

EVALUATION OF BIOACTIVE CONSTITUENTS AND *IN-VITRO* ANTIOXIDANT ACTIVITY OF DIFFERENT EXTRACTS OF *PIPER BETLE* L. LEAVES.

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ABSTRACT

Antioxidant potential of various extracts of *Piper betle* L. leaves viz. pet ether, aqueous, ethyl acetate and ethanol was determined by the Fentons reagent (hydroxyl radical scavenging activity) and FRAP Assay (Fe^{3+} reducing power). All the extracts of *Piper betle* L. showed the different amount of phytochemical constituents and reducing capacity, whereas ethanol extract exhibited the highest level of hydroxyl radical scavenging activity 90.11 ± 0.275 at $10 \mu\text{g/ml}$, followed by ethyl acetate 55.05 ± 0.601 at $5 \mu\text{g/ml}$, aqueous 50.02 ± 0.339 at $5 \mu\text{g/ml}$ and pet.ether 45.43 ± 0.049 at $5 \mu\text{g/ml}$, likewise, Ethyl acetate exhibited high reduction ability of 1.299 ± 0.019 at $50 \mu\text{g/ml}$ followed by aqueous 0.440 ± 0.014 at $50 \mu\text{g/ml}$, pet ether 0.366 ± 0.021 at $50 \mu\text{g/ml}$

and ethanol extract 0.128 ± 0.018 at $5 \mu\text{g/ml}$. The order of antioxidant activity in *Piper betle* L. extracts displayed from higher to lower level as ethanol, aqueous, ethyl acetate and pet ether extracts. The antioxidant potential of *Piper betle* L. leave extracts significantly correlated with the phytochemical constituents particularly phenolic, alkaloid and flavonoid present in the extract. DMSO taken as control showed highest antioxidant power in the present study.

KEYWORDS: *Piper betle* L., Antioxidant, Fenton's reaction and FRAP.

INTRODUCTION

Plants are invaluable sources of new drugs and have been intensively studied for phytochemicals for their antitumor properties. Despite great progress in medicine, use of herbal medicines is still popular. Herbal medicines have been in use since ancient times for general health and for specific diseases.^[1] Herbal plants used in traditional medicine contain a

wide range of bioactive compounds that can be used to treat contagious diseases.^[2-4] There is an enormous historical legacy regarding the use of plant preparations in folk medicine.^[5] Scientific studies on plants used in ethnomedicine have led to the discovery of many valuable drugs such as taxol, navelbine, camptothecin, vincristine, etc.

Piper betle L. the green gold of india is commonly known as the betel vine and belongs to the family *Piperaceae* i.e. the Black Pepper family.^[6] It is an important medicinal and recreational plant in Southeast Asia. The most probable place of origin of betel vine is Malaysia but today the plants are also cultivated in India, Srilanka, Bangladesh, Burma and Nepal.^[7-8] The deep green heart shaped leaves of betel vine are popularly known as Paan in India. It is also known as Nagaballi, Nagurvel, Saptaseera, Sompatra, Tamalapaku, Tambul, Tambuli, Vaksha Patra, Vettilai, Voojangalata etc in different parts of the country.^[9-10] The leaves are the most valued plant part and in the past were routinely used as a chewing agent to prevent halitosis. The phenolic constituent allylpyrocatechol from the leaves showed activity against obligate oral anaerobes responsible for halitosis.^[11] Medicinally the leaves are useful in catarrhal and pulmonary affections.^[12] The leaf extract has significant stimulatory influence on pancreatic lipase activity in experimental rats.^[13] The leaf extract inhibits radiation induced lipid per-oxidation. The extract also increased the activity of superoxide dismutase activity in a dose dependent manner, indicating elevation of antioxidant status in Swiss albino mice.^[14] *Piper betle* leaves also afforded a significant hepato-protective effect and improved the tissue antioxidant status by increasing the levels of non enzymatic antioxidants (reduced glutathione, vitamin C and vitamin E) and the activities of free radical-detoxifying enzymes in liver and kidney of ethanol-treated rats.^[15] Several workers have reported on the different biological activities of *Piper betle* in various *in vitro* and *in vivo* test models. Preclinical experiments have shown that betel leaf possess anti oxidant,^[16-20] anti-bacterial,^[21] anti-cariogenic,^[22] anti-fungal,^[23] anti- larval, anti- protozal, anti-filarial, anti-allergic, anti diabetic,^[24] anti-inflammatory,^[25-26] hepatoprotective, anti microbial,^[27] anti-ulcer,^[28] anti-fertility,^[29-33] Cardioprotective,^[34] anti-hyperlipidaemic, anti-platelet, vasorelaxation, immunomodulatory radio protective effects.^[35]

The present study aims to assess the preliminary phytochemical constituents and antioxidant activity of various extracts of *Piper betle* L. leaves.

