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ORIGINAL ARTICLE

Efficacy of Salivary and Diastase Extracts of *Piper betle* in Modulating the Cellular Stress in Placental Trophoblast during Preeclampsia

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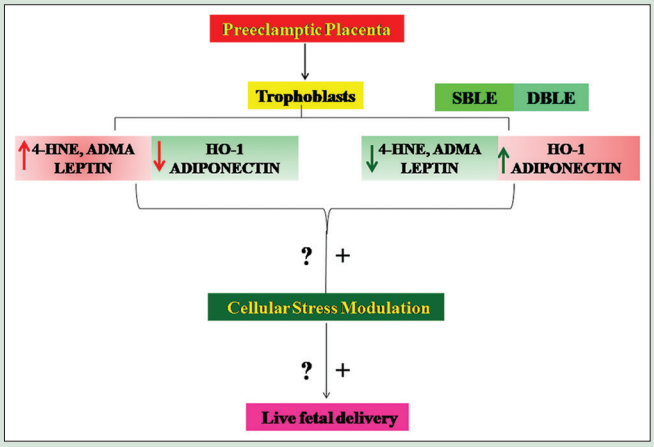
ABSTRACT

Background: Betle leaf (BL) is an ancient herb with a potential nontoxic natural antioxidant efficacy. Oral intake and chewing betel leaves have an effort on the moving parts of salivary gland and induce salivation and detoxification; hence plays a vital role in oral hygiene. BL produces and activates various salivary enzymes which include α-amylase, lipase, lysozyme, and lactoperoxidase. BL possess antibacterial, antioxidant, anti-diabetic, immunomodulatory, and antihypertensive properties. Hence, it can be effective in the treatment of hypertensive disorder of pregnancy like preeclampsia (PE). **Objective:** In this context, two extracts of BL (Salivary BL extract [SBLE] and diastase BL extract [DBLE]), proved to impart efficient radical scavenging activity in our previous research work were utilized to analyze their efficacy on the level of 4-hydroxy nonenal (4-HNE), heme oxygenase-1 (HO-1), asymmetric dimethylarginine (ADMA), adiponectin/leptin in the trophoblast isolated from placental tissue of both normotensive and preeclamptic patients. **Materials and Methods:** Trophoblasts were isolated from both subjects and incubated with different extracts of *Piper betle* (saliva and diastase) to assess the cellular stress in placental trophoblast during PE. **Results:** Results demonstrate that extracts of BL exhibit a significant role in regulating 4-HNE, ADMA, leptin, HO-1, and adiponectin in preeclamptic trophoblasts. Still, SBLE showed relatively higher efficiency in defining the level of 4-HNE, ADMA, leptin, HO-1, and adiponectin in preeclamptic trophoblasts than DBLE, suggesting that it may be attributed to the interaction of the phytochemicals present in BL with the components of saliva. **Conclusion:** Hence, BL may be recommended as the effective natural remedy in the management of pathological complications of PE; which may preclude low birthweight babies and ensure live fetal delivery.

Key words: Adiponectin, diastase, leptin, saliva, trophoblast

SUMMARY

- Our present study clearly indicates that extracts of *Piper betle* prepared with saliva or with diastase may impart an efficient role in managing preeclamptic stress reflected from the expression of signaling proteins in the isolated trophoblast. Hence, chewing of BL may be recommended as the natural remedy to overcome the pregnancy stress.



Abbreviations Used: 4-HNE: 4-hydroxy-nonenal, ADMA: Asymmetric dimethylarginine, BL: Betel leaf, BLE: Betel leaf extract, CO: Carbon monoxide, DB: Diastase with betel leaf, DBLE: Diastase betel leaf extract, ELISA: Enzyme-linked immunosorbent assay, eNOS: Endothelial nitric oxide synthase, FBS: Fetal bovine serum, GSH: Reduced glutathione, HBSS: Hank's balanced salt solution, HO-1: Heme oxygenase-1, iNOS: Inducible nitric oxide synthase, NO: Nitric oxide, NOS: Nitric oxide synthase, PE: Preeclampsia, ROS: Reactive oxygen species, SB: Saliva with betel leaf, SBLE: Salivary betel leaf extract.

