Myanmar Health Sciences Research Journal, Vol. 27, No. 3, 2015

Antioxidant Activity of Betel Leaf (*Piper betle* Linn.)

May Thandar Htun[*], *Wai Mi Aung, Aye Aye Phyu, Aye Thida Htun, Swe Zin Aung, Zin Nwe Soe, Swe Swe & Yi Yi Myint*

Department of Medical Research (Upper Myanmar)

Piper betle Linn. belongs to the family Piperaceae is an evergreen and perennial creeper with glossy heart-shaped leaves and white catkin. The leaves of *Piper betle* Linn. have been traditionally known for its various therapeutic uses such as stimulant, antitussive, carminative, expectorant, antihyperglycemic, antibacterial and antifungal. In the present study, the leaves of *Piper betle* Linn. were collected from cultivated sources in Kyaukse Township, Mandalay Region. Botanical identification of plant was done by morphological examinations. Phytochemical characters were studied by using reference analytical methods. The different extracts were obtained by percolation and reflux method. Antioxidant activities of fresh juice, ethanol extract and aqueous extract of *Piper betle* Linn. was determined by using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay and ascorbic acid was used as specific standard. While IC_{50} of ascorbic acid was 1.66 µg/ml, the concentration of *Piper betle* Linn. needed for 50% inhibition (IC_{50}) was found to be 92 µg/ml, 94 µl/ml, 95 µg/ml for ethanol extract, fresh juice and aqueous extract, respectively. The antioxidant activity of ethanol extract of *Piper betle* Linn. was found to be slightly more than fresh juice and aqueous extract. From above data, fresh juice of *Piper betle* Linn. possesses antioxidant activity which demonstrates the possibility of the use of this plant as supplementary medicine.

Key words: Antioxidant activity, *Piper betle* Linn., DPPH assay, IC_{50}

INTRODUCTION

Oxidative damage in the human body plays an important causal role in disease initiation and progression. Damage from reactive oxygen species (ROS) including free radicals has been linked to some neuro-degenerative disorders (Alzheimer's disease and Parkinson's) and cancers. ROS include free radicals such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and non-free-radical species such as H_2O_2 and singlet oxygen. These molecules are exacerbating factors in cellular injury and aging process.

Antioxidants, or antioxidation agents, reduce the effect of dangerous oxidants by binding together with these harmful molecules, decreasing their destructive power. Antioxidantscan also help repair damage already persistent by cells. Certain antioxidant enzymes are produced within the body. The most commonly recognized of these naturally occurring antioxidants are superoxide dismutase, catalase, and glutathione.[1]

The most commonly used antioxidants at present are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tert-butylhydroquinone. However, BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis. Therefore, the development and utilization of more effective anti-oxidants is desired. Much attention has been spotlighted on the antioxidative compounds present in plants because of safety

*To whom correspondence should be addressed.
Tel: +95-943015121
E-mail: mthanhtun@gmail.com

concerns associated with synthetic anti-oxidants.[2] A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials. Plants, which are rich in phenolic components, are of interest as sources of natural antioxidants. Phenolics display a wide variety of structures, ranging from simple moieties containing a single hydroxylated aromatic ring to highly complex polymeric substances.[3]

The betel (*Piper betle* Linn.) is the leaf of a vine belonging to the Piperaceae family. World Health Organization (WHO) figured out the betel plant as a mild stimulant and for its medicinal properties.[4] The betel plant is an evergreen perennial creeper, with glossy heart-shaped leaves and white catkin. The betel plant originated from South and South East Asia.

The leaves of *Piper betle* Linn. have been traditionally known for its various therapeutic uses such as stimulant, antitussive, carminative, astringent (juice of leaves with oil), expectorant, stomachic, relieving gastrointestinal disorders, hepatoprotective, antihyperglycemic, neuroprotective, antibacterial, antifungal, antihistaminic and antiulcer.[5-7]

The present investigation was undertaken to estimate the antioxidant potential of *Piper betle* Linn. extracts through DPPH (1, 1-diphenyl-picryl hydrazyl) *in vitro* assay model.

