Research Papers

Extract of *Piper Betle* Facilitates Significant Modulation on the Redox Status in Preeclamptic Placental Trophoblast

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ABSTRACT

Introduction and Aim: Preeclampsia (PE), a condition associated with incomplete trophoblast invasion leads to redox status alteration. Effective strategies for the management of PE without any side effects are lacking, and the available synthetic drugs seem to cause harmful effects to mother and fetus. Herbal supplements may help to overcome the clinical manifestations of PE, need of the hour. *Piper betle* (PB) leaf contains several biologically active compounds with protective effects. However, the effect of PB for the treatment of hypertensive disorder like preeclampsia is seldom explored. Extracts of betle leaf-BLE (aqueous-ABLE, PBS-PBLE, saliva-SBLE, diastase-DBLE) are utilized to examine its effect on redox changes in placental trophoblast.

Materials and Methods: Three different concentrations of BLE were used to evaluate the optimal concentration having potent radical scavenging activity. Following this, incubation time exhibiting maximum efficiency was standardized by analyzing the trophoblast viability upon incubation with BLEs at the optimum concentration of 1mg/mL.

Results: All the extracts of BL (1mg/mL/1h) showed variations in their radical scavenging activity in the order as follows SBLE>DBLE> PBLE>ABLE. It inferred that SBLE is more efficient in modulating redox status in preeclamptic placental trophoblast which is followed by DBLE, may be due to the presence of antioxidants in saliva.

Conclusion: Reveals that chewing of the betle leaf during pregnancy facilitates a significant change in the oxidants-antioxidants status in the trophoblast. Hence dietary supplementation of BL orally in particular by chewing might effectively render beneficiary effect in the management of oxidative stress and also help to overcome the other related complications of PE.

Key Words: Antioxidants, Betle leaf extract (BLE), Preeclampsia, Redox status, Trophoblast.

INTRODUCTION

Trophoblasts, the major cell type of the placenta, attach to and invade into the uterine lining, begin the process of pregnancy. Trophoblasts have a potent invasive capacity which is modulated by endometrium. Recent studies have shown that invasion of cytotrophoblast into the uterus is a unique differentiation pathway for proper implantation (1). Researchers revealed that any abnormalities in trophoblast reflect the changes in the placenta leading to pregnancy complications like preeclampsia (2). Preeclampsia is a hypertensive disorder that develops after twenty weeks of pregnancy and associated with the defective manifestation of the placenta. It is the second most frequent cause of death in pregnant women, and 10-15% of maternal deaths are due to preeclampsia and eclampsia (3) leading to severe complications for mother and fetus, especially premature births. In the mother, preeclampsia may cause premature cardiovascular disease, such as chronic hypertension, ischemic heart disease, and stroke, later in life (4). Moreover, failure in proper placental development leads to inadequate fetal nutrition resulting in growth-restricted neonates (5) and increased oxidative product with low antioxidant level.

Subsequently altered redox status is the key factor involved in the development of preeclampsia; sup-

plementing women with antioxidants during pregnancy may help to counteract oxidative stress and thereby prevent or delay the onset of preeclampsia (6) Since many of the synthetic drugs are known to stimulate deleterious effects, natural plant extracts are believed to initiate beneficiary effect on pregnant women. *P.betle* may be one such plant that may facilitate the placental cell to counter the oxidative stress created during preeclampsia.

Betle leaf is a well-known ethnomedicinal plant consumed in the Asian countries (7). It is a heart shape leaf comes under the family Piperaceae, and its botanical name is *Piper betle* (8). The plant leaf has wide variety of secondary metabolites such as phenolic compounds (chavicol, hydroxyl chavicol), volatile oils like safrole, eugenol, isoeugenol, eugenol methyl ester (9) According to Dorman *et al*. (10) chavibetol, eugenol, methyl eugenol and carvacrol of BL plays a crucial role in antioxidant effectualness. Rathee (11) also reported on the O2-. radicals scavenging activity betle leaf due to the presence allylpyrocatechol. This was further confirmed by Sripradha (12) that treatment with BL elevates the level of antioxidants. The plant leaf exerts various pharmacological activities like antimicrobial, antidiabetic, antioxidant, hepato-protective, antitumor, antimutagenic and antihelminthic activities (13). However, the facilitating effect of betle leaf on hypertensive disorder like preeclampsia is seldom explored.

