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ANTIOXIDANT, CYTOTOXIC AND PHYTOCHEMICAL PROPERTIES OF THE ETHANOL EXTRACT OF *PIPER BETLE* LEAF

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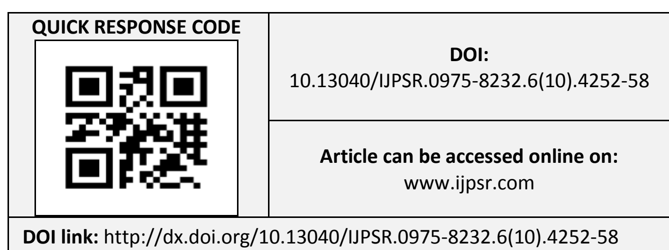
ABSTRACT: The use of natural products for the treatment of various diseases has a long history. In the present study, the anti-oxidative, cytotoxic and phytochemical properties of ethanol extract of a local variety of *Piper betle* leaves cultivated in Potia, Bangladesh has been investigated. The antioxidant and cytotoxic activity of the *Piper betle* ethanol extract were evaluated by DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging method and brine shrimp lethality bioassay method, respectively. Preliminary phytochemical group tests were also done to investigate the phytochemical properties of the extract. Leaf extract showed significant DPPH free radical scavenging effect compared to standard antioxidant ascorbic acid. IC_{50} value of ascorbic acid and leaf extract was found 1.81µg/ml and 151.36µg/ml respectively. In brine shrimp lethality bioassay LC_{50} value of *Piper betle* ethanol extract was found 274.63862µg/ml with 95% confidence limit where the lower and upper limits were 198.39 µg/ml and 387.18µg/ml respectively, which indicates that the *Piper betle* leaf extract has promising cytotoxic effect. Phytochemical analysis was found to be positive for alkaloid, glycosides, terpenoids, flavonoids, tannins and saponins. The present study demonstrates that ethanol extract of *Piper betle* Linn. Leaf has significant antioxidant and cytotoxic effect.

INTRODUCTION: *Piper betle* commonly called betel leaf is a climbing shrub or small tree indigenous to tropical Asia, Australasia, and the Pacific and grown mostly in Bangladesh, India, China, Bhutan, and Malaysia [1-3]. The betel plant is an evergreen and perennial creeper, with glossy heart-shaped leaves and white catkin. The medicinal properties of betel leaf are well known since time immemorial. Traditionally the leaves are used to treat various diseases like halitosis, boils and abscesses, conjunctivitis, constipation, swelling of gums, cuts and injuries. The essential oil contained in the leaves is known to possess anti-bacterial, anti-protozoan and anti-fungal properties.



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Antioxidants are type of molecules that neutralize harmful free radicals, produced through a chain of reactions [4] that damage living cells, spoil foods; degrade materials such as rubber, gasoline, lubricating oil. Antioxidants terminate these chain reactions through the removal of free radical intermediates and inhibition of other oxidation reactions [5]. This is why plants and animals maintain complex systems of multiple antioxidants, such as glutathione, vitamin C, and vitamin E along with some enzymes like catalase, superoxide dismutase and various peroxidases.

The use of antioxidants in pharmacology is intensively studied as oxidative stress might be an important part of many human diseases particularly stroke and neurodegenerative incidents. Antioxidants, therefore, are routinely added to meals, oils, foodstuffs, and other materials to prevent free radical damage. Recently there has been an upsurge of interest in the therapeutic

potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. A lot of new plant species have been investigated in the search for novel antioxidants [6-9] other than well known and traditionally used natural antioxidants from tea, wine, fruits, vegetables and spices [10] but there is still a demand to find more information on the antioxidant potential of plant species. Cytotoxicity usually gives a preliminary idea on the anticancer effect of plant extract containing bioactive compounds those are toxic to living body at higher doses and pharmacologically beneficial at lower doses.

Scientists have been proving that all the natural things are not good for health. Different retrospective studies done over the last 20 years indicated that the incidence of deaths occurring due to exposure to plants (as a proportion of total patients poisoned by traditional plant medicine) was about 1.5% in France, 5% in Belgium, 6.5% in Italy, 7.2% in Switzerland and 6% in Turkey [11]. There is, therefore, a need to have an understanding of the risks posed by herbal medicines so as to ensure that such products could be used safely. However, study on cytotoxicity of plant extract could give us an idea of the anticancer as well as toxic profile of studied plant extract.

