



Research article

Strengthening antioxidant defense & cardio protection by *Piper betle*: An in-vitro studyHardik Savsani^a, Abhay Srivastava^b, Sarita Gupta^b, Kirti Patel^{a,*}^a Department of Pharmacology, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodra, Gujarat, 390001, India^b Molecular Endocrinology and Stem Cell Research Laboratory, Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodra, Gujarat, 390005, India

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ABSTRACT

Introduction: The purpose of this research work was to evaluate *Piper betle* ethyl acetate extract (PBEA) for its free radical scavenging, antioxidant, anti-apoptotic activities and its role in protecting against oxidative cardiac cell injury.

Methods: The Free radical scavenging activity and antioxidant potential of PBEA were evaluated using various non-cellular methods (1,1-Diphenyl-2-picrylhydrazyl, β -carotene bleaching, superoxide anion, hydroxyl radical, hydrogen peroxide, Reducing power, Total phenolics and Total flavonoids). PBEA was standardized with Eugenol by GC-FID analysis. Furthermore, PBEA was also assessed for its cytoprotective effect against 100 μ M H₂O₂ in H9c2 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Intracellular reactive oxygen species scavenging and anti-apoptotic activity of PBEA was assessed by using 2', 7'-Dichlorofluorescein diacetate and Annexin- Propidium Iodide, respectively.

Results: PBEA exhibited radical scavenging and antioxidant defense response at different magnitudes of potency. Eugenol, a cardiac protective bioactive molecule in PBEA was found to be 43.43 ± 1.46 mg/g of PBEA extract. Further, pre-incubation of H9c2 cells with 10 μ g/ml PBEA for 24 h exhibited remarkable cytoprotective effect against H₂O₂ induced oxidative stress. PBEA at 10 μ g/ml dose with 24 h contact with H9c2 cells significantly enhanced the activity of cellular defense system and significantly decreased intracellular ROS ($P < 0.001$) and apoptosis ($P < 0.01$) thereby protecting against the cytotoxic effects of H₂O₂.

Conclusion: These outcomes indicated that PBEA could shield against oxidative and apoptotic cardiac cell injury in invitro studies. Thus, PBEA might be a desirable antioxidant of natural origin that has future clinical implications in both health care and food industry.

1. Introduction

Colligate role of calcium signaling, oxidative stress and mitochondrial dysfunction is pathogenesis of cardiovascular diseases [1]. ROS production and calcium signaling regulation can be deliberated as bidirectional. Acceleration of oxidative stress by the disparity between inadequate antioxidant defenses system and systemic manifestation of reactive oxygen species (ROS), that are constantly produced, transformed and consumed in all living organisms as an upshot of aerobic life [2]. Calcium is converse with versatile system and pathway, among them also with metabolic byproduct of oxygen including hydroxyl radical, superoxide anion, hydrogen peroxide which can damage cellular and sub cellular organelle [1, 3, 4]. Dysfunctional calcium overload and oxidative stress

through mitochondria and endoplasmic reticulum in cardiomyocytes develop fibrosis, apoptosis, inflammation, ischemia/reperfusion damage, hypertrophy as well as structural and functional cardiac remodeling ultimately leads to atherosclerosis, myocardial infarction, arrhythmias, hypertension, cardiomyopathy, heart failure and emerging death in the old age people [5, 6, 7].

Accordingly, oxidative stress modulation looks highly engaging from a therapeutic standpoint. Oxidative cardiac cell injury can be prevented through scavenging oxidants via involvement of the enzymes (SOD, CAT, GPx) and non-enzymes (GSH, Vit. C, Vit. E and β -carotene) antioxidant defense systems [8]. Former reports strongly evoked that foods containing constituent like antioxidants and radical scavengers have potential prophylactic action against cardiovascular disorders accelerated

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by ROS. Antioxidants prevent or repair free radical induced cell damage by inhibiting or delaying the induction or propagation of oxidative chain reaction thereby promoting decomposition, scavenging activity and preventing radicals formation [8, 9].

Thus lookout for adequate, nontoxic natural compounds along with high antioxidant potential has been consolidating in recent times. Moreover, this suitable strategy is to build high endogenous antioxidant enzyme level by dietary and plant-derived antioxidants intake. Natural antioxidant containing Indian dietary constituents and medicinal plants have impregnable defensive effect against many disorders [10, 11]. Therefore, consumption of various natural compounds containing high antioxidant may bring down the risk of life-threatening cardiovascular disorders influenced by ROS and intracellular calcium overload.

The genus *Piper* contains above 700 species of plants and is spread in both hemispheres. Out of these, about 30 species have been reported in India. *Piper betle* Linn. (Known as Paan) belongs to the plant family Piperaceae [12]. This plant is economically, ethnobotanically, ethnomedicinally and traditionally important in the whole world [13]. Although known from prehistoric times, scientific involvement in *Piper betel*, an evergreen vine, was restricted because of its association with the oral cancer, on consumption of betel quid with tobacco [14]. Now a days, an elicited response in exploring this member of the natural medicine for therapeutic potential, have started to establish the medicinal capability of this plant using a rationale driven scientific approach. *Piper betle* contains considerably large number of useful secondary metabolites and versatile phytoconstituents, such as hydroxychavicol, α -Pinene, Eugenol, β -Caryophyllene, chavibetol, A-cadinene, β -sitosterol, piperol A, piperol B piperbetol and methyl piperbetol [15] which has been cited throughout the world for various biological activity such as anti-inflammatory, radicals scavenging activity, inhibiting lipid peroxidation, antioxidant [16], anti-cancer, platelet activating factor [15], immunomodulatory, anti-infective, anti-mutagenic [17], and anti-diabetic [18]. Moreover, *Piper betle* containing Eugenol exhibits the most powerful antioxidant activity, radical-scavenging activity as well as inhibition of the intracellular calcium release and extracellular calcium entry [19, 20, 21].

However, the non-cellular and cellular mechanisms of ethyl acetate extract of *piper betle* have not yet been subject to serious scientific study. Thus an attempt was made to assess the potential cardio protective effect of ethyl acetate extract of *piper betle* against H_2O_2 induced oxidative cardiac injury on H9c2 (2-1) cells via cell viability, cellular antioxidant activity, intracellular ROS accumulation and cell apoptosis.

