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Biomolecule protective, antioxygenic and anxiolytic properties of *Piper betel* leaves extract.

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Abstract

Piper betel L. is the leaf of a vine belonging to the Piperaceae family. This study was aimed at investigating the biomolecule protective, *in vitro* antioxidant and anti-anxiety properties. The hydro-alcohol extract was examined for radical scavenging ability against 2, 2-diphenyl-2-picryl hydrazyl (DPPH), 2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals, metal cheating inhibition, DNA and protein damage protection assay, anti lipid peroxidation, reducing power and nitric oxide radical scavenging activities. The tests employed for anti-anxiety property are open field test (OFT) and elevated plus maze test (EPM) conducted in mice. The IC_{50} values for metal chelating power, ABTS radical scavenging, NO scavenging and *in vitro* lipid peroxidation were 272.3µg ml-1,343.3µg ml-1, 309.1µg ml-1 and 146.3µg ml-1 respectively. The plant extract provided protection against DNA and protein damage and this was comparable to gallic acid, the standard. The animals receiving the extracts also showed an increase in the time of stay and number of entries in the open arm of the EPM with increased time mobile in the OFT. The study suggests that the extract of *Piper betel* possesses antioxidant and anti-anxiety properties, providing significant protection against oxidative damage to major biomolecules.

Keywords: Piper betel L, DPPH, ABTS, open field test, elevated plus maze test.

Introduction

Oxidative stress has been implicated in depression, anxiety disorders and high anxiety levels [1-2]. Oxidative stress can alter neurotransmission, neuronal function and overall brain activity resulting in cell death [3]. Oxidative stress has been associated with several diseases which are specific for nervous system impairment including neurodegradative and psychiatric diseases, such as schizophrenia, and anxiety [4]. Anxiety is a normal emotional response to a threat or potential threat. When this emotion is inappropriate and persistent, it is classified as pathological [5]. Anxiety is controlled by the nervous system and that GABAergic and serotonergic systems play an important role in the regulation of anxiety [6].

The *Piper betel* is the leaf of a vine belonging to the Piperaceae family. It has analgesic and cooling properties and biological activities described for the essential oil include antifungal, antiseptic and anthelmintic effects [7]. Carminative, aphrodisiac and anticancerous properties of *P. betel* essential oil have also been reported [8].The *Piper betel* leaves extract possess antioxidant, antimicrobial and anti-inflammatory effects [9]. *In vivo* studies also showed that *Piper betel* extracts improve antioxidant status in oxidative stress condition [10-11]. The objective of the present study was to evaluate the free radical scavenging, protection of biomolecules and anxiolytic properties of hydro-alcohol extract of *Piper betel* leaf.

Materials and methods

Chemicals

Ethanol was used as solvent for extraction of antioxidant compounds. NaOH, HCl, DPPH, BHA, gallic acid, Folin-Ciocalteu reagent, $FeCl_2$, ferrozine, potassium ferricyanide, EDTA, ascorbic acid, TPTZ(2,4,6,-tripyridy-striazine), TCA, $FeCl_3$, Na_2CO_3, catechin, thiobarbituric acid (TBA) were of analytical grade and were stored at prescribed conditions in the laboratory.

Plant material

100gms of crushed plant material was used for extraction. This sample was soaked overnight in 70% ethyl alcohol. This extraction was repeated by adding fresh solvent, for another 48 hrs. The pooled extract was subjected to filtration through normal filter paper followed by Whatman No.1. This filtered 70% alcohol extract was subjected to flash evaporator followed by lyophilization. The lyophilized samples were analyzed further for its antioxidant activities by various *in vitro* assays and anxiolytic activity.

Animal experiment:

Animal studies were conducted according to the institute animal ethical committee regulations approved by the committee for the purpose of the control and supervision of experiments on animals. Male mice weighing 25-30 g were selected from the stock colony, Defence Food Research Laboratory, Mysore, India, housed in an acryl fiber cage in a temperature controlled room temperature (25 ± 2º C) and was maintained in 12 h light/ dark cycle with free access to diet and drinking water.