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INTRODUCTION

Betel leaf (BL) is a potential nontoxic natural antioxidant.[1] The intake and chewing of betel leaves have an effect on the moving parts of salivary gland and induce salivation[2] which is the first step of digestion. Saliva contains various enzymes involved in the breakdown of food (diastase) and also fights bacteria in the mouth. BL is consumed orally for its mild stimulant and medicinal properties.[3] Leaf extracts and purified compounds of BL have antiseptic, antibacterial, antioxidant, anti-inflammatory, anti-cancer, and immunomodulatory efficacy.[4] It contains various phytochemicals such as alkaloids, flavonoids, steroids, saponins, and tannins,[5] and it also contains sugar, diastases, and essential oil. Ayurveda also states BL and its components act as heartbeat regulators in relaxing the blood vessels thereby reducing hypertension. BL is heart-shaped; belonging to the family Piperaceae and the taxonomical classification is given in Table 1.

When betel leaves are chewed, it induces salivation and also it may have some interaction with salivary diastase revealed from the previous study where the extraction of BL with diastase and saliva exhibited significant free radical scavenging activity. This may be attributed to the effect of proteolytic enzyme or carbohydrate digesting enzyme of saliva on

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Table 1: Taxonomical classification of betel leaf

Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Magnoliopsida*
Order: *Piperales*
Family: *Piperaceae*
Genus: *Piper*
Species: *Betle*
Binomial name: *Piper betle* L.

plant extract which can effectively stimulate the free radical reducing property and antioxidant activity of the herbal extract via promoting the breakdown of complex materials into simpler one.[6] However, the efficacy of salivary and diastase extracts of BL in the management of preeclampsia (PE), a serious pregnancy-specific hypertensive disorder with proteinuria and placental insufficiency is seldom explored.

PE is a pregnancy disorder, and it is pathologically associated with inadequate trophoblast invasion and defective remodeling of spiral arteries in the placenta. The placenta is a unique organ regarded as a selective passive filter. Now it has been recognized to play a vital role in fetal nutrition, and it adapts by responding to maternal nutritional cues, intrinsic nutrient-sensing signaling pathways, and fetal-demand signals.[7] It consists of trophoblasts, principal cell types exhibiting functions such as protection, nutrition, and respiration of the fetus.[8] Since trophoblast cells of the placenta are more sensitive to biological fluctuations in materno-fetal interface, the present study is focused to ascertain the impact of BL extract (BLE) on various signaling proteins.

4-hydroxy-2-nonenal (4-HNE) is the modified protein, an oxidative stress marker and it is the major end-product of fatty acid oxidation. It is generated by the propagation of free radical reaction mechanism imparting oxidative stress.[9] Studies also reported that expression of 4-HNE alters antioxidant defense mechanism by reacting with histidine of the glutathione-S-transferases apart from inducing damage of reduced glutathione (GSH) and GSH peroxidase resulting in altered vascularization.[10]

Heme oxygenase-1 (HO-1) ubiquitously distributed, the highly inducible enzyme catalyzes the first and rate-limiting step in heme catabolism toward biliverdin, carbon monoxide (CO), and free iron.[11] In particular, HO-1 exerts a direct antioxidant effect by degrading pro-oxidant heme and preventing intracellular iron accumulation. The cellular functions of HO-1 are exhibited via the catabolic products of heme imparting the vasodilatory (alter blood pressure), anti-inflammatory and anti-apoptotic function.[12] The product of HO-1-decreases blood pressure by stimulating the release of nitric oxide (NO) from intracellular stores and heme another product exhibits pro-oxidant and pro-inflammatory properties. Hence, dietary antioxidants rich in phytochemicals that can induce HO-1[13] are of high demand to combat the cellular stress. Syahidah *et al.*[14] demonstrated the presence of various phytochemicals; biologically active compounds in BL, which raises our concern to elucidate the antioxidant efficacy of BL during PE.