MATERIALS AND METHODS

Reagent and Chemicals

All the chemicals and solvents were of analytical grade used in research at Pinnacle Biomedical Lab (PBRI) Bhopal.

Preparation of *Piper betle* L. Extracts

Fresh and disease free leaves of *Piper betle* L. were collected from the local market at Bhopal city of M.P. during the month of September to October. The plant was acknowledged by a senior Botanist Dr. Sanjay Sahay, Professor, Department of Botany, Govt. Science and Commerce College Benazir, Bhopal. After identification of plant a specimen was procured in herbarium record maintained at Govt. Science and Commerce College Benazir, Bhopal, M.P. The leaves were washed thoroughly 2 to 3 times in sterile distilled water. The leaf materials were then air dried under shade at room temperature for 8 days and finely powdered. The coarsely powdered dried leaves were cited in a container with 1000 mL pet. Ether solvent for defating and permissible to stand at room temperature for a period of at least 3 days with recurrent agitation until the soluble matter has dissolved.^[36-37] Similarly, the defatted leaf extract was further placed in the same container after utterly washing of container with distilled water. The leaf extract was further soaked in the container with distilled water to make an aqueous extract of *Piper betle* L. leaves.

Phytochemical analysis

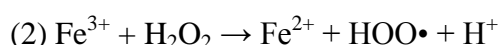
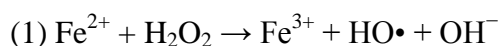
The phytochemicals showed difference in their presence or absence in different solvent extract. That is, presence of carbohydrates and saponins was detected positive in ethanol and aqueous extracts and negative for pet.ether and ethyl acetate extract of *Piper betle* leaves. The test for steroids and terpenoids were positive in ethanol extract. The test for flavonoids gave positive result in all extracts. The test for phenolic compounds was positive in pet. ether, ethyl acetate and ethanol extracts. The test for tannin was positive only in ethyl acetate and ethanol extracts. All preliminary and main tests were conducted to screen the presence of bioactive compounds. The results are discussed in the table 1.

Anti oxidant capacity

Hydroxyl Radical Scavenging Activity Assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction.^[38-39] Reaction mixture contained 60 μ L of 1.0 mM FeCl_3 , 90 μ L of 1 mM 1,10-phenanthroline, 2.4 mL of 0.2 M phosphate buffer (pH 7.8), 150 μ L of 0.17 M H_2O_2 , and 1.5 mL of extract at

various concentrations. After incubation at room temperature for 5 min, the absorbance of reaction mixture was noted at 560 nm. The hydroxyl radicals scavenging activity was calculated according to the following equation and compared with ascorbic acid as standard: where was the absorbance of blank (without extract) and was the absorbance of tested samples. Iron (II) is oxidized by hydrogen peroxide to iron (III), forming a hydroxyl radical and a hydroxide ion in the process. Iron (III) is then reduced back to iron (II) by another molecule of hydrogen peroxide, forming a hydro peroxyl radical and a proton. The net effect is a disproportionate of hydrogen peroxide to create two different oxygen-radical species, with water ($H^+ + OH^-$) as a byproduct.



Ferric Reducing Antioxidant Potential (FRAP) Assay

Ferric reducing power of *Piper Betle L.* extracts were determined using FRAP assay.^[40-41] This method is based on the reduction of colourless ferric complex (Fe^{3+} tripyridyltriazine) to blue-colored ferrous complex (Fe^{2+} tripyridyltriazine) by the action of electron donating antioxidants at low pH. The reduction was monitored by measuring the change of absorbance at 593 nm. The working FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer, pH 3.6, with 1 volume of 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 mM HCl and with 1 volume of 20 mM ferric chloride. All the required solutions were freshly prepared before their uses. 100 μ L of samples (mg/mL) were added to 3 mL of prepared FRAP reagent. The reaction mixture was incubated in a water bath for 30 min at 37°C. Then, the absorbance of the samples was measured at 593 nm. The difference between absorbance of sample and the absorbance of blank was calculated and used to calculate the FRAP value. FRAP value was expressed in terms of mmol Fe^{2+} /g of sample using ferric chloride standard curve. All measurements were calculated from the value obtained from triplicate assays.

Statistical Analysis

Results were expressed as mean value \pm SD. Regression analysis was performed to calculate the dose-response relation between the extracts. Linear regression analysis was performed to find out the correlation coefficient. Statistical significance was evaluated employing t-test and which were considered to be significant.