2. Materials and methods

2.1. Chemicals

The cell culture Dulbecco's Modified Eagle Medium (DMEM), Eugenol, α -tocopherol and β -carotene were purchased from HiMedia Laboratories (Mumbai, India). 2', 7'-Dichlorofluorescein diacetate (DCFH-DA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Propidium Iodide (PI), Quercetin, Curcumin, Gallic acid and L-Ascorbic acid were purchased from Sigma (St. Louis, MO, USA). Annexin-V was purchased from BD Biosciences (San Jose, CA, USA). Pen Strep was purchased from Thermo Pierce (Waltham, MA, USA). All other biochemical reagents were of analytical reagent (AR) grade.

2.2. Plant material

The aerial part (leaves) of *Piper betle* was accumulated from West Bengal of India. The plant leaves was identified by Dr. Padmanabhi S. Nagar (Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Gujarat, India) and a voucher specimen (BARO 20610) was stored in herbarium section of Botany Department, The Maharaja Sayajirao University of Baroda, Gujarat, India.

The leaves of *piper betle* were washed with water, shorted and dried with air and then fine powder was prepared by grinding. The leaves powdered (30g) were extracted with ethyl acetate solvent (250 ml) at 70 °C using Soxhlet extractor till colorless solvent were seen in siphon tube. After extraction, ethyl acetate was evaporated completely using rotary evaporator (Büchi® Rotavapor R-100). *Piper betle* Ethyl acetate extract (PBEA) was protected from light and stored at 4 °C until further experiment.

2.3. DPPH radical scavenging assay

DPPH-Radical scavenging capacity was determined according to the method reported by [22]. 1000 μ g/ml PBEA stock solution was prepared by dissolving extractive yield of PBEA (10 mg) in methanol (10 ml). PBEA stock solution was series diluted into 10, 25, 50, 100, 200 and 500 μ g/ml with methanol. In each concentration, 1 ml of PBEA solutions and methanol (Blank) were mixed with 1.5 ml of DPPH (0.25 mM). The reaction mixture was shaken vigorously for 2 min and incubates at room temperature for 30 min in dark place. Ascorbic acid, Quercetin, α -tocopherol and Curcumin were used as standard and for the blank, sample was substituted by methanol. Disappearance of DPPH color was determined by measuring the absorbance at 517 nm against methanol with a spectrophotometer (Shimadzu - 1800). The DPPH radicals scavenging activity was determine by the following equation: Scavenging activity = $[A_0 - A_1/A_0] \times 100$ where A_0 is the absorbance of the blank (without extract) and A_1 is the absorbance of the sample (extract or standard).

2.4. β -carotene linoleic acid assay

The antioxidant activity of the PBEA was estimated by β -carotene bleaching method [23]. A stock solution of β -carotene-linoleic acid mixture was prepared by β -carotene in chloroform (0.5 mg/ml) was added in to linoleic acid (25 μ L) and Tween 80 (200 mg) emulsifier mixture. After chloroform evaporation, distilled water (100 ml) was added slowly along with vigorous shaking. Prepared mixture (4 ml) was added into 0.2 ml of PBEA solution (500 μ g/ml). Zero minute absorbance was immediately measured at 470 nm using a spectrophotometer (Shimadzu - 1800). After emulsion integral was incubated at 50 °C for 120 min, absorbance was measured. Devoid of β -carotene in mixture as blank was primed for background correction. Ascorbic acid, Quercetin, α -tocopherol and Curcumin were used as standards. The bleaching rate (R) of β -carotene was determined according to the following equation: $R = \ln(a/b)/t$ where $\ln$ is the natural log, a is the 0 min absorbance, b is the 120 min absorbance. % inhibition of sample as compare to control represented as antioxidant activity (AA), which was determined by following equation: $AA = [(R_{control} - R_{sample})/R_{control}] \times 100$.

2.5. Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of the PBEA was analyzed by the method of [24]. O_2^- anions were created in 3 ml of Tris-HCl buffer (16 mM, pH 8.0) reaction mixture containing equal ratio of NBT (50 μ M), NADH (78 μ M) and PBEA solution (10, 25, 50, 100, 200 and 500 μ g/ml). Reaction was initiated by adding 1 ml of PMS solution (10 μ M) and incubated at 25 °C for 5 min. The absorbance was measured at 560 nm using a spectrophotometer (Shimadzu - 1800) against the corresponding blank. The capacity of scavenging the anion radical was measured by the following equation:

Super oxide anions scavenging activity (%): $[(A_{control} - A_{sample})/A_{control}] \times 100$

Where $A_{control}$ was the absorbance of blank and A_{sample} was the absorbance of sample.

2.6. Hydroxyl radicals scavenging activity

Generated hydroxyl radicals by Fenton reaction was measured by the inhibition of deoxyribose degradation [25, 26]. The Fenton reaction mixture (1.0 ml) consist of 100 μ L of 2-deoxy-Dribose (28 mM in 20 mM KH_2PO_4 -KOH buffer, pH 7.4), 500 μ L of PBEA solution (10, 25, 50, 100, 200 and 500 μ g/ml), 200 μ L of FeCl_3 (200 μ M): EDTA (1.04 mM) (1:1 v/v), 100 μ L of H_2O_2 (1.0 mM) and 100 μ L of ascorbic acid (1.0 mM). 1.0 mL of Trichloroacetic acid (TCA) (2.8%) and 1 ml of Thiobarbituric acid (TBA) (1%) were added to the Fenton reaction mixture after incubation for 1 h at 37 °C and placed in a boiling water bath for 20 min for color development. For control sample, deionized water was used instead of methanol because methanol itself as Hydroxyl radical scavenger. After cooling, absorbance was measured at 532 nm using spectrophotometer (Shimadzu - 1800), against a blank sample containing devoid of deoxyribose. Ascorbic acid used as standard and percentage inhibition was calculated by comparing test and blank sample.

2.7. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of PBEA was estimated according to the method of [27]. H_2O_2 solution (40 mM) was prepared in phosphate buffer saline (PBS) (pH 7.4) at 20 °C and concentration was measured at 230 nm using spectrophotometer (Shimadzu - 1800). H_2O_2 solution was added in to PBEA solution at a final concentration of 10, 25, 50, 100, 200 and 500 μ g/ml at 20 °C. After 10 min absorbance of H_2O_2 was determined in a Spectrophotometer (Shimadzu - 1800) at 230nm against blank solutions containing PBEA solution (10, 25, 50, 100, 200 and 500 μ g/ml) in PBS without H_2O_2 . Hydrogen peroxide scavenging activity is determined by the following equation: % scavenged = $[(A_0 - A_1)/A_0] \times 100$ where A_0 is the absorbance of the control and A_1 is the absorbance of the sample (extract or standard).

