DOSE DEPENDENT EFFECT OF *PIPER BETLE* LINN. LEAF EXTRACT ON ERYTHROCYTES OF EXPERIMENTAL MICE

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ABSTRACT

Background:

Objective: *This study examined the dose – dependent effect of oral administration of Piper betle leaf extract on lipid peroxidation, antioxidants, antioxidant enzymes and membrane – bound ATPases in mice.*

Method: *Adult female mice weighing 30 ± 2 grams were administered different doses (0.2, 0.4, 0.6, 0.8, & 1.0 gm / day) of betel leaf extract orally for 15 days. Plasma, erythrocytes and erythrocyte membrane were separated and used for the assay of thiobarbituric acid reactive substances (TBARS), superoxide dismutase, catalase in RBC hemolysate, ascorbic acid and vitamin E in plasma and membrane – bound ATPases (Na^+ / K^+ - ATPase, Ca^{2+}- ATPase, Mg^{2+} - ATPase) in erythrocyte membrane were measured.*

Results: *A significant reduction in TBARS and significant increase in ascorbic acid, vitamin E, super oxide dismutase, catalase and membrane – bound ATPases were observed in mice fed 0.2 gm / day. The extract of Piper betle leaf at the low dosage of 0.2 gm / day for 15 days provides better antioxidant potential as well as membrane stabilizing action in Swiss mice over controls.*

Key words : *Piper betle, antioxidants, membrane – bound ATPases, lipid peroxidation*

INTRODUCTION

Reactive oxygen species (ROS) are responsible for oxidative damage of biological macromolecules such as DNA, carbohydrates and proteins [1]. These processes are discussed as pathobiochemical mechanisms involved in the initiation and progression phase of various diseases [2]. Some of the most relevant ROS are: peroxyl radicals ($ROO^{\cdot}$), the nitric oxide radical ($NO^{\cdot}$), the superoxide anion radical ($O^{\cdot}{}_2^-$), singlet oxygen ($^1O_2$), peroxy nitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2). ROS are either radicals (molecules that contain at least one unpaired electron) or reactive non – radical compounds, capable of oxidizing biomolecules. Therefore, these intermediates are also called oxidants or pro - oxidants [3]. The superoxide radical anion appears to play a central role, since other reactive intermediates are formed in reaction sequences starting with $O^{\cdot}{}_2^-$. H_2O_2 is a non – radical reactive species and can easily diffuse between living cells. It is efficiently converted to water by the enzyme catalase, a process which determines its half – life. The peroxyl radical ($ROO^{\cdot}$) is relatively long lived (seconds) with a considerable diffusion path length in biological systems. It can be generated in the process of lipid peroxidation which is initiated by the abstraction of a hydrogen atom from poly unsaturated fatty acids (PUFA); the hydroxyl radical is capable of starting this reaction sequence [4, 5]. While *Piper* species are reported to have wide spectrum of biological activity but its antioxidant potential has not been established so far, making it important and interesting to analyze the antioxidant potential of *Piper betle* leaf extract in the present study.

Many unknown and lesser - known plants are used in folk and tribal medicinal practices in India, with their medicinal values not known much to the scientific world. *Piper betle* (Family - Piperaceae) is one such plant, which is commonly known as "pan" and often chewed by Indians resulting in habit formation of this practice. Medicinally *Piper betle has* been attributed with properties – like anti poisonous [6] and wound healing [7]. Extracts of *Piper betle* leaves also possess antimicrobial, antifungal, anti-inflammatory and antiplatelet activities [8, 9]. Ethanol extracts of *Piper betle* leaf exhibited gastrocytoprotective properties on experimentally induced gastric lesions [10] and betel nut along with the betel leaves significantly reduced the infiltration activity and abolished the surface anesthetic activity of betel leaf [11]. Studies on the physiological effects have shown that the initial effects of chewing betel with areca nut and other adjuncts can cause excitation of the salivary glands and also irritation to the mucous membrane of the mouth. Betel leaf alone apparently does not induce tumors and it seems to be the combined usage of betel nut and tobacco which provides the carcinogenic stimulus [12]. Prolonged consumption of betel leaves will cause cancer of the mouth, upper aero digestive tract and stomach [13].

