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Vol. 8(23), pp. 2307-2314, 4 June, 2014
DOI: 10.5897/AJMR2014.6713
Article Number: 652F32E45198
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Anti-candidal activity of *Piper betle* (L.), *Vitex negundo* (L.) and *Jasminum grandiflorum* (L.)

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Received 15 February, 2014; Accepted 19 May, 2014

The discovery of antimicrobials from traditional medicinal plants is gaining importance. The objectives of this study were to determine the anti-candidal activity of young and mature leaves of *Piper betle* collected from dry and wet zones of Sri Lanka, leaves and roots of *Vitex negundo* and leaves of *Jasminum grandiflorum* and the determination of minimum inhibitory concentrations (MIC). Water and ethanolic extracts of plant material were tested against standard cultures of *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis*. The MICs of active extracts were determined. Ethanolic extracts of young leaves of *P. betle* showed significantly higher ($p < 0.05$) anti-candidal activity against all *Candida* species bioassayed, while mature leaves showed less activity. MICs of ethanolic extract of young leaves of *P. betle* were within 0.64 - 3.2 mg/mL. There was no significant difference between the activity of leaves of *P. betle* collected from wet zone and dry zone ($p > 0.05$). Water extracts of leaves of *P. betle* and water and ethanolic extracts of leaves and roots of *V. negundo* and leaves of *J. grandiflorum* did not show a significant anti-candidal activity. Hence, young leaves of *P. betle* can be used as an anti-candidal agent since betel leaves are used in masticatory mixtures.

Key words: Candidiasis, *Piper betle*, young and mature leaves, MIC.

INTRODUCTION

Much focus is being given to traditionally-utilized medicinal plants for their antimicrobial properties, due to the shortcomings in most of the widely used synthetic drugs, *viz*; the evolution of resistance by microorganisms to prevailing antimicrobials, potential health hazards, side effects and the loss of public reliance (Livermore, 2004). In the recent past, research has proven the potential of herbal products in the development of novel, safe,

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Abbreviations: MIC, Minimum inhibitory concentration; **DMSO,** dimethyl sulfoxide; **MHA,** Mueller Hinton agar; **BSAC,** British Society for Antimicrobial Chemotherapy.

effective and potent antimicrobials (Rai and Mares, 2003).

Most antimicrobial and medicinal properties of plants can be attributed to secondary metabolites (Cowan, 1999). Young leaves, young stems and reproductive plant parts have been observed to contain higher concentrations of secondary metabolites while mature plant parts have lower concentrations (Taiz and Zeiger, 2006). In addition, more secondary metabolites are produced when the plant is under stress including water stress and pathogen attack (Taiz and Zeiger, 1991). Controversially, it is also reported that water stress leads to low concentrations of secondary metabolites in plants (Gutbrodt et al., 2011).

Piper betle L. (Piperaceae) is a perennial, evergreen vine with stout, semi-woody stems. It has the vernacular names 'bulath' in Sinhala, betel vine or betel pepper in English and 'ilaikkodi', 'nirvalli' in Tamil. The plant grows well in warm, humid climates and is indigenous to and cultivated in Sri Lanka, India, Malay Peninsula, Philippines and East Africa, exhibiting a wide-spread distribution.

The active compounds isolated from the leaves are hydroxychavicol, hydroxychavicol acetate, chavibetol, cadenine, piperbetol, methylpiperbetol, piperol A and piperol B. In addition, the alkaloid arakene, terpenens, sesquiterpenes, cineol, eugenol, methyl ether and caryophyllene also are present in the leaves (Jayaweera, 1982; Datta et al., 2011; Pin et al., 2010). The major components of *P. betle* essential oil are known to be eugenol and acetyleugenol (Prakash et al., 2010). The leaves of *P. betle* are well recognized in Ayurveda for their antimicrobial and antioxidant properties (Datta et al., 2011).