The practice of chewing betle leaf is common after a feast and even after normal food renders complete digestion, and it also acts as prophylaxis for digestion (14). When betle leaves are chewed, it induces salivation. Saliva secretion comprises 99% water, small quantities of various organic and inorganic compounds, bacteria, epithelial cells and gingival fluid. In addition to its other host-protective qualifications, saliva constitutes the first line of defense against free radical-mediated oxidative stress, since the process of mastication and digestion of ingested foods promotes a variety of reaction. The antioxidant defense system of saliva includes various molecules and enzymes (15), and the major enzyme may be diastase.

An imbalance in the level of antioxidant defense systems and the generation of reactive oxygen species (ROS) leads to oxidative stress ultimately resulting in the damage of macromolecules and indicators of this includes lipid peroxidation products such as malondialdehyde (MDA) and lipid hydroperoxides (LHP). To protect the cells from free radicals induced oxidative stress, living systems have developed strong defense mechanisms that include antioxidants and in extreme conditions by signaling molecules (16). The sum of endogenous and food-derived antioxidants is represented by the total antioxidant activity (TAC) of the system which is a measure of both water-soluble such as and fat-soluble antioxidants (17). Such an oxidative imbalance in trophoblast ultimately results in abnormal placentation and placental dysfunction leading to preterm delivery (18) and other clinical complications of PE. Herbal supplements may be the only solution to overcome the manifestations of preeclampsia.

The present study was aimed to investigate the free radical scavenging activity of various extracts (aqueous, PBS, saliva and diastase) of *P.betle* L. Free radical scavenging activities of the extracts were assessed against DPPH and hydroxyl radicals and to evaluate the effective role of various extracts of BL on normotensive and preeclamptic placental trophoblast assessment of viability, oxidant/antioxidant status were performed, which has not been previously reported.

MATERIALS AND METHODS

Collection and Authentication of *P.betle*

P.betle plant saplings were purchased from Tamilnadu Horticulture Institute, Chennai and the plants were grown in a well-defined soil and packed in a pot with appropriate water and sunlight. The young leaves were collected from the grown plant and authenticated by the Siddha Central Research Institute, Chennai (Central Council for Research in Ayurveda and Siddha, New Delhi, under the Ministry of Health & Family Welfare, Government of India).

Preparation of Betle Leaf Extracts

Aqueous betle leaf extract was prepared by boiling 10% (w/v) leaves the deionised distilled water and allowed to concentrate to about 90% (w/v). The leaves were sieved out and the crude aqueous extract obtained was filtered. 1mL aliquots of the crude extract were dried over night using speed vacuum concentrator. The dried pellets of the crude aqueous extract samples were refrigerated at -40^{o}C until further use. The pellets were then weighed, dissolved and diluted to suitable concentration as mentioned below.

Different concentrations of BLE (1, 2 and 3mg/ml) were prepared with distilled water, PBS of pH 7.4, 0.1% diastase and 1:10 diluted saliva. The isolated trophoblasts of normotensive and preeclamptic placenta were incubated with various concentrations of BLE at different time intervals (1h, 2h, and 3h). Following the incubation, cell viability was assessed and the effective concentration (1mg/ml and 1h) at the time having maximum efficiency was utilized for further studies.

Selection of Subjects

The study was carried out for a period of 6 months. The placental samples were obtained from a private hospital at Chennai. Informed consent was obtained from the subjects, and the study was approved by Ethical Committee (IEC/S/BWC/0610/ 2014). Placenta was collected from both normal (n = 10) and preeclamptic (n = 10) pregnant women in the age group of 20-40 years, post delivery. Patients with preeclampsia were defined by the following laboratory criteria: blood pressure greater than 140/90 mmHg but less than 160/110 mmHg, proteinuria > 300 mg/L, and xanthine oxidase activity of approximately 2.6 units/ mg protein. Patients with severe preeclampsia and other severe maternal complications were excluded from the study.

Isolation of Trophoblast

Third-trimester villous trophoblast cells, which were used for the comparison, were isolated from term placentas by the method of Douglas (19). Briefly, placental villi were cut and thoroughly washed to remove blood. After that, they were incubated for four times in a digestion medium composed of HBSS, containing trypsin and deoxyribonuclease at 37°C for 30 min in a water bath with continuous shaking. The dispersed cells were layered on the top of a discontinuous 5%–70% percoll gradient and centrifuged at 507 Xg for 25 min. The intermediate layers (density between 1.048 and 1.062) containing cytotrophoblast cells were removed and washed, and the cell viability was determined by trypan blue exclusion. Following trophoblast isolation, cells were seeded at a density of approximately 1.6 × 106 cells per well in 6-well plate. The complete culture medium constituted of M199, 2 mM glutamine, 10% FBS. All the experiments were performed on the same day of trophoblast isolation to overrule the influence of the cultivation process.