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An earlier report on the anti – inflammatory and anti - diabetic activity of leaf extracts prompted us to evaluate its dose – dependent action. We therefore, studied the antioxidant and – membrane stabilizing action on experimental mice. Results from enzymatic and non – enzymatic antioxidant activities and membrane - bound ATPases are presented.

MATERIALS AND METHODS

Plant Material: *Piper betle* Linn (Syn: Chavica betle Miq.) popularly known as "vetrelei" in Tamil, "betel" in English and "Nagavalli" in Sanskrit was purchased from local market, Chennai, Tamil Nadu, India.

Chemicals: Chemicals used were of analytical grade.

Experimental animals: Adult female Swiss mice weighing approximately 30 ± 2 grams were obtained from the Central Animal Facility of Sri Ramachandra Medical College and Research Institute, Deemed University, Chennai were used in this study. The animals were maintained 12 hour light / 12 hour dark cycle and fed on a pellet diet (Hindustan Lever Ltd., India) and water *ad libitum*. The animals were maintained in their respective groups for 15 days. All studies were conducted in accordance with the National Institute of Health "Guide for the care and Use of Laboratory animals [14].

Experimental design: Thirty-six adult healthy female mice were divided into six groups of six animals each.

Group I - Control (2ml of saline / day)

Group II - 0.2 gms of betel leaf in 2 ml of saline / day.

Group III - 0.4 gms of betel leaf in 2 ml of saline / day.

Group IV - 0.6 gms of betel leaf in 2 ml of saline / day.

Group V - 0.8 gms of betel leaf in 2 ml of saline / day and

Group VI - 1.0 gms of betel leaf in 2 ml of saline / day.

Betle leaf extract was given orally for 15 days; the mice were fasted overnight and sacrificed by cervical decapitation. Plasma was separated using EDTA as an anti coagulant. The hemolysate and erythrocyte membrane was isolated according to the procedure of Dodge *et al* [15] with a change in buffer according to Quist [16]. Plasma was used for the analysis of ascorbic acid [17] and vitamin E [18]. RBC hemolysate was used for the estimation of TBARS [19], protein [20] and assay of superoxide dismutase [21], catalase [22], and erythrocyte membrane was used for the assay of Na^+ / K^+ - ATPase [23], Ca^{2+} - ATPase [24], Mg^{2+} - ATPase [25].

Statistical analysis: Statistical analysis of the data was performed using student's't' – test and

$p < 0.05$ was considered as significant.

Results

Table 1 shows the level of TBARS, in erythrocyte membrane, ascorbic acid and vitamin E in plasma of experimental animals. Significant reduction ($p < 0.001$) in the level of TBARS was observed in group II mice (0.2 gm / day) when compared to control, whereas concomitant increase ($p < 0.001$) was observed in the levels of plasma ascorbic acid and vitamin E in group II animals. Significant increase in the levels of TBARS and significant decrease in the levels of plasma ascorbic acid and vitamin E was observed in all the other groups of experimental mice (i.e. group III to VI).

Table 2 shows the activities of superoxide dismutase and catalase in RBC hemolysate of experimental animals. Significant increase ($p < 0.01$) in the activities of superoxide dismutase and catalase was observed in group II mice (0.2 gm / day) when compared to control. Significant decrease was observed in all the other groups of experimental mice (i.e. group III to VI).

Table 3 shows the activities of Na^+ / K^+ - ATPase, Ca^{2+} - ATPase, Mg^{2+} - ATPase in erythrocyte membrane of experimental animals. Increased activities ($p < 0.05$) were observed in group II mice (0.2 gm / day) when compared to control. Significant decrease was observed in all the other groups of experimental mice (i.e. group III to VI).