2.8. Reducing power assay

The Fe^{3+} reducing power of the PBEA was resolute by the technique of [28] with slight modifications. 750 μ L of PBEA solution (10, 25, 50, 100, 200 and 500 μ g/ml) were mixed with 750 μ L of phosphate buffer (0.2 M, pH 6.6) and 750 μ L of $[\text{K}_3\text{Fe}(\text{CN})_6]$ (1% w/v), followed by incubating for 20 min at 50 °C in a water bath. 750 μ L of chilled trichloroacetic acid (TCA) solution (10%) was added for the stopping of reaction mixture and then centrifuged at 4000 rpm for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and add 100 μ L of FeCl_3 solution (0.1% w/v). After 10 min, absorbance was determined at 700 nm with spectrophotometer (Shimadzu - 1800).

2.9. Determination of total phenols and flavonoids

The total phenolics content of the PBEA was estimated according to the Folin & Ciocalteu method described by [29]. Briefly, aliquots of 1 mg/ml PBEA solution were prepared in 80% Methanol. This 0.5 ml of PBEA solution (1 mg/ml) was mixed with 2.5 ml FCR (diluted 1:10, v/v) and 10 min later, 2 ml of sodium carbonate (7.5%, w/v) was added. The reaction mixture was incubated at room temperature for 90 min and the dark blue color changes due to phenol oxidation were determined by spectrophotometer (Shimadzu - 1800) at 765 nm. Standard curve was plotted using seven different concentrations (0–200 mg/l) of Gallic acid (GA), the levels of total phenolic contents in PBEA was resolute and the results were articulated as gallic acid equivalents (mg gallic acid/g dried extract).

The total flavonoids content of PBEA was estimated according to the aluminum chloride method described by [30]. Briefly, aliquots of 1 mg/ml PBEA solution were prepared in 80% Methanol. This 0.5 ml PBEA solution (1 mg/ml) was mixed with 1.5 ml methanol (95%), 0.1 ml of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (10%), 0.1 ml of CH_3COOK (1 M) and 2.8 ml of deionized water, followed by incubating for 40 min at room temperature.

Absorbance of the reaction mixture was then determined by spectrophotometer (Shimadzu - 1800) at 415 nm against deionized water blank. Standard curve was plotted using seven different concentrations (0–200 mg/l) of quercetin, the levels of total flavonoids content in PBEA was resolute and the results were articulated as quercetin equivalents (mg gallic acid/g dried extract).

2.10. Standardization of PBEA by GC-FID analysis

PBEA was standardized with Eugenol by gas chromatography with flame ionization detector (Perkin Elmer GC clarus 500) and analyzed by TotalChrom Navigator clarus 500 software. Instrument is coupled with a split/splitless injector, run in a split-mode and ZB-5 capillary column (cross bond 5% diphenyl/95% dimethyl polysiloxane, 30 m $\times$ 250 μ m) was used throughout the analysis. The GC-FID parameters were used in the analysis on the basis of the boiling point and affinity towards the stationary phase of the drug. Eugenol has a boiling point of about 254 °C. 1 μ L sample was manually injected at an inlet temperature and detector temperature of 240 °C and 300 °C, respectively. After injection, the oven temperatures was increased quickly from 60 °C and hold for 2 min then programmed within 25min–310 °C at a rate of 10 °C per min and hold for 8 min. Carrier gas nitrogen (0.942 mL/min), synthetic air (450 mL/min), hydrogen (45 mL/min) were fed to the FID.

2.11. Cell culture and treatment

Rat heart cell line H9c2 (2-1) (National Centre for Cell Science, Pune) was cultured in DMEM high glucose media plus 10% FBS, and Pen Strep in a humidified 5% CO_2 atmosphere at 37 °C. Cells were nearly 80% confluent, numbers of cell were counted using a haemocytometer and the abilities of the cells to exclude trypan blue. Cells were detached by trypsinization and seeded overnight at 10,000 cells/well in a 96-well plate. In the control group, cells were not treated with any drug, but in PBEA group, different concentrations of PBEA solution were treated for 24 h before the exposure of 100 μ M H_2O_2 for 1 h.

2.12. MTT assay

Subsequently, 20 μ L MTT solutions (5 mg/mL in PBS) was added to 200 μ L media with final conc. 500 μ g/ml in each well and incubated in a dark for 4h at 37 °C to allow for formazan formation. The MTT solution was removed, and the insoluble blue dark formazan crystals were dissolved in 200 μ L DMSO. After incubation for 10 min on a shaker, absorbance of all culture well was determined at 570 nm using microplate reader (BIO-RAD 680 XR, CA, USA).

2.13. Intracellular reactive oxygen species (ROS) levels assay

The generated ROS was visualized by fluorescent microscopy using 2',7'-dichloro fluoresceindiacetate (DCFH-DA) staining according to [31]. To detect H_2O_2 induced intracellular ROS accumulation, the cells were plated in 12-wellplates. In the control group, cells didn't treat with any drug, but in PBEA group 10 μ g/ml of PBEA solution were treated for 24 h and 100 μ M H_2O_2 was added to the plate and incubated at 37 °C for another 1 h. Cells were stained for 30 min in the dark with 10 μ g/ml DCFH-DA and 1 μ g/ml DAPI, then take out the extracellular compounds by washed twice with PBS. DCFH-DA green fluorescence was observed using florescent microscope (Zeiss CLSM 510). The relative cell fluorescence intensity was examined by Image J software (NIH, Bethesda, MD) and then plotted as previously reported by [32].

2.14. Cellular antioxidant enzyme profile

1×10^6 cells/well were seeded and cultured overnight. Cells were exposed with or without PBEA solution (10 μ g/ml) in 24 well culture plates for 24 h. Adherent cells were harvested in 1.5 ml centrifuge tubes

after 1 h H₂O₂ exposure and centrifuged at 1000 rpm for 10 min at 4 °C. Ice-cold 0.1 % Triton X-100 lysis buffer was used for cell pellets homogenization and cells were again centrifuged at 10000g for 15 min at 4 °C. Supernatant were used for assay of Lipid peroxidation, endogenous antiperoxidative enzymes like Superoxide dismutase (SOD) and Catalase, Reduced glutathione (GSH) were determined by the method of [33, 34, 35] and [36], respectively, which were normalized by total protein content.

