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Antioxidant and antimicrobial potential of selected varieties of *Piper betle* L. (Betel leaf)

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ABSTRACT

Piper betle L., is an evergreen perennial creeper belonging to family Piperaceae and is known to possess numerous medicinal properties. Current study focuses on evaluating antioxidant and antimicrobial potential of betel leaf. For the present study, distilled water, hexane, acetone and ethanolic extracts of two varieties of betel leaves: *Meetha paan* and *Banarasi paan* were used. Biochemical tests such as proximate analysis (moisture, ash, protein, lipids, minerals viz., sodium and potassium), antioxidant activity tests (DPPH radical scavenging activity, total phenolics, ascorbic acid, reducing power) and antimicrobial test (antibacterial and antifungal susceptibility test) against four pathogens viz., *B. subtilis, E. coli, A. niger* and *S. cerevisiae* were determined. Ethanolic extract had the highest antioxidant activity (89.46% inhibition), while the aqueous extract exhibited lowest antioxidant activity (62.03% inhibition). With increasing concentration (5, 10, 25 and 50 µg/mL), the reducing power of leaf extracts also increased. The ascorbic acid was not significant in *Banarasi paan* (5.21mg/100 g) and *Meetha paan* (5.20mg/100 g). The highest antibacterial activity of ethanolic extract (*Banarasi paan*) may be attributed to the presence of phytosterols in the leaf varieties. Antioxidant and antimicrobial potential study will help to build a database and promote the utilization of betel leaf as a medicinal herb.

Key words: *Piper betle*, piperaceae, antioxidant, antimicrobial, biochemical.

INTRODUCTION

Naturally occurring herbs are being used for a long time in food and for medicinal purposes throughout the world. Although, modern approach towards lifestyle has isolated us from the natural way of life, mankind so far has been dependent on natural resources for its every need. We have been ignoring the rich natural heritage inherited from our ancestors. This ignorance has aggravated numerous health issues in our day to day lives such as digestive problems, aging problems, etc. However, as the menace of synthetic medicines and food additives in the form of preservatives, coloring agents,

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and antioxidants kept on increasing, mankind is becoming increasingly aware of the natural resources and its benefits. Several researches are now being directed to explore natural herbs for their nutraceutical, antimicrobial and nutritive potential. One such herb of importance is *Piper betle* L., commonly known as betel leaf.

Betel leaf belongs to the family Piperaceae. It has more than 100 varieties, all over the world of which about 40 of them are found in India. It grows in dry, loam and clay soils that contain high amount of detritus, maintaining a pH of 7-7.5. These heart shaped leaves are aromatic because of the presence of essential oils and its taste ranges from sweet to pungent (Pradhan et al. 2013). It is locally known as '*paan*' in Hindi (India) and is mostly consumed in the form of mouth freshener or appetizer in India.

The betel leaves are nutritive and possess an insecticidal and antitumor activity (Gundala and Aneja 2014), antioxidant activity (Jaiswal et al. 2014), neuroprotective activity (Chan and Wong 2014), antidiabetic and antihelmintic activity (Shah et al. 2016), antimicrobial activity (Nouri and Nafchi 2014) and many more. The leaves also contain a variety of biologically active components like hydroxychavicol, chavicol, piperbetol, chavibetol, piperol A, methylpiperbetol, and piperol. The key component of the leaf is a volatile oil known as betle oil (Kumari and Rao 2015, Widawati and Riandi 2015).

The present study was designed to evaluate the nutraceutical properties of two selected, popularly consumed varieties of *Piper betle* L., namely, *Meetha* and *Banarasi*. The antioxidant and antimicrobial potential of these varieties were analyzed. The database is aimed to help promote the utilization of betel leaf in nutraceutical and functional food development.

MATERIALS AND METHODS

SAMPLE COLLECTION

Healthy and young betel leaves were procured from the local market of Midnapore, West Bengal and Varanasi, Uttar Pradesh, India. The local names of different varieties taken for the present study were: *Meetha paan* (Midnapore, West Bengal, India) and *Banarasi paan* (Varanasi, Uttar Pradesh, India). The leaf varieties were authenticated in the Department of Botany, Lovely Professional University, Punjab (India).