MATERIALS AND METHODS:

Collection of plant leaves:

Fresh plant leaves of *Piper betle* were collected from the Potia, Chittagong, Bangladesh. The plant leaves were collected in the sterilized polythene bag. The polythene bags were sterilized with 95% ethanol. Then the bags were properly tied and labeled. After that the collected samples were brought to the laboratory.

Preparation of leaf extract:

The fresh *Piper betle* leaves were washed with distilled water, air dried at room temperature for about 10 days, ground into powder (500 gm) and extracted with ethanol, being stirred and macerated at room temperature (23 ± 5^0C) for 15 days. The ethanol was evaporated under reduced pressure below 50^0C through rotary vacuum evaporator (Bibby RE200, Sterlin Ltd., England). The concentrated extract (52 gm blackish-green) was stored at 4^0C for further use.

In vitro assay for antioxidant activity of leaf extract:

The antioxidant activity of *Piper betle* extract was assessed in comparison to standard antioxidant ascorbic acid (BDH, England) depending on the scavenging effect of 1, 1-diphenyl- 2-picrylhydrazyl (DPPH)-free radical. The whole procedure was administrated according to established procedure by Braca *et al.*[19-20]. Ascorbic acid solution (5 ml) and different concentrations of extract (20, 40, 60, 80, 100, 200, 400 and 800μg/ml in methanol) solutions (5 ml) were mixed with 3 ml of 0.4 mM (0.004 %) DPPH solution. The mixtures were kept in dark for 30 minutes to measure the absorbance at 517 nm using UV-Visible Spectrophotometer (UV-1601 Shimadzu, Kyoto, Japan) and ascorbic acid was used as a positive control. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The degree of decolorization of DPPH from purple to yellow indicated the scavenging efficiency of the extract. The scavenging activity against DPPH was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [(A - B) / A] \times 100$$

Where,

A is absorbance of control (DPPH solution without the sample),

B is the absorbance of DPPH solution in the presence of the sample (extract/ ascorbic acid).

The scavenging activity (%) or % inhibition was then plotted against log concentration and from the graph IC_{50} (Inhibition concentration 50) value was calculated by linear regression analysis [21].

Assay for cytotoxicity of leaf extract:

Cytotoxic activity of plant extract was determined by Brine Shrimp lethality bioassay as described by Meyer *et al.* [22]. Shrimp eggs were added to the artificial “sea water” (38 gm sea salt pure Sodium chloride weighed, dissolved in 1 liter of distilled water adjusted to pH 8.5 using 1N NaOH and was filtered off to get clear solution) in the larger compartment of an unequally dividend tank which was darkened by covering it with Aluminum foil [23]. The chamber was kept under illumination using a table lamp for 48 hrs for the eggs to hatch into

shrimp larvae. The illuminated compartment attracts shrimp larvae (nauplii) through perforations in the dam. 10 shrimp larvae were added to 10 ml of sea water in 10 test tubes and 1000, 800, 600, 400, 200, 100, 80, 60, 40 and 20µg/ml solutions of extracts, prepared from 10 mg/ml of crude through serial dilution, were added to these test tubes. Each concentration was tested in triplicate. A control containing 10 ml of DMSO solvent was used for each solvent.

The test tubes were maintained under illumination. After 24 hours have elapsed, survivors were counted with the aid of a 3X magnifying glass. From the % lethality of brine shrimp, the probits were calculated for each concentration by using computer software "BioStat-2006". Probits were then plotted against corresponding log concentration of leaf extract to get LC_{50} (lethal concentration 50) value through regression analysis.

Phytochemical analysis:

Test for Alkaloids:

To the extract added 1% HCl and 6 drops of Mayer's reagent and Dragendroff's reagent. An organic precipitate indicated the presence of alkaloids in the sample [24].

Test for Flavonoids:

5 ml of dilute ammonia solution were added to a portion of aqueous filtrate of each plant extract followed by addition of conc. H_2SO_4. A yellow coloration is observed which confirms the presence of flavonoids and it disappears on standing [25].

Test of glycosides:

Dissolve small amount of an alcoholic extract of the fresh or dried material in one ml of water. Add a few drops of aqueous NaOH solution. Yellow color indicates the presence of glycoside [25].