Experimental design:

The extracts of *Piper bete/* leaves were separately suspended in a vehicle comprising 1% w/w Tween 20 in distilled water. Various doses viz., 100, 200 or 400 mg kg^{-1} of 70% ethanol extract of *Piper bete/* were prepared by suspending the dried extracts in vehicle. Six mice were taken in each group. The doses of extracts were so adjusted as to administer 0.25 ml of the suspension of extracts. Diazepam 1 $mgkg^{-1}$ suspended in the vehicle was used as standard anxiolytic. The suspending vehicle (0.25 ml) was used as control.

Total phenol content

The total phenol content of different extracts was measured using colorimetric Folin –Ciocalteu method of Singleton & Rossi [12]. The reaction mixture consisted 5ml of diluted sample to which 3 ml of distilled water and 0.5ml Folin –Ciocalteu reagent was added. After 3minutes, add 2ml of 20% Na_2CO_3 solution and placed the tubes in boiling water bath for one min, cooled and the absorbance was measured at 760 nm. Standard graph was prepared by using different concentration of gallic acid.

Total flavonoid content

The flavonoid content of different extracts was measured using a modified method of Zou, Lu & Wei [13]. 0.5ml of sample was mixed with 0.5 ml of 2% $AlCl_3$ and incubated for 10mins and the absorbance was measured at 415 nm. The measurement was compared to a standard graph using catechin.

Determination of reducing power (Fe^{3+} - Fe^{2+} transformation ability)

The reducing power of different extracts was measured using a method of Oyaizu [14]. Various concentrations of the extracts in 1ml of water were mixed with phosphate buffer (2.5 ml, 0.2 M pH6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50ºC for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged al 3000xg for 10 min. upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and freshly prepared $FeCl_3$ solution (0.5ml, 0.1%). The absorbance was measured at 700nm.

DPPH radical scavenging activity

The assay is based on the measurement of the scavenging ability of antioxidant towards the stable radical DPPH using a method of Chen et al [15]. DPPH radical react with suitable reagent, the electrons become paired off and the solution looses color depending on number of electrons taken up. A volume of 2ml of sample was added to 2ml of phosphate buffer (0.02M, pH 6) and 2ml of 0.2mM DPPH in ethanol. The mixture was shaken and left for 30 min. at R.T. and the absorbance was measured at 517 nm.

ABTS radical scavenging assay

ABTS assay, the method of Re et al [16] was adopted. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The solution was then diluted by mixing 1 ml ABTS $^{+}$ solution with methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min.

Nitric oxide radical scavenging activity

The assay is based on the measurement of the scavenging ability of antioxidant towards the NO radical using a method of Ilavarasan et al [17]. Sodium nitroprusside (5mM, 1ml) in phosphate buffer saline (PBS) incubated at 25ºC for 150 min with samples. 0.5 ml of the samples was mixed with 0.5 ml of Griess reagent. The absorbance was measured at 546 nm.

Chelating effects on ferrous ions

The ability of the leaf extracts to chelate ferrous ions was estimated by the method of Dinis et al [18]. Briefly, 2 ml of various concentrations of the extracts in methanol were added to a solution of 2 mM $FeCl_2$ (0.05ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml). The mixture was shaken vigorously and left at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm.

Inhibition of lipid peroxidation

The anti-lipid peroxidation assay was estimated by Liuet al [19] with modification. Liver excised from adult male Wister rats, was homogenized (20 g 100 ml^{-1}tris buffer) in 0.02 $molL^{-1}$, tris buffer (pH 7.4). Liver homogenate was incubated with 200µM of AAPH with or without extract in a total volume of 1 mL of phosphate buffer (pH 7.4). After incubation at 37 ºC for 60 min, 1 ml of 20% TCA and 1ml of TBA was added to the reaction mixture and boiled for 15 min. The TBA reactive substances (TBARS) were calculated from the absorbance at 535 nm where BHA was used as the positive control.