Vascular dysfunction due to differential expression of endothelial nitric oxide synthase (NOS) and inducible NOS was observed to be the pathophysiological event existing during PE. Lumicisi *et al.*[15] already showed that NO synthesized by NO synthase is involved in regulating trophoblast motility and invasion. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NO synthesis; hence, accumulation of ADMA is associated with the development of PE. Inhibition of NO synthesis predictably results in increased blood pressure, along with a reduction in trophoblast invasion[16] and the pathogenesis of PE can be implicated by synthesis of NO and CO.[17] Hence, the present study is focused to determine whether the extracts of BL cater to the demand of preeclamptic placental trophoblast via assessing the ADMA expression.

In addition, the effect of extracts of BL on the expression of two important adipokines-leptin and adiponectin were analyzed in placental trophoblast.[18] Adya *et al.*[19] explored that adipokines enhance angiogenesis both by the activation capillary tube formation in endothelial cells and by elevating the mobilization of vascular progenitor cells from bone marrow. In placenta and endometrial tissues, leptin and adiponectin play a vital role in the regulation of cell proliferation/differentiation, local angiogenesis, immune tolerance, and inflammatory processes, which altogether regulate the trophoblast invasiveness. These adipokines synthesized by placenta play a crucial role in the first steps of embryo implantation and thus act as the key components of the fetal-maternal interface. Adipokines can create a favorable environment for embryo implantation and fetal-maternal metabolism, communication, and gestation.[18]

Leptin is a novel placental trophoblast-derived hormone in humans.[20] Placental leptin is transferred to the fetus and helps in regulating the development and growth. It plays a potential role in satiety and energy homeostasis, and it determines many of the reproductive functions such as appropriate implantation and the growth of the embryo.[21] In severe PE with hypertension, maternal uteroplacental blood flow is impaired ultimately resulting in altered placental circulation causing chronic disturbance of nutrient supply. This finally leads to growth restriction resulting in low birth weight babies one on the major consequences of PE.[22] On the other hand, placental hypoperfusion also produces local hypoxia, which consequently augments leptin gene expression in the placenta. It is an important physiological regulator of growth and plays major role in the development of hypertension and adverse pregnancy outcome like PE.[23] In this context, an attempt was made to ascertain the effect of BLE on the expression of leptin in preeclamptic trophoblast.

Adiponectin has insulin-sensitizing, anti-inflammatory, and anti-proliferative effects. At the embryo-maternal interface, adiponectin promotes differentiation, and invasion of human trophoblastic cells. Adiponectin exerts anti-proliferative effects on trophoblast cells[24] and it is necessary for the endocrine action in the placenta.[25] According to Dos Santos *et al.*[18] metabolic abnormalities and its related alteration in energy balance may end up with the pregnancy-related complications and altered fetal growth. This may lead to low birthweight babies as in the case of PE and low level of adiponectin associated with decreased insulin sensitivity and increased energy expenditure.[25] Hence, assessment of the efficacy of BLE on the expression of adiponectin in placental trophoblasts was executed which may give an insight into the impact of adiponectin on the clinical complications and low birthweight fetus.

Hence, the present study focuses on the elucidating the potential role of the two extracts of *Piper betle* such as diastase and salivary extracts of BL such as diastase BL extract (DBLE) and Salivary BL extract (SBLE), respectively, on the placental trophoblasts of both normal and preeclamptic patients, by ascertaining the level of 4-HNE, HO-1 and ADMA and adipokines such as adiponectin and leptin before and after incubating the isolated trophoblast with DBLE and SBLE and to ensure the potency of both the extracts in defeating stress-related complications like PE.

MATERIALS AND METHODS

Collection and authentication of *Piper betle*

P. betle plant saplings were purchased from Tamil Nadu 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 and Family Welfare, Government of India).