Test for Steroids:

2 ml of acetic anhydride was added to 0.5gm of ethanolic extract of each sample with 2 ml of H_2SO_4. The color change from violet to blue or green indicated the presence of steroids [26].

Test for Tannins:

5 ml of extract was added to few drops of 1% lead acetate. A yellow precipitate indicated the presence of tannins [26].

Test for Terpenoids:

5 ml of each extract was added to 2 ml of chloroform and 3 ml of conc. H_2SO_4 to form a monolayer of reddish brown coloration of the interface was showed to form positive result for the terpenoids [25].

Test for Saponins:

The extract with 20 ml of distilled water was agitated in a graduated cylinder for 15 minutes. The formation of 1cm layer of foam indicated the presence of saponins [24].

RESULTS AND DISCUSSION:

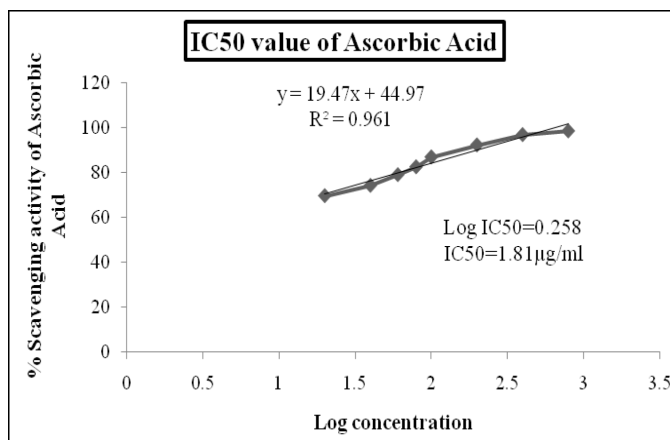
Assay for anti-oxidative activity of *Piper betle* leaf extract:

The DPPH free radical scavenging activity of the *Piper betle* leaf extract and ascorbic acid is shown in **Table 1.**

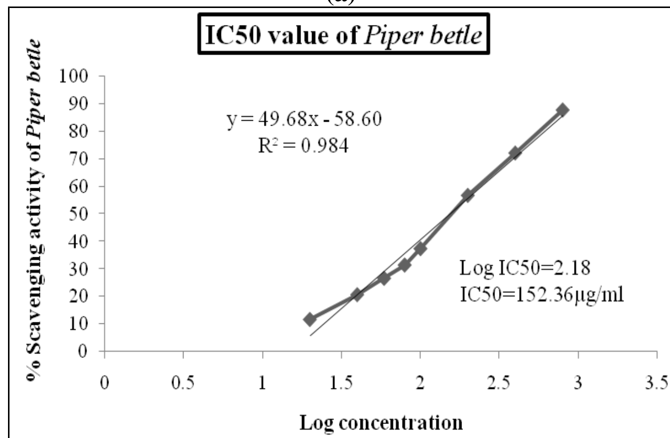
TABLE 1: DPPH FREE RADICAL SCAVENGING ACTIVITY OF ASCORBIC ACID AND *PIPER BETLE* LEAF EXTRACT

Test material	Concentration (µg/ml)	% Scavenging Activity	IC_{50} (µg/ml)
Ascorbic acid (Standard)	20	69.54128	IC_{50} = 1.81 µg/ml
	40	74.12844	
	60	78.89908	
	80	82.38532	
	100	86.78899	
	200	92.11009	
	400	96.69724	
	800	98.34862	
Piper betle leaf extract	20	11.81619	IC_{50} = 151.36 µg/ml
	40	20.78774	
	60	26.69584	
	80	31.50984	
	100	37.41794	
	200	56.67396	
	400	71.99124	
	800	87.52735	

Both ascorbic acid and *Piper betle* ethanol extract showed dose dependent activity. Among the eight different concentrations used in the study (20, 40, 60, 80, 100, 200, 400 and 800µg/ml) ascorbic acid showed 69.541289%, 74.12844%, 78.89908%, 82.38532%, 86.788996%, 92.11009%, 96.69724%, 98.34862% scavenging activity where highest scavenging activity was 98.34862% at concentration 800µg/ml. On the other hand, *Piper betle* ethanol extract showed 11.81619%, 20.78774%, 26.69584%, 31.50984%, 37.41794%, 56.67396%, 71.99124%, and 87.52735% scavenging activity at the above mentioned eight different concentrations where highest scavenging activity of *Piper betle* ethanol extract was 87.52735% at concentration 800µg/ml. % of scavenging activity or % of inhibition was plotted against log concentration and from the graph IC_{50} (Inhibition concentration 50) value was calculated by linear regression analysis. IC_{50} value of ascorbic acid and *Piper betle* ethanol extract was found 1.81µg/ml and 151.36 µg/ml, respectively **(Fig.1)**.