RESULT AND DISCUSSION

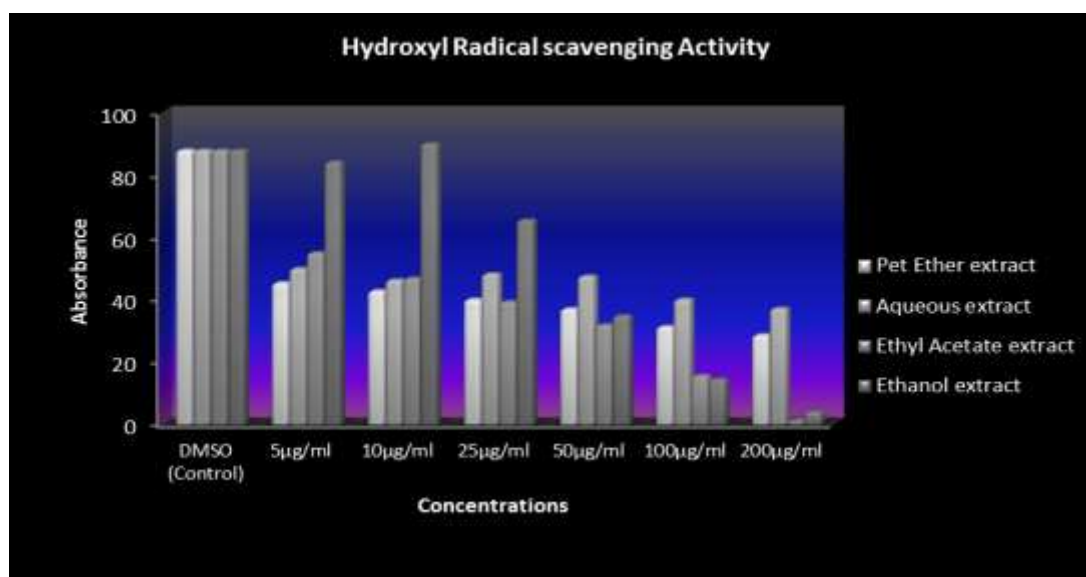
The *Piper betle* leaf extracts confirmed the presence of various useful bioactive constituents. All the tests performed for the presence of phytochemicals revealed positive results (Table 1). Antioxidant potential of different extracts of *Piper betle* L. demonstrated the potent antioxidant activity by using Fentons and FRAP Radical Scavenging Assay. Table 3 represents the measurement of reductive ability based on the principle of reduction (low pH) of ferric tripyridyl triazine complex to ferrous. Increase in absorbance indicates increased reducing power. Ethyl Acetate extract exhibited overall maximum reduction activity in concentration of 50µg/ml. Ethanol extract showed minimum reduction power. The ethanolic extract exhibited maximum hydroxyl radical scavenging activity followed by ethyl acetate, aqueous and pet ether extract simultaneously (Table 2 & Graph 1). However when the similar extracts were subjected for FRAP assay the ethyl acetate exhibited remarkable high reduction ability (Table 3 & Graph 2). Our results are in accordance with the findings of P Maisuthisakul,^[42] who reported Ethyl acetate to be the best solvent for the extraction of anti oxidant compounds from betel leaves due to its nonpolar components and also confirmed relationship between yield, total phenolic content and antioxidant activities for Betel leaf extracts. Arembewala et, al (2006)^[43] repoted the antioxidant activity extracts obtained from the leaves of *P. betle* had profound antioxidant activity as judged by DPPH_E scavenging assay and the scavenging effects of *P. betle* extracts on DPPH radicals decreased in the following order, cold ethanolic extract (CEE) > essential oil (EO) > hot water extract (HWE). Our results are also in accordance with the findings of Islam et al. (2010),^[44] who reported antioxidant activity of chloroform and ethyl acetate extracts of *Piper betle* L. leaves with IC values 9.187µg/ml and 4.56 µg/ml, respectively. Similarly Mokhtar et. al, (2008)^[45] reported that the three extracts of *P. betle* demonstrated considerable ferric reducing capabilities, with the ethyl acetate extracts having the strongest activity. Our findings are also in accordance with the findings of Dasgupta and Baratati (2004)^[46] who reported that aqueous extracts of three local varieties of *P. betle* had significant antioxidant activity. The extracts were found to have different levels of antioxidant activity in the systems tested. The antioxidant activities of the three varieties were in the order Kauri>Ghanagete>Bagerhati.

Table 1: Showing presence of different primary and secondary metabolites in crude extracts of *Piper betle* leaves.

