ejpmr, 2016,3(11), 644-648

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

CARDIOPROTECTION OF AN INDIAN MEDICINAL HERB BY ASSESSING MITOCHONDRIAL AND LYSOSOMAL MARKER ENZYMES

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Article Received on 17/09/2016 **Article Revised on 09/10/2016** **Article Accepted on 30/10/2016**

ABSTRACT

This study was carried out to examine the effect of *Millingtonia hortensis* against myocardial infarction(MI) induced by isoproterenol in rat model. Isoproterenol induced rats (20mg/100g bwt, subcutaneously) showed a significant decrease in the activities of heart mitochondrial enzymes (Malate dehydrogenase, Succinate dehydrogenase and α-keto glutarate dehydrogenase). The activities of lysosomal enzymes (β-NAG, Cathepsin-D and ACP) were found to be increased in ISO induced rats. Pretreatment with Silver nanoparticles (AgNPs) synthesized from *Millingtonia hortensis* significantly increased the activities of mitochondrial enzymes and decreased the activities of lysosomal enzymes in the heart tissue of ISO induced rats. Plant alone treated rats showed normal effect as that of control rats. This study showed that *Millingtonia hortensis* protects the heart against ISO induced MI by altering mitochondrial and lysosomal enzymes.

KEYWORDS: Myocardial infarction, isoproterenol, mitochondrial enzymes, lysosomal enzymes, cardioprotection.

INTRODUCTION

Mitochondria is traditionally known as the powerhouse organelle in eukaryotic cells, charged with the role of energy production. For the functional activity of mammalian cells production of ATP produced by oxidative phosphorylation in the inner mitochondrial membrane is the major source. Dysfunction of respiratory chain (RC) complexes and highly heterogeneous group of diseases with very bad prognosis is due to ATP synthase which predominantly affect muscles, brain and heart.

Myocardial infarction (MI) is the principle cause of death in both developed and developing countries accounting for roughly 20% of all worldwide death as per year due to myocardial damage. Despite major therapeutic advances it remains the major cause of death in India and increased mortality due to CVD is expected to double by 2020. Hence the prevention of MI is of utmost importance in the reduction of mortality rate. A newer dimension to the management of MI is the increasing awareness that could be the most direct way to reduce mortality and morbidity from MI.[1]

Isoproterenol is extensively used as a model for assessing cardioprotective drugs and studying myocardial consequences of ischemic disorders which include myocardial ischemia, necrosis, hypoxia and fibroblastic hyperplasia which is strongly similar to local myocardial infarction-like pathological changes seen in human myocardial infarction.[2]

The use of traditional medicine is common and plants have a large source of novel active biological compounds with different activities, including anti-viral, anti-cancer, anti-bacterial, anti-inflammatory, and cardioprotective activities.[3] Active principles of traditional plants have been claimed to be useful for the control of ischemia and pathological changes. Natural products are considered as the richest source for potential drugs, *Millingtonia hortensis* was used in the present study with an aim to ameliorate cardiovascular disease (CVD), particularly MI with less/no toxic effects.

MATERIALS AND METHODS

SYNTHESIS OF AgNPs

The leaves of *Millingtonia hortensis* were collected and authenticated by the botanist from BSI, TNAU campus, Coimbatore. The leaves were shade dried and powdered and used for the synthesis of AgNPs. For the synthesis of silver nanoparticles, 2mM Silver nitrate solution was used. 30ml of plant extract was added to 100ml of aqueous solution of 2mM silver nitrate and incubated. Dark brown colour indicates the formation of silver nanoparticles.

ANIMALS AND ETHICAL APPROVAL

Male Wistar rats (150-200g) were used in the study. The animals were fed with normal pellet procured from AVM cattle and poultry feed, Coimbatore and water *ad libitum*. The rats were acclimatized to laboratory conditions for 10 days before the commencement of the experiment. The clearance of the ethical committee for experimentation on animals were obtained before the start of the experiment.(331/2016/IAEC).

