EVALUATION OF ANTIOXIDANT AND CARDIOPROTECTIVE ACTIVITIES OF BRIDELIA RETUSA ON ISOPROTERENOL INDUCED MYOCARDIAL NECROSIS IN ALBINO RATS

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ABSTRACT
The present study was undertaken to evaluate the antioxidant and protective myocardial necrosis effects of extracts of Bridelia retusa in isoproterenol induced rats. Different concentration of chloroform, ethyl acetate and methanol extracts of Bridelia retusa (Euphorbiaceae) were investigated for in-vitro antioxidant activity by thiocyanate method. The methanol extract of Bridelia retusa exhibited maximum inhibitory activity of peroxidation, over the other organic extracts, which was subjected to further in-vivo antioxidant and myocardial necrosis studies. Methanol extract of Bridelia retusa was administered to rats at two different doses of 250 mg/kg and 500 mg/kg for 90 days to evaluate oxidative stress parameters like ferric reducing ability of plasma (FRAP) thio barbituric acid reactive substance (TBARS), reduced glutathione (GSH) and antioxidant enzyme levels of superoxide dismutase (SOD) & catalase (CAT). Myocardial necrosis and myocardial oxidative stress were evident in the isoproterenol (85 mg/kg, s.c, two doses at 24 h interval) administered rats for 30 days by significant rise in myocardial TBARS, myocardial GSH, plasma TBARS and plasma lactate dehydrigenase (LDH) along with reduction of myocardial SOD, myocardial CAT and myocardial glutathione peroxidase (GPX). Further myocardial necrosis was evident from the light microscopic changes. It observed that methanol extract of Bridelia retusa conferred antioxidant and myocardial protective effects by histopathological and biochemical observations in rats. It can be fulfilled
that methanol extract of *Bridelia retusa* had significant antioxidant activity, which capacity be helpful in preventing (or) slowing the diverse oxidative stress- related diseases. Further, the results suggest that chronic administration of methanol extract of *Bridelia retusa* prevents oxidative stress and myocardial necrosis in isoproterenol induced in rats.

**Keywords**: *Bridelia retusa*, myocardial necrosis, isoproterenol, antioxidant, oxidative stress.

**INTRODUCTION**

Oxidative stress owed to increase in free radical generation (or) impaired endogenous antioxidant mechanism is an important factor that has been implicated in various diseases like diabetes mellitus, ageing, atherosclerosis, cancer, Alzheimer’s disease and also in myocardial infarction [1]. Ischemic heart diseases, particularly acute myocardial infarction/ myocardial necrosis, remain the leading cause of death in both developed and developing countries as seen over the past quarter century [2]. Reduction of mortality rate and prevention of myocardial infarction and myocardial necrosis are utmost importance. Reactive oxygen species, which possess highly reactive and toxic properties can be generated as a result of ischemia, myocardial infarction, myocardial necrosis and exacerbate the degree of myocardial damage sustained by the ischemic myocardium [3, 4]. Isoproterenol [L-β-(3, 4-dihydroxy phenyl)-α-isopropyl amino ethanol hydrochloride], a β-adrenergic agonist has been reported to show many metabolic and morphologic aberrations in the heart tissue of the experimental animals similar those observed in human myocardial infarction [5]. It induces myocardial necrosis by a multiple step mechanism [6].

A growing body of evidence is emerging which suggests that reactive oxygen derived free radicals play a crucial role in the pathogenesis of isoproterenol- induced myocardial necrosis [7]. In pathological or diseased conditions, such as myocardial necrosis, diabetes, stroke and others, the production of free radicals may override the scavenging effects of antioxidants leading oxidative stress [8, 9]. There is evidence that antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is an increasing interest in the protective biochemical function of natural antioxidants contained in vegetables, fruits and medicinal herbs [10]. Several medicinal plants have been suggested to be related to their antioxidant activity [11]. Flavonoids and other polyphenolic compounds were present in the plant, which may be responsible for the antioxidant activity [12]. Ekdania (*Bridelia retusa* L) is a monoecious, deciduous medicinal plant belonging to family Euphorbiaceae. *Bridelia retusa* is one of the most important medicinal plants used by tribal in the treatment of urinary
problems. The root bark of the plant is valuable astringent. In the indigenous herbal system of medicine *Bridelia retusa* is used for diabetics [13].