SAMPLE PREPARATION

With the help of a chilled mortar and pestle, fresh leaves (10 g) were ground and dissolved in 100 mL of respective solvents i.e., distilled water, hexane, acetone, and ethanol (Himedia, India). The extracts were incubated in a shaker incubator (Remi Co., Model CIS 18 Plus, Mumbai, India) overnight at 28 ºC. Further, the samples were centrifuged at 10,000 rpm for 10 min. All the prepared samples were stored at -20 ºC until further analysis.

PHYTOCHEMICAL SCREENING

Different biochemical assays were performed on both varieties of betel leaves extracted with hexane, ethanol, acetone and distilled water for checking the presence of moisture, ash, carbohydrate, protein, lipids, total soluble solid (TSS), vitamin C, total phenolic, sodium and potassium.

PROXIMATE ANALYSIS

Proximate analysis of samples was carried out according to Association of Offical Analytical Chemists (AOAC) international methods (AOAC 2004). Moisture was determined by drying to a constant weight at 105 °C. Ash content was carried out at 550 °C (method 923.03). Crude protein (N × 6.25) content was determined by the micro-Kjeldahl procedure (method 960.52). Crude lipid content was quantified by extracting the sample with petroleum ether in a Soxhlet apparatus. Total soluble solids (TSS) content for the extract was determined with the help of hand refractrometer of range 0-32 ºBrix (Model ERMA) as described

in Ranganna (2007). The potassium and sodium content of leaves was determined by the method described in Ranganna (2007).

DETERMINATION OF ANTIOXIDANT ACTIVITY

Free radical scavenging activity was determined using DPPH radical on the basis of scavenging ability of the extracts of betel on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals (Jenitha and Anusuya 2016). The reducing power was determined by ferricyanide-ferric chloride method as described by Shiban et al. (2012). Total phenolic content of the sample was determined spectrophotometrically at 765 nm by using folin-ciocalteu's reagent (Rasane et al. 2015). Ascorbic acid content was determined using 2,6-dichlorophenol indophenol dye (AOAC 2004).

DETERMINATION OF ANTIMICROBIAL ACTIVITY

Antifungal and antibacterial susceptibility test

Two different betel leaf extracts were tested against four microorganisms viz; *Escherichia Coli* (gram negative), *Aspergillus niger* (fungi), *Bacillus subtilis* (gram positive), and *Saccharomyces cerevisiae* (yeast). Agar well diffusion method was used for testing anti-microbial activity of four sample extract. Antibacterial activity was determined by measuring the zone of inhibition (mm) around the well (Chakraborty and Shah 2011).

STATISTICAL ANALYSIS

Means and standard deviations of three replicates were determined for all the analysis in the present study. Significant difference of mean values was assessed by one-way analysis of variance (ANOVA) followed by Duncan's LSD test using the commercial statistical package SPSS ver.11.5 (SPSS Inc., Chicago, IL, USA) at a significance level of ($p \leq 0.05$).

RESULTS AND DISCUSSION

NUTRITION AND PHYTOCHEMICAL PROFILE OF BETEL LEAF

The nutritional and phytochemical profile of the betel leaf was analyzed and the result is shown in the Table I. The composition of lipids, vitamin C and total ash, including sodium and potassium content showed no significant ($p>0.05$) difference in both the varieties (*Meetha* and *Banarasi*) of leaves. There was slightly significant difference in the carbohydrates and protein amongst the two varieties of the leaves. The results were in accordance with the findings of Shah et al. (2016). The presence of the five industry' standard proximate composition reveals the nutritive value of the leaves (Table I). The essential oil contained in the leaves is known to possess antifungal, antibacterial and antiprotozoan properties with the potential to kill or inhibit the growth of disease causing bacteria. The significant vitamin and mineral content of leaves also adds to its nutritive value (Guha 2006). Betel leaf oil contains a phenol called chavinol, which has antiseptic properties. It was also reported by Dwivedi and Tripathi (2014) that, the chavinol in betel leaf is an aromatic compound responsible for the spicy odor of the leaves. Total soluble solids

TABLE I
Phytochemical screening of betel leaf.