CHEMICALS

Isoproterenol used in the present study was obtained from Sigma-Aldrich. All other chemicals were of analytical grade.

EXPERIMENTAL DESIGN

30 rats were divided into five groups of six animals in each group.

GROUP I: Control rats received only standard pellet for 30 days.

GROUP II: Toxicity induced rats were administered with isoproterenol (20mg/100g b.wt) twice at an interval of 24hrs dissolved in normal saline.

GROUP III: The rats were pretreated with AgNPs of *Millingtonia hortensis*-(100mg/kg b.wt po) for 30 days and at the end of 28^{th} and 29^{th} day isoproterenol(20mg/100g b.wt) was injected subcutaneously twice at an interval of 24hrs.

GROUP IV: The rats were pretreated with standard drug verapamil(0.1g/100g b.wt po) for 30 days and at the end of 28th and 29th day isoproterenol(20mg/100g b.wt) was injected subcutaneously twice at an interval of 24hrs.

GROUP V: The rats were pretreated with AgNPs of Millingtonia hortensis (100mg/kg b.wt po) for 30 days.

After the experimental regimen of 30 days, the animals were sacrificed under mild chloroform anaesthesia. Blood was collected by cardiac puncture and serum was obtained by centrifugation at 2500rpm. The serum was used for biochemical estimations.

ISOLATION OF HEART MITOCHONDRIAL FRACTION

Heart mitochondria were isolated by the method of Takasawa et al.,(1984). The heart tissue was put into ice cold 50mM Tris HCl (pH 7.4) containing 0.25M sucrose and homogenised. The homogenate was centrifuged at 2000 rpm for 20 minutes and the supernatant obtained was centrifuged at 4000 rpm for 15 minutes. Then the pellet was washed with 10mM tris HCl (pH 7.8) containing 0.25M sucrose and finally resuspended in the same buffer for the assay of mitochondrial marker enzymes.

ASSAY OF MALATE DEHYDROGENASE (MDH)[4]

The reaction mixture contained the following reagents and enzyme in a total volume of 0.3 ml. 75 µM of phosphate buffer, 0.15µM of NADH and 0.76µMof oxaloacetate. The reaction was carried at 25 ºC and was started by the reagents by the addition of enzyme preparation .The control tubes contained all reagents except NADH. The change in OD at 340 nm was measured for 2 minutes at interval of 15 seconds.The activity of the enzyme was expressed as nanomoles of NADH oxidized/min/mg protein.

ASSAY OF SUCCINATE DEHYDROGENASE (SDH)[5]

Added 1.0ml of phosphate buffer, O.lml of EDTA, 1.0ml of KCN and made up to 2.9ml with water. Note the extinction at 455nm, then started the reaction by addition of enzyme and followed the change in extinction during the first two min. Initial rates were taken as a measure of activity. A blank rate (all reagents except succinate) must be determined separately.In this determination, 1 mole of succinate reduces 2moles of potassium ferricyanide. Concentration of potassium ferricyanide rates can be measured by following the reaction at 420nm (s =1.03x 10 cm). The enzyme activity is expressed as nanomoles of succinate produced/min/mg protein.

ASSAY OF ISOCITRATE DEHYDROGENASE (IDH)[5]

0.3 ml of Tris-HCl buffer was taken in a test tube and 0.2 ml of trisodium isocitrate, 0.3ml of manganese chloride and 0.2 ml of the mitochondrial suspension were added. A control tube was also prepared simultaneously. 0.2 ml of co-enzyme solution was added to the test and 0.2 ml of saline was added to control tubes. After mixing well, both the tubes were incubated for 60 mins. 1 ml of colour reagent (DNPH) was added to both the tubes followed by 0.5 ml of EDTA. The tubes were kept at room temperature for 20 minutes and 10 ml of 0.4 N NaOH was added to the tubes. A blank was run simultaneously. The colour was measured at 390 nm. A standard curve was prepared using a - ketogulutarate. The activity of isocitrate dehydrogenase is expressed as nanomoles of a - ketogulutarate formed/ hr / mg protein .