MATERIALS AND METHODS

The present study was laboratory-based experimental study. Identification of plant, extractions of different extracts and determination of phytochemical constituents were done at Pharmacology Research Division and antioxidant activity testing at Experimental Medicine Research Division, Department of Medical Research (Upper Myanmar).

This study was conducted from August to November, 2014.

Data analysis

The percent inhibition was calculated by the formula. The results were analyzed by using SPSS 20 (Statistical Package for Social Science) software. Then, IC_{50} values were calculated.

Plant materials and identification

The plant materials used in this study were collected from cultivated in Taung Bot Village, Kyaukse Township, Mandalay Region. The morphological characters of the plant were examined at Pharmacology Research Division, Department of Medical Research (Upper Myanmar) and verified according to the characters of ovaries and fruits, bracts, petioles of fertile branch leaves, lamina and pistillate spikes described in the reference monograph Dassanyake.[8]

Preparation of plant extracts

The leaves of *Piper betle* Linn. were air-dried for one week and then reduced into coarse powder by using powdering machine. The powdered material (100 gm) was percolated with 95% ethanol for a week by percolation method.[9]

The leaves powder were moistened with sufficient quantity of the solvent and allowed to stand for 4 hours in a well-closed vessel. Then, the content was packed in a percolator and sufficient amount of the solvent was added to saturate the materials. When the liquid began to drop from the percolator, the outlet was closed and sufficient amount of the solvent was added to leave a layer above the powdered sample and the content was allowed to macerate for 24 hours. The percolation process was allowed to proceed slowly until the percolate measured about three-quarters of the required volume. Then, this liquid extract was evaporated by using waterbath to give a dry mass.

For aqueous extract, 50 gm of powdered leaves of *Piper betle* Linn. was extracted by means of reflux apparatus. The aqueous extract was also evaporated by using the

waterbath to get the dryness. Fresh juice of betel leaves was prepared by using clean motor and pestle.

Phytochemical analysis of Piper betle Linn. leaves

Qualitative analysis for the active constituents was done by the quality control methods according to WHO.[10]

Detection of alkaloids

The dried powdered samples (1g) were boiled with 10% HCl (10 ml) for about 10 minutes, allowed to cool and filtered. The filtrate was divided into 4 portions and tested separately with Dragendorff's reagent, Mayer's reagent, Hager's reagent and Wagner's reagent. Observations were made to see the coloured precipitates.

Detection of carbohydrates

Molisch's test: Two drops of 10% alcoholic α-naphthol solution were added to 1.5 ml of aqueous extract, and then mixture was shaken well. This test tube was inclined at an angle of 45˚ and 1 ml of concentrated sulphuric acid was poured in slowly along the sides of the tube and allowed to stand so that it formed a separate layer at the bottom of the tube without mixing. A red ring was formed between the two layers.

Detection of glycosides

When 0.5 ml of 10% lead acetate was added to 1.5 ml of aqueous extract of the powdered sample, a white precipitate was produced.

Detection of phenols

To 1 ml of aqueous extract of the powdered sample, a few drops of 1% ferric chloride solution was added. Brownish green colour was formed.

Detection of amino acids

Ninhydrin test: The dried powdered sample (3 gm) was boiled with distilled water (25 cm^3) for about 10 min and then filtered. An aliquot portion of filtrate was transferred to a filter paper with the help of micro-pipette and allowed to dry. Then the filter paper was sprayed with ninhydrin reagent and dried at 110ºC in oven and then purple colour appeared.

Detection of saponins

The aqueous extract (2 ml) is diluted with distilled water and made up to 20 ml. The suspension is shaken in a graduated cylinder for 15 min. Formation of 2 cm layer of foam was produced.

Detection of starch

Iodine 1.5 gm and 7.5 gm of potassium iodide were dissolved in 50 ml of distilled water and added 2-3 ml of an aqueous extract of the powdered sample. A blue colour was produced.

Detection of tannins

To 1 ml of aqueous extract of the powdered sample, a few milliliters of 2% sodium chloride solution was added and shaken and a few drops of gelatin solution was added. A white precipitate was produced.

Detection of flavonoids

The dried powdered sample (1g) was boiled with 10 ml of 95% alcohol and filtered. A few drops of concentrated hydrochloric acid was added into a test tube containing a few milliliters of alcoholic extract of the powdered sample followed by small pieces of magnesium turnings. A pink colour was observed.