Preparation of betel leaf extracts

Aqueous BLE was prepared by boiling 10% (w/v) leaves the deionized distilled water and allowed to concentrate to about 90% (w/v). The leaves were sieved out and the crude aqueous extract obtained was filtered. A volume of 1 mL aliquots of the crude extract were dried overnight using speed vacuum concentrator. The dried pellets of the crude aqueous extract samples were refrigerated at −40°C until further use. The pellets were then weighed, dissolved, and diluted to suitable concentration as mentioned below. The isolated trophoblasts were incubated with 1 mg/ml concentration for 1 h, following the incubation, cell viability was assessed, and further experiments were carried out.

Selection of subjects

The study was carried out for 6 months. The placental samples were obtained from a private hospital in Chennai. Informed consent was obtained from the subjects, and the study was approved by the 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 2040 years, postdelivery. Patients with PE were defined on the basis of the following laboratory criteria: Blood pressure >140/90 mm Hg but <160/110 mm Hg, proteinuria >300 mg/L, and xanthine oxidase activity of approximately 2.6 units/mg protein. Patients with severe PE and other severe maternal complications were excluded from the study.

Isolation of trophoblast

Third-trimester villous trophoblast cells, which were used for comparison, were isolated from term placentas by the method of Douglas and King.[26] Briefly, placental villi were cut and thoroughly washed to remove blood. Thereafter, they were incubated four times in a digestion medium composed of Hank's balanced salt solution, containing trypsin and deoxyribonuclease for 30 min at 37°C in a water bath with continuous shaking. The dispersed cells were layered on top of a discontinuous 5%–70% percoll gradient, and centrifuged for 25 min at 507 ×g. The intermediate layers (density between 1.048 and 1.062) containing cytotrophoblast cells were removed and washed, and 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% fetal bovine serum. All the experiments were performed within a day of trophoblast isolation to overrule the influence of the cultivation process.

Assessment of cell viability

The viability of the isolated trophoblast was assessed by the trypan blue exclusion method.[27] 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.

Quantification of 4-hydroxynonenal

4-HNE was assessed by the enzyme-linked immunosorbent assay (ELISA) kit (MBS161454, My Biosource, USA) following the manufacturer's instructions.

Quantification of heme oxygenase-1

The level of HO-1 was quantified using HO-1 ELISA kit (ADI-EKS-800, Enzo Life Sciences, New York, USA) according to the manufacturer's instructions.

Estimation of asymmetric dimethylarginine

The level of ADMA in the placental trophoblast was quantified using ADMA ELISA kit (MBS264847, Life Science Inc, USA) according to the manufacturer's instructions.

Quantification of leptin by enzyme-linked immunosorbent assay

The level of leptin in the placental trophoblast was quantified using Leptin ELISA kit (11-LEPHU-E01, ALPCO, India) according to the manufacturer's instructions.

Quantification of adiponectin by enzyme-linked immunosorbent assay

The level of adiponectin in the placental trophoblast was quantified using total Adiponectin ELISA kit (47-ADPHUT-E01, ALPCO, India) according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using Statistical Software package version 7.0 ((IBM SPSS statistics Advanced Analytics Solutions and Services provider in the UK and Ireland, Presidion London Office, Bracknell, UK). Student's t-test was used to ascertain the significance of variations between normotensive and preeclamptic placental trophoblast. All data were presented as mean ± standard deviation differences were considered statistically significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

Quantification of 4-hydroxynonenal

Figure 1(left side): depicts the level of 4-HNE from the placental trophoblasts isolated from normal and preeclamptic women upon incubation with SBLE and DBLE. There was a significant elevation in the level of 4-HNE preeclamptic trophoblasts (67%, $P < 0.001$) when compared to normotensive trophoblast. Incubating both the samples with SBLE and DBLE a substantial decrease in the level of 4-HNE in the preeclamptic trophoblasts was observed by 26% ($P < 0.01$) and 23% ($P < 0.01$) respectively.

Quantification of heme oxygenase-1

A significant decrease in the level of HO-1 by 38% ($P < 0.001$) was seen in preeclamptic subjects comparing to normal due to its increased utilization to overcome the maternal complication like hypertension [Figure 2]. On incubating normotensive and preeclamptic trophoblasts to salivary and diastase extracts of betel leaf, a significant elevation (29% and 24%) in HO-1 was detected in both, where as less significant alteration was found in normotensive trophoblasts after incubation.

