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PHARMACOLOGICAL EFFICACY OF METHANOLIC EXTRACT OF THE PLANT *GINKGO BILOBA*, AGAINST ISOPROTERENOL INDUCED CARDIAC TOXICITY IN RATS

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ABSTRACT: *Ginkgo Biloba* is a potent antioxidant dietary source for human health. Oxidative stress through generation of free radicals damages the myocardium in different experimental condition. The present research was designed to evaluate the cardio protective role of chronic oral administration of *Ginkgo biloba* leaf extract against Isoproterenol induced myocardial injury. Male Wistar albino rates were randomly divided into five groups (n = 6) and treated as per treatment protocol with three different dose of *Ginkgo biloba* extract (125, 250, and 500 mg/kg b.w.) orally for thirty days. At the end of the treatment all the rats (except control rats) were administered with Isoproterenol (85 mg/kg) two consecutive days and subjected to biochemical and histopathological estimation. Isoproterenol (group II) induced the oxidative myocardial damage via alteration in the endogenous antioxidant enzymes and myocardial marker enzymes. Ginkgo biloba extract in all three dose (group III, IV and V) shows protective mechanism via decreasing thiobarbituric acid reactive substance (TBARS) and enhancing the endogenous antioxidant enzymes (reduced glutathione (GSH), superoxide dismutase (SOD) and catalase). Thus, the study shows that *Ginkgo biloba* extract exhibits significant antioxidant activity and protect the heart from free radical mediated toxicity of Isoproterenol.

INTRODUCTION: Myocardial infarction (MI) is the acute condition of necrosis of the myocardium that occurs as a result of imbalance between coronary blood supply and myocardial demand. [1] Oxidative stress resulting from increased production of free radicals is associated with decreased levels of antioxidants in the myocardium and plays a major role in cardiovascular diseases. [2] Damage to the myocardial cells arises due to the generation of toxic reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and hydroxyl radical.[3]

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Isoproterenol (ISO) is an adrenergic agonist and acute administration of ISO in experimental animals causes necrosis to heart muscle. [4] ISO damages the myocardial via calcium accumulation in cytosolic membrane, generation of reactive oxygen species and procogulant activity. [5] ISO causes the patchy pathological changes in the myocardial tissue, which is almost clinically relevant to myocardial infarction of ischemic heart disease. [6]

Phytopharmaceutical are gaining importance in allopathic as well as traditional medicine owing to their non-addictive and less toxic nature. Drugs to enhance the endogenous antioxidant enzymes to protect the heart from stress have been paid more attention. Natural antioxidants play a major role to reduce the oxidative stress by scavenging the excess free radicals. [7] Administration of antioxidants during ischemic reperfusion injury (IRI) ameliorates the severity of IRI through

augmentation of endogenous antioxidants, which might be a promising loom to treat heart disease. [8, 9]

Several natural products have been reported to have protective roles against ISO-induced MI in rats. *Ginkgo biloba* L. (Family-Ginkgoaceae) is the one of oldest existing seed plants, also known as "living fossils." It contains Flavonol and flavone glycosides, Ginkgolides, Diterpen lactones, ascorbic acid, Sesquiterpenes, Catechin and Iron-based superoxide dismutase. [10] *Ginkgo biloba* has been reported to have memory enhancer, antidepressant, antimicrobial, hepatoprotective, anticoagulant, anti-infammatory, cytotoxic, anti-stress, anti-ulcer, anti-tubercular and anti-aging properties. [11-22] *Ginkgo biloba* also known for its antioxidant activity and effective scavenger of oxidative radicals. [23] *Ginkgo biloba* are popular for their nutritional and medicinal values but have no studies conducted in direction of protective role of *Ginkgo biloba* extract against ISO induced Cardiotoxicity may be via antioxidant system. Hence, the present study was undertaken to find out the cardio protective potency of methanolic extract of *Ginkgo biloba* leaves.

MATEIALS AND METHODS:

Drugs and Chemicals: Leaves of *Ginkgo biloba* was collected from Darjeeling, West Bengal, India, and were authenticated by Dr. S. K. Mahajan M Sc, PhD, department of botany, Govt. P. G. Collage, Khargone, M.P., India. All chemicals were of analytical grade purchased from sigma chemicals, USA.

Extract preparation: Dried leaves of Ginkgo biloba were coarsely powdered and 1 kg of this powered plant material was extracted with the help of the soxhlet apparatus using methanol as a solvent. The solvent from the methanolic extract was removed under vacuum distillation; dried material was kept in a desiccators. A suspension of the leaves in 5% Tween 80(Vehicle) was made daily.

Experimental animals: Male Wistar albino rats of body weight 150-200 g were obtained from the Institute Animal House. The rats were acclimatized in the department animal house at an ambient temperature of 25°C, under a 12hour dark -12 hour light, cycle, for the whole period of the study. The animals were fed with a standard pellet diet and water *ad libitum.* The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experimental on Animals (CPCSEA), New Delhi, India and the research protocol was approved by the Institute animal ethical committee (1151/ac/07/CPCSEA).