Detection of steroids

Liebermann-Burchard's test: A few drops of acetic anhydride solution were added to 1 ml of petroleum ether extract of the powdered sample followed by a few drops of concentrated sulphuric acid. A green colour developed.

Detection of cyanogenetic glycoside

Distilled water (5 ml) was added to 0.5 g of dried powdered sample followed by a few drops of concentrated sulphuric acid and sodium picrate and then it was boiled. A pink colour developed.

Antioxidant activity testing

Antioxidant activity of plant extracts was determined by DPPH method. The extracts

used in this assay were 95% ethanol extract, aqueous extract and fresh juice. The stock solution was prepared by dissolving plant extract with 95% ethanol at concentration of 1mg/ml. From this stock solution, different concentrations (50, 100, 150 and 200 µg/ml) were prepared. The sample solutions were prepared by mixing thoroughly with 1 ml of 60 µM DPPH solution (2.36 mg of DPPH in 100 ml of 95% ethanol) and 1 ml of different concentrations of each plant extracts which was prepared from stock solution and were mixed vigorously by a vortex mixer.

Ascorbic acid was used as standard and prepared as in sample solution. Blank solution was prepared without the addition of DPPH solution. All solutions were allowed to stand at room temperature (27ºC) for 30 minutes after which measurement of absorbance was done at 517 nm using UV-Visible 1601PC, Shimadzu Spectrophotometer. Absorbance measurements were done in triplicate and the absorbance was calculated to obtain the percent inhibition by using the following formula:

$$\%\text{Inhibition} = \frac{\text{DPPH alone} - (\text{Sample} - \text{Blank})}{\text{DPPH alone}} \times 100$$

% Inhibition=Percent inhibition of sample extract to be tested
DPPH alone=Absorbance of 60 µM DPPH solution
Sample=Absorbance of test sample solution
Blank=Absorbance of blank solution

RESULTS

Identification of plant

The betel plant is vine climbing with adventitious roots and stem is woody. The ovaries and fruits concrescent of betel plant are fused with spike axis. Bracts are distinct. Petioles of fertile branch leaves 1-1.7, lamina not or very shallowly cordate, shining at least on upper side. Pistillate spikes about as long as their peduncles and leaves are bright green. These characters are also mentioned for *Piper betle* L. belongs to the family Piperaceae by Dassanyake.[8] Due to this above characters, the plant was identified as *Piper betle* Linn.

Extraction of Piper betle Linn. leaves

The yield percentage of different extracts of *Piper betle* Linn. leaves by different solvents were 16 for distilled water and 7.4 for 95% ethanol.

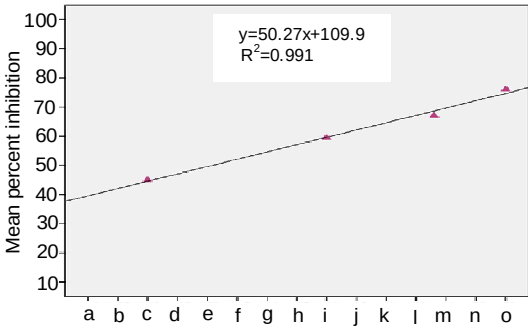
Table 1. Phytochemical analysis of leaves powder of *Piper betle* Linn.

Type of compound	Reagent	Results
Alkaloids	Dragendroff's ragent	+
	Mayer's reagent	+
	Hager's reagent	+
	Wagner's reagent	+
Carbohydrates	10% α-napthol and H_2SO_4	+
Glycosides	10% lead acetate solution	+
Phenolic compounds	5% Ferric chloride solution	+
α-amino acid	Ninhydrin reagent	+
Saponins	Distilled water	+
Starch	I_2 and KI_2 solution	+
Tannins	1% Gelatin and 2% NaCl solution	+
Flavonoid	Conc: HCL, Mg turnings	-
Steroids	Acetic anhydride solution and conc: H_2SO_4	+
Cyanogenetic glycoside	Distilled water, conc: H_2SO_4, sodium picrate	-