Secondary Metabolite	Extract 2 (Pet. Ether)	Extract 3 (Aqueous)	Extract 4 (Eth. Acetate)	Extract 1 (Ethanol)
Carbohydrates	-	+	-	+
Flavanoids	+	+	+	+
Tannins	-	+	+	+
Saponin	-	+	-	+
Phenols	+	+	+	+
Glycosides	+	+	+	+
Alkaloids	-	+	+	+
Proteins	-	-	-	+
steroids	-	-	-	+
terpenoids	-	-	-	+

Table 2: Showing hydroxyl radical scavenging activity of different extracts of *Piper betle* L. leaf

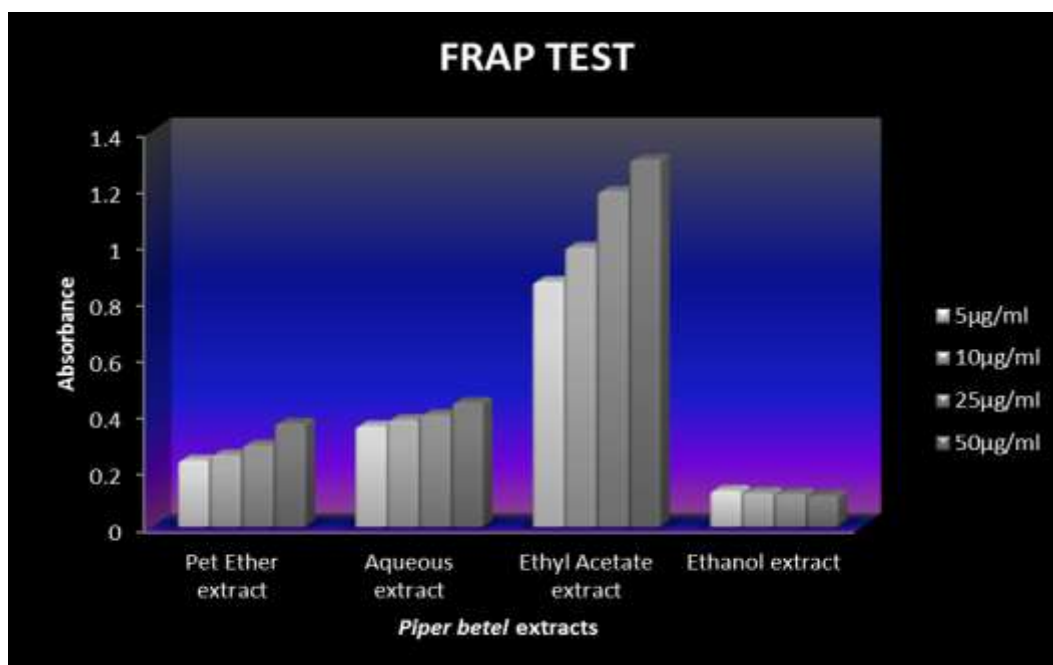
Concentrations	Pet Ether extract	Aqueous extract	Ethyl Acetate extract	Ethanol extract
DMSO (Control)	87.97±0.374	87.97±0.374	87.97±0.374	87.97±0.374
5µg/ml	45.43±0.049	50.02±0.339	55.05±0.601	84.24±0.608
10µg/ml	42.87±0.091	46.34±0.466	46.88±0.438	90.11±0.275
25µg/ml	40.12±0.268	48.26±0.5232	39.34±0.466	65.56±0.311
50µg/ml	37.10±0.282	47.55±0.544	31.89±0.129	34.89±0.304
100µg/ml	31.34±0.466	40.13±0.615	15.67±0.4454	14.56±0.424
200µg/ml	28.68±0.226	37.22±0.197	01.11±0.5586	04.12±0.489



Graph 1: Graph showing hydroxyl radical scavenging activity of different extracts of *Piper betle* leaf

Table 3: Showing reductive ability of different extracts of *Piper betle* leaf

Concentrations	Pet Ether extract	Aqueous extract	Ethyl Acetate extract	Ethanol extract
5µg/ml	0.235±0.014	0.356±0.015	0.867±0.024	0.128±0.018
10µg/ml	0.254±0.019	0.378±0.021	0.989±0.0212	0.121±0.0212
25µg/ml	0.289±0.014	0.396±0.021	1.187±0.0106	0.117±0.019
50µg/ml	0.366±0.021	0.440±0.014	1.299±0.019	0.112±0.0141

**Graph 2: Graph showing reductive ability of different extracts of *Piper betle* leaf**

CONCLUSION

Based on the above study it can be concluded that *Piper betle* which has been used in traditional medicine since time immemorial can be a good source for the production of antioxidant drugs which will be natural and without side effects. Future work of the current study includes the further screening and pharmacological evaluation of the factors responsible for antioxidant properties.

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