Decoction of stem bark liquor is used for diarrhoea, ear ache and prevents pregnancy. Extract from the stem bark has, anti-cancer and hypotensive properties. Stem bark paste is applied to wounds, and bark juice taken internally in case of snake bite [14]. *Bridelia retusa* has some medicinal properties and is used in the treatment of diarrhoea, dysentery, haemorrhoids, haemorrhage, menorrhagia, leucorrhoea, arthritis, diabetes, wounds, ulcers and poison [15]. Bark is good for dealing of urinary concretions and a liniment with gingerly oil in rheumatism, lumbago, hemiplegia and anti-fertility activity [16]. The modern pharmacological studies of *Bridelia retusa* showed that it has a broad range of physiological activity and pharmacological effects, such as anti-viral, hypoglycemic properties [17], antibacterial, conjunctivitis [18], anti-inflammatory [19], anti-fungal, blood pressure, cholesterol [20]. The different phytoconstituents found in fruit pulp and seeds of *Bridelia retusa* are gallic acid, β-sitosterol, and allagic acid [14]. Apart from this bark consists of triterpenoid ketone, 16-40% tannins, 2.76 % condensed tannins, 12.20% tannic acid, 12.49% total phenols, 10.38% crude proteins, 69.31% neutral detergent fibre (NDF), 28.34% acid detergent fibres (ADF) and minerals like copper, iron, phosphorus, manganese, calcium, magnesium, zinc [21, 22]. It also contains bisalabone sesquiterpenes (isochaminic acid), elimicin, sesamin, and cumic acids [20].

Based on these reports the studies have been designed to examine the extract of *Bridelia retusa* exerts a invitro and in vivo antioxidant activities. In order to investigate the chronic different doses of methanol extract of *Bridelia retusa* was administered to animal and assess changes in lipid peroxidation level, ferric reducing ability of plasma level, reduced glutathione level and antioxidant enzyme activity, such as SOD & catalase. Further study was undertaken to evaluate the protective effect of chronic oral administration of methanol extract of *Bridelia retusa* on isoproterenol induced myocardial oxidative stress and necrosis in rat.

**MATERIAL AND METHODS**

**Collection of Plant Material**

The bark part of *Bridelia retusa* (Euphorbiaceae) was collected in the month of November, from forest area of Tirumala hills, Tirupathi and Chittoor district. It was shade dried away from sunlight and stored suitably. The plant material was taxonomically identified by Pharmacognosy expert.
Extraction
The bark of *Bridelia retusa* was powdered with a mechanical grinder and passed through Sieve no. 40 which was individually extracted with chloroform, ethyl acetate and methanol by continuous soxhlet extraction method. The solvents were removed by rotary vacuum evaporator, the remaining mass of extracts were concentrated and dried. The extracts were stored in desiccators for further phytochemical, myocardial necrosis and antioxidant studies.

Animals
Male albino rats Wistar strain (200-250 gm) were housed in polypropylene cages and maintained in a controlled environment (28-32°C) with 12:12 hr of light and dark cycle. Each day fed normal laboratory diet. The protocol was approved by Institutional Animal Ethical Committee constituted for the purpose.

Total antioxidant activity in vitro
The antioxidant activity was determined according to the thiocyanate method with some modifications [23]. Various concentrations (10, 20, 40, 80, 160 µg/ml) of three different extracts were individually added in 2.5 mL of 0.04 mol/L potassium phosphate buffer (pH 7.0) and 2.5 mL of linoleic acid emulsion. Linoleic acid emulsion (50 mL) was prepared with 350 mg of tween-20 and 310 µl of linoleic acid and 0.04 mol/L potassium phosphate buffer (pH 7.0). The mixed solution (5 mL) was incubated at 37ºc in dark phase. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer after reaction with FeCl₂ and thiocyanate every 12 hours. Further, the same solutions without extracts were used as control samples. The % inhibition of lipid peroxidation was calculated.

Acute toxicity study
Acute toxicity study [24] was carried out by using OECD guide lines No. 423. Three female rats of the same age group and weight were taken in a single dose up to the highest dose of 2000 mg/kg orally. The animals were observed for 1 h, 4 h and after every 24 h up to 15 days for any mortality or gross behavioral changes.

In vivo antioxidant activity without drug induced method
Wistar albino male rats (200-250 gm) were housed in polypropylene cages and maintained in a controlled environment (28-32°C) with 12:12 h of light and dark cycle. Each day fed normal laboratory diet. The rats were divided into three groups with six rats each after acclimatization of seven days. Group-1 animals were not given any materials and treated as
normal control. In group-2, normal animals were administered with methanol extract of *Bridelia retusa* at a dose of 250 mg/kg body weight. Group-3 animals received methanol extract of *Bridelia retusa* at a dose of 500 mg/kg body weight. The methanol extract of *Bridelia retusa* was given once in a day, orally for 90 days continuously to rats. All the animals were sacrificed by decapitation on 91st day of the experiment. The liver, kidneys and heart were removed, washed in cold saline and stored in liquid nitrogen for further biochemical studies.