Estimation of asymmetric dimethylarginine

ADMA is a molecule capable of affecting angiogenesis in pregnancy related complication like preeclampsia and hence there a substantial elevation (70%, $P < 0.001$) in PT was observed when compared to NT [Table 2]. A decline in the level of ADMA 29% and 24% was found in preeclamptic trophoblasts on incubation with SBLE and DBLE. The polyphenols and other phytonutrients of betel leaf express anti-angiogenic and antihypertensive effect property and when mixed with saliva and diastase its efficacy is further increased.

Quantification of leptin

Figure 1(right side): demonstrates a marked elevation in the level of leptin (55%) was observed in preeclamptic placental trophoblasts when compared to normal subjects as demonstrated [Figure 1] (right side). SBLE and DBLE incubation considerably decrease the level by 25% and 21% in trophoblast of preeclampsia indicating the efficacy of both the extracts on overcoming the hypoxia and its mediated complications like vasoconstriction and hypertension.

Quantification of adiponectin

The level of adiponectin in placental trophoblast of normotensive and preeclamptic subjects was given in [Figure 3]. The level of adiponectin was significantly decreased by 47% in preeclamptic trophoblast when compared with normotensive. The level of adiponectin was found to be increased by 28% and 26% in preeclamptic placental trophoblast upon incubation with SBLE and DBLE when compared to cells without incubation.

DISCUSSION

Most of the antihypertensive agents in routine use have been documented to be teratogenic, i.e., it crosses the placenta and reach the fetal circulation.[27] They fail to protect the woman from high blood pressure and are ineffective in the management of hypertensive disorder of pregnancy like PE. Hence, the demand for a natural remedy which can be consumable is more. We have already established that SBLE and DBLE were found to be effective in maintaining the altered redox status of trophoblasts in our previous research work.[10] Hence, their efficiency in regulating the expression of few key signaling proteins that are instantly responding to cellular stress was quantified. The expression of key signaling proteins and the effect of BLE on the same signaling proteins were discussed below.

The level of 4-HNE is greatly associated with the generation of reactive oxygen species. Hence 4-HNE and its conjugates in tissues are frequently used as markers of oxidative stress. During pregnancy vascular diseases like PE, lipid peroxide-induced damage is an active contributor. It is extensively implicated in the pathogenesis of PE through the induction of pro-inflammatory signaling cascades, and it can diminish the bioavailability of NO, a vasoactive agent through the modulation of NOS activity.[28] Consistent with this study, there is an elevation in the level of 4-HNE (67% $P < 0.001$) in preeclamptic placental trophoblast was noted, and the extracts of BL such as SBLE and DBLE are potential enough in decreasing the level of 4-HNE (26% and 23%; ($P < 0.01$), respectively) [Figure 1]. Regulation of 4-HNE by the extracts of BL may be associated with the polyphenols present in BLE and the impact of salivary enzymes, in particular, amylase which may play a key role in promoting the antioxidants activity of BL. It also reveals that it may ultimately prevent the isolated placental trophoblasts from 4-HNE induced cellular damage and hence their intake may promote proper growth and development of the fetus.

The expression of HO-1 is indicative of sufficient placental development and proper fetal growth, indicating that their reduced expression in the trophoblast is associated with PE.[11] Ahmed *et al.*[29] reported the reduced expression of HO-1 in the preeclamptic placental tissue. Coherent with their studies, we also observed a decrease in the level of HO-1 (38%, $P < 0.001$) suggesting that it may be extremely utilized to protect the placental trophoblast from oxidative insult. Hence, administering heme oxygenase or its metabolites might be an intriguing new therapy in the treatment of PE.[11] In addition, another research study reported that induction of HO-1 and the release of bioactive metabolites such as CO and bilirubin imparts a significant role in down-regulating the factors involved in the etiology of PE.[30] Consistent with this, we have observed a significant increase in HO-1 in preeclamptic placental trophoblast on incubation with diastase and the salivary extracts of BL and their impression in regulating the level of stress markers [Figure 2]. This may enhance the vascularization and uteroplacental perfusion ultimately leading to proper trophoblast invasion to overcome the complications of PE.