+=Detected, -=Not detected

Table 2. Results for determination of antioxidant activities

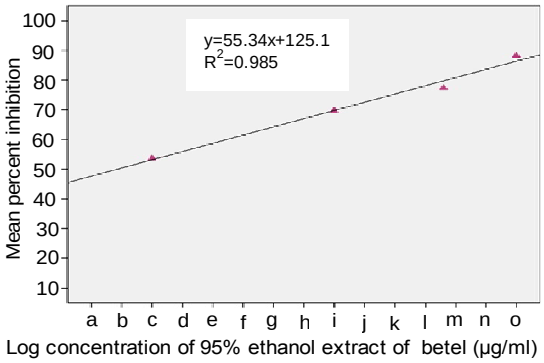
	Inhibition percent (mean ± SD) in various concentrations (n=3)				IC_{50}
Extract	*50 µg/ml*	*100 µg/ml*	*150 µg/ml*	*200 µg/ml*	
Aqueous	44.98 ±0.65	59.44 ±1.37	67.08 ±2.14	76.11 ±0.97	95
95% Ethanol	53.68 ±1.02	69.68 ±1.42	77.42 ±0.41	88.28 ±0.55	92
Fresh juice	48.08 ±0.45	74.86 ±1.41	85.79 ±0.99	90.38 ±1.05	94
Standard	*1 µg/ml*	*2.5 µg/ml*	*5 µg/ml*	*7.5 µg/ml*	
Ascorbic acid	40.01 ±0.36	56.19 ±1.64	76.00 ±1.334	84.00 ±1.99	1.66



a = -1.40 d = -1.25 g = -1.10 j = -0.95 m = -0.80
b = -1.35 e = -1.20 h = -1.05 k = -0.90 n = -0.75
c = -1.30 f = -1.15 i = -1.00 l = -0.85 o = -0.70

The regression coefficient (R^2) was 0.991 and IC_{50} was 95 µg/ml.

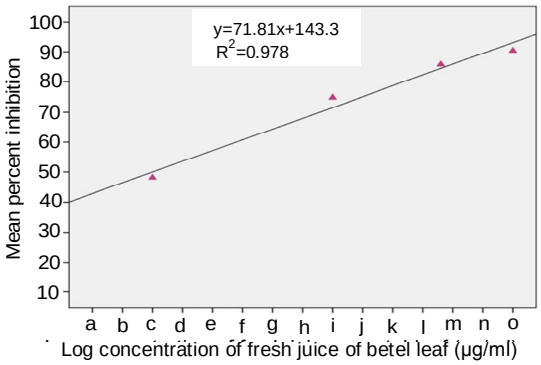
Fig. 1. Linear regression of % inhibition with log concentration of aqueous extract



a = -1.40 d = -1.25 g = -1.10 j = -0.95 m = -0.80
b = -1.35 e = -1.20 h = -1.05 k = -0.90 n = -0.75
c = -1.30 f = -1.15 i = -1.00 l = -0.85 o = -0.70

The regression coefficient (R^2) was 0.985 and IC_{50} was 92 µg/ml.

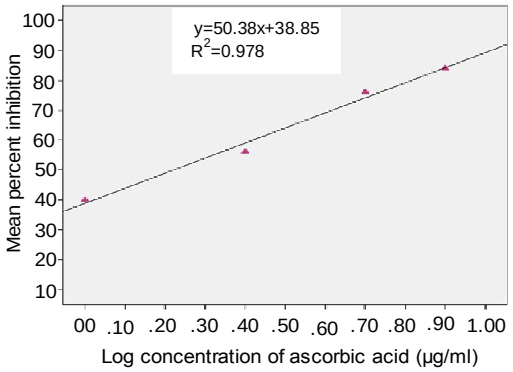
Fig. 2. Linear regression of % inhibition with log concentration of 95% ethanol extract



a = -1.40 d = -1.25 g = -1.10 j = -0.95 m = -0.80
b = -1.35 e = -1.20 h = -1.05 k = -0.90 n = -0.75
c = -1.30 f = -1.15 i = -1.00 l = -0.85 o = -0.70

The regression coefficient (R^2) was 0.978 and IC_{50} was 94 µg/ml.