**Ferric reducing ability of plasma (FRAP) assay**
Total plasma antioxidant capacity was measured according to the FRAP method [25]. The blood samples were collected from the rat retro-orbital venous plexus into heparinized glass tubes at 0, 45 and 90 days of treatment. Briefly, 3 mL of freshly prepared and warm 37°C FRAP reagent [1 mL (10 mM) of 2,4,6 tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, 1 mL 20 mM FeCl$_2$.6H$_2$O, 10 mL of 0.3 M acetate buffer pH 3.6)] was mixed with distilled water (0.375 mL) and test samples (0.025 mL). The absorbance of developed colour in organic layer was measured at 593 nm. The temperature was maintained at 37°C. The readings at 180 seconds were selected for the calculation of FRAP values. Ferrous sulphate (FeSO$_4$.7H$_2$O) was used as a standard for calibration and the data was expressed as nM Fe$^{2+}$/Liter.

**Preparation of rat heart, liver and kidney homogenate**
Tissue homogenate was prepared in a ratio of 1 gm of wet tissue to 10 times (w/v) 0.05 M ice cold phosphate buffer (PH 7.4) and homogenized by using a Teflon homogenizer. 0.2 ml of sample homogenate was used for estimation of TBARS (thiobarbituric acid reactive substance). The remaining part of the homogenate was divided into two parts. First part was mixed with 10% trichloro acetic acid (1:1), centrifuged at 5000g (4°C, for 10 min) and the supernatant was used for GSH estimation. The second part of the homogenate was centrifuged at 15,000 g at 4°C for 60 minutes and the supernatant was used for superoxide dismutase, catalase and protein estimation.

**Thiobarbituric acid reactive substance (TBARS) estimation**
Lipid peroxide was measured by the method of established by Liu [26]. Acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulphate (8.1%) were added to 0.1 ml of processed tissue samples and then heated at 100°C for 60 min. The
mixture was chilled and 5 ml of n-butanol-pyridine (15:1) mixture with 1 ml of distilled water was added and vortexed vigorously. The organic layer was separated after centrifugation at 4000 rpm for 10 min. Absorbance was measured at 532 nm using a spectrophotometer and concentration of TBARS was expressed as nmol/g tissue.

**Estimation of superoxide dismutase (SOD)**

SOD activity was analysed by the method described by Kakkar et al [27]. Assay mixture contained supernatant (0.1 ml), sodium pyrophosphate buffer (pH 8.3, 0.052 M, 1.2 ml), phenazinemethosulphate (186 µm, 0.1 ml), nitro blue tetrazolium (300 µM, 0.3 ml), NADH (Nicotinamide Adenine Dinucleotide reduced disodium salt, 750 µM, 0.2 ml). Reaction was started by addition of NADH. The reaction was stopped by the addition of 0.1 ml of glacial acetic acid after incubation at 30°C for 90 sec. Reaction mixture was stirred forcefully with 4.0 ml of n- butanol. Mixture was endorsed to stand for 10 min, centrifuged and the butanol layer was separated. The chromogen colour strength in the butanol was measured at 560 nm by spectrophotometrically and concentration of SOD was expressed as units/mg of protein.

**Estimation of catalase (CAT)**

Catalase activity was measured by the method of [28]. Supernatant (0.1 ml) was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by the adding of 1.0 ml of recently prepared 30 mM hydrogen peroxide. The rate of decomposition of H₂O₂ was measured spectrophotometrically from changes in absorbance at 240 nm. The catalase action was expressed as units/mg of protein.

**Estimation of reduced glutathione (GSH)**

Reduced glutathione was measured according to the method of Ellman [29]. The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. The supernatant (0.01 ml) was separated from this solution and mixed with 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5’5-dithio, bis (2-nitrobenzoic acid) and 0.4 ml double distilled water. Mixture was vortexed and the absorbance read at 412 nm within 15 min. The glutathione concentration was determined by µg/g tissue.

**Myocardial protective and antioxidant effects of Bridelia retusa**

Seven days after acclimatization, the rats were divided into four groups with six rats each. Group-1 animals were served as control and were fed normal saline daily for 30 days. In group-2, normal animals were administered isoproterenol (subcutaneous route) in saline at
the end of the 30 day. The methanol extract of Bridelia retusa was fed by oral gavage everyday at a fixed time for 30 days in two different doses of 250 mg/kg and 500 mg/kg for groups 3 and 4. Changes in body weight and intake patterns of water and food in all groups were noted throughout the experimental period at regular intervals. At the end of the 30-days treatment period, rats were administered two doses of 85 mg/kg B/W; s.c isoproterenol (dissolved in distilled water) 24 h apart and euthanised 24 h after the last injection for groups- 2, 3 and 4 [30].