ISOLATION OF HEART LYSOSOMAL FRACTION

The lysosomal fraction of the heart tissue was isolated by the method of Wattiaux (1977). Fresh heart tissue was homogenized in ice cold 0.25 M sucrose solution. The homogenate was filtered and centrifuged at 3000 rpm for 10 minutes. The pellet was removed and recentrifuged. The supernatants were combined and centrifuged again at 15000 rpm for 20 minutes. The lysosomal pellet was suspended in 1.15% potassium chloride and used for the assay of lysosomal marker enzymes namely Cathepsin D, N- acetyl glucosaminase (NAG) and Acid phosphatase (ACP).

ASSAY OF ACID PHOSPHATASE (ACP)[6]

Pipetted 4.0ml of the buffered substrate (citrate buffer and disodium phenyl phosphate) into a test tube and incubated at 37°C for 5 minutes. Added 0.2ml of sample and incubated further for exactly 60 minutes. Removed and containing 4.0ml buffered substrate and 0.2ml of the sample 1.8ml phenol reagent was added immediately added 1.8ml of diluted phenol reagent. At the same time a control was set up. To 4.0ml of the supernatant and 4.0ml of working phenol solution, 2.0ml of sodium carbonate was added. Incubated all the tubes at 37°C for 15 mins. Read the colour developed at 700nm. The activity of ACP in tissue homogenate was expressed as n moles of phenol liberated/min/mg of protein.

ASSAY OF N-ACETYL-GLUCOSAMINASE ACTIVITY (NAG)[7]

To 0.2 ml of sample, 0.2 ml of buffered substrate (4-Nitrophenyl-N-acetyl glucoseamine in citrate buffer)was added and incubated at 37º C for 40 minutes. At the end of incubation period, the reaction was arrested by the addition of 2.2 ml of 0.2 M glycine buffer and the colour was read at 420 nm.The activity is expressed as nano moles of p- nitrophenol liberated / minute /mg-protein in tissue sample, in serum and urine the activity is expressed as n moles of p-nitrophenol liberated/ minute / L of sample.

ASSAY OF CATHEPSIN-D [8]

0.9 ml of buffered substrate(sodium acetate buffer and haemoglobin) was mixed with 0.1 ml of enzyme preparation and incubated for 2 hours at 37 °C. The reaction was stopped with 1.0 ml of TCA and the samples were centrifuged for 10 minutes. To the control tubes, the enzyme preparation was added after the addition of TCA. To 1.0 ml of supernatant, 1.0 ml of 5 % sodium hydroxide and 4.5 ml of alkaline copper reagent were added. After 10 minutes, 0.5 ml of Folin's phenol reagent was added and the colour developed was read at 640 nm after 15 minutes. The standards were treated similarly. Enzyme activity is expressed as µmoles of tyrosine liberated / hour / mg protein at37°C.

STATISTICAL ANALYSIS

The results were presented as mean± standard deviation. Statistical analysis was carried between the experimental groups using one way analysis of variance (ANOVA) employing statistical package for social science (SPSS Version 16.0). Post hoc testing was performed for inter-group comparisons using Duncan's multiple range test. The level of significance was set as ($p<0.05$).

RESULTS AND DISCUSSION

EFFECT OF SILVER NANOPARTICLES SYNTHESIZED FROM *Millingtonia hortensis* ON MITOCHONDRIAL MARKER ENZYMES

The activities of MDH, SDH and IDH in the heart mitochondria of ISO induced rats were significantly decreased when compared to control rats. Rats administered with AgNPs synthesized from *Millingtonia hortensis* and standard drug verapamil showed an increase in the activities of MDH, SDH and IDH compared to ISO alone treated rats (TABLE:1). AgNPs alone treated rats showed normal activity as that of control rats.