Phytochemicals	*Banarasi*	*Meetha*
Moisture (%)	84.83 ± 0.38^{b}	86.48 ± 0.27^{a}
Ash (%)	4.30 ± 0.07^{a}	4.20 ± 0.03^{a}
Carbohydrate (%)	5.03 ± 0.03^{a}	5.20 ± 0.05^{b}
Protein (%)	3.20 ± 0.03^{b}	3.34 ± 0.05^{b}
Lipids (%)	0.80 ± 0.04^{a}	0.78 ± 0.02^{a}
TSS (ºBrix)	23.00 ± 0.08^{a}	23.03 ± 0.06^{a}
Total phenolics (mg/100 gm)	13.08 ± 2.03^{b}	15.03 ± 0.04^{a}
Vitamin C (mg/100 gm)	5.21 ± 0.01^{a}	5.20 ± 0.06^{a}
Sodium (mg/100 gm)	11.37 ± 0.44^{a}	11.35 ± 0.03^{a}
Phosphorus (mg/100 gm)	41.54 ± 0.07^{a}	40.33 ± 0.02^{b}

Mean values with different superscripts on the same row differ significantly (Duncan's LSD test, $p < 0.05$).

(TSS) of the betel leaves was found to be 24.0 °Brix in the *Banarasi* variety and 23.0 ºBrix in the *Meetha* variety. Similar results for the TSS on *Piper betle* L., were obtained in the study carried out by Guha (2006) and Pradhan et al. (2013).

ANTIOXIDANT ACTIVITY AND ASCORBIC ACID CONTENT

Free radical reactions occurring in the body are associated with numerous diseases and health problems such as cardiovascular diseases, neurological diseases, cancer and pulmonary diseases. These free radicals play a major role in the aging process. Antioxidants are the potential solution to encounter such anomalies. Naturally occurring antioxidants such as total polyphenols, vitamins, etc., present in herbs are involved in scavenging these free radicals. For the present study antioxidant activity of different betel leaf extracts were screened by free radical scavenging activity, measurement of reducing power, total polyphenol content and ascorbic acid content. The antioxidant activity is attributed to the phenol and flavonoid content of the leaves. Higher the content, the more is the antioxidant and radical scavenging activity. Studies by Rintu et al. (2015) revealed that variety with maximum total phenolic content exhibited maximum antioxidant and free radical scavenging activities. The present study was also in accordance with the above results.

FREE RADICAL SCAVENGING ACTIVITY

Free radicals are reactive oxygen species generated by cellular oxidative pathway. An imbalance in the generation and removal of these reactive species damages the biomolecules of cells, resulting in chronic diseases (Lee et al. 2009). Shah et al. (2016) also reported that betel leaf extracts exhibited free radical scavenging activity. The free radical scavenging activity was found to be maximum in the ethanolic extract (89.46% inhibition) and lowest (62.03% inhibition) in the distilled water extract. Similar observations were reported by Shah et al. (2016) and thus the ethanolic extract can be used in extending the shelf life of various products. Table II summarizes the DPPH scavenging activity and the descending order of the extracts can be arranged as follows Ethanolic *Meetha* (EM) > Ethanolic *Banarasi* (EB) > Acetonic *Banarasi* (AB) > Acetonic *Meetha* (AM) > Hexane *Banarasi* (HB) > Hexane *Meetha* (HM) > Distilled Water *Banarasi* (DWB) > Distilled Water *Meetha* (DWM). The results are comparable with that reported by Dasgupta and De (2004) for *Piper betle* L. Jaiswal et al. (2014) reported high antioxidant activity in the leaves because of the presence of phenolic compound hydroxyl chavicol (4-allyl pyrocatechol) and is proving to be a better preservative. Shah et al. (2016) reported that hydroxyl chavicol is effective against several strains of fungi and thus acts as a preservative.