2.15. Detection of apoptosis with annexin V-PI

Annexin V-PI dual staining was utilized to make a distinction between apoptotic and necrotic cell death by fluorescent microscopy as described by [37]. Briefly, 1×10^5 cells were plated in 12-wellplates and exposed to PBEA solution (10 µg/ml) for 24 h. Followed by 100 µM H₂O₂ was added and incubated at 37 °C for another 1 h. Cells were washed with cold PBS and resuspended in 1X binding buffer, after which they were incubated with Annexin-V for 15 min in dark at room temperature (RT) followed with addition of PI in the same tubes to a final concentration of 2 µg/ml for 15 min at RT as per manufacturers protocol. These, cells were observed using florescent microscope (Zeiss CLSM 510). The relative cell fluorescence intensity was examined using Image J software (NIH, Bethesda, MD) and then plotted.

2.16. Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by dunnett post hoc test and the values of P < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Yield of Piper betle ethyl acetate extract

As per previously mentioned process parameter of extraction, we observed 6.8% extractive yield.

3.2. Free radical scavenging and antioxidant activity of PBEA

Engagement of free radicals and calcium overload in the etiology of cardiovascular disorders represent promising avenue for recent developments in biomedical science [38]. Endogenous and exogenous antioxidant (dietary antioxidant from natural product) intake may be important strategy to stop or reverse the promotion or progression of the cardiovascular diseases through intervention and interception of free radical and calcium overload [39].

The Phytochemicals from natural products cover a diverse range of chemical entities containing Phenolic compounds such as phenolic acids, flavonoids, diterpenes, volatile oil and tannins like phytochemicals from natural products have usual consideration for their high antioxidative activity. Especially crude extracts of foods and medicinal plants have been heavily used in the food industry due to the presence of high amount of phenolics; because of their ability to impede lipids oxidation and thereby greater the quality and nutritional importance of food [40].

Phytochemical investigations have revealed that *Piper* species are mostly affluent in polyphenolics, flavonoids and essential oil which shows antioxidant properties. *Piper longum*, *Piper nigrum*, *Piper sarmentosum* are widely used medicinal and aromatic plants. *Piper betle* is one of endemic species of *Piper* in India, which used in betle quid and as medicinal herb in traditional medicine. Nonetheless, there is still lack of information regarding antioxidant and supplementary biological activity of this species. In order to implement more than one antioxidant methods, is required to understand the various oxidation aspects during the evaluation of antioxidant activity. In this circumstance, the antioxidant activity of the ethyl acetate extract of *Piper betle* leaves and well known antioxidant were compared.

Table 1. Free radicals scavenging ability and the inhibition of β-carotene linoleic acid oxidation by PBEA and standards antioxidant compounds in DPPH and β-carotene linoleic acid assays. Data are presented as mean ± SEM (n = 3).

Sample	DPPH (IC ₅₀) (µg/ml)	β-carotene bleaching (% inhibition)
Ascorbic acid	53.66 ± 0.43	89.31 ± 1.19
Quercetin	11.41 ± 0.14	80.6 ± 2.17
α-Tochoferol	19.96 ± 0.16	83.21 ± 0.93
Curcumin	22.83 ± 0.21	91.58 ± 1.21
PBEA	100.1 ± 0.32	73.64 ± 0.84

Free radical scavenging (hydrogen donors), antioxidant activity of foods and complex biological system was evaluated by the simple and rapid 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The IC₅₀ value (the concentration with scavenging activity of 50%) of PBEA, Ascorbic acid, Quercetin, α-tocopherol and Curcumin was found to be 100.1, 53.66, 11.41, 19.96 and 22.83 µg/ml; respectively for DPPH radical scavenging activity (Table 1). PBEA has the ability to reduce the stable radical DPPH to the yellow-coloured diphenyl picrylhydrazine through Electron transfer/hydrogen donating ability of PBEA contributing radical scavenging activity due to presence of phenolics compounds like eugenol, hydroxychavicol and iso-eugenol. However, The DPPH radical scavenging activity of PBEA was found to be moderate than standard component. Quenching of different radicals and reaction capacity of the extract are depending on stereo selectivity of the radicals and the extract solubility in different solvent system.

In another perspective, β-carotene bleaching assay, Potency of PBEA for inhibiting the formation of dienehydroperoxides from linoleic acid oxidation was estimated based on the discoloration of yellowish color of a β-carotene solution. In this experiment, the effect of PBEA on oxidation of β-carotene/linoleic acid is shown (Table 1). A result was indicating that the oxidation of β-carotene by hydroperoxides reduced through the presence of antioxidants in the PBEA. However, β-carotene bleaching activity of PBEA was found to be submaximal than standard component. Thus, antioxidant activity of the PBEA might be attributed to decrease degradation rate of β-carotene through neutralizing the linoleate-free radical and other free radicals formed in the system.

Superoxide anion radicals engage in the production of powerful and dangerous other reactive oxygen species such as hydroxyl radical, hydrogen peroxide, and singlet oxygen in living system. Regarding data presented in Figure 1a, the scavenging activity increased with increasing concentration up to a certain limit (between 100–500 µg/ml for PBEA). The highest scavenging activity was found to be 98% and 97% at 500 µg/ml concentration for PBEA and Ascorbic acid, respectively. Those results recommend that the PBEA could prevent or ameliorate oxidative stress via dose dependent superoxide anion radical scavenging activity.

We also tried out the scavenging action of PBEA on hydroxyl radical via deoxy-ribose degradation. Hydroxyl radical is generating carcinogenesis, mutagenesis, and cytotoxicity through damaging all biomolecule found in the living cells and leads to DNA strand breakage. To attack on the substrate deoxyribose, hydroxyl radicals were generated by reaction of Ferric-EDTA together with H₂O₂ and ascorbic acid. Regarding our data existing in Figure 1b, the scavenging activity increased with increasing concentration up to a certain limit (between 0.6 – 1 µg/ml for PBEA), and over the limit reach a plateau state (data not shown). The IC₅₀ value of PBEA and α-tochoferol as a positive control was found to be 0.6 and 0.4 µg/ml, respectively was extremely efficient on hydrogen radical scavenging. PBEA inhibited free radical mediated deoxyribose damage remarkably.

