2014) for a single cultivar, but they have not considered different cultivars of betel. Therefore, this study investigated the anti-candidal activity of available *P. betle* cultivars as an extension of the previous work.

A study by Nanayakkara et al. (2014), revealed that ethanolic crude extracts from young betel leaves showed higher anti-candidal activity compared to mature leaves. The reason can be an increase in the activity of polyphenol oxidase and peroxidase enzymes through which the phenolic compounds can be converted into quinones with leaf maturation, resulting in young leaves having more antimicrobial phenolics than mature leaves (Figueiredo et al., 2008). In addition, the rates of cyclization and dehydration of the compounds increase upon leaf maturation. Further, the differences in the anti-candidal activity of young leaves of *P. betle* over mature leaves can also be attributed to the type of secretory structure in the leaves and the mechanism of releasing secretion. However, Sivareddy 2019 showed contrary results than Nanayakkara et al 2014 wherein mature leaves had better anti-candidal activity in ethyl acetate crude extract of young leaves, which can probably be explained by the use of ethyl acetate, a solvent not used in the study by Nanayakkara et al., 2014 and the differences of the extraction method as well as the chemotype variation of the *P. betle* used for the studies. According to Arambewela et al. (2005) *P. betle* young leaves contained high eugenol (13.41%) and phellandrene (10.66%), which possess several antimicrobial properties and are also used in the production of essential oils, while low safrole (27.98%), Allylpyrocatechol diacetate (1.17%), and chavibetol acetate (2.02%) than mature and harvesting stages leaves. Most of the anti-candidal studies were carried out for betel oil and crude aqueous extract, while few records exist on the ethanolic crude extract (Arambewela et al., 2005; Himratul-Aznita et al., 2011).

The ethanolic extracts of all the studied betel cultivars and similar to the previous study young *P. betle* leaves showed a significant anti-candidal activity in the current study. No significant difference could be observed among the anti-candidal activity of young *P. betle* leaves of the five betel cultivars and previously tested betel type ($p > 0.05$). However, Ethanolic extract of the 'Ratadalu' cultivar has shown a relatively higher mean diameter of the inhibitory zone than the others. The reason may be due to the quantity variation of each active secondary metabolite in betel cultivars. Although the betel cultivars studied by Arambewela et al. (2005), were slightly different from the current study, the studied betel cultivars contain similar chemical constituents but in different quantities, according to the type of betel cultivar agree with the above results.

According to Chakraborty & Shah (2011), the presence of flavonoids, alkaloids, terpenoids, tannins, phenols, and several other secondary metabolic compounds can be the reason for the observed anti-candidal activity from all crude extracts. It has been reported earlier that *P. betle* extracts containing high concentrations of fatty acids like palmitic acid, stearic acid, and hydroxyl fatty acid esters showed potential antimicrobial activity against diverse pathogenic microorganisms (Arifin et al., 2015). Eugenol, hydroxychavicol, phellandrene, safrole, allylpyrocatechol diacetate, and chavibetol acetate were major compounds present in betel leaves in secretory structures (Shah et al., 2016). Amount and the content of secondary metabolic compounds in oil bodies may cause the varying anti-candidal activity in betel types. Hydroxychavicol exhibited fungicidal activity against both *Candida* species and the reduction in the number of CFU per milliliter (Sivareddy et al., 2019). Studies have suggested that Hydroxychavicol may alter the cell membrane structure, resulting in the disruption of the permeability barrier of microbial membrane structures and thereby being responsible for their anti-candidal activity. There has been evidence on the antifungal activities of *P. betle*. A study reported that *P. betle* gel inhibited the growth of dermatophytes that cause ringworm and growth of *Candida* species more effectively than tolnaftate and with a similar inhibitory effect to that of clotrimazole (Ali et al., 2016). Furthermore, the free radical scavenging activity was found to be higher in the ethanolic extract than the distilled water extract (Sarma et al., 2018).

In the microbiological study, the ethanolic crude extracts of different betel cultivars showed activity against *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilopsis*, and *C. tropicalis*. According to the results of minimum inhibitory concentration for ethanolic crude extracts of betel cultivars, 'SuduNagawalli', 'KahaNagawalli', 'Maneru', 'Narammali', and previously tested type crude extract inhibited the growth of *C. albicans*, *C. glabrata*, *C. parapsilopsis*, and *C. krusei* at 6.4 mg/ml. As expected, at this concentration, 'Ratadalu' type inhibited the growth of all tested *Candida* species.

The MIC values obtained in this experiment slightly deviated from the results of Nanayakkara et al. (2014). This may be due to differences in the crude preparation procedure, and the differences in types and amounts of the

active compounds remain in the crude sample.

Nanayakkara et al. (2014) reported that *C. tropicalis* and *C. albicans* are the most resistant isolates, while *C. parapsilosis* is the most susceptible to the extract. In the current study, *C. tropicalis* and *C. krusei* were more resistant to the crude extracts, giving the highest MIC value. Variation of MIC values between different *Candida* species could be attributed to the composition of the cell wall of *Candida* spp. *Candida albicans* is known to be the major human pathogen, and its pathogenicity is aided by the virulent features, including the production of germ tubes, biofilm formation, and enzyme secretion, including phospholipase, protease, and esterase activity (Nanayakkara et al., 2014).