Malate dehydrogenase is found in the outer membrane of mitochondria and is vulnerable to free radical attack. The enzyme is involved in catalysing reversible oxidation of malate to oxaloacetate by reducing NAD^+ to NADH. Malate dehydrogenases, which have EC numbers catalyse the reaction which oxidizes malate. Pyruvate in the mitochondria acts on pyruvate carboxylase producing oxaloacetate, which is a citric acid cycle intermediate. To get the oxaloacetate out of the mitochondria, oxaloacetate is reduced to malate by malate dehydrogenase , and traverses the inner mitochondrial membrane. Malate is oxidized back to oxaloacetate by cytosolic malate dehydrogenase in the cytosol. Finally, oxaloacetate is converted to phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (PEPCK).

Isocitrate dehydrogenase is a predominant enzyme in the heart which catalyzes the oxidative decarboxylation of isocitrate, forming alpha-ketoglutarate (α-ketoglutarate) and CO_2. This is a two-step process, which involves oxidation of isocitrate to oxalosuccinate, followed by the decarboxylation to the ketone, forming alpha-ketoglutarate. In humans, IDH exists in three isoforms: IDH3 the third step of the citric acid cycle which catalyses NAD+ to NADH in mitochondria. IDH1 and IDH2 catalyses the same reaction outside mitochondria and use NADP+ as a cofactor instead of NAD+. They localize to the cytosol as well as the peroxisome and mitochondria[9]

Succinate dehydrogenase is a flavoprotein which is involved on the production of ATP in mitochondria, and it is sensitive to free radicals. It is located at the inner surface of the inner membrane of mitochondria. The enzyme is involved in the oxidation of succinate to fumarate by the coenzyme Q as an electron acceptor. Isolated SDH incorporated in liposomes can produce ROS, via its FAD, which can be reduced in the absence of electron acceptor.[10] In sub mitochondrial particles, ROS production can be inhibited by carboxin, an inhibitor of SDH.[11] However, the same inhibitor also suppressed antimycin-induced ROS production whereas the ROS production was supported by NADH oxidation. These two reactions are thought to be independent of SDH. Therefore, it is unclear whether the enzyme SDH as part of intact respiratory chain is capable of ROS generation.

Reduction in the activities of tricarboxylic acid cycle enzymes in ISO induced rats may be due to excessive production of free radicals upon ISO treatment.Prince (2013)[12] has also reported that the activity of TCA cycle

enzymes such as MDH, SDH and IDH were significantly decreased in ISO induction.

Table: 1: Effect of AgNPs, synthesized, from *Millingtonia* hortensis on Mitochondrial Marker Enzymes

GROUPS	Units		
	MDH	SDH	IDH
I	329.90 ± 1.99	237.40 ± 2.84	749.38 ± 3.69
II	152.61 ±2.26 [a]	145.98 ± 3.10 [a]	541.98 ± 3.82 [a]
III	200.85 ± 2.26 [a,b]	206.05 ± 1.73 [a,b]	660.23 ± 3.31 [a,b]
IV	279.85± 2.28 [a,b,c]	211.81 ± 2.66 [a,b]	689.05 ± 3.10 [a,b,c]
V	327.03 ± 1.81	235.56 ± 2.85	748.40± 3.23

Values are expressed by mean ± SD of six samples in each group
1U for MDH = nmoles of NADH oxidized/min/mg protein
1Ufor SDH = nmoles of succinateoxidized/min/mg protein
1Ufor IDH= nmoles of α-keto glutarate formed/hr/mg protein.

Group Comparison:
a - GI vs GII, GIII, GIV, GV
b - GII vs GIII, GIV
c - GIII vs GIV

EFFECT OF SILVER NANOPARTICLES SYNTHESIZED FROM *Millingtonia hortensis* ON LYSOSOMAL MARKER ENZYMES

The activities of the enzymes ACP β-NAG and Cathepsin-D were significantly increased in the ISO treated rats when compared to control rats (FIGURE 1-3). There was a significant decrease in the activities of enzymes in silver nanoparticle synthesized from *Millingtonia hortensis* and standard drug verapamil treated rats. There was no significant difference between control rats and silver nanoparticle alone treated rats.