TOTAL POLYPHENOLIC CONTENT

Polyphenols have assembled huge popularity due to their antioxidant properties and defending

TABLE II
Antioxidant activity of betel leaf extracts.

Samples	Free radical scavenging activity (% inhibition)
AM	82.28 ± 0.43^{d}
AB	85.34 ± 0.32^{c}
HM	76.40 ± 0.55^{f}
HB	78.73 ± 0.70^{e}
EM	87.34 ± 0.32^{b}
EB	89.46 ± 0.54^{a}
DWM	62.03 ± 0.05^{h}
DWB	74.68 ± 0.56^{g}

AM: acetone *meetha*, AB: acetone *banarasi*, HM: hexane *meetha*, HB: hexane *banarasi*, EM: ethanol *meetha*, EB: ethanol *banarasi*, DWM: distilled water *meetha*, DWB: distilled water *banarasi.*

Mean values with different superscripts on the same column differ significantly (Duncan's LSD test, $p < 0.05$).

actions against ultraviolet radiation or aggression by pathogens, parasites and predators. It has one or more aromatic rings with one or more aromatic group (Dai and Mumper 2010). The polyphenolic content of the betel leaf was 15.03 and 13.08 TAEmg/100 g, in *Meetha* and *Banarasi* varieties respectively, and varied significantly ($p<0.05$) amongst the varieties. For the antioxidant activity, the major components of *Piper betle* L. are chavinol, chavibetol, allylprotocatechol and eugenol (Jaiswal et al. 2014, Dwivedi and Tripathi 2014). The presence of phenolic compound contributes to the antioxidant activity of the leaves and allows them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, heavy metal chelators and hydroxyl radical quenchers (Jha et al. 2011).

MEASUREMENT OF FERRIC REDUCING ANTIOXIDANT POWER

Table III depicts the measurement of the reductive ability of leaf extracts. Reducing power of the extract is mainly due to the phenolic compounds and flavonoids present in raw formulations. Phenolic compounds and flavonoids have the ability to donate electrons and act as reductones and play a major role in the reducing power of the extracts (Gat and Ananthanarayan 2015). Shah et al. (2016) has shown that an ethyl acetate extract of betel leaf showed maximum Fe^{2+} ion reducing ability owing to its high phenolic content. Maximum activity was observed in the AM variety (25µg/mL) and the lowest was observed in the EB variety (5µg/mL). It was observed that along with the increase in concentration, there was an increase in absorbance. Higher absorbance indicates that the leaves possess strong reducing power. Study by Chakraborty and Shah (2011) using four extracts (methanol, aqueous, petroleum ether and ethyl acetate) have revealed similar results.

ASCORBIC ACID CONTENT

Various physiological functions including the synthesis of collagen, neurotransmitters and carnitine are carried out by vitamin C and it is regarded as the most important antioxidant. It is a strong reducing agent, and it is absence may lead to delay in wound healing and failure in repairing fractures (Molnar et al. 2014). The healing action is attributed to free radical scavenging activity (Shah et al. 2016). In the *Banarasi* and *Meetha* variety of leaf, vitamin C content was found to be 5.21 and 5.20 mg/100 g, respectively as illustrated in Table I. Similar data were recorded in a study carried out by Guha (2006) for *Piper betle* L. leaves. The presence of vitamin C indicates that, the leaves will act as superoxide neutralizer, oxygen quencher, a hydroxyl radical scavenger and promote antioxidant activity (Aguirre and May 2008). Saravanan et al. (2003) investigated and reported that, consumption of betel leaf increased the serum antioxidants like vitamin C and vitamin E. The leaves should

TABLE III
Reduction power of betel leaf extracts.