MICs of different betel type extracts show that 'Ratadalu' type can suppress the growth of all tested *Candida* species more than other types under a concentration of 6.4 mg/ml. Based on the results of morphological, microscopic, and phytochemical screening, 'Ratadalu' may have a higher content of oil bodies that contain candidal growth-suppressing secondary compounds than others. The current study selected the best betel type that can suppress the candidal growth. However, further studies are required to define the anti-candidal mechanism and formulation of a cream against superficial candidal infection.

According to the results obtained from agar well diffusion bioassay on crude extracts with different solvents, all the tested crude extracts inhibited the growth of *Candida* species tested. They showed a clear zone of inhibition against the tested *Candida* sp. This may be because of the presence of high concentrations of fatty acids and sterols. However, when all the tested solvent crude extracts were treated with previously obtained MIC 6.4 mg/ml for all the tested *Candida* species, all of them inhibited the growth. When the tested crude extracts were treated with concentrations lower than MIC (3.2 mg/ml), growth of all the tested *Candida* species was inhibited by the crude extracts obtained from the solvents of chloroform, ethyl acetate, and methanol. Therefore, the solvent may have to be changed for the extraction according to the industrial requirements.

Further, the efficacy of these leaf extracts was tested by obtaining their MIC values. It showed that the MIC value of fluconazole (62.5 µg/ml) was twice that of the ethyl acetate extract of mature betel leaves (0.125 mg/ml). In a study conducted by Ali et al. (2010) using hydroxychavicol isolated from the chloroform extract soluble fraction of the aqueous extract of leaves of *P. betle*, the MIC values obtained for *Candida* spp. ranged from 0.015 to 0.5 mg/ml; higher MIC values were observed against *C. albicans* as reflected in our study. However, a study by Makkar et al. (2023) showed lower MIC values, and another study by Nanayakkara et al. (2014) showed higher MIC values.

In the current study, ethyl acetate young leaves betel crude extract showed higher anti-candidal activity than the other solvent crude extracts, which is in agreement with the results of Sivareddy et al. (2019). However, this requires more studies to confirm the effect of ethyl acetate solvent on the increased release of secondary metabolites from betel leaf. The low candidal activity of the iso-propyl

alcohol crude extract leaf may be improved via the use of different extraction procedures, considering the polarity of the active compounds, since the solvent is available and the cheapest of the solvents. In addition, the purification of the active compound from betel crude extracts may lead to higher anti-candidal activity.

Junairiah et al. (2017) studied the anti-candidal activity and the anti-bacterial activity of *P. betle* L var Nigra for methanol, n-hexanes and ethyl acetate crude extracts and found that the methanol extract was the best solvent to be used compared to n-hexane and ethyl acetate (Junairiah et al., 2017). In the current study, methanol and ethyl acetate crude extracts gave relatively higher inhibition than the ethanolic crude, which may be due to the variation of the used betel cultivar.

The crude extracts of *P. betle* from different solvents can be seen as a potential source for useful drugs. The higher MIC values obtained from young *P. betle* leaves in the present study can be attributed to the use of crude extracts of the leaves when compared with the purified form of the standard drug. Further studies are going on in order to isolate, identify, characterize, and elucidate the structure of the bioactive compounds. The antimicrobial activities of these plants for the treatment of diseases as claimed by traditional healers, were also being investigated (Tadeg et al., 2005).

The parameters that are reported here can be considered as distinctive enough to identify and decide the authenticity of the drug in the herbal industry. These studies can also help manufacturers with the identification and selection of raw materials for drug production.

CONCLUSION

Piper betle is a well-known medicinal plant cultivated worldwide and widely used in traditional herbal medicine. Its leaves are rich in nutrients, antioxidants, and diverse phytochemicals with recognized nutraceutical properties. The present study demonstrates that microscopic anatomical evidence is sufficient to distinguish variations among *P. betle* cultivars. Phytochemical screening of ethanolic crude extracts prepared from young leaves revealed the presence of alkaloids, tannins, phenolic compounds, flavonoids, steroidal glycosides, terpenes, anthraquinones, and steroids, which may collectively contribute to the observed therapeutic activities. The findings also highlight the potential for further research focusing on the isolation and identification of bioactive compounds from *P. betle* leaves using chromatographic and spectroscopic techniques. Among the tested extracts, ethyl acetate and chloroform crude extracts of *P. betle* exhibited superior anticandidal activity against *Candida albicans* compared with extracts obtained using other solvents. These results suggest that *P. betle* crude extracts represent a promising source of novel antifungal agents and may help address the growing issue of drug-resistant *Candida* species by supporting the use of traditional medicinal resources. Further investigations involving detailed chemical profiling and minimum inhibitory concentration (MIC) determination of ethyl acetate, chloroform, and methanolic extracts are

recommended to better evaluate their efficacy relative to standard antifungal drugs. Such studies may also assist herbal medicine manufacturers in the identification and selection of suitable raw materials for drug development.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS' CONTRIBUTIONS

Conceptualization, Supervision: **A.D.S.N.P.A., C.L.A., G.J.P., J.A.M.S.J.**; Investigation, Data curation, Formal analysis, Writing—original draft: **W.G.M.L.**; All authors have read, approved, and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data and materials analyzed in this study are not publicly available but can be obtained from the corresponding author upon reasonable request

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