**Preparation of plasma from blood**
After the experimental period the animals were sacrificed under mild chloroform aesthetic condition by cervical decapitation. The blood (2 ml) was aspirated from the left ventricle, collected in a heparinised vial, centrifuged at 3000× g for 30 min and the plasma stored at –20°C for estimation of plasma lactate dehydrogenase (LDH) and thio-barbituric acid reactive substance (TBARS). Hearts were removed and stored in liquid nitrogen for biochemical estimation.

**Preparation of heart homogenate**
Heart tissue were carefully exercised and homogenized in cold 1.15% Kcl- 10 mM phosphate buffer with ethylene diamine tetra acetic acid (EDTA, pH 7.4) and centrifuged at 12,000 g for 60 minutes. The supernatant was used for the assay of marker enzymes (glutathione peroxidase, catalase and superoxide dismutase), protein estimation and thiobarbituric acid reactive substances (TBARS) content.

**Myocardial TBARS**
Lipid peroxidation was measured by the method of [26]. It was calculated using a molar extinction coefficient of 1.56 ×10^5 M^−1 cm^−1 and expressed as nM/gram tissue mass.

**Myocardial catalase**
Catalase activity was measured by the method [28]. Activity of catalase was expressed as U/mg of protein.

**Myocardial GSH**
Reduced glutathione was measured according to the method of Ellman [29]. Data were expressed as µg/gram tissue mass.
Myocardial GPx

GPx activity was determined by the method described by Paglia and Valentine [31]. The reaction mixture consisted of 400 µl 0.25 M potassium phosphate buffer (pH 7.0), 200 µl supernatant, 100 µl GSH (10 mM), 100 µl NADPH (2.5 mM) and 100 µl glutathione reductase (6 U/mL). Reaction was started by adding 100 µl hydrogen peroxide (12 mM) and absorbance measured at 366 nm at 1-min intervals for 5 min. It was calculated using a molar extinction coefficient of 6.22 x 10^3 M^-1 cm^-1. Data were expressed as mU/mg of protein.

Plasma LDH

LDH was estimated by the method of [32]. Cuvettes (1 ml) in potassium phosphate buffer (pH 7.0) using 20 µl sample. Reduction in NADH was monitored at 340 nm against the appropriate controls every 15 s for 60 s. Data were expressed as mU/mL.

Plasma TBARS

Plasma TBARS were estimated by the same procedure used for measuring myocardial TBARS [26], using 0.2 ml plasma. Data were expressed as nM/mL.

Histopathology study

Myocardial tissue was fixed in 10% formalin, routinely processed and embedded in paraffin. Paraffin sections (3 µM) were cut on glass slides and stained with hematoxylin and eosin (H&E) and periodic acid schiff reagent and examined under a light microscope by a pathologist.

Statistical analysis

The data were expressed as mean ± S.E.M, which for biochemical and physiological parameters were analysed statistically using one way ANOVA followed by Dunnet-t-test using the SPSS statistical software for comparison with control group. P<0.05 was measured as significant.

RESULTS

Acute Toxicity Study

Rat when fed with aqueous ethanol extract of *B. retusa* up to 2000 mg/kg, p.o. exhibited no mortality or any sign of gross behavioral changes when observed initially for 72 h, and finally up to 15 days.
Total antioxidant activity in vitro

All the extracts were shown effective total antioxidant activity during the whole incubation time of 12 h. Methanol extract was exhibited strong antioxidant activity than the ethyl acetate, & chloroform extracts and comparable to that of standard α-tocopherol. The ethyl acetate extract effect showed similar antioxidant activities of α-tocopherol. Less activity was shown by chloroform extract. The order of antioxidant activity in different extracts was methanol > ethyl acetate > chloroform (Figure 1).

In vivo antioxidant activity without drug induced method

Ferric reducing ability of plasma (FRAP) assay

The FRAP level of rat after administration of methanol extract of Bridelia retusa at two different doses over a period of 90 days is shown in Figure 2. In control group, there was no significant change in FRAP value on days 45 (1032 nmol Fe2+/lit) and 90 (1034 nmol Fe2+/lit) when compared with zero (O) day (1012 nmol Fe2+/lit). There was a significant (p<0.05; p<0.01) increase in FRAP values in groups 2 and 3 on days 45 (1223 and 1415 nmol Fe2+/lit) and 90 (1417 and 1517 nmol Fe2+/lit) when compared with 0 day (1032 and 1034 nmol Fe2+/lit). Maximum enhancement was obtained in group 4 after 90 days of treatment.

Estimation of TBARS

The effect of two different doses of methanol extract of Bridelia retusa on the lipid per oxidation and endogenous antioxidants of liver, heart and kidney of rats is shown in Figure 3. A significant (p< 0.05; p<0.01) decrease in TBARS concentration in liver (318, and 246 nm/gm wet wt tissues) and kidney (264 and 260 nm/gm wet wt tissue tissues) for groups 2 and 3 was observed when compared with control group (427 nm/gm and 268 nm/gm wet wt tissue). However, there was slight (p<0.05; p<0.01) decreased change of TBARS level in heart tissue for treated groups 2 and 3 (294 and 288 nm/gm wet wt tissue) when compared with control group (300 nm/gm. wet wt. tissue).