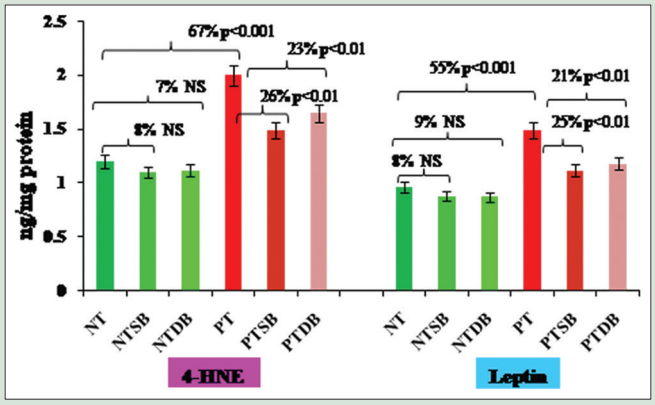

Figure 1: Level of 4-hydroxy nonenal and leptin were assessed in normotensive and preeclamptic placental trophoblast with and without diastase with betel leaf and saliva with betel leaf. Values are expressed as means ± standard deviation ($n = 10$)

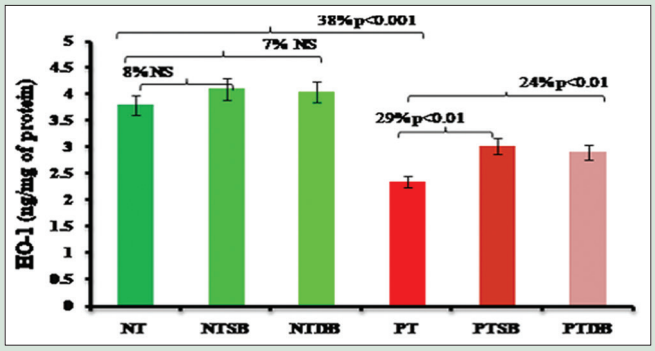

Figure 2: Level of heme oxygenase-1 was assessed in normotensive and preeclamptic placental trophoblast with and without diastase with betel leaf and saliva with betel leaf. Values are expressed as means ± standard deviation ($n = 10$)

Table 2: Level of asymmetric dimethylarginine was assessed in normotensive and preeclamptic placental trophoblast with and without DB and SB

Parameters	NT	NTSB	NTDB	PT	PTSB	PTDB
ADMA (mM/mg protein)	0.03±0.0028	0.028±0.0027	0.027±0.0.026	0.051±0.0048	0.036±0.033	0.039±0.0038

Values are expressed as means±SD (n=10). SD: Standard deviation; ADMA: Asymmetric dimethylarginine; NT: Normotensive placental trophoblast; NTDB: Normotensive placental trophoblast with DBLE; NTSB: Normotensive placental trophoblast with SBLE; PT: Preeclamptic placental trophoblast; PTDB: Preeclamptic placental trophoblast with DBLE; PTSB: Preeclamptic placental trophoblast with SBLE; SBLE: Salivary BL extract; DBLE: Diastase BL extract; SB: Salivary BL; DB: Diastase BL

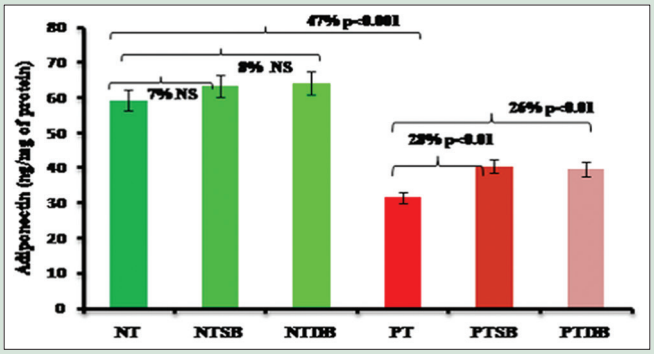

Figure 3: Level of adiponectin was assessed in normotensive and preeclamptic placental trophoblast with and without diastase with betel leaf and saliva with betel leaf. Values are expressed as means ± standard deviation ($n = 10$)