H₂O₂ is very toxic if it goes through cellular membranes, which can generate hydroxyl radicals through combine with reactive iron in the cells. The scavenging of hydrogen peroxide by PBEA was augmented in a dose-dependent manner as illuminated in Figure 1c. The IC₅₀ value of PBEA and Quercetin was found to be 46.17 µg/ml and 84.57 µg/ml, respectively. These results imply that PBEA having abundant

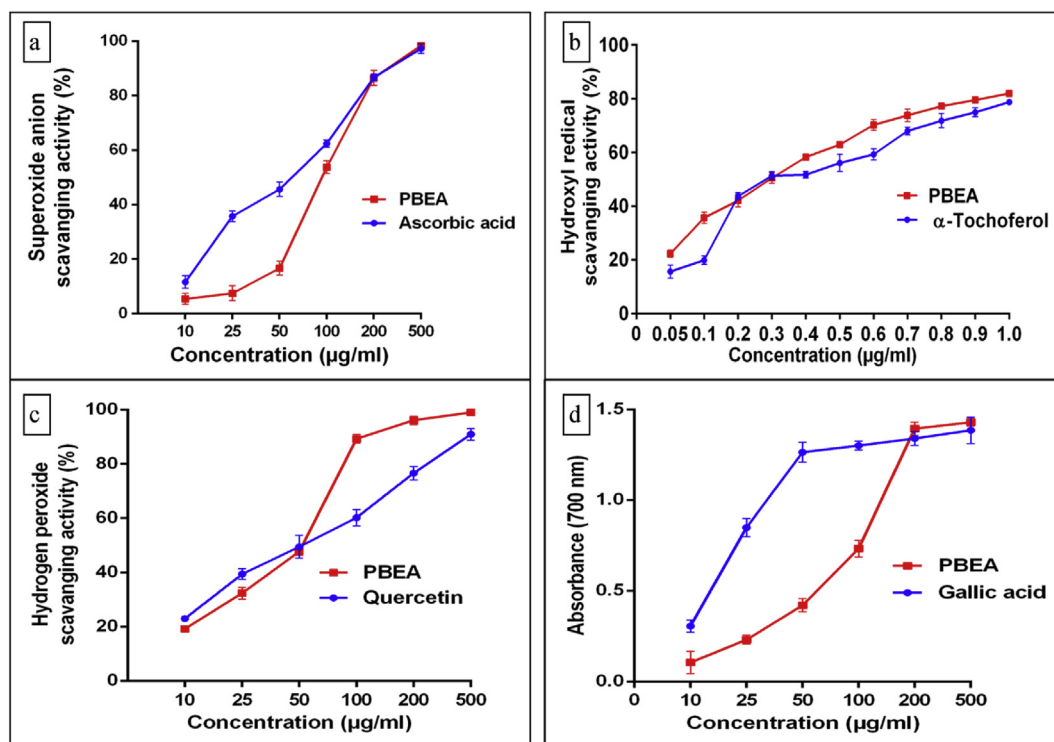


Figure 1. Superoxide anion radical scavenging (a), hydroxyl radical scavenging (b), hydrogen peroxide scavenging (c) and reducing power (d) properties of PBEA and standard antioxidants at various concentrations. Data are presented as means $\pm$ SEM (n = 3).

antioxidative phytochemicals (electron donating/radical quenching) like hydroxychavicol, chavibitol and allylpyrocatechol could also shield against cell lysis.

For the measurement of the reducing power, we looked into antioxidant potency of PBEA via reducing capacity of the Fe^{3+} /ferricyanide complex in the presence of PBEA. As mentioned in Figure 1d, showed the reductive capacity (as indicated by absorbance at 700 nm) of PBEA proportional to Gallic acid as standard. Reducing activity of PBEA increased in dose-dependent manner to certain level and subsequently leveled off with more augment in concentrations with high correlation index ($r^2 = 0.998$) which is similar to the antioxidant activity. The reducing power, $\text{RP}_{0.5}$ AU (defined as the effective concentration (μg per ml) that produces 0.5 absorbance unit at 700 nm for reducing power) of PBEA was 73.3 $\mu\text{g}/\text{ml}$. The outcome elucidate that polyphenolic content of the PBEA which may act as good electron and hydrogen donors, thereby producing more stable products and terminate radical chain reaction. It is very essential to determine the molecular mechanisms of the radical scavenging activities of extract for better perceptive of the mode of action.

3.3. The amount of total phenolics, flavonoids and standardization of PBEA

Being plant secondary metabolites, the phenolics or polyphenols reveal significant free radical scavenging and antioxidant activities by chelating redox-active metal ions, terminating lipid free radical chains, and inhibiting the transfer of hydroperoxide into reactive oxyradicals [41]. Flavonoids and its subclass have powerful antioxidant activity which depends on the existence of free OH groups, especially both 3-OH and 5-OH. As per report of Bors et al., a variety of oxidation-triggering radicals have been scavenged by the capability of different phenolics substance. Therefore it would be beneficial to evaluate the total phenolic and flavonoid content of the PBEA. Total phenolic and flavonoid contents of PBEA were estimated by the method of Folin-Ciocalteu's assay and

Table 2. Amount of total phenolics, flavonoids and the qualitative-quantitative analysis of Eugenol carried out by GC-FID from PBEA.

Compound	Amount compound
Total phenolic	54 $\pm$ 2.19
Total flavonoid	45.22 $\pm$ 1.65
Eugenol	43.43 $\pm$ 1.46

Total phenolic content was articulated as mg Gallic acid equivalents/g dried extract.

Total flavonoid content was articulated as mg Quercetin equivalents/g dried extract.

Eugenol was articulated as mg/g of dried PBEA extract.

aluminum chloride assay, respectively. Total phenolic contents of PBEA was determined through a linear Gallic acid standard curve ($y = 0.012x + 0.008$; $r^2 = 0.998$) and articulated in mg of Gallic acid equivalents while flavonoid contents of PBEA was determined through a linear Quercetin standard curve ($y = 0.003x - 0.013$; $r^2 = 0.995$) and articulated in mg of Quercetin equivalents (Table 2). As shown in Table 2, PBEA has 54.0 mg Gallic acid equivalent and 45.22 mg Quercetin equivalent, as phenolic and flavonoids, respectively.

Phenolics compound containing volatile oils are extensively occurring in the plant kingdom especially in leaves and herbs. For standardization of the extract, GC-FID analysis was done using commercially available Eugenol as a standard. Eugenol is a phenylpropene, an allyl chain-substituted guaiacol and an important constituent of volatile oil present in *Piper betle*. The GC-FID Chromatogram of PBEA revealed peak at the retention time 14.8 min using the proposed procedure as previously outlined (Figure 2 and Table 2). The chemical fingerprint thus affirms the standardization and validation of the extract used in the study. Our total phenolics and total flavonoids data, affirmed that the excellent antioxidant and free radical scavenging activity of PBEA might be attributed to its phenolics compound and flavonoids compound.