The antifungal activity of hydroxychavicol isolated from the chloroform extraction of aqueous leaf extract of *P. betle* has been investigated by Ali et al. (2010). They have documented the antifungal effect of the leaf extract on yeasts, *Aspergillus* spp. and dermatophytes. Bruised leaves are applied on cuts and wounds as an antiseptic (Jayaweera, 1982). Due to the strong pungent aromatic flavour, betel leaves are used as a masticatory agent by certain Asian communities (Datta et al., 2011).

Vitex negundo L. (Verbenaceae) is a slender, deciduous tree growing up to heights of about 8 m. It is known as 'nika', 'nirgundi' in Sinhala and as 'nir-nochchi', 'nochchi' in Tamil. This plant is native to many countries including Zanzibar, Mozambique, Madagascar and Sri Lanka. The chemical constituents of leaves of *V. negundo* include flavonoids, nishindine, 1-sabinene, 1-α-pinene, viridiflorol, beta-caryophyllene, 4-terpineol, gamma-terpinene, caryophyllene oxide and sesquiterpenes including copaene (Singh et al., 1999; Jayaweera, 1982). Gautam et al. (2008) have carried out a phytochemical investigation of the methanolic extract of leaves of *V. negundo* to identify the major chemical constituents, that is, negundoside, agnuside, vitegnoside, 7,8 dimethyl herbacetin 3-rhamnoside, 5,3'-dihydroxy-7,8,4'-trimethoxy flavanone, 5-hydroxy-3,6,7,3',4'-pentamethoxy flavone, 5,7 dihydroxy- 6,4' dimethoxy flavonone and 5 hydroxy-7,4' dimethoxy flavones. The leaves and roots are known to have antimicrobial properties. In Sri Lanka, *V. negundo* is used in the treatment of toothache, eye diseases and rheumatism (Vishwanathan and Basavaraju, 2010).

Jasminum grandiflorum L. (Oleaceae) is a climbing shrub with green stems. It is known as 'saman pichcha', 'desaman' in Sinhala and as 'kodimalligai', 'pichi' in Tamil. *J. grandiflorum* is a common plant grown in Sri Lankan home gardens and is especially reckoned for its fragrant flowers. The active compounds of the leaves include the alkaloid jasminine, salicylic acid and an astringent principle.

In addition, Sadhu et al. (2007) have identified secoiridoid glucosides, 2″-epifraxamoside and demethyl-2″-epifraxamoside and the secoiridoid jasminanhydride from aerial parts of *J. grandiflorum*. In traditional Ayurveda, the leaves of the plant are used as a remedy to treat ulcers in the mouth and to relieve toothache. Also, the fresh juice of leaves is used to soften corns (Jayaweera, 1982).

The current study was carried out with the aim of determining the anti-candidal activity of leaves of *P. betle*, leaves and roots of *V. negundo* and leaves of *J. grandiflorum.* Further, the effects of leaf maturity and the dry and wet zone climatic conditions relating to anticandidal activity of *P. betle* were investigated.

MATERIALS AND METHODS

Microbial isolates

Standard cultures of five *Candida* spp., namely *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258), *Candida parapsilosis* (ATCC 22019) and *Candida tropicalis* (ATCC 13803) were used in the study. Sub culturing was done on sterile Sabouraud's Dextrose Agar (SDA) and the plates were incubated at 37°C for 24 h. The resultant colonies were kept at 4°C for short-term storage.

Identification of plants

Herbarium specimens of the three test plants were prepared with twigs carrying reproductive plant parts and the specimens were identified to be *P. betle*, *V. negundo* and *J. grandiflorum* by comparing with the specimens at the National Herbrium of the Royal Botanic Gardens, Peradeniya, Sri Lanka. The three specimens were deposited at the Herbarium of the Department of Botany, University of Peradeniya, Sri Lanka.

Plant material

Water extracts of leaves of *P. betle* were prepared with leaves collected from Kandy (Central province) and for the preparation of ethanolic extracts, young and mature leaves of *P. betle* were collected from 8 locations each from the dry zone (Central province,

Table 1. Volumes of the stock solution added to obtain the dilution range of the extract by Andrews (2005 and 2001) methods.

Volume of the stock solution (µl)	Concentration of final solution (mg mL^{-1})
a) 2560	12.8
1280	6.4
640	3.2
320	1.6
b) 256	1.28
128	0.64
64	0.32
32	0.16
0	0

a) Andrews (2005); b) Andrews (2001).