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity

DPPH radical scavenging ability was determined by the method of Viturro *et al.* (20). The scavenging activity was expressed as mg/mL.

Hydroxyl Radical Scavenging Assay

Hydroxyl radical scavenging activity of the extracts was determined according to the method reported by Klein *et al*. (21).

Assessment of Cell Viability

The viability of the isolated trophoblast was assessed by trypan blue exclusion method (22). Briefly, 10 µL of the isolated cells was mixed with 0.4% trypan blue solution and was allowed to react for 5 min in a moist chamber. The viable unstained cells were then counted using a hemocytometer. The results were expressed as % of viability.

Estimation of Lipid Peroxide (LPO)

LPO was determined by thiobarbituric acid (TBA) reaction using the method of Ohkawa *et al.* (23). The LPO content was expressed as nanomoles of MDA/ mg of protein.

Estimation of Total Antioxidant Capacity (TAC)

TAC analysis was performed by the method of Prieto *et al.* (24). The total antioxidant activity was expressed as Trolox equivalent in mmol/L.

Statistical Analysis

Data were analyzed using statistical software package version 7.0. Student's t-test was used to ascertain the significance of variations between normotensive and preeclamptic placental trophoblast. All data were presented as mean ± SD. Differences were considered significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

Figure 1: 2,2-Diphenyl-1-Picrylhydrazyl (DPPH.) radical scavenging activity of different extracts of *Piper betle*. Values are mean ± SD

ABLE: Aqueous extract of betle leaf; PBLE: Phosphate buffered saline extract of betle leaf

DBLE: Diastase extract of betle leaf; SBLE: Salivary extract of betle leaf

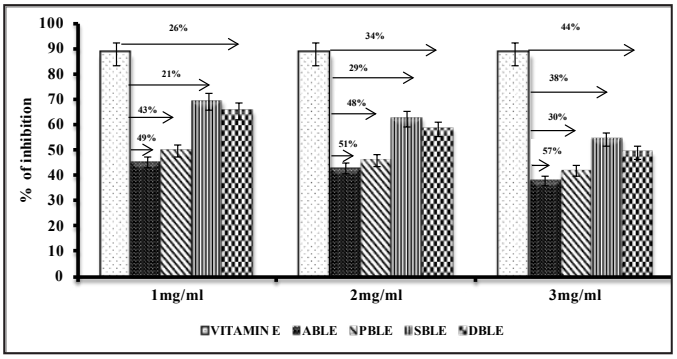
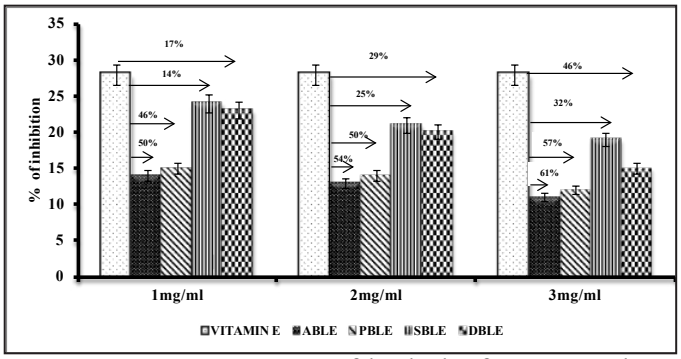


Figure 1 represents the DPPH radical scavenging activity. The scavenging activity of various extracts of betle leaf in a dosage-dependent manner was characterized by increasing scavenging activity with 1% concentration. The extracts radical scavenging activity were effective in the order was found to be SBLE > DBLE > PBLE > ABLE. SBLE exhibited strongest DPPH radical scavenging activity compared to other extracts. All the extracts of *P.betle*, when added to the reaction mixture scavenge hydroxyl radicals in a concentration-dependent manner. Vitamin C was taken as control showed highest antioxidant power in the present study.

Figure 2: Hydroxyl (.OH) radical scavenging activity of ABLE, PBLE, DBLE and SBLE. Values are mean ± SD



ABLE: Aqueous extract of betle leaf; PBLE: Phosphate buffered saline extract of betle leaf

DBLE: Diastase extract of betle leaf; SBLE: Salivary extract of betle leaf

Hydroxyl radical is the most reactive oxygen centered species and causes severe damage to the adjacent biomolecule. The scavenging of the hydroxyl radicals may be due to the presence of a hydrogen donating ability phenolic compounds in the extracts. The decreasing order of hydroxyl radical scavenging activity of the extracts **(Figure 2)** was found to be SBLE > DBLE > PBLE > ABLE. SBLE and DBLE exhibited highest DPPH radical scavenging activity compared to other extracts.