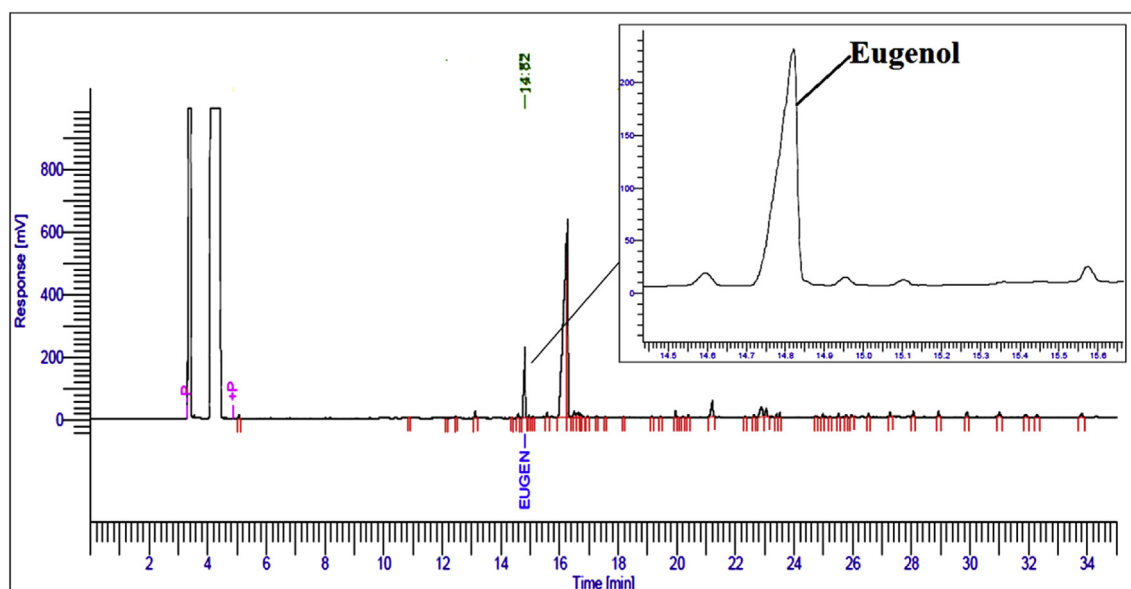


Figure 2. GC-FID chromatogram for standardization of PBEA via eugenol as standard.

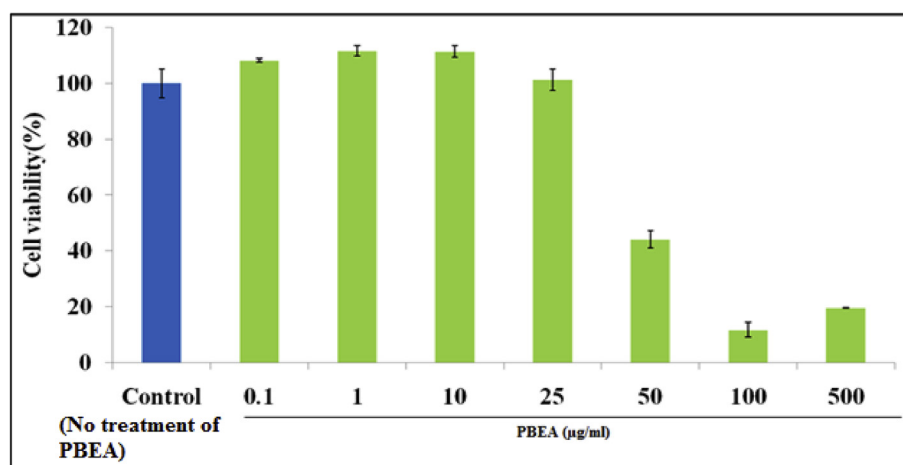


Figure 3. Cytotoxicity activity of PBEA. H9c2 cells were incubated with different concentrations of PBEA for 24 h. Then, cell viability was estimated by the MTT assay. Data are presented as mean $\pm$ SEM (n = 3).

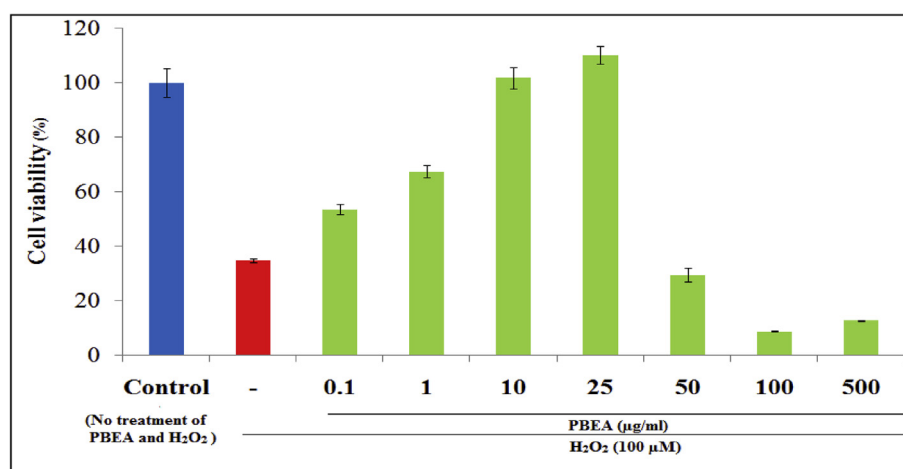


Figure 4. Cytoprotective activity of PBEA. H9c2 cells were incubated with or without different concentrations of PBEA for 24 h and exposure of 100 μM H₂O₂ for another 1 h. Then, cell viability was estimated by the MTT assay. Data are presented as mean $\pm$ SEM (n = 3).