North Central province and North Western province) and wet zone (Southern province, Sabaragamuwa province, Western province and Central province) of Sri Lanka. Each second leaf counting from the leaf bud was taken as a young leaf while each fifth leaf counting from the leaf bud was taken as a mature leaf. Each sample comprised 12 leaves collected from 6 betel vines.

For the preparation of water and ethanolic extracts, leaves and roots of *V. negundo* and leaves of *J. grandiflorum* were collected from Kandy (Central province).

The plant material was cleaned to remove adhering soil, dust, debris and other alien material, washed well with distilled water and air-dried at room temperature (27 ± 1°C).

Preparation of extracts

Water extracts

Leaves of *P. betle* were cut into pieces (0.5 x 0.5 cm). Twenty grams of the leaves were boiled in 80 mL of distilled water, until the final volume reached 10 mL, according to traditional Ayurvedic practice. The extract was filtered using Whatmann No. 1 filter paper and the filtrate was transferred to sterile glass vials.

The same procedure was carried out for the preparation of water extracts of leaves and roots of *V. negundo* and leaves of *J. grandiflorum,* separately.

Ethanolic extracts

Young and mature leaves of *P. betle* were cut into pieces (0.5 x 0.5 cm) and 20 g of each sample was weighed (SETRA EL - 410s). The sample was soaked separately for 10 min in 200 mL of 99% ethanol at room temperature (27 ± 1°C). Using the vacuum infiltration technique where soluble compounds are extracted to the solvent under a vacuum, the plant parts were stirred with the same aliquot of alcohol in a magnetic stirrer (STUART CB 161) at a speed of 3 revolutions per second, at room temperature for one hour. Subsequently, the extract obtained was filtered through a sterile Whatmann No. 1 filter paper and the filtrate was collected. Alcohol was removed from the ethanolic extract using a rotary evaporator (STUART RE 300) at a speed of 25 rounds per minute at 40°C (Rangama et al., 2009). The total amount of extract obtained from 20 g of leaves of *P. betle* was dissolved in 6 mL of dimethyl sulfoxide (DMSO) and was subjected to the agar well diffusion bioassay as described below. The extract was freeze-dried and stored at -70°C, for the determination of the minimum inhibitory concentration.

The same procedure was carried out for the preparation of ethanolic extracts of leaves and roots of *V. negundo* and leaves of *J. grandiflorum.*

Agar well diffusion bioassay

The previously isolated 24 h standard cultures were used to prepare broth cultures (McFarland 0.5 standard). Twenty five milliliters each of sterile Mueller Hinton Agar (MHA) was poured into sterile 90 mm Petri dishes and were left to set followed by allowing to dry at 44°C. From each liquid microbial culture prepared, 2 ml was pipetted onto separate MHA plates and spread evenly by swirling the plate. The excess liquid culture was pipetted out using a micropipette. The plates were dried at 44°C for 10 min. Using a sterile 8 mm cork borer, equidistant wells of 8 mm in diameter and 4 mm in depth were bored on each MHA plate. Each well was sealed at the bottom with 5 µL of molten MHA and was labelled for the purpose of identification. Approximately 200 µL of each water extract was directly loaded into its corresponding well, ensuring that the wells neither overflowed nor were loaded below the top. The ethanolic extracts of the plant material were dissolved in 6 ml of DMSO and 200 µL of each extract was loaded into wells separately. The plates were left at room temperature (27 ± 1°C) for 30 min to allow the solutions to diffuse into MHA and were subsequently incubated at 37°C for 24 h. After incubation, the zone of inhibition around each well was measured. The above procedure was carried out in quadruplicate for each *Candida* isolate, separately.

Determination of the minimum inhibitory concentration (MIC) of the extracts

MIC of young leaves of *P. betle* was determined using both the Andrews (2001) method and the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2005) method. Since the leaf extract of *P. betle* was saturated at a concentration of 1.28 mg/mL in the Andrews (2001) method, BSAC method was subsequently followed to test for higher concentrations of the extract.