(a)



(b)

FIG.1: COMPARATIVE IC_{50} VALUES OF REFERENCE ANTIOXIDANT AND STUDIED PLANT *PIPER BETLE* LEAF EXTRACT: (A) ASCORBIC ACID (B) *PIPER BETLE*.

The IC_{50} value obtained for *Piper betle* extract and ascorbic acid **(Fig.1)** indicates that *Piper betle* leaf extract possess efficiency to neutralize free radicals higher than ascorbic acid **(Fig. 2)**.

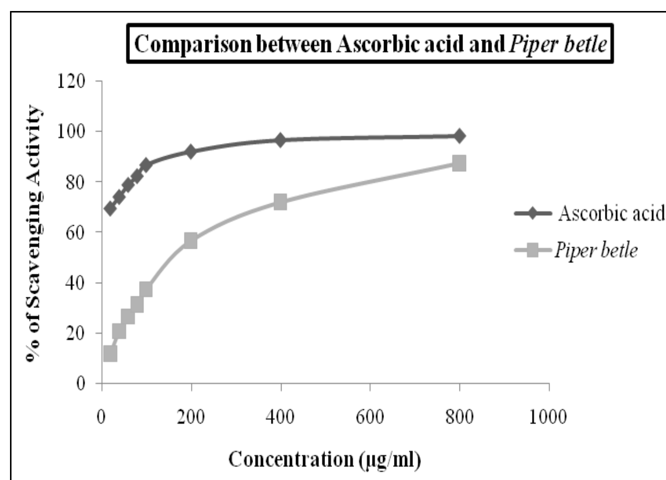


FIG. 2: COMPARATIVE % SCAVENGING ACTIVITIES OF *PIPER BETLE* LEAF EXTRACT AND ASCORBIC ACID.

Different research suggests that most of the plant extracts showing antioxidant activity are due to the presence of phenolic compounds [27-28]. Phenolic natural compounds such as flavonoids **(Table 4)** possess antioxidant activity due to their redox properties which allow them to act as reducing agents and singlet oxygen quencher. In addition, they have metal chelating potentials. The phenolic compounds, identified in the extract might contribute to the antioxidant activity of *Piper betle* leaf extract.

Assay for cytotoxicity of *Piper betle* leaf extract: Percentage lethality of brine shrimp at ten different concentrations (20 to 1000µg/ml) of *Piper betle* ethanol extract is presented in **Table 2**. Plant extract showed lethality in a dose dependent manner [29]. More specifically 0, 0, 10, 10, 20, 40, 50, 70, 90 and 100% mortality of brine shrimp was observed at 20, 40, 60, 80 100, 200, 400, 600, 800 and 1000µg/ml concentrations respectively **(Table 2)**.

From the % lethality of brine shrimp, the probits were calculated for each concentration by using computer program "BioStat-2006". Response (%) or lethality (%) was then plotted against corresponding log concentration of plant extract. From this plot, LC_{50} (lethal concentration 50) value was found by regression analysis using computer

program "BioStat-2006". LC_{50} value of *Piper betle* ethanol extract was found 274.63862µg/ml with 95% confidence limit where the lower and upper limits were 198.39 and 387.18µg/ml respectively (**Table 3 and Fig.3**).

TABLE 2: BRINE SHRIMP CYTOTOXICITY OF *PIPER BETLE* LEAF ETHANOL EXTRACT

Dose (µg/ml)	Log dose	Total (n)	Alive	Death	% Lethality	Actual%*	Probit Y*
20	1.30	10	10	0	0	2.5	0.0036
40	1.60	10	10	0	0	2.5	0.0239
60	1.78	10	9	1	10	10	0.0591
80	1.90	10	9	1	10	10	0.1025
100	2.00	10	8	2	20	20	0.1497
200	2.30	10	6	4	40	40	0.3723
400	2.60	10	5	5	50	50	0.6504
600	2.78	10	3	7	70	70	0.7890
800	2.90	10	1	9	90	90	0.8640
1000	3.00	10	0	10	100	97.5	0.9079

*ProbitY were calculated using statistical software "Biostat 2006"
*Actual % = Actual formulas (n is the number of animals in a group): For the 0% dead, 100 (0.25/n), for the 100% dead, 100 (n-.25) /n.