DNA damage protective activity

DNA damage preventive property of Piper betel leaves was checked using pBR322 plasmid. Plasmid is treated with AAPH the radical inducer in presence of plant extract and checked on 1% agarose according to Russo et al [20] with minor modifications. In brief, the experiment was performed in a 10µl in a micro centrifuge tube containing 200ng of plasmid pBR322 DNA AAPH was added at final concentration of 200 mM ml-1 with various concentrations of plant extracts (1 µl of 1mg ml-1 concentration). Along with plant extract, known antioxidants such as gallic acid were also used at the concentration of 100 µM. The reactions were initiated by incubating the tubes for 60 min in incubator. After incubation the reaction mixture along with gel loading dye (6X) was loaded on to 1% agarose gel and run at 200v for 1hr. Untreated pBR322 plasmid DNA was used as a positive control in each run of gel electrophoresis.

Inhibition of protein oxidation

Protein oxidation was assayed as described previously Kwon et al [21] with minor modifications. Oxidation of BSA (5µg) in phosphate buffer was initiated by 200 mM AAPH and the inhibitory properties of extracts were measured at a fixed concentration (1mg ml-1). After incubation for 2 hours at 37ºC, 0.02% BHT was added to prevent the formation of further peroxyl radical. The samples were then analyzed with normal SDS-PAGE.

Acetylcholinesterase activity

Acetylcholinesterase inhibitory property was measured following the method of Ellman et al. following Howes [22]. Electric eel AchE was used for assay, the reaction mixture contained 0.02 ml AchE (19.93 unit/ml buffer, pH 8), 0.01ml plant extract, 1ml buffer, 0.01 ml 0.5mM DTNB and 0.02 ml. 0.6mM acetyl thiocholine iodide solution. The reaction mixture was incubated at 37^0C for 20 min. The absorbance was measured at 412 nm immediately.

Elevated plus-maze test

The test procedure and scoring methodology for the elevated plus-maze test have been described by Kulkarni [23].In brief, the apparatus was composed of two open (30 x 5 x 0.25 cm) and two enclosed (30 x 5 x 15 cm) arms that radiated from a central platform (5 x 5 cm) to form a plus sign. The plus-maze was elevated to a height of 40 cm above floor level by a single central support. The mice were injected with drugs or vehicle and, sixty minutes later, the trial was started by placing an animal on the central platform of the maze facing an open arm. The number of entries into, and the time spent in, each of the two types of arm, were counted during a 5 min test period. The open-arm entries and open-arm time were used as indices of anxiety. A mouse was considered to have entered an arm when all four paws were on the arm.

Open Field test

Spontaneous motor activity was evaluated in open field test have been described by Bhattacharya & Satyan [24]. The open field apparatus is made up of black plexiglass and consisted of square 56 cm x 56 cm. The entire floor of the apparatus was divided into 16 squares of identical dimension. The entire room, except the open field was kept dark during the experiment. One hour after vehicle/standard/extract treatment each animal was placed at one corner of the apparatus and the behavioral aspects were noted in the next 5min.

Statistical analysis

Experimental results are expressed as means ± SD and was analysed by one-way ANOVA. All measurements were replicated three times. The data were analyzed by windows excel. The IC_{50} values were calculated from linear regression analysis. The groups treated with extracts and diazepam was compared with the control group. P values <0.05 were considered statistically significant. All behavioral recordings were carried out using ANY MAZE software from Columbus Instruments, Ohio, USA..

Results and Discussion

Antioxygenic and biomolecule protective properties

Total phenolic and flavonoid content

Flavonoids as one of the most diverse and widespread group of natural compounds are likely to be the most important natural phenolics. There is a correlation between phenolic compounds and antioxidant activity [25]. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities [26]. The total phenolic contents of plant extract were 140µg gallic acid equivalent mg^{-1} and the total flavonoid contents of plant extract was 25.1 µg catechin equivalent mg^{-1} of sample.

Determination of reducing power (Fe^{3+} - Fe^{2+} transformation ability)

The presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron [27]. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig 1 shows that the reducing power of *Piper bete*/leaves extract increased with the increase of concentration as in the case of gallic acid, the standard.