From the radical scavenging activities, 1mg/mL concentration of various extracts of *P.betle* was found to be the effective concentration having the significant radical scavenging property, and the SBLE is the most efficient.

Figure 3: Inner Figure 3 represents the Viability of normotensive and preeclamptic placental trophoblast. Outer figure shows effect of ABLE, PBLE, SBLE and DBLE on the viability of normotensive and preeclamptic placental trophoblast at different time intervals and the values are expressed as means ± SD (n = 10) and the "p" values are given in Table 1.

NT, normotensive placental trophoblast; PE, preeclamptic placental trophoblast.

ABLE: Aqueous extract of betle leaf; PBLE: Phosphate buffered saline extract of betle leaf

DBLE: Diastase extract of betle leaf; SBLE: Salivary extract of betle leaf

Figure 3: Inner figure represents the ability of placental trophoblast to maintain cell viability assessed by Trypan blue staining. Cell viability in preeclamptic placental trophoblast was decreased, which is proved by 80% viable cells when compared to normotensive placental trophoblast, where 95% viable cells were observed. It represents the 16% difference between normotensive and preeclamptic placental trophoblast viability.

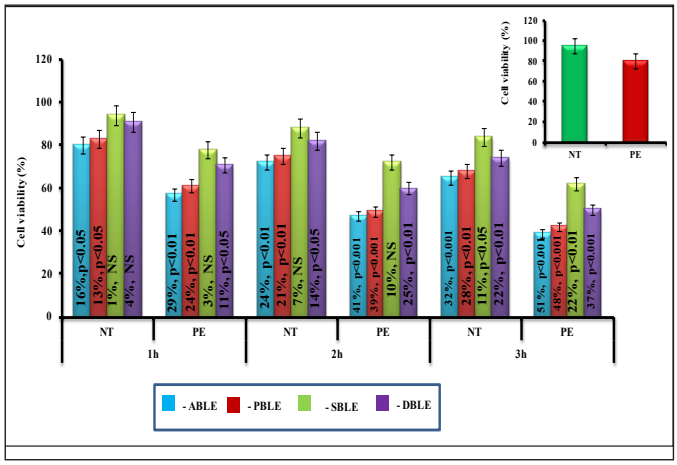


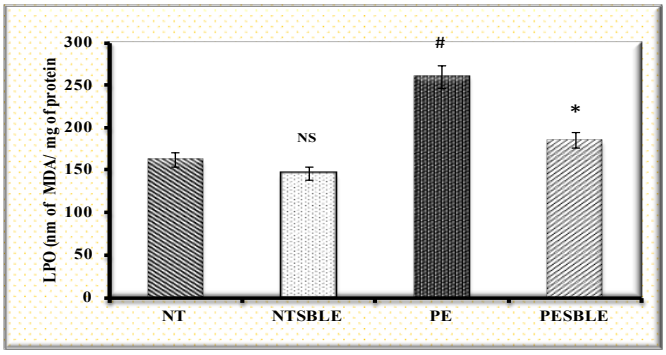
Figure 3: Outer figure represents the effective duration of incubation of the isolated normotensive and preeclamptic placental trophoblasts with 1 mg/ml of different BLE (ABLE, PBLE, DBLE and SBLE) at different times of incubation (1hr, 2hrs and 3hrs) and the effective time of incubation was standardized. Percentage of significance for the comparative analysis is given in Table 1

Table 1: Percentage of Significance for the Comparative Analysis for Figure 3

Comparision	NT/PE	with ABLE			with PBLE			with SBLE			with DBLE		
		1h	2h	3h	1h	2h	3h	1h	2h	3h	1h	2h	3h
NT		15.7895	24.2105	31.5789	12.6316	21.0526	28.4211	1.05263	7.36842	11.5789	4.21053	13.6842	22.1053
	15.7895	p<0.05	p<0.01	p<0.001	p<0.05	p<0.01	p<0.01	NS	NS	p<0.05	NS	p<0.05	p<0.01
PE	p<0.05	28.75	41.25	51.25	23.75	38.75	47.5	2.5	10	22.5	11.25	25	37.5
		p<0.01	p<0.001	p<0.001	p<0.01	p<0.001	p<0.001	NS	NS	p<0.01	p<0.05	p<0.01	p<0.001

Values represent the percentage of significance for 4 different extracts (ABLE, PBLE, SBLE and DBLE) at a different time interval (1h, 2h and 3h) between normotensive and preeclamptic placental trophoblast. In the table, a column filled with dark grey demonstrates the effective incubation time in preserving the cell viability. DBLE secured the second position in preserving cell viability. Other two extracts ABLE and PBLE were found to be moderately efficient in preserving cell viability.