Andrews (2001, 2005) methods

Different volumes of the stock solution were added to 20 mL of sterile molten MHA maintained at a temperature of around 45°C, as indicated in Table 1 and mixed well. Each solution was transferred into a sterile 90 mm Petri dish which was marked into 5 partitions on the reverse side.

Table 2. Attributes of the ethanolic extracts of the plant material.

Extract	Solubility in water	Solubility in DMSO	Form		Colour *
			After rotary evaporation	After freeze drying	
Young leaves of *P. betle*	Partial	Complete	Sticky semi-solid	Sticky powder	Dark green
Mature leaves of *P. betle*	Partial	Complete	Sticky semi-solid	nd	Dark green
Leaves of *V. negundo*	Partial	Complete	Sticky semi-solid	nd	Dark brownish green
Roots of *V. negundo*	Complete	Complete	Sticky semi-solid	nd	Brownish
Leaves of *J. grandiflorum*	Partial	Complete	Sticky semi-solid	nd	Green

nd = No data as further experiments were not carried out for these extracts since they did not have a significant anti-candidal activity; *The colour of the extracts after rotary evaporation and after freeze drying was the same.

Table 3. Inhibition by water and ethanolic extracts of the plant material.

Isolate	Average radius of zone of inhibition (mm)						
	***P. betle* leaves**	***V. negundo* leaves**		***V. negundo* roots**		***J. grandiflorum* leaves**	
	WE	**WE**	**EE**	**WE**	**EE**	**WE**	**EE**
C. albicans	0.2±0.61	0	1.8±0.41	0	0.4±0.25	0.2±0.41	0.1±0.20
C. glabrata	0.2±0.61	0	0.8±0.42	0	0	0	0
C. krusei	1.7±0.41	0	0.8±0.75	0	0	0	0
C. parapsilosis	0	0	2.6±0.86	1.8±1.61	0	0	0.8±0.99
C. tropicalis	0.4±0.55	0	1.7±0.68	0	0	0	0.3±0.82

n=6, WE - water extract, EE - ethanolic extract, data for EE of leaves of *P. betle* is given in Figure 2.

MHA was allowed to solidify and the plates were subsequently dried at 44°C. Similarly, agar dilution plates were prepared using the negative control DMSO.

Inoculation of the plates

Liquid microbial cultures were prepared and the turbidity was made similar to that of the McFarland 0.5 standard solution. Within 30 min of inoculum preparation, 1 µL each of the inoculum was placed on the corresponding partitions on the MHA set plates prepared as mentioned above. These were allowed to be absorbed into the agar before incubation at 37°C overnight (Andrews, 2001). The above procedure was carried out for the agar dilution plates of the negative control, that is, DMSO in place of the leaf extract. The entire experiment was carried out in quadruplicate.

Statistical analysis

The data was subjected to statistical analysis of variance (ANOVA) using the SAS software (v6.12).

RESULTS

Attributes of the water extracts

The water extract of leaves of *P. betle* was dark green in colour, while the extracts of the leaves and roots of *V. negundo* were dull dark green and brownish, respectively. The colour of the water extract of the leaves of *J. grandiflorum* was light green.

Attributes of the ethanolic extracts

On the other hand, the ethanolic extracts differed in their solubility, form and colour markedly in comparison to the water extracts. Table 2 illustrates the above differences, *viz*; solubility of the ethanolic extracts in water, in DMSO and their form and colour.

Agar well diffusion bioassay:

The activity of the water extracts of leaves of *P. betle* and water and ethanolic extracts of the leaves and roots of *V. negundo* and the leaves of *J. grandiflorum*, against *Candida* spp. is depicted in Table 3. The ethanolic extract of young leaves of *P. betle* showed a significant anti-candidal activity, which was greater than that of mature leaves, with a significant difference ($p < 0.05$; Figures 1 and 2). No significant difference could be observed between the anti-candidal activity of *P. betle* leaves collected from the wet and dry zones ($p > 0.05$; Figure 2).