DPPH radical scavenging activity

DPPH assay is one of the most widely used methods for screening of antioxidant activity of plant extracts [28]. Result shows the ability of scavenging of DPPH radicals by the extract at various

concentrations. The IC_{50} values of plant extract was 436 µgml^{-1}and that of the standard BHA was 10.4 µg ml^{-1}.The results showed that extract is a free radical inhibitor as well as a primary antioxidant that react with free radicals which may limit the occurrence of free radical damage in human body (Fig 1).

ABTS assay

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, decreases with the scavenging of the proton radicals [29]. The *Piper betel* leaves was fast and effective scavengers of the ABTS radical and this activity was comparable to that of vitamin C. Higher concentrations of the extracts were more effective in quenching free radicals in the system. Ascorbic acid has shown higher antioxidant activity (% inhibition) as compared to *Piper betel* leaves with IC_{50} values of 343.3µgml^{-1} and 22.6µg ml^{-1} respectively (Fig 1).

Metal chelating power

The ferrous ion chelating effect was shown by 70% ethanol extract and EDTA (Fig 1). Ferrozine can quantitatively form complexes with Fe^{2+}. It is reported that chelating agents, who form ϭ- bonds with metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of metal ion [30]. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red colour complex. Therefore, measurement of colour reduction allows estimating the metal chelating activity of the co-existing chelator. The extracts of *Piper betel* leaves and the standard EDTA showed metal chelating power; with IC_{50} values, 272.3 µg ml^{-1} and 26.7µg ml^{-1} respectively.

Nitric oxide radical scavenging activity

The betel leaves extract effectively reduced the generation of nitric oxide from sodium nitroprusside. *In vitro* inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent [31]. The percentage scavenging activity increased with increasing concentration of the extract. The IC50 value of *Piper betel* leaf extract was found to be 309.1µgml^{-1}and that of standard BHA was 49.1µg ml^{-1}(Fig 1).

Prevention of Lipid peroxidation

Rat liver homogenate was used and induction of lipid peroxides by AAPH -induced generation of ROS, especially hydroxyl radical and peroxyl radical, which are capable of inducing lipid peroxidation leading to the production of malondialdehyde (MDA) was studied [32]. The *Piper betel* leaves extract was able to prevent lipid peroxidation, with the IC_{50} value, 146.3µg ml^{-1} (Fig 1). At the same time IC_{50} value of standard BHA was 16.3µg ml^{-1}.

DNA damage protective activity

Oxidative modification of DNA has been suggested to contribute to aging and various diseases including cancer and chronic inflammation. DNA damage protection studies were performed using hydro-alcohol extract. All the doses of plant extracts provided DNA damage protection; however, the protection provided at the dose of 16 µg was comparable to that of standard gallic acid (Fig 2). The super coiled form was the predominant band when the normal plasmid DNA pBR322 was run on an agarose gel. Exposure of the DNA to AAPH which induces generation of ROS especially hydroxyl and peroxyl radicals resulted in DNA cleavage as evident from the loss of the super coiled form of the DNA in the control incubations [32]. The *Piper betel* leaves extract was able to protect this damage to a large extent.

Inhibition of Protein oxidation

Oxidation of cellular proteins has been described under many pathological conditions. The vulnerability of various amino acid residues of proteins to oxidation varies with ROS. The protection by the *Piper betel* leaves extract against protein oxidative damage was determined by the oxidation of BSA initiated by AAPH. AAPH is a water soluble initiator, which decomposes at physiological temperature producing alkyl peroxyl radicals with oxygen to initiate the protein oxidation [33]. The study shows that two hours after the incubation, the BSA was completely degraded by 200 mM AAPH in positive control as studied by SDS-PAGE electrophoresis (lane 2, Fig 3). Among all the leaf extracts, 20 µg of hydro-alcohol extract significantly protected the protein oxidation compared to negative BSA control at the concentration of 1mg ml^{-1}.

Acetylcholine esterase inhibition

AChE inhibitors have extensively been studied and this has resulted in the discovery and development of several natural and synthetic AChE inhibitors [34].The Piper betel leaves extract has been shown to cause moderate inhibition of acetylcholine esterase with IC50 of 215.6µg ml-1 (Fig 1). The ability of Piper betel leaves extract to inhibit AChE may cause memory improvement and hence the extract may be examined for its effectiveness at treating memory impairments such as those caused by Alzheimer's disease.