Cartwright *et al.*[16] demonstrated that the concentration of ADMA during PE was significantly elevated compared to normotensive controls. Coherent with this, we observed a significant elevation in the ADMA (70%, $P < 0.001$) in preeclamptic trophoblasts which may be associated with excessive levels of oxidative damage and stress markers in placental tissues [Table 2]. On incubation of trophoblast with SBLE and DBLE, decrease in the level of ADMA was noted which may be attributed to the presence of polyphenols such as hydroxychavicol, allylpyrocatechol, chavibetol, piperbetol, arecoline, charvacol, caryophyllene, piperitol, and eugenol[14] in BLE, the main effectors in the vasoregulation thereby preventing hypertension.

In addition, adipokines such as leptin and adiponectin were assessed in preeclamptic trophoblast as their ratio (leptin/adiponectin ratio) may serve as a clinically valuable marker for detecting some pathologies in pregnancy and both these adipokines can modulate invasion of trophoblasts and supply of nutrients to the fetus.[18]

Leptin regulates placental nutrient transport, placental angiogenesis, trophoblastic mitogenesis, and immunomodulation that are crucial for development and adequate placental function.[31] According to Poston,[32] leptin has a vital role in the physiology of placenta and trophoblast invasion. Another study reported that leptin is increased under placental hypoperfusion, thus promotes the development of hypertension and adverse pregnancy outcome like PE. Therefore, leptin is described as the vital physiological regulator of fetal growth.[23] Coherent with this studies there is a significant increase in the level of leptin (55%, $P < 0.001$) in the preeclamptic placental trophoblast when compared with normal was observed. Whereas, on incubation of preeclamptic trophoblast with SBLE and DBLE; there is a decrease in the level of leptin (25% and 21%) when compared with trophoblast without incubation [Figure 1]. This clearly indicates that both the extracts of *P. betle* may modulate the level of leptin and thereby it can efficiently restore the metabolic abnormalities and energy balance. This may ensure the proper supply of energy and nutrients for growth and development of the fetus; and may be helpful in combating low birthweight babies as seen in PE.

Like leptin, adiponectin also plays a central role in the regulation of fetal growth by modulating placental nutrient transport.[33] Abnormal expression of adiponectin is involved in the pathogenesis of PE,[34] consistent with this there was a decrease in the level of adiponectin (47%, $P < 0.001$) in the placental trophoblast of PE was observed. This may decrease the fat store, thereby limiting the fetal growth leading to the complications of PE.[35] Incubation of trophoblasts with both diastase and salivary extracts of BL are more or less equally efficient (26% and 28%, respectively) in enhancing the level of adiponectin when compared with normal trophoblasts [Figure 3] which may be due to the efficient interaction of various bioactive components of BL with diastase and the enzyme of saliva depicting chewing BL helps in interaction of phytochemicals of BL with saliva. A study reported that HO-1-mediated increase in adiponectin provides the vascular system with tolerance/resistance to oxidative stress.[36] Coherently, increase in the expression of HO-1 and the mediated adiponectin elevation was noted in preeclamptic placental trophoblast on incubation with BLE, depicting that flavonoids present in BLE impart an effective vasoprotective role in preeclamptic placental trophoblast.

CONCLUSION

Overall, results reveal that this protective role of BLE may be attributed to the interaction of biologically active components of saliva with the BL flavanoids. Consistently, studies also demonstrated that saliva possesses immunomodulators, antioxidants, anticoagulants, and many biologically active compounds.[37,38] Sujatha *et al.*[39] were also reported that BLE prepared with saliva enhance the antioxidant potency of due to the conversion of inactive large compounds of BL into active smaller components.

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Nil.

Conflict of interest

There are no conflicts of interest.

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