Determination of the MIC

Table 4 gives the MIC and the average radius of zone of

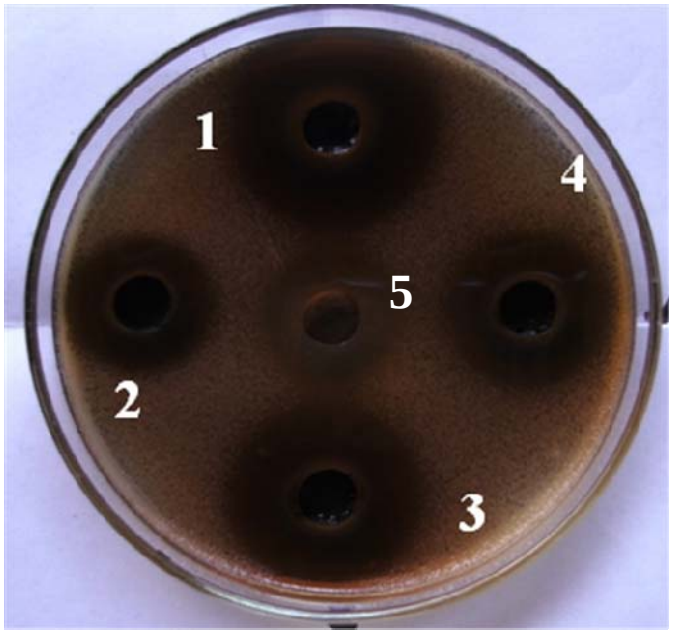

Figure 1. Zones of inhibition given by young leaf extract of *P. betle* from wet zone (1), mature leaf extract of *P. betle* from wet zone (2), young leaf extract of *P. betle* from dry zone (3), mature leaf extract of *P. betle* from dry zone (4) and the negative control DMSO (5).