Anxiolytic properties

Elevated plus maze test

Anxiety is a normal emotional response to a threat or potential threat. When this emotion is inappropriate and persistent, it is classified as pathological. The EPM is one of the most popular animal tests for research on behavioral pharmacology of anxiety. It involves spontaneous or natural aversive stimuli, i.e., height, unprotected opening, and novelty. Several plants that are used in folk medicine to diminish anxiety are reported to bring about an increase in the exploration of the open arms in the EPM test [35].

In the Elevated plus maze test, *Piper betel* leaves extract at 100mg kg^{-1} body weight did not show any significant change. At the same time, with the higher doses; 200 and 400mg kg^{-1} body weight there was a significant increase in the time spent in the open arms. Diazepam (1mg kg^{-1} body weight), a standard anxiolytic produced a significant increase in the time spent and number of entries in the open arms of elevated plus maze (Figures 4 and 5).

Open field test

In the Open field test anxiety was induced in rodents by forced confrontational situations where they prefer to move along peripheral squares of the apparatus (Thigmotaxis) instead of central squares. Anxiolytics make the animal preferably to move in central squares and increase time spent in centre squares [36]. In this test, Piper betel leaves extract at doses of 100mgkg-1 and 200mg kg-1 body weight, did not show any significant increase in the total locomotion and central locomotion. However with the dose of 400mgkg-1 body weight and diazepam, (1mg kg-1 body weight) there was a significant increase in the total locomotion and central locomotion (Figure 6)., shape and number comparatively similar to that of standard treated. Where as pancreas present in the group of animals treated with aqueous extract 200 mg/kg showed normal appearing exocrine pancreas but islets number was found less compared to alcoholic extract treated and size was also reduced. And pancreas of diabetic control animal showed partially damaged or even destroyed pancreatic lobules, acini, cells, islet size, shape and number. The photomicrographs of histopathological section of pancreas are presented in figure (3,4,5,6,7).