TABLE 3: CALCULATION OF LC_{50} VALUE, REGRESSION EQUATION, CONFIDENCE LIMIT AND CHI SQUARE BY PROBIT ANALYSIS.

Log10 LC50	LC50 (µg/ml)	95% confidence limit (µg/ml)	Regression equation	Chi square
2.43876	274.63862	198.39-387.18	Y=61.14x-96.49	2.28011

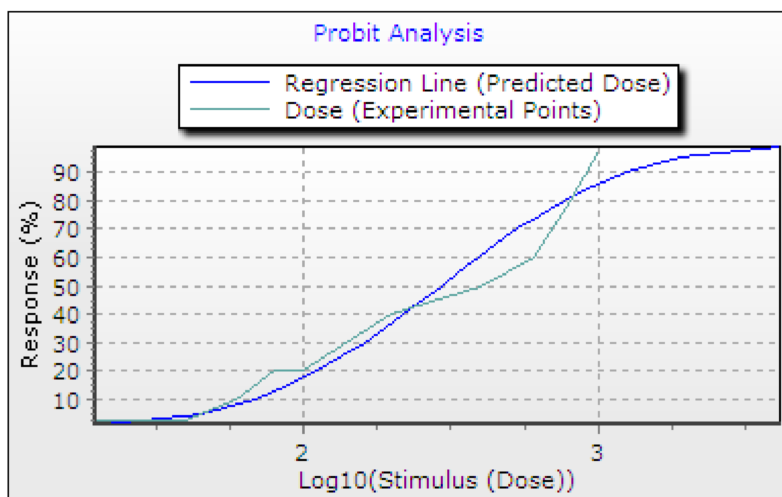


FIG.3: REGRESSION LINE FOR DETERMINING THE LC_{50} VALUE OF ETHANOL EXTRACT OF *PIPER BETLE*

Preliminary Phytochemical Screening:

The present study carried out on the plant samples revealed the presence of medicinally active metabolites. The phytochemical characters of both the plants investigated are summarized in the **Table 4** given below.

TABLE 4: OBSERVATION ON PHYTOCHEMICAL GROUP TESTS

Secondary Metabolite	Name of the test	Observation	Result
Alkaloids	Mayer's test	Creamy white precipitate	++
	Hager's test	Yellow crystalline precipitate	++
	Wagner's test	Brown precipitate	++
Glycosides	General test	Yellow color	++
Cardiac glycosides	Legal's test	Pink to red color	++
	Baljet's test	Yellow orange color	++
Anthraquinone glycoside	For O-glycoside	Rose pink in the aqueous layer	++

	For C-glycoside	Rose red coloration in aqueous layer	++
	For aglycones	Bright pink coloration	++
Terpenoids	Salkowsky test	Red color	++
Flavonoids	General test	Rose pink in the aqueous layer	++
	Specific test	Orange to red color	++
Tannins	$FeCl_3$ test	Brownish green color	++
Saponins	Frothing test	Change observed	++

N.B. "++" stands for the presence and "--" indicates the absence of secondary metabolites.

Qualitative secondary metabolite tests **(Table 4)** showed that *Piper betle* possess alkaloid, terpenoids, steroids and flavonoids. Plants produce a huge variety of secondary compounds as natural protection against microbial and insect attack. Many of these compounds have been used in the form of whole plants or plant extracts for food or medical applications in man because plants are the natural reservoir of many antimicrobial, anticancer agents, analgesics, anti-diarrheal, antifungal as well as various therapeutic activities [30]. Presence of such alkaloid and flavonoids might have some role in showing antioxidant and cytotoxic properties of the *Piper betle*.

CONCLUSIONS: The results of the study demonstrate that the ethanol extract of *Piper betle* leaf exhibits very potential antioxidant and cytotoxic effect in experimental models which support the claims by traditional medicine practitioners. These results can be strong scientific evidence to use this plant variety as a useful source of antioxidant references. However, further studies are still necessary to elucidate a mechanistic way how the plant contributes in these pharmacologic properties. Phytochemical investigation in order to isolate the active fraction and eventually the pure compound will be a future study.

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