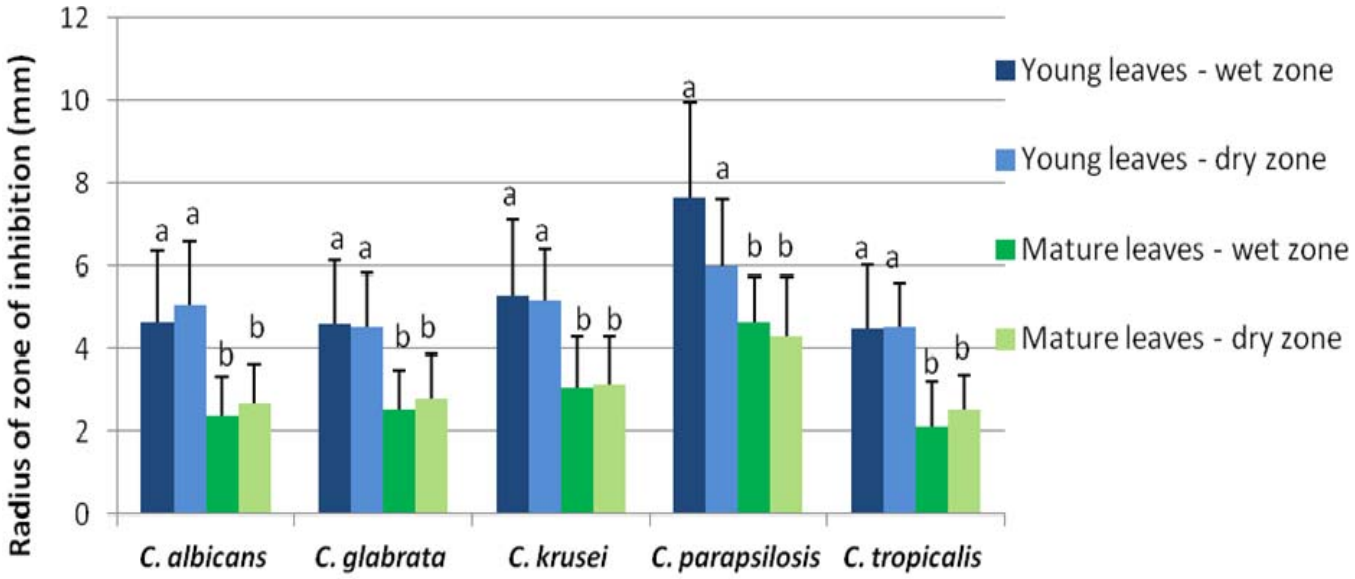

Figure 2. Effect of ethanolic extracts of young and mature leaves of *P. betle* collected from the wet and dry zones against different *Candida* species. $p < 0.05$ between anti-candidal activity of young and mature leaves, $p>0.05$ between anti-candidal activity of leaves collected from dry and wet zones; n=4. Within each isolate, columns with the same letter are not significantly different ($p > 0.05$), by SAS Software (v6.12).

inhibition given by the ethanolic extract of young leaves of *P. betle* against each *Candida* sp. tested, together with the comparison with Fluconazole which is a commercially available antimicrobial drug. As depicted in Table 4, a negative correlation was observed between the MIC values obtained and the average radius of the zone of

Table 4. Average radius of zone of inhibition and the MICs of the ethanolic extract of leaves of *P. betle*.

Isolate	Average radius of zone of inhibition (mm)	MIC (mg/mL)	MIC for Fluconazole (mg/mL) *
C. albicans	4.6	1.6	0.128
C. glabrata	4.6	0.8	0.064
C. krusei	5.3	1.6	0.064
C. parapsilosis	7.7	0.64	0.016
C. tropicalis	4.5	3.2	> 0.128

*Kanatiwela et al. (2010). Fluconazole is a standard antimycotic drug used against *Candida* spp.

inhibition, v*iz;* when the MIC value was 0.64 mg/mL (the lowest) against *C. parapsilosis* the average radius of zone of inhibition was 7.7 mm (the highest), while when the MIC value was 3.2 mg/mL (the higest) against *C. tropicalis,* the average radius of the zone of ihinhibition was 4.5 mm (the lowest).

DISCUSSION

Many plants produce antimicrobial compounds mainly as a defense mechanism against stresses, pathogen attack, etc. (Taiz and Zeiger, 1991). As *Candida* spp. evolve resistance towards available antimycotic agents, much focus is being given to the investigation of novel, potent antimicrobial compounds mainly from natural sources including plants (Rai and Mares, 2003). The current study investigated the anti-candidal activity of three plants against standard cultures of 5 *Candida* species. This is the first record where the effects of leaf maturity and climatic conditions (dry and wet zones) have been investigated for anti-candidal activity of plant material.

According to studies carried out in the same laboratory, the ethanolic extract of leaves of *P. betle* proves to be a more potent anti-candidal agent when compared with *Pongamia pinnata*, *Tephrosia purpurea* and *Mimusops elengi* which also are used in traditional oral health care. The leaves, pods and roots of *T. purpurea* and bark of *M. elengi* had not shown a significant anti-candidal activity (Rangama et al., 2009) while the roots of *P. pinnata* has exhibited an MIC of 6.4 mg/mL against *C. albicans* while all other standard cultures were resistant against the extract (Kanatiwela et al., 2010). Hence, comparatively, leaves of *P. betle* prove to be a potential anti-candidal agent.

According to the results obtained in the current study, the ethanolic extract of young leaves of *P. betle* (variety 'val bulath') have a significant anti-candidal activity against all the five *Candida* species investigated: *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*. Ethanolic extract of young leaves of *P. betle* showed a higher anti-candidal activity as compared to mature leaves of *P. betle* which indicates that young leaves of *P. betle* have a higher amount of secondary metabolites as compared to mature leaves. This could be accepted as primarily antimicrobial compounds are secondary metabolites in plants (Taiz and Zeiger, 2006). It is inferred that secondary metabolites accumulate more in tissues with a higher probability of being attacked. Young leaves are more prone to attack by pathogens than mature leaves because of the fragility of their physical defenses such as the lack of a thick waxy cuticle and low cell wall rigidity. In addition, with leaf maturation, an increase in the activity of polyphenol oxydase and peroxidase enzymes occurs through which the phenolic compounds can be converted into quinones. Hence, young leaves may have more antimicrobial phenolics than mature leaves. Also, the rates of cyclization and dehydration of the compounds increase upon leaf maturation. In addition, 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. Members of family Piperaceae (*P. betle*) contain osmophores which are external secretory structures. Plants with external secretory structures release their secretions upon organ maturation due to trichome cuticle disruption (Figueiredo et al., 2008).