Estimation of superoxide dismutase (SOD)

The administration of methanol extract of Bridelia retusa at two different doses caused slight significant (p<0.05) increase of SOD levels at 250 mg/kg body weight (421, 326 and 479 units/mg of protein) in liver, kidneys and heart when compared with control (390.8, 310 and 475 units/mg of protein). However, the level of SOD in the liver, kidneys and heart found to be highly significantly (p< 0.01) increased at the doses of 500 mg/kg (462, 322 and 481 units/mg of protein) when compared with control. The results were mentioned in Figure 4.
Estimation of catalase

The treatment of methanol extract of *Bridelia retusa* at two different doses to normal rats for 90 days induced a dose dependent increase in the level of catalase in the liver, kidneys and heart. The results are significantly ($p<0.01$) increased more at 500 mg/kg body weight (321, 438 and 58.2 units/mg of protein) for liver, kidneys and heart when compared with normal (285, 431 and 54.2). The results have been mentioned in Figure 5. However, the level of CAT in the liver, kidneys and heart of the extract treated rats at 250 mg/kg B/W (292, 434 and 58.2 units/mg of protein) was found to be less significantly increased ($p< 0.05$) when compared with control.

Estimation of reduced glutathione (GSH)

There was slight significant ($p<0.05$) changes and increased reduced glutathione level in liver (117 mg/gm. wet wt. of tissues), kidneys (127 mg/gm. wet wt. of tissues) and heart (382 µg/gm. wet wt. of tissues) for group 2, as compared with control group of liver, kidneys and heart (115 mg/gm, 125 µg/gm and 380 µg/gm. wet wt. of tissues, respectively). Simultaneously, the results are more significantly ($p< 0.01$) increased at 500 mg/kg body weight dose of the treatment of methanol extract for liver (119 & 121 units/mg of protein), kidneys (129 & 131 units/mg of protein) and heart (384 & 388 units/mg of protein), when compared with control group (Figure 6).

Myocardial Protective and antioxidant effects of *Bridelia retusa*

Myocardial level of TBARS and catalase

As shown in Figure 7, there was significant ($p< 0.001$) increase in myocardial TBARS in the group 2 (isoproterenol induced rat) when compared with group 1 (control). Significant ($p<0.01$) decrease in the level of myocardium TBARS was observed in groups 3 and 4 in comparison to the isoproterenol induced rat group 2. Administration of methanol extract of *Bridelia retusa* at 250 mg/kg (group 3) has shown less significant ($p< 0.05$) decrease in myocardial TBARS levels. In isoproterenol induced rat group (group 2), there was a significant ($p< 0.001$) decrease in myocardial catalase activity compared to vehicle control group (group-1). In group 3, there was less significant ($p<0.05$) increase in the level of myocardial catalase activity compared to isoproterenol induced rat (group 2). However, more significant ($p< 0.01$) increase in myocardial catalase activity was seen in groups 4 (Figure 7).
Myocardial SOD
Significant (p< 0.01) reduction of myocardial SOD activity was observed in group-2 (isoproterenol induced rat), when compared to control group (group-1). Myocardial SOD increased significantly (p< 0.01) in rats treated with 500 mg/kg methanol extract of Bridelia retusa (group 4). However, there was less significant (p< 0.05) increase in the level of myocardial SOD activity in methanol extract of Bridelia retusa at 250 mg/kg (group-3) in compared to isoproterenol induced rats, which has been illustrated in Figure 8.

Myocardial GSH and myocardial GPX
In group-2, significantly (p< 0.01) decreased myocardium GSH was observed in comparison to control rats (group 1). There was less significant increase (p< 0.05) and change in GSH levels in group-3 (methanol extract of Bridelia retusa at 250 mg/kg) following isoproterenol administrations, when compared to group-2 (isoproterenol induced). However, more significant (p< 0.01) increase in myocardial GSH activity was observed in groups 4 (methanol extract of Bridelia retusa at 500 mg/kg). Significant (p< 0.001) reduction in GPX activity was observed in isoproterenol-induced rats without treatment of plant extracts (group-2) when compared with control. Animals of group 3 exhibited slight significant (p< 0.05) increase in myocardial GPX activity when compared to control induced rat group (group-2), but there was greater significant (p<0.01) change and increase of GPX activity was observed in group 4 in comparison to isoproterenol induced rat group (Figure 9).