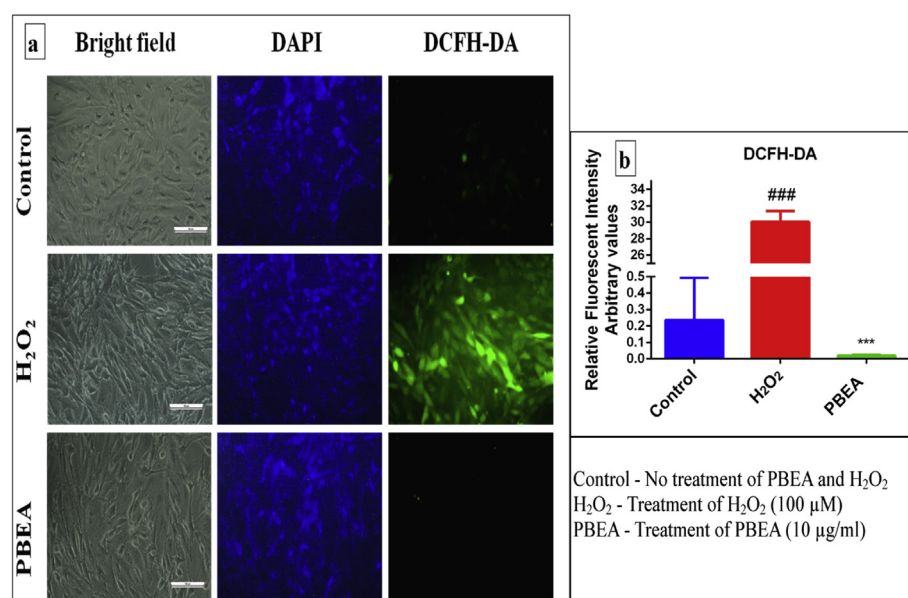


Figure 5. Effect of PBEA on H₂O₂ induced intracellular accumulation of ROS using DCFH-DA fluorescence. H9c2 cells were pretreated with or without PBEA (10 μg/ml) for 24 h before exposure to 100 μM H₂O₂ for 1 h (a) DCFH-DA staining for intracellular ROS. (b) Histogram showing the ROS level in H9c2 cells after treatment with H₂O₂ in presence or absence of PBEA compared to control groups. Scale bar = 100 μm. Data are presented as mean ± SEM (n = 3). ### Significantly different from control, P < 0.001. *** Significantly different from H₂O₂, P < 0.001.

3.4. Effect of PBEA on H₂O₂ induced H9c2 cell viability

Aim of the present examination was to determine the cardio protective effect of PBEA against oxidative cell death. H₂O₂ induced cell death of H9c2 cells was determined by MTT assays. Cells incubated with the dose of (0.1, 1.0, 10 or 25 μg/ml) PBEA had no perceptible cytotoxic or inhibitory effect on the growth of H9c2 cells (Figure 3). The 50% cell viability (IC₅₀) for 24 h pretreatment of PBEA was 48.56 ± 0.34 μg/ml (data not shown). Our experiment demonstrated that the viability of cells exposed to 100 μM H₂O₂ for 1 h was 34.84 ± 0.67% of the control group (Figure 4), while pretreatment with PBEA (0.1, 1.0, 10 or 25 μg/ml) protected cells from H₂O₂ induced damage, restoring cell survival to 53.48 ± 1.88%, 67.45 ± 2.09%, 101.81 ± 3.86%, and 110.19 ± 3.10%, respectively. However, the higher concentrations of PBEA were found ineffective. Our experiments have noticeably revealed a protective effect of PBEA, which is recognized to have antioxidant effect, on H₂O₂ exposed H9c2 cells. In addition, PBEA concentrations of 0.1–25 μg/ml had no cytotoxic effect in H9c2 cells (Figure 3). Our data designated that the protective action of PBEA was dose dependent and that 10 and 25 μg/ml of PBEA treatment were adequate to provide a significantly protective effect against H₂O₂ induced cytotoxicity. On basis of this result, we decided a low dose of PBEA (i.e. 10 μg/ml) with equal effect for further study.

3.5. PBEA attenuated H₂O₂ induced oxidative stress in H9c2 cells

H₂O₂ as the main resource of ROS is drawn in multiple factors induced oxidative stress in cardiovascular diseases. The intracellular ROS estimation explores the degree of cellular oxidative stress. Cell permeable DCFH can directly oxidize to fluorescent DCF by pro-oxidant. The Fenton reaction between H₂O₂ and ferrous ion causes conversion of H₂O₂ in to HO·, the most highly reactive species of ROS and also decompose to peroxy radicals and produce lipid peroxides under biological conditions, thus increasing fluorescence. In the present experiment, the intracellular ROS generation was significantly (p < 0.001) higher in H₂O₂ treated H9c2 cells compared to the untreated control (Figure 5a). However, pretreatment of H9c2 cells cultured with 10 μg/ml of PBEA for 24 h and followed by H₂O₂ exposure significant reduced ROS generation as evidenced by decreased DCF fluorescence intensity (Figure 5b). This outcome proves that PBEA contain natural antioxidants preventing or

Table 3. Effect of PBEA on activities of malondialdehyde (MDA), reduced glutathione (GSH) content, superoxide dismutase (SOD) and catalase (CAT) in cells. H9c2 cells were pretreated with PBEA (10 μg/ml) for 24 h before exposure to 100 μM H₂O₂ for 1 h and the antioxidant defense systems were determined. Data are presented as mean ± SEM (n = 3).

Enzymes	Control	H ₂ O ₂	PBEA
MDA	1.18 ± 0.09	4.10 ± 0.30 ^{###}	1.59 ± 0.06 ^{***}
GSH	461.86 ± 16.38	184.32 ± 7.78 ^{###}	297.95 ± 15.85 ^{***}
SOD	22.38 ± 1.48	6.37 ± 1.27 ^{###}	13.91 ± 0.68 ^{***}
CAT	6.31 ± 0.22	2.53 ± 0.12 ^{###}	4.33 ± 0.40 [*]

MDA and GSH are articulated as nmol/mg protein. SOD and CAT are articulated as U/mg protein. ### Significantly different from control, P < 0.001. *** Significantly different from H₂O₂, P < 0.001. * Significantly different from H₂O₂, P < 0.05.

delaying complimentary oxidative insults through decrease the steady-state generation of ROS in H9c2 cells culture. In addition, sequestering ROS and/or maintaining the cell and cellular machinery in their appropriate redox state by antioxidant enzymes and their changeable activity can be measured as biomarkers of antioxidant response.

Different antioxidant components are present as antioxidant defense systems and their antioxidant capacities depend upon which free radicals or oxidizers are generated in the body. Defense mechanisms against oxidative injury plays pivotal role in the cardiac cell injury and it is protected by enzymes (SOD, CAT and GPx) and non-enzymes defenses (GSH, vitamins C, E and β-carotene). SOD, Catalase and GSH are critically involved in protection against various forms of oxidative injuries through the detoxification of ROS. As shown in Table 3, H₂O₂ exposed H9c2 cell showed significantly increased lipid peroxidation levels (246.36%) but decreased GSH (60.09%), SOD (71.50%) and Catalase (59.78%) activities. However, pre-incubation with PBEA at the indicated concentration (10 μg/ml) for 24 h showed decreased lipid peroxidation (61.05%) whereas increased GSH content (61.65%), SOD (118.21%) and Catalase (70.69%) activities were observed respectively. In present study, PBEA treated H9c2 cells were significant reduced ROS generation. In cellular antioxidant assay we have shown that pre-incubation of cells with PBEA before H₂O₂ exposure indicate that the integrity of PBEA treated cells was confined against the oxidative affront.