DISCUSSION

Oxidative stress is a state of imbalance between generation of reactive oxygen species (ROS) like hydroxyl and superoxide radicals, and the level of antioxidant defense systems. Oxidative stress results in the damage of macromolecules including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates. Lipid peroxidation is oxidative deterioration of polyunsaturated fatty acids and it involves reactive oxygen species and transition metal ions [26]. Peroxidation of membrane system are the foremost consequences of free radical damage and the efficiency of plant extracts in inhibiting lipid peroxidation *in vivo* is a very good measure of assessment of antioxidant potential. The lowest dose of 0.2 gm / day inhibited lipid peroxidation in erythrocytes, indicating anti-peroxidative effect of betel leaf extract. Lipid peroxide contents were increased in other groups (group III and group VI) which indicate high doses of betel leaf extract produce toxic effect in the experimental mice (Table 1). The increased lipid peroxidation by higher doses of betel leaf extract is due to fall in total radical – trapping capacity of blood plasma and marked reduction in plasma levels of antioxidants such as vitamin E and C was evident in the present study.

Table1. Effect of different doses of *Piper betle* leaf extract on the levels of TBARS in erythrocytes, ascorbic acid and vitamin E in plasma of control and experimental animals after 15 days of treatment.

Statistically significant values were expressed as mean ± S.D of 6 mice from each group.

Groups	**TBARS** (nmoles / ml)	**Ascorbic acid** (mg / dl)	**Vitamin E** (mg / dl)
Group-I-Control (2 ml saline / day)	1.78 ± 0.14	1.6 ± 0.17	1.9 ± 0.10
Group-II- 0.2 gms / day)	1.50 ± 0.1***	1.9 ± 0.09***	2.1 ± 0.07***
Group-III- 0.4 gms / day)	1.89 ± 0.09*	1.4 ± 0.10*	1.8 ± 0.09NS
Group-IV- 0.6 gms / day)	1.97 ± 0.12 **	1.38 ± 0.11**	1.8 ± 0.11**
Group-V- 0.8 gms / day)	2.30 ± 0.19***	1.30 ± 0.08***	1.7 ± 0.12***
Group-VI-1.0 gms / day)	2.81 ± 0.23***	1.28 ± 0.11***	1.5 ± 0.16***

Group II to VI were compared with group I (Control). * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, NS - Non- significant

Table 2. Effect of different doses of *Piper betle* leaf extract on the activities of superoxide dismutase and catalase in erythrocytes of control and experimental animals after 15 days of treatment.

Statistically significant values were expressed as mean ± S.D of 6 mice from each group.

Groups	**Superoxide dismutase** (Units / min / mg protein)	**Catalase** (mmoles of H_2O_2 utilized / min/ mg Hb)
Group-I-Control (2 ml saline / day)	3.01 ± 0.28	7.5 ± 0.6
Group-II- 0.2 gms / day)	5.21 ± 0.19***	8.3 ± 0.4**
Group-III- 0.4 gms / day)	3.20 ± 0.07NS	7.9 ± 0.7 NS
Group-IV- 0.6 gms / day)	2.69 ± 0.12**	6.9 ± 0.3**
Group-V- 0.8 gms / day)	2.06 ± 0.08***	6.4 ± 0.8**
Group-VI-1.0 gms / day)	1.88 ± 0.14***	6.0 ± 0.7***

Groups II to VI were compared with group I (Control). NS - Non significant, ** - $p < 0.01$, *** - $p < 0.001$

Table 3. Effect of different doses of *Piper betle* leaf extract on the activities of membrane – bound ATPases in erythrocyte membrane of control and experimental animals after 15 days of treatment.

Statistically significant values were expressed as mean ± S.D of 6 mice from each group.

Groups	**Na^+/K^+ ATPase@**	**Ca^{2+} ATPase@**	**Mg^{2+} ATPase@**
Group-I-Control (2 ml saline / day)	0.0549 ± 0.003	0.0238 ± 0.002	0.0779 ± 0.004
Group-II- 0.2 gms / day)	0.0620 ± 0.008*	0.0298 ± 0.006*	0.0818 ± 0.002*
Group-III- 0.4 gms / day)	0.0532 ± 0.009NS	0.0222 ± 0.003NS	0.0751 ± 0.003NS
Group-IV- 0.6 gms / day)	0.0501 ± 0.004*	0.0201 ± 0.004*	0.0746 ± 0.005*
Group-V- 0.8 gms / day)	0.0498 ± 0.005*	0.0198 ± 0.003*	0.0732 ± 0.002*
Group-VI-1.0 gms / day)	0.0490 ± 0.003**	0.0188 ± 0.005*	0.0711 ± 0.004*

@ - μmoles of Pi liberated / hr / mg protein.