Plasma TBARS
The methanol extract of Bridelia retusa treated groups of 3 and 4 were significantly (p< 0.05 and p< 0.001) decreasing the level of plasma TBARS when compared with isoproterenol induced group (group-2). Isoproterenol induced (group-2) animals showed significantly (p< 0.001) increased plasma TBARS level than the control and plant extracts treated groups (Figure 8).

Plasma LDH
Plasma LDH was increased significantly (p< 0.01) in isoproterenol induced group in comparison to control (group-1). Significant (p< 0.01) reduction of LDH was observed only in group-4 (methanol extract of Bridelia retusa at 500 mg/kg) when compared with group 2 (Figure 10). However, less significant (p<0.05) decrease of Plasma LDH was observed with methanol extract of Bridelia retusa at 250 mg/kg (group 3).
Light microscopical changes

Figure 11(A), shows the H&E light micrograph of control heart showing normal architecture. There was confluent necrosis of cardiac muscle fibers with infiltration of acute and chronic inflammatory cells along with extravasation of red blood cells in isoproterenol induced group [Figure 11(B)]. In group- 3, there was focal necrosis of muscle fibers with acute and chronic inflammation [Figure 11(C)]. However, there was only occasional loss of myofibers and inflammation was observed in group 4 with minimal in comparison to other groups [Figure 11 (D)]. Myocardial necrosis was also unremarkable in those groups.

Figure. 1. % Inhibition of lipid peroxidation of standard, α-tocopherol and different doses of extracts [chloroform, ethyl acetate, methanol and hydro alcohol (7:3 ratios)] of Bridelia retusa in the linoleic acid emulsion.

Figure 2. Changes in rat total antioxidant capacity of plasma (FRAP) measured by Fe2+ equivalent after administration of methanol extract of Bridelia retusa. Values are mean ± s.d (n=6). Groups *P< 0.05; **P< 0.01 (one way analysis of variance) compared with control.
Figure 3. Changes of thiobarbituric acid reactive substances (TBARS; nmol/gm wet wt) levels in rat liver, kidneys and heart following oral administration of methanol extract of *Bridelia retusa*. Values are mean ± s.d. (n=6). Groups- 2 (methanol extract of *Bridelia retusa* 250 mg/kg), 3 (methanol extract of *Bridelia retusa* 500 mg/kg) compared with group 1 (control rats). **P< 0.01, *P< 0.05.

Figure 4. Changes of superoxide dismutase (SOD; 10-1 U/mg protein) levels in rat liver, kidneys and heart following oral administration of methanol extract of *Bridelia retusa*. Values are mean ± s.d. (n=6). Groups- 2 (methanol extract of *Bridelia retusa* 250 mg/kg), 3 (methanol extract of *Bridelia retusa* 500 mg/kg) compared with group 1 (control rats). **P< 0.01, *P< 0.05.
Figure 5. Changes of catalase (CAT; 10$^4$ U/mg protein) levels in rat liver, kidneys and heart following oral administration of methanol extract of Bridelia retusa. Values are mean ± s.d. (n=6). Groups- 2 (methanol extract of Bridelia retusa 250 mg/kg), 3 (methanol extract of Bridelia retusa 500 mg/kg) compared with group 1 (control rats). **P< 0.01, *P< 0.05.

Figure 6. Changes of reduced glutathione (GSH; µg/ gm. wet wt.) levels in rat liver, kidneys and heart following oral administration of methanol extract of Bridelia retusa. Values are mean ± s.d. (n=6). Groups- 2 (methanol extract of Bridelia retusa 250 mg/kg), 3 (methanol extract of Bridelia retusa 500 mg/kg) compared with group 1 (control rats). **P< 0.01, *P< 0.05.
Figure 7. Effect of methanol extract of *Bridelia retusa* on myocardial level of TBARS (nM/g of tissue) and myocardial level of CAT (U/mg of protein) during isoproterenol induced oxidative stress in rats. Values are mean ± s.d (n=6). Group-2 (isoproterenol induced) compared with Group-1 (control rats). Groups- 3 (methanol extract of *Bridelia retusa* 250 mg/kg), Group-4 (methanol extract of *Bridelia retusa* 500 mg/kg) compared with group-2 (isoproterenol induced rats). **P< 0.01, *P< 0.05.

Figure 8. Effects of methanol extract of *Bridelia retusa* on plasma TBARS (nM/mL) and myocardial level of SOD (U/mg of protein) during isoproterenol induced oxidative stress in rats. Values are mean ± s.d (n=6). Group-2 (isoproterenol induced) compared with group-1 (control rats). Groups- 3 (methanol extract of *Bridelia retusa* 250 mg/kg), 4 (methanol extract of *Bridelia retusa* 500 mg/kg) compared with group-2 (isoproterenol induced rats). **P< 0.01, *P< 0.05.
Figure 9. Effect of methanol extract of *Bridelia retusa* on myocardial level of GSH (µg/g of tissue) and myocardial level of GPX (mU/mg of protein) during isoproterenol induced oxidative stress in rats. Values are mean ± s.d (n=6). Group-2 (isoproterenol induced) compared with group-1 (control rats). Groups- 3 (methanol extract of *Bridelia retusa* 250 mg/kg), 4 (methanol extract of *Bridelia retusa* 500 mg/kg) compared with group-2 (isoproterenol induced rats). **P< 0.01, *P< 0.05.