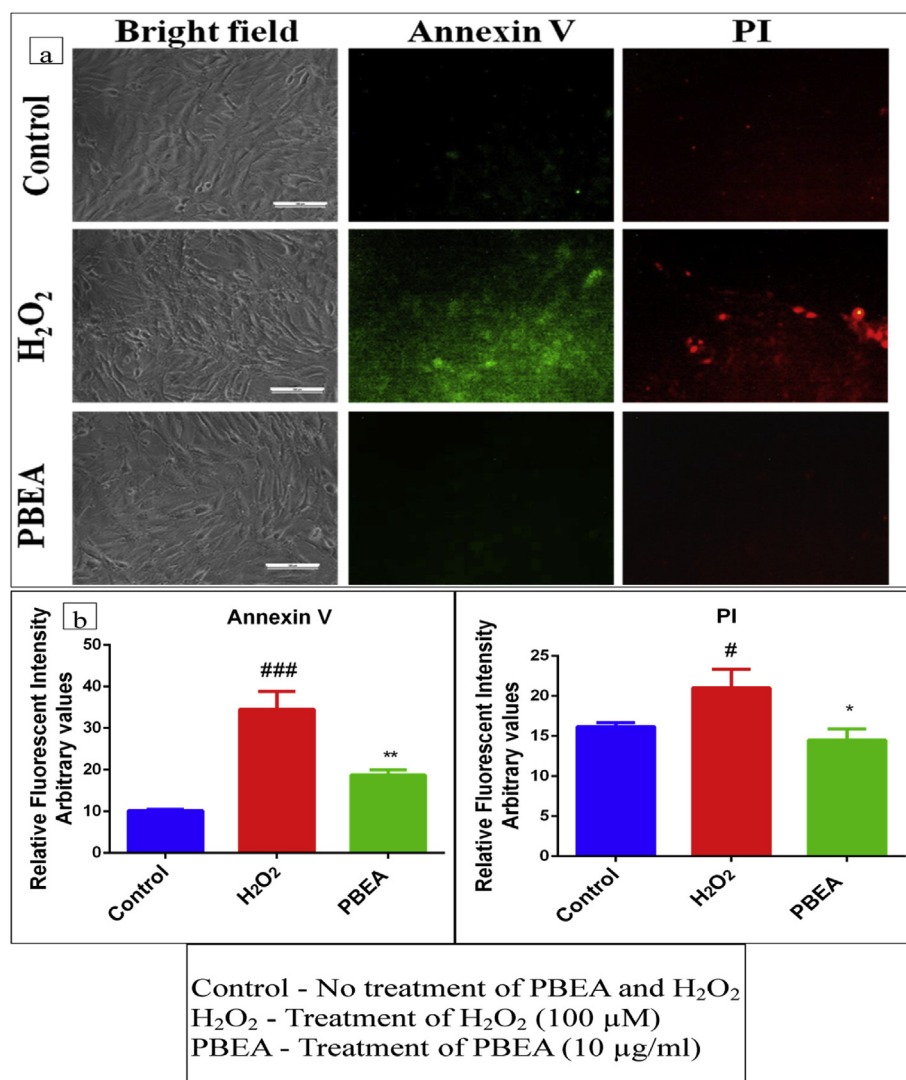


Figure 6. Effect of PBEA on H₂O₂ induced Apoptosis using Annexin V and PI fluorescence. H9c2 cells were pretreated with or without PBEA (10 μg/ml) for 24 h before exposure to 100 μM H₂O₂ for 1 h. (a) Annexin V and PI staining for Apoptosis (b) Histogram showing the Apoptosis level in H9c2 cells after treatment with H₂O₂ in presence or absence of PBEA compared to control groups. Scale bar = 100 μm. Data are presented as mean ± SEM (n = 3). ### Significantly different from control, P < 0.001. # Significantly different from control, P < 0.05. ** Significantly different from H₂O₂, P < 0.01. * Significantly different from H₂O₂, P < 0.05.

3.6. Protective effect of PBEA on H₂O₂ induced apoptosis in H9c2 cells

We continued our studies to finding apoptotic and necrotic cells both qualitatively and quantitatively by Annexin-V/PI dual staining. Moreover, Von Harsdorf reported that H₂O₂ induced cardiomyocyte apoptosis is mediated by ROS. H9c2 Control cell did not show Annexin-V staining with negligible apoptotic nuclei. On the other hand, cell when exposed to H₂O₂ for 1 h, demonstrated significant increase in the apoptotic nuclei (red) with Annexin-V binding to phosphatidylserine (green). Nevertheless, pre-incubating of H9c2 cells cultured with 10 μg/ml of PBEA for 24 h and later exposed to H₂O₂ significantly inhibited apoptosis (Figure 6a). It was observed that both Annexin-V and PI fluorescent intensities when quantified manifested significant reduction after PBEA pretreatment (Figure 6b). In this study, PBEA protects H9c2 cells against apoptosis induced by H₂O₂ through detoxification of free radical, through the ROS-dependent signaling pathway and by enhancing the intracellular antioxidant defense grid.

Taken together, these data strongly suggest that PBEA protects H9c2 cells against H₂O₂ induced cardiac cytotoxicity through decreasing ROS production, apoptosis and increasing antioxidant defense system.

The present study has revealed that ethyl acetate extract of *piper betle* showed prominent super oxide anion free radical scavenging activity and marked antioxidant defense against different reactive oxygen species, although with different efficiencies. Standardization of PBEA using

eugenol as biomarker gives the confirmation for antioxidant activity, thus neglecting any future bias. Moreover, this extract can shield apoptosis generated through oxidative insults as seen by significant cytoprotection in cardiac H9c2 cell line thus concluding significant cardioprotective property of PBEA. Further studies exploring molecular mechanism in appropriate animal models are necessary for adopting PBEA for cardio protection.

Declarations

Author contribution statement

Hardik Savsani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Abhay Srivastava: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Sarita Gupta: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kirti Patel: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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