Figure 4 : Level of LPO in normotensive and preeclamptic placental trophoblast with and without SBLE. Values are expressed as means ± SD (n = 10).



NT, normotensive placental trophoblast; NTSBLE, normotensive placental trophoblast with SBLE; PE, preeclamptic placental trophoblast; PESBLE, preeclamptic placental trophoblast with SBLE.

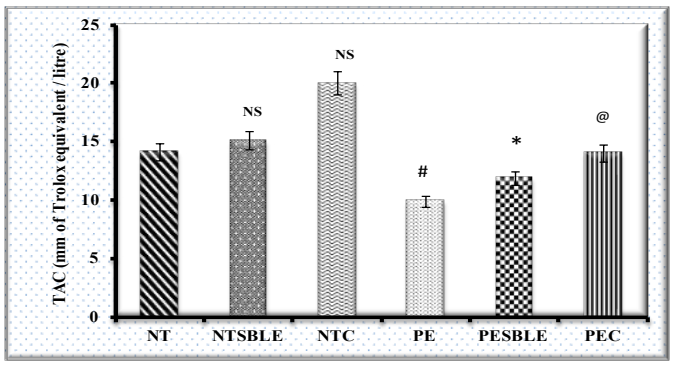
#p < 0.001 when compared to normal trophoblast without SBLE.

*p < 0.01 when compared to preeclamptic trophoblast without SBLE.

NS not significant when compared to normal trophoblast without SBLE.

The extent of lipid peroxidation was determined by measuring the release of MDA Figure 4, the level of MDA was significantly higher in preeclamptic placental trophoblast by 60% (p < 0.001) than normotensive placental trophoblast. In preeclamptic placental trophoblast, SBLE significantly decreased the level of MDA by 29% (p < 0.01) when compared to preeclamptic placental trophoblast without SBLE.

Figure 5: Level of TAC in normotensive and preeclamptic placental trophoblast with and without SBLE. Values are expressed as means ± SD (n = 10).



NT, normotensive placental trophoblast; NTSBLE, normotensive placental trophoblast with SBLE; NTC, normotensive placental trophoblast with Vitamin C; PE, preeclamptic placental trophoblast; PESBLE, preeclamptic placental trophoblast with SBLE; PEC, preeclamptic placental trophoblast with Vitamin C.

#p < 0.001 when compared to normotensive trophoblast without SBLE.

*p < 0.01 when compared to preeclamptic trophoblast without SBLE.

@p < 0.05 when compared to preeclamptic trophoblast without SBLE.

NS not significant when compared to normotensive trophoblast without SBLE.

Figure 5, shows that the total antioxidant capacity of the SBLE it was determined to assess the antioxidant capacity of SBLE in placental trophoblast and the level of TAC was significantly lower in preeclamptic placental trophoblast by 30% (p < 0.001) than normotensive placental trophoblast. In preeclamptic placental trophoblast, SBLE significantly increased the level of TAC by 21% (p < 0.01) when compared to preeclamptic placental trophoblast without SBLE.

DISCUSSION

Preeclampsia, multisystem disease of unknown etiology and its pathophysiology may begin with abnormal placentation resulting in systemic dysfunction, and deficient spiral artery remodeling leads to placental ischemia. In parallel, oxidative stress markers like free radicals, oxidized lipids are released into the maternal circulation which is responsible for the insufficient trophoblast invasion and plugging of maternal vessels in the periphery (25). However, de-

fects in these processes can ultimately lead to severe complications during pregnancy and resulting in hypertensive disorder like PE. Treatment of the disease like preeclampsia with modern medicine is often associated with serious side effects that affect both mother and fetus: herbal remedies having a synergistic effect to overcome the defective trophoblast functions and traditional method of consumption may be a suitable alteration for these patients.