Lysosomes are membrane bound structure which play an important role in the secretion and transport processes. Lysosomal enzymes ACP, β-NAG and Cathepsin-D are involved in the degradation of cellular constituents. Cathepsin –D is a lysosomal aspartic protease found in all animal cells. ACP is an important and critical enzyme in biological process responsible for biosynthesis of energetic macromolecule, metabolism and detoxification.

The heart lysosomal enzymes were significantly increased in MI. The increase may be due to membrane deterioration by ISO induced lipid peroxidation. Aministration of AgNPs synthesized from *Millingtonia hortensis* normalised the increased lysosomal activities by inhibiting lipid peroxidation thereby reducing lysosomal damage.

Our results are in consistent with the results of Sathish *et al*., (1973)[13] who have reported that the effect of Nicorandil and Amlodipine on lysosomal hydrolyses during MI in rats.

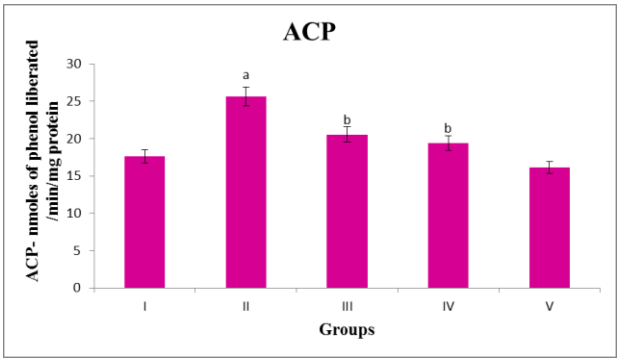

Figure: 1 Effect of AgNPs Synthesized from *Millingtonia hortensis* ON ACP

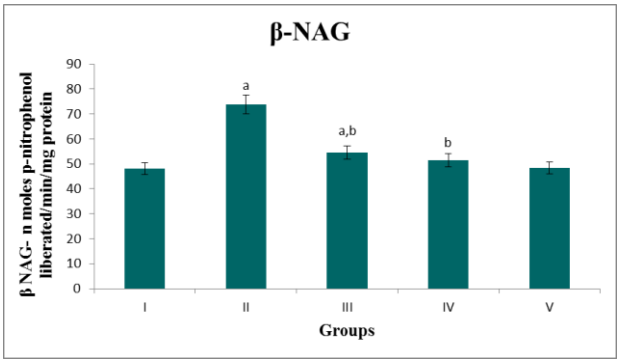

Figure: 2: Effect of AgNPs Synthesized from *Millingtonia hortensis* ON β-NAG

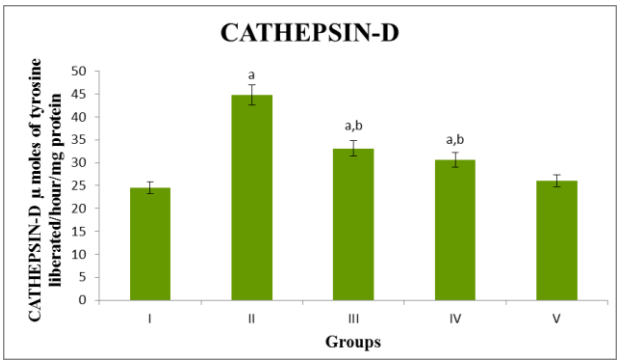

Figure: 3: Effect of AgNPs Synthesized from *Millingtonia hortensis* on Cathepsin-D

Values are expressed by mean ± SD of six samples in each group
Group Comparison:
a - GI vs GII, GIII, GIV, GV
b - GII vs GIII, GIV
c - GIII vs GIV

CONCLUSION

From the present study it can be concluded that administration of AgNPs synthesized from *Millingtonia hortensis* was able to reduce the damage to the lysosome caused by ISO and also preserves the membrane integrity in ISO induced myocardial damage in rats.

ACKNOWLEDGEMENT

I thank the management of PSG College of Arts & Science for providing the facilities to carry out this work.

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