Fig. 3. Linear regression of % inhibition with log concentration of fresh juice



The regression coefficient (R^2) was 0.989 and IC_{50} was 1.66 µg/ml.

Fig. 4. Linear regression of % inhibition with log concentration of ascorbic acid

Phytochemical analysis of leaves powder of Piper betle Linn.

The results of phytochemical analysis of leaves powder from *Piper betle* Linn. are shown in Table 1.

Determination of antioxidant activities by 1, 1-diphenyl-picryl hydrazyl (DPPH) assay

The IC_{50} values of leaf extracts were 92 µg/ml for 95% ethanol extract, 94 µl/ml for fresh juice and 95 µg/ml for aqueous extract. The IC_{50} value of ascorbic acid was 1.66 µg/ml (Table 2).

DISCUSSION

The yields of crude extracts of plant constituents were different. This difference may be due to different solubility of powder leaves in respective solvents and the power of vaporization of solvents. In this study, the yield of aqueous extract was higher than 95% ethanol extract.

Therefore, the constituents in leaves powder are more soluble in water. Soxhlet extraction method can get high yield percentage of extract. But the constituents may be degraded because of heating in Soxhlet extraction method. So, percolation method is more convenient for extraction of thermolabile constituents from plant materials.

It was observed that the sample leaves powder possess alkaloids, carbohydrate, glycosides, phenols, steroids, saponins and amino acid. In antioxidant activity testing, all the extracts of *Piper betle* L. showed DPPH scavenging effect. But the IC_{50} values observed were found to be lower than that of standard ascorbic acid. The IC_{50} values of betel leaf extracts were 92 µg/ml, 94 µl/ml, 95 µg/ml for ethanol extract, fresh juice and aqueous extract respectively. The DPPH radical scavenging effects of the extracts of leaves of *Piper betle* Linn. decreased in the following order: 95% ethanol extract > fresh juice > aqueous extract.

Conclusion

The results of this study indicated that betel leaf extracts are rich in antioxidant activity. It may be due to the constituents of phenolic compounds which are believed to have the ability of free radical scavenging and anti-oxidation. Hence, this plant can be used to discover bioactive natural products that may help in the development of supplementary medicine from medicinal plants.

ACKNOWLEDGEMENT

We would like to express our sincere gratitude to Dr. Kyae Mhon Htwe, Deputy Director/Head, and staff from Experimental Medicine Research Division, DMR (UM) for the successful completion of this work.

REFERENCES

1. Mates JM, Perez GC & Nudezde CI. *Clinical Biochemistry* 1999; 32: 595-603.
2. Imaida K, Fukushima S, Shirai T, Ohtaui M, Nakanishi K & Ito N. *Carcinogenesis* 1983; 4: 7895-7899.
3. Dixon R & Paiva NL. Stress-induced phenyl-propanoid metabolism, *Plant Cell Biology* 1995; 7: 1085.
4. Swapna NL, Ammani K & Prasad HKR. Antioxidant activity of Mokkathotapapada leaves of *Piper betel* L. *Free Radicals and Antioxidants* 2012; 2(4): 68-72.
5. Prokopczyk B, Rivenson A & Hoffmann D. A study of betel quid carcinogenesis IX. Comparative carcinogenicity of 3-(methyl-nitrosamino) propionitrile and 4-(methylnitro-samino)-1-(3-pyridyl)-1-butanone upon local application to mouse skin and rat oral mucosa. *Cancer Letters* 1991; 60(2): 153-157.
6. Sharma DC. Betel quid and areca nut are carci-nogenic without tobacco. *The Lancet Oncology* 2003; 4(10): 587.
7. Santhaikumari P. Antihyperglycemic activity of *Piper betle* leaf on streptozotocin-induced diabetic rats, *Journal of Medicinal Food Spring* 2006; 9(1): 108-112.
8. Dassanyake MD. A Revised Handbook to the Flora of Ceylon, Amerind Publishing Co. Pvt. Ltd. 1987; 6: 274-289.
9. British Pharmacopoeia. The Pharmaceutical Press, HMSO, London: The general medical council, 2007.
10. WHO. Quality control methods for medicinal plant materials, Geneva: World Health Organi-zation, 1998.