Figure 10. Effect of methanol extract of *Bridelia retusa* on Plasma LDH (mU/mL) during isoproterenol induced oxidative stress in rats. Values are mean ± s.d (n=6). Group-2 (isoproterenol induced) compared with group-1 (control rats). Groups- 3 (methanol extract of *Bridelia retusa* 250 mg/kg), 4 (methanol extract of *Bridelia retusa* 500 mg/kg) compared with group-2 (isoproterenol induced rats). **P< 0.01, *P< 0.05.
Figure 11. Light microscopical changes (Histopathology).

(A) Light micrograph of control rat heart. Normal architecture of myocytes (H&E, 10X) (Normal); (B) Isoproterenol (ISO) group showing focal confluent necrosis of muscle fiber with acute and chronic inflammation and myophagocytosis along with extravasation of red blood cells (10X, H&E); (C) *Bridelia retusa* extract (250 mg/kg) + Isoproterenol (ISO) group, showing focal necrosis of muscle fiber with acute and chronic inflammation (10X H&E); (D) *Bridelia retusa* extract (500 mg/kg) + Isoproterenol (ISO) group, showing occasional loss of muscle fiber with focal acute and chronic inflammation (10X H&E).

DISCUSSION

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage [33]. A substance that protects cells from damage by inhibiting oxidation is known as antioxidant. Antioxidants have anti-aging effect because they are scavengers of free radicals (and other reactive oxygen species) which are linked with human diseases including cancer, cardiovascular disease [34]. This current study reveals that, invitro antioxidant activities of chloroform, ethyl acetate, methanol extracts of *Bridelia retusa* were determined by using the thiocyanate method. From this method, the amount of peroxides formed in emulsion during incubation is resolute spectrophotometrically by measuring absorbance at 500 nm. High absorption is an indication of high concentration of peroxides formed. Therefore, low absorbance indicates high antioxidant activity [35]. All extracts and standard
of α-tocopherol were showed to reduce the formation of peroxides at diverse concentrations. However, utmost inhibition of peroxidation was found with methanol extract over the other organic extracts. In the present study, FRAP test measured total antioxidant capacity determined by non-enzymatic antioxidants. Several methods have been developed to assess the total antioxidant capacity of serum or plasma because of the difficulty in measuring each antioxidant component separately in the serum or plasma [36]. One of these is the FRAP, which measures the reduction of ferric to ferrous iron in the presence of water soluble exogenous antioxidants [25]. The significant (p<0.05) increase in FRAP level after oral administration of methanol extract of *Bridelia retusa* indicates the presence of bio-available antioxidants in the plant. Plasma concentrations of thiobarbituric acid reactive substances (TBARS) are an index of lipid peroxidation and oxidative stress. The present study showed the depletion in the lipid peroxidation as observed by significant decrease in the TBARS level of the liver, heart and kidneys in the plant extract treated groups when compared with control group. Superoxide dismutase (SOD) is a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. SOD metabolizes the superoxide radical anion. It is an effective defense of the cell against endogenous and exogenous generation of superoxide [37]. The ROS scavenging activity of SOD is effective only when it is followed by the action of CAT and GPX, because of the dismutase activity of SOD generates hydrogen peroxide from the superoxide ion, which is more toxic than oxygen derived free radicals and required to be scavenged further by CAT and GPX [38]. Generally, results for the kidney have shown fewer changes in antioxidant activity compared to liver [39]. The administration of methanol extract of *Bridelia retusa* significantly increased the level of SOD& catalase in liver, kidneys and heart. This shows the antioxidant nature of the extract. GSH metabolism plays a vital role in many biological processes; such as the detoxification of xenobiotics. The methanol extract of *Bridelia retusa* has been found to increase GSH level in liver, kidneys and heart.