Groups II to VI were compared with group I (Control). * - $p < 0.05$, ** - $p < 0.01$, NS - Non significant

Superoxide anion (O_2) reacts with water to form hydrogen peroxide (H_2O_2), which in turn is responsible for the generation of hydroxyl radicals. Hydroxyl radicals attack membrane fatty acids inducing lipid peroxidation. Catalase is believed to be the most effective defensive agent against high concentration of H_2O_2. Interestingly, in the present study, the higher doses of the drug decreased the activity of catalase in group III to VI and the lower doses in group II increased the catalase activity, suggesting that these dose dependent differential actions of the plant extract on lipid peroxidation are probably mediated by catalase (Table 2).

Apart from enzymatic antioxidants, non- enzymatic antioxidants namely vitamin A, E, C and glutathione are important for cellular system in curtailing reactive oxygen species. The most important antioxidants, ascorbic acid and vitamin E were studied to evaluate the antioxidant potential of different doses of betel leaf extract in experimental mice. Ascorbic acid is reported to be associated with better scavenging action in *in vivo* than the antioxidant enzymes, because it is present in both extracellular as well as in the intracellular fluids [27]. The low levels of ascorbic acid was found in group III to group VI animals might be due to high lipid peroxidation products formed in these groups or rapid removal of ascorbic acid and vitamin E in the body.

Vitamin E may protect cellular components against peroxidative damage via the free radical scavenging mechanism or as a constituent of the membrane [27]. The antioxidant properties of a- tocopherol result from its ability to quench both singlet oxygen and peroxides [28]. Within the membrane, vitamin E is the only protective agent that can act against the toxic effects of oxygen radicals [29]. In the present study a low dose of betel leaf extract has antioxidant potential but at higher doses vitamin E levels in group III to VI decreased dramatically (Table1). This damage to the membranes was discernible in the slightly lower activity of membrane – bound ATPases in animals fed with higher doses of the extract.

Membranes are vital for biological system and their integrity is essential for normal functioning of cells and damage to membrane organization is an initial step in cell death. Peroxidation of membrane lipids initiates a loss of membrane – bound enzyme activity and cell lysis [30], alters membrane permeability and cell function [31]. Abnormal lipid peroxides affect membrane – bound ATPases activities and their levels were decreased due to the excessive production of thiobarbituric acid reactive substances [32]. The membrane bound enzymes such as Na^+ / K^+ ATPase, Mg^{2+} ATPase and Ca^{2+}ATPase are responsible for the transport of sodium / potassium, magnesium and calcium ions respectively, across the cell membranes at the expense of ATP on hydrolysis [33]. Na^+ / K^+ ATPase, which is an integral membrane protein and it is responsible for a large part of energy consumption constituting the basic metabolic rate[34].The reduced activity of Na^+ / K^+ ATPase indicate changes in the membrane under a pathological conditions [35]. Na^+ / K^+ ATPase is reduced, whereas Mg^{2+} ATPase activity is inhibited in photosensitivity – induced damage in plasma membrane [36]. Ca^{2+}-ATPase, the enzyme responsible for active calcium transport, is extremely sensitive to hydroperoxides and this may lead to its inhibition. Hence high concentrations of betel leaf extract causes loss of membrane – bound enzymes activity (Table 3).

From the results it is evident that, betel leaf extract exhibits dose –dependent actions. At low concentrations it is anti-peroxidative, whereas at higher concentrations it induces oxidative damage to biological membranes as indicated by the elevated levels lipid peroxides in erythrocytes and lower activity of superoxide dismutase, catalase and membrane - bound ATPases. Low doses of betel leaf extract have beneficial effect rather than high doses of the extract. The result of this study shows that a daily administration of *Piper betle* at the dosage of 0.2 gm / day for 15 days provides better antioxidant potential as well as membrane stabilizing action. However further investigations are needed to assess the efficacy of *Piper betle* in oxidative stress induced pathological conditions.

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