Samples conc. (µg / mL)	AM	HM	EM	DWM	AB	HB	EB	DWB
5	0.70 ± 0.004^{d}	0.25 ± 0.03^{d}	0.13 ± 0.34^{c}	0.36 ± 0.43^{a}	0.65 ± 0.03^{c}	0.22 ± 0.23^{d}	0.12 ± 0.23^{c}	0.33 ± 0.44^{b}
10	0.89 ± 0.12^{c}	0.27 ± 0.04^{c}	0.13 ± 0.20^{c}	0.37 ± 0.10^{a}	0.68 ± 0.43^{c}	0.26 ± 0.14^{c}	0.15 ± 0.33^{b}	0.34 ± 0.10^{b}
25	0.97 ± 0.22^{a}	0.29 ± 0.23^{b}	0.15 ± 0.02^{b}	0.39 ± 0.32^{a}	0.77 ± 0.12^{a}	0.29 ± 0.44^{a}	0.17 ± 0.43^{ab}	0.36 ± 0.04^{ab}
50	0.95 ± 0.03^{b}	0.34 ± 0.20^{a}	0.16 ± 0.35^{a}	0.38 ± 0.08^{a}	0.72 ± 0.06^{b}	0.33 ± 0.52^{b}	0.19 ± 0.12^{a}	0.39 ± 0.22^{a}

AM: acetone *meetha*, AB: acetone *banarasi*, HM: hexane *meetha*, HB: hexane *banarasi*, EM: ethanol *meetha*, EB: ethanol *banarasi*, DWM: distilled water *meetha*, DWB: distilled water *banarasi.*
Mean values with different superscripts on the same column differ significantly (Duncan's LSD test, $p < 0.05$).

be stored in a favorable condition to ensure the quantity retention in order to treat various diseases.

ANTIMICROBIAL ACTIVITY

Antibacterial susceptibility test

Antimicrobial activity was seen with respect to all the leaf extracts viz; aqueous, hexane, acetone and ethanol. The notable antibacterial effect was observed in relation to aqueous, hexane, acetone and ethanolic extracts. All extracts were effective against the test microorganisms.

Ethanolic extract of *Banarasi* variety reported highest antibacterial activity against *Bacillus subtilis*. Datta et al. (2011) reported that ethanolic and methanolic extracts of *Piper betle* L., possesses antimicrobial activity against pathogens (gram positive and negative). It was also shown by Agarwal et al. (2012) that aqueous, acetonic, methanolic and ethanolic extracts of *Piper betle* L., possess antimicrobial activity against both gram positive and gram negative pathogens with ethanolic extract having maximum activity. A zone of inhibition (mm) of Ethanolic extract of *Banarasi* and *Meetha* variety of leaves against various pathogens (*B.subtilis*, *E. coli,* and *S. cerevisiae*) is shown in Figure 1a, b, and c. Methanolic and ethanolic extract exhibited potent antibacterial activity against various pathogens. However, Kaur and Mondal (2014) showed that *Piper betle* L., extract are inactive against *E. coli* and *S. aureus*. The susceptibility of gram positive bacterial strains was found to be more because of simple cell wall along with small pores in the outer layer of the cell which possess a natural sieve effect against large molecules (Sugumaran et al. 2011). The antibacterial activity may be due to sterol, which is found in abundance in the betel leaf extracts and due to the surface interaction of the leaf extracts with the cell wall of bacteria thereby, destroying the bacterial components. The antibacterial activity is attributed to polyphenols (Tan and Chan 2014).

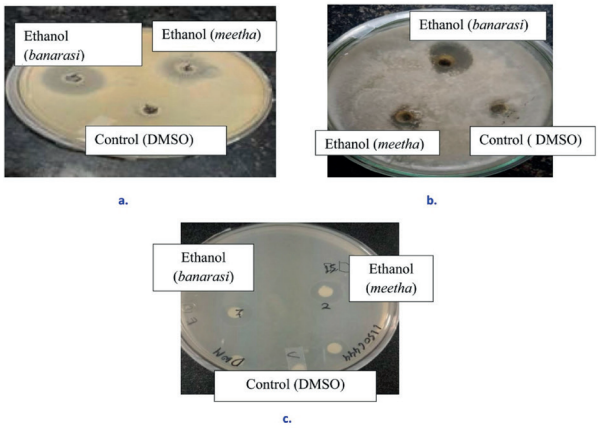

Figure 1 - Zone of inhibition (mm) of ethanolic extracts on **a**. *Bacillus subtilis*, **b**. *E. coli*, **c**. *Saccharomyces cerevisiae*.