During myocardial infarction, reactive oxygen species like hydroxyl, hydrogen peroxide and superoxide radicals are produced in enormous amount which contributes to myocardial tissue injury [40]. Some factors proposed to explain the mechanisms of isoproterenol-induced damage to cardiac myocytes include hypoxia due to myocardial hyperactivity and depletion of energy reserves, coronary hypotension, calcium overload, and excessive production of oxygen-free radicals resulting from the oxidative metabolism of catecholamines [41].
Isoproterenol-induced cardio toxicity was explained by the formation of oxygen-free radicals and sulfhydryl reactivity through a variety of its oxidation products [42]. Myocardial antioxidants inhibit or delay the oxidative damage to lipids, DNA, carbohydrates, and sub cellular proteins. Although the exact mechanisms and interactions among various antioxidants are not fully unstated, it is probable that one antioxidant may equilibrate with another to establish a cellular redox potential and thus all endogenous antioxidants may act in concert to protect against oxidative insult. Antioxidants are usually classified as endogenous antioxidants and exogenous antioxidants. Many substances have been suggested to act as endogenous antioxidants. Lipid peroxide is an important pathogenic event in myocardial infarction and the accumulated lipid peroxides reflects the various stages of the disease and its complications [43]. It is reported that enhanced lipid peroxidation in serum/plasma and heart of isoproterenol treated rats when compared to control. Increased levels of lipid peroxides injure blood vessels, causing increasing adherence and aggregation of platelets to the injured sites [44]. Significant rise observed in the level of lipid peroxides in the heart tissue of isoproterenol-administered rats, and has suggested an enhanced oxidative stress in experimentally induced myocardial infarction condition [45, 46]. The rats fed with methanol extract of Bridelia retusa showed significant decrease in the level of lipid peroxidation in the heart tissue as compared to that of isoproterenol induced group (group-2), indicating that the presence of antioxidant property.

Activities of antiperoxidative enzymes (SOD & CAT) were also significantly decreased in the heart tissue of isoproterenol injected rats as compared to control (group-1). In myocardial necrosis, the production of superoxide radicals modulates catalase and superoxide dismutase, ensuing in the loss of activity and gathering of superoxide anion, thus damaging the myocardial cell [47]. The methanol extract of Bridelia retusa treatment increases & scavenges the level of SOD & CAT and reduces myocardial damage caused by free radicals.

Isoproterenol treated rats showed significant decrease in the level of heart GSH, when compared with control group. A similar observation was also reported previously [48], the mechanism of which is unclear. The reduced glutathione is involved in many important cellular functions, ranging from the control of physicochemical properties of cellular proteins and peptides to the detoxification of free radicals [49]. The administration of methanol extract of Bridelia retusa for 30 days resulted in significantly increases the GSH levels, which protects the myocardial membrane from oxidative damage. GPx offers protection to the
cellular and subcellular membranes from the peroxidative damage by eliminating hydrogen peroxide and lipid peroxide. Inhibition of this enzyme leads to the accumulation of these oxidants and makes myocardial cell membranes more susceptible to oxidative damage [50].

GPx was significantly lower in the heart tissue of group-2 rats (isoproterenol induced) as compared to normal controls, which was in accordance with the report [7], reflecting an increased oxidative stress in isoproterenol-induced myocardial necrosis. The methanol extract of *Bridelia retusa* pre-treatment increases the activities of GPX, but more significant change was observed in group 4, when compared to control induced rat group.

Significant elevation noticed in the levels of LDH, and TBARS in plasma of group 2 (isoproterenol-induced rats), which is in line with earlier reports [51, 52], is an indication of the severity of isoproterenol-induced necrotic damage to the myocardial membrane. It was reported that the amount of enzymes present in plasma is directly proportional to the number of necrotic cells present in the cardiac tissue [53]. In the present study, the prior administration of methanol extract of *Bridelia retusa* was found to be significantly prevents the elevation levels of plasma TBARS and plasma LDH for groups 3 and 4, respectively. Degree of myocardial necrosis and loss of muscle fibre were significantly less in methanol extract of *Bridelia retusa* treated groups form the light microscopic changes of histopathology study.

**CONCLUSION**

In conclusion, the current study showed that the methanol extract of *Bridelia retusa* possesses sturdy antioxidant properties as evidenced by the significant raise in the level of SOD and CAT in the liver and kidney. However, there was a decrease in the level of TBARS and an increase in GSH level in the liver and kidney, but there was refusal significant change of TBARS, SOD, CAT and GSH in the heart. The plant might be helpful in preventing (or slowing) the development of various oxidative stress-related diseases of main organs like liver, kidney and heart. Further, the results of the myocardial necrosis study conclude that the prior administration of methanol extract of *Bridelia retusa* for 30 days prevents the isoproterenol-induced myocardial necrosis and oxidative stress in rats. The overall cardio protective effect of plant extract is probably related to a counteraction of free radicals by its antioxidant property, or to its capability to preserve near to normal status the performance of free radical enzymes (SOD and CAT) and the level of GSH and LDH in plasma, which protect myocardial membrane against oxidative damage by decreasing lipid peroxidation in
heart tissue as well as plasma. Histopathological findings further confirmed the cardio protective effect of *Bridelia retusa*.

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