P.betle is such a plant, and the leaf extracts of PB has antioxidant (26), anti-hypercholesterol (27), and vasodilatory actions. PB showed vasorelaxation on isolated perfused mesenteric artery preparation (28), which may be due to the presence of various polyphenols compounds like hydroxy chavicol, chatecol, allylpyrocatecol which inhibits lipid peroxidation. This could be attributed to its ability to scavenge free radicals involved in initiation and propagation steps of lipid peroxidation (29).

DPPH is a stable nitrogen-centered free radical commonly used for testing radical scavenging activity of plant extracts. The DPPH free radical scavenging activity of a compound indicates its hydrogen-donating tendency. A high correlation between DPPH radical scavenging activities and total polyphenolics has been reported by many researchers (30). Similarly, the antioxidant activity of plant extracts is also correlated with their hydroxyl radical scavenging activity (31). Coherent with this our present study also evidences on the antioxidant property for different extracts of *P. betle*. 1mg/mL was found to be the effective concentration having the radical scavenging property, and this was fixed as the effective concentration to assess the cell viability at various time intervals.

Oral intake and chewing of betle leaves induce salivation (32), and saliva provides a medium which might enhance the activity of the bioactive components. The various active components are capable enhancing the normal growth and development of cells. Our study on cell viability demonstrates that the extract of the betle leaf is crucial in precluding the cell death of isolated trophoblast. Incubation of trophoblast with SBLE and DBLE were more potent in maintaining the cell viability when compared to PBLE and ABLE.

Chewing of BL stimulates the salivary secretion and saliva may activate the components of BL to enhance the antioxidant property that helps in competing altered redox status. The patho-biochemical mechanisms involved in the initiation and progression phase of various diseases are reactive oxygen species (ROS). Excessive production of free radicals and its associated damages result in the loss of antioxidant status. During preeclampsia oxidative damage due to antioxidant insufficiency plays a vital role in trophoblast dysfunction and vasoconstriction which further complicates pregnancy (16). Consistent with this there is a significant increase in the level of LPO along with a decrease in the level of TAC in preeclamptic placental trophoblast when compared to normotensive which may be due to the increased utilization of antioxidants to counter balance the free radical-induced oxidative damage. The diastase extract of the betle leaf was found to have potential antioxidant efficacy in the management of hypertensive mediated OS-AO imbalance.

Antioxidants from natural compounds of medicinal plants are useful in inhibiting or preventing the deleterious consequence of oxidative stress. Badrul *et al.* (33) earlier demonstrated that *P.betle* leaves are rich in polyphenolic compounds, such as flavonoids, tannins, and phenolic acids. These are responsible for the multiple biological effects of BL which includes antioxidant activity. When BL is chewed it mixes with saliva which might enhance the bioactive property of the components present in BL. The efficiency of plant extracts in inhibiting lipid peroxidation is an excellent measure of assessment of antioxidant potential. The high scavenging property of *P. betle* may be due to hydroxyl groups existing in the phenolic compounds, and these are responsible for the antioxidant activity. The antioxidant activity is exhibited by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (34). Consistent with this study we observed that the various extract of BL could decrease the level of LPO and quench the free radicals along with increasing the total antioxidant capacity. Among the different extracts of BL, SBLE counterbalances the preeclampsia mediated oxidative stress effectively. This may be attributed to its ability to scavenge free radicals which may be due to the presence of secondary metabolites in BL and its interaction with salivary antioxidants. Diastase is also equally potent in counter balancing free radicals and the associated pregnancy induced hypertension.

Sujatha *et al.* (35) reported that the antioxidant potency of the betle leaf aqueous extract was poor. However, we found that when the aqueous extract was mixed with diastase in vitro or in vivo by the process

of chewing the antioxidant properties increased significantly. Hence, these enzymatic/salivary extracts from leaves could be used as a potential water-soluble antioxidant source which can be effective in the management of hypertensive disorder like PE. BL itself has higher antioxidant potency when leaves are chewed the antioxidant efficacy of BL further enhanced by of saliva. Alternatively, a diastase-BL mixer may be prepared and made available to pregnant women for minimizing the complication that might arise due to pregnancy.

The present study clearly indicates that the salivary extract of *P. betle* was found to be more potential in maintaining the altered redox status of trophoblasts. It may be concluded that the active components present in SBLE and DBLE might be involved in the induction of antioxidant efficacy. Therefore, dietary supplementation of BL orally in particular by chewing might effectively render beneficiary effect in the management of preeclamptic mediated oxidative stress and may help to overcome the other complications of PE.

Conflict of Interest: The authors report no conflict of interest.

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