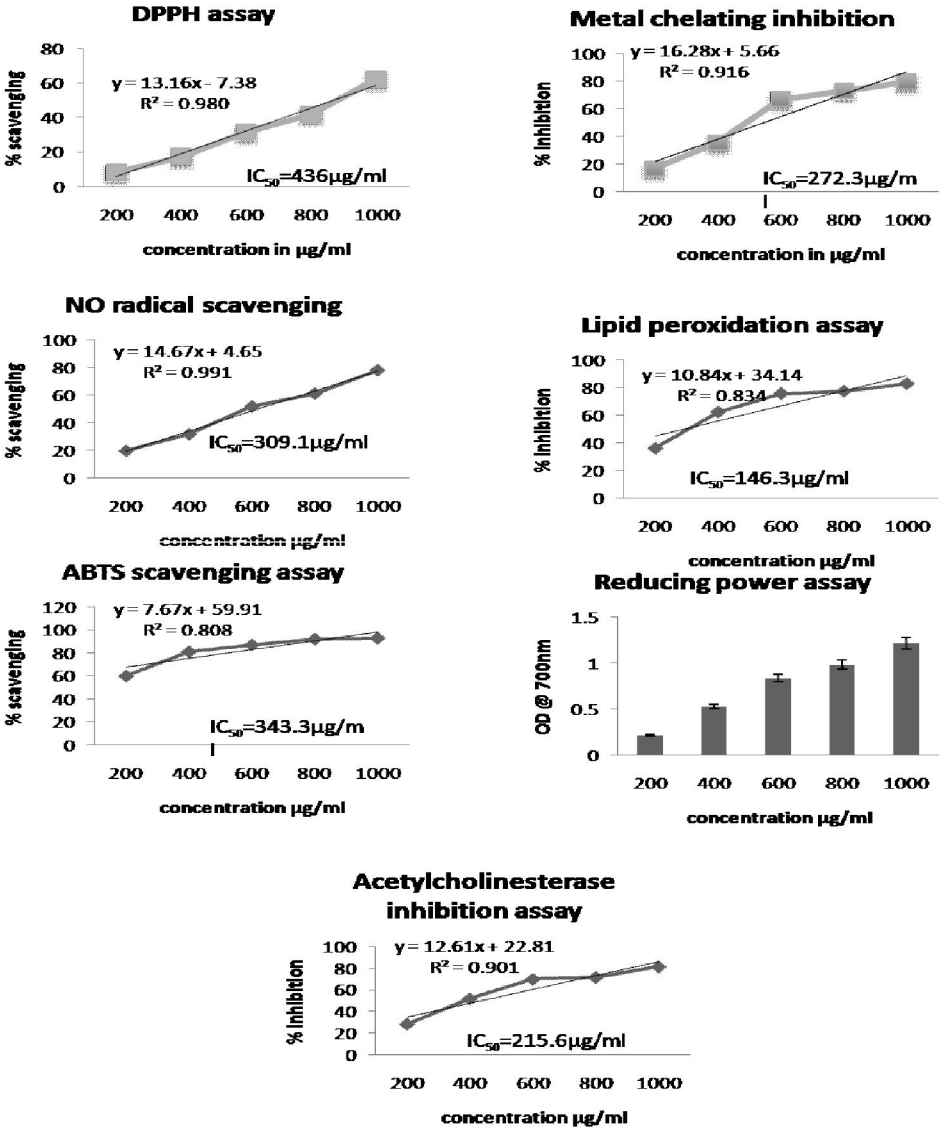

Figure 1: Effect of hydro-alcohol extract of *Piper betel* leaves (0-1000µg ml-1) on an array of *in vitro* antioxidant assays (n=3)

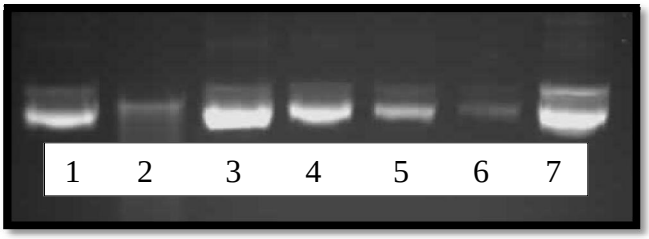

Figure 2: Effect of hydro-alcohol *Piper betel* leaves extract and gallic acid against 200mM of AAPH induced pBR322 DNA strand breakage. 1. control: DNA; 2. + ve control: DNA+AAPH; 3. DNA+AAPH+ 16µg hydro-alcohol *Piper betel* leaves extract; 4. DNA+AAPH+ 12µg hydro-alcohol *Piper betel* leaves extract; 5. DNA+AAPH+8µg hydro-alcohol *Piper betel* leaves extract; 6. DNA+AAPH+4µg hydro-alcohol *Piper betel* leaves extract; 7. DNA+AAPH+ 4µg gallic acid .

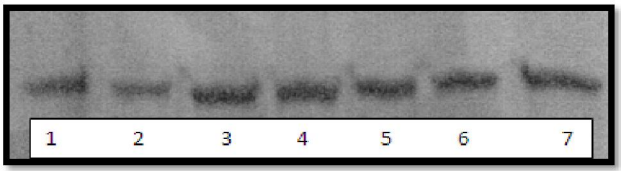

Figure 3: Effect of hydro-alcohol extract of *Piper betel* leaves and gallic acid against 200mM of AAPH induced BSA oxidative fragmentation. 1. BSA; 2. BSA + AAPH; 3.BSA + AAPH + 5µg hydro- alcohol *Piper betel* leaves extract; 4.BSA + AAPH + 10µg hydro-alcohol *Piper betel* leaves extract; 5.BSA + AAPH + 15µg hydro-alcohol *Piper betel* leaves extract; 6.BSA + AAPH + 20µg hydro-alcohol *Piper betel* leaves extract; 7.BSA + AAPH +Gallic acid 5µg