Hoque et al. (2012) observed antibacterial activity against some spoilage microorganisms and food borne pathogens using ethanolic extract. The zone of inhibition is maximum for ethanolic extracts which is in accordance with the results of Agarwal et al. (2012). The results also suggested that compounds present in the betel leaf extract possess antibacterial properties against various pathogens. Antibacterial properties possessed by an ethanolic extract would be fruitfully utilized to extend the shelf-life of food or food products.

INHIBITION ZONE

Inhibition zone was found to be the highest for the DWM. Similar results were found in the study carried out by Chakraborty and Shah (2011). Various studies narrated that the betel leaf extract contains betel oil that possess antioxidant and antimicrobial properties (Jaiswal et al. 2014, Nouri and Nafchi 2014). Figure 2 depict the inhibition zone produced by different leaf extracts against *B. subtilis, E. coli, A. niger* and *S. cerevisiae*.

Previous studies on *Piper betle* L., extracts having high concentration of fatty acids like stearic acid, palmitic acid and hydroxy fatty acid esters showed positive antimicrobial activity against a wide range of pathogens (Khan and Kumar 2011).

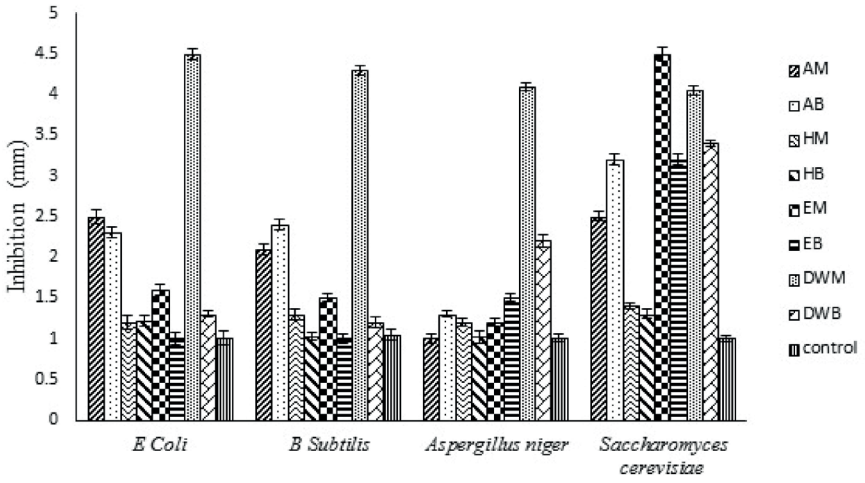

Figure 2 - Inhibition zone (mm) produced by different betel leaf extracts against various pathogens. Where, AM- Acetone *Meetha*, AB- Acetone *Banarasi*, HM- Hexane *Meetha*, HB- Hexane *Banarasi*, EM- Ethanol *Meetha*, EB- Ethanol *Banarasi*, DWM- Distilled Water *Meetha*, DWB- Distilled Water *Banarasi*

Ethanolic extract showed maximum inhibition zone against *E. coli* followed by *S. aureus*. All extracts (ethanol, distilled water, hexane and acetone) showed inhibition zone (mm) against all fungi, bacteria and yeast in the present study. Hexane, acetone and aqueous extracts showed higher inhibition than the ethanolic extracts. Lowest inhibition was observed in the EB and EM against *B. subtilis* and *E. coli* which indicates higher effectiveness. Deshpande and Kadam (2013) reported that the minimum inhibitory concentration (MIC) of ethyl acetate, methanol, petroleum ether extracts was low as compared to chloroform extract, and further stated that, the lower the minimum inhibitory concentration (MIC), the more effective is the betel leaf extract.

CONCLUSIONS

The present study unveiled the antimicrobial potential of *Piper betle* L. extracts. Ethanolic extracts showed the most effective result. The leaves are a good source of natural antioxidant for the pharmaceutical industry, that will accomplish desirable therapeutic outcomes and can be of great potential as a health care resource for fighting against various pathogens.

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