In the current study, no significant difference was observed between the anti-candidal activity of ethanolic extract of leaves of *P. betle* collected from the dry and the wet zones. Therefore, it can be inferred that the dry zone environment has not significantly affected the production and composition of secondary metabolites in the leaves of *P. betle*, during this study. The work of Gutbrodt et al. (2011) also shows that water stress leads to low concentrations of secondary defense compounds in plants which are severely stressed. However, according to Taiz and Zeiger (1991), the production of secondary metabolites is increased when the plant is under stress including water stress and pathogen attack.

A significant anti-candidal activity was not observed in the ethanolic and water extracts of leaves and roots of *V. negundo* and leaves of *J. grandiflorum*. The low anti-candidal activity of the ethanolic extract of the leaves of *V. negundo* may be improved via the use of a different solvent and a different extraction procedure, considering the polarity of the active compounds. Also, the purification of the active compound may lead to a higher anti-candidal activity.

The MIC values obtained for the ethanolic extract of

young leaves of *P. betle* against the five *Candida* spp. ranged from 0.64 to 3.2 mg/mL. In a previous study done using hydroxychavicol isolated from the chloroform extraction 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 (Ali et al., 2010). The higher MIC values obtained in the current study can be attributed to the fact that in the previous study (Ali et al., 2010) the bioassay was carried out with the purified compound hydroxyl-chavicol, while the current study used the crude extracts of the leaves of *P. betle*. In addition, in the study by Ali et al. (2010), the water extracts were tested while the current study used the ethanolic extracts. Hence, due to differences in the solvent and in the extraction procedure, there may be differences in the types and amounts of the active compounds extracted. Several studies suggest that the phenolics 2-hydroxychavicol (4-allylpyrocatechol) and chavibetol are the major active principles isolated from the ethanolic extract of the leaves of *P. betle* (Jitesh et al., 2006).

It is reported that the ethanolic extract of leaves of *P. betle* shows antimicrobial activity against *C. albicans* (Napisah et al., 2011) whereas literature is minimal on the anti-candidal activity of the ethanolic extract of the leaves of *P. betle* against non-albicans. To obtain precise MIC values, a concentration series within the range obtained in the current study can be further investigated. For more accurate and precise results, the isolation and characterization of the active compounds in the ethanolic extract of the leaves of *P. betle* is required.

The variation of MIC values between different *Candida* species could be attributed to the composition of the cell wall of *Candida* spp. (Odds, 2004). It can be inferred that C*. tropicalis* and *C. albicans* are the most resistant of the isolates while *C. parapsilosis* is the most susceptible to the extract. *C. albicans* is known to be the major human pathogen and its pathogenecity is aided by the virulence features including the production of germ tubes, bio-film formation and protein secretion including phospholipase, protease and esterase activity. Further studies are necessary to explain the high resistance of *C. tropicalis* to the ethanolic extract of leaves of *P. betle.* Non-albicans including *C. tropicalis* too are emerging human patho-gens although to a lesser extent as compared to *C. albicans* (Ellepola and Samaranayake, 2000).

It could be concluded that the ethanolic extract of young leaves of *P. betle* has significant anti-candidal activity against all the *Candida* spp. bioassayed. In addition, this was the first study investigating the effect of leaf maturity and climatic conditions on the anticandidal activity of *P. betle.* It was revealed that young leaves possess higher anticandidal activity as compared to mature leaves. Therefore, young leaves are more effective to be used for commercial antimicrobial formulations, as com-pared to the use of mature leaves. Hence, the use of young leaves of *P. betle* has a potential to replace drugs to which *Candida* spp. have evolved resistance, by incor-porating the active compounds to tablets, disinfectants, toothpastes, mouth washes, creams, *etc.* in the pharmaceutical and cosmetic industries.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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