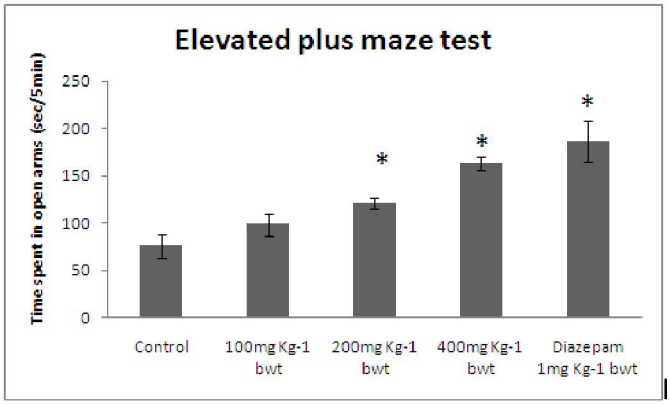

Figure 4: Effect of hydro-alcohol *Piper betel* leaves extract on the time spent in the open arms of the elevated plus-maze during a 5 min test in mice. The plant extracts, diazepam or control, were injected 60 min prior to test. Data are presented as mean values (±SD.) from group of six mice.*P < 0.05 compared with vehicle-treated control.

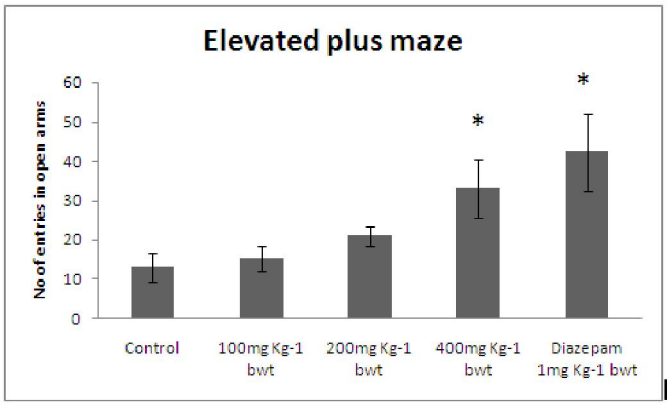

Figure 5: Effect of hydro-alcohol *Piper betel* leaves extract on the number of entries in the open arms of the elevated plus-maze during a 5 min test in mice. The plant extracts, diazepam or control, were injected 60 min prior to test. Data are presented as mean values (±SD.) from group of six mice.*P < 0.05 compared with vehicle-treated control.

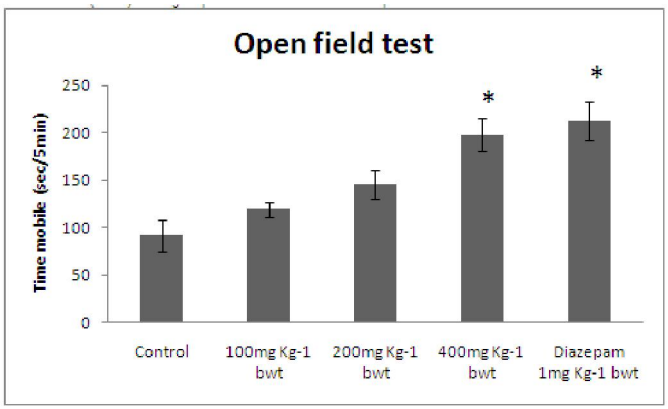

Figure 6: Effect of hydro-alcohol *Piper betel* leaves extract on the total locomotion during a 5 min test in mice. The plant extracts, diazepam or control, were injected 60 min prior to test. Data are presented as mean values (±SD.) from group of six mice.*P < 0.05 compared with vehicle-treated control.

Conclusion

In order to characterize antioxidant and anxiolytic activities of a plant extract, it is subjected to various tests that evaluate the range of activities such as scavenging of the reactive oxygen species, metal ion chelation; inhibition of lipid peroxidation and acetylcholine esterase inhibitory activity wherein the extract demonstrated good antioxidant activity. The extract also exhibited protection against protein oxidation and DNA damage followed by anxiolytic properties. The study also showed a dose dependent free radical scavenging and anxiolytic activities. This study indicated the potential of the Piper betel leaf extract as a source of natural antioxidants or nutraceuticals that could be of use in food industry with potential application to reduce oxidative stress in living system with consequent health benefits.

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