Experimental Protocol: The rats were divided into 5 groups (6 in each group) and fed with the suspension of *Ginkgo biloba* leaves extract of three doses (125 mg/kg, 250 mg/kg and 500 mg/kg) by oral gavages once a day for 4 weeks (6 days/week). At the end of the treatment period rats from all groups except control group were administered Isoproterenol (ISO) 85 mg/kg i.p., for two consecutive days to induce myocardial injury. After 48 hours of the first dose of ISO the rats were sacrificed, hearts and blood samples were collected and immediately frozen in liquid nitrogen for biochemical estimation. [24]

Treatment protocol:

The groups studied were:

Group GI: Control, Vehicle + saline injected rats

Group GII: Vehicle + ISO treated rats (85 mg/kg)

Group GIII: 125 mg/kg of MEGB + ISO treated rats (85 mg/kg)

Group GIV: 250 mg/kg of MEGB + ISO treated rats (85 mg/kg)

Group GV: 500 mg/kg of MEGB + ISO treated rats (85 mg/kg)

Estimation of biochemical parameters

The following biochemical parameters were studied in the heart homogenate.

Myocardial thiobarbituric acid reactive substances (TBARS): TBARS levels in the myocardium were determined by the method described by Ohkawa et al (1997). [25] Hearts were homogenized with 10 ml of Trichloroacetic acid (TCA). 0.2 ml of whole homogenate was taken to which 0.2 ml of 8.1% Sodium lauryl sulfate, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA were added. Volume was made up to 4 ml with double distilled water. It was heated at 95^0C for 60 min.

After cooling, 1 ml of double distilled water and 5 ml of butanol: pyridine mixture was added and centrifuged at 4000 rpm for 10 min in a cold centrifuge. The organic layer was separated and absorbance was observed at 532 nm in a spectrophotometer.

Myocardial reduced glutathione (GSH): Myocardial GSH was estimated by the method of Ellman et al, (1959). [26] The reaction mixture contained 0.1mL of supernatant, 2.0ml of 0.3M phosphate buffer (pH- 8.4), 0.4 ml of double-distilled water and 0.5ml of 5, 5 dithiobis 2-nitrobenzoic acid (DTNB). The reaction mixture was incubated for 10 min and the absorbance was measured at 412 nm. Data are expressed as mole per gram wet weight.

Superoxide dismutase (SOD): SOD levels in the hearts were determined by McCord and Firdovich method (1969) and modified by Kakkar et al, (1984). [27] A sample (100µl) was added to sodium pyrophosphate buffer (pH-8.3), followed by addition of 0.1mL of 186 M phenazine methosulfate, 0.3mL of 300mM nitroblue tetrazolium and 0.2ml of 780M NADH. The reaction mixture was incubated for 90 second at 30^0C and the reaction was stopped by adding 1.0ml of acetic acid, 4.0ml of n-butanol was then added and centrifuged at 3000 g for 10min. The absorbance of the organic layer was measured at 560 nm. Data are expressed as units per mg protein.

Estimation of Catalase: Catalase level was estimated by the method described by Aebi et al. [28] Sample (50 µl) was added to a 3.0-ml cuvette that contained 1.95ml of 50mM phosphate buffer (pH 7.0). Then 1.0ml of 30mM hydrogen peroxide was added and changes in absorbance were followed for 30 s at 240 nm at an interval of 15 s. Catalase levels are expressed as units per mg protein.

Estimation of Protein: Protein estimation for the tissue samples were done by the method of Bradford (1976). [29] Sample (2µl) was made up to 20 µl with double-distilled water, 50µl of 0.1N NaOH and 1mL of Bradford reagent were added, vortexed and kept for 10min and the absorbance was measured at 595nm.

Histological examination: The rat hearts were removed, washed immediately with saline and then fixed in 10% buffered formalin. The hearts were embedded in paraffin section cut at 5 µ stained with hematoxylin and eosin. These sections were then examined under the light microscope for histological changes.

Statistical analysis: All values are expressed as mean ± SEM for 6 animals in each group. Data for various biochemical parameters were analyzed using one-way analysis of variance followed by Tukey's multiple comparison tests (graphPad Version 3.06, La Jolla, CA, USA). Significance is set at $p < 0.05$.

RESULTS: The results obtained in the different groups subjected to *in-vivo* ischemic reperfusion injury are presented below.

Myocardial TBARS: Myocardial TBARS in GII group (68.65±1.28) was significantly higher than that in control group (44.82±1.54). In GIV and GV treated groups there was a significantly lower TBARS ($53.76±2.46^b$ and $44.45±1.95^c$) respectively, whereas in the GIII group the TBARS shows no significant change ($59.25±1.46^a$) in comparison to GII group (**Table 1).**

Myocardial GSH: Myocardial GSH level was significantly lower in GII group (217.66±5.81) in comparison to that of the control group (353.85±3.69). There was a significant increase in the levels of GSH in the GV ($330.4±4.83^c$), whereas in there was slight increase in the levels of GSH levels GIII ($304.5±7.57^b$) and GIV ($312.58±6.31^b$); in comparison to the GII group (**Table 1**).

The blood GSH levels of the ISO treated animals showed (64.33±1.11) a significant decrease ($p < 0.01$) as compared to normal control (73.16±1.29) group. Here also MEGB at the dose of 500 mg/kg provided a highly significant ($p < 0.001$) increase (71.8±1.14) in blood GSH as compared to Group II, III, IV (**Table 2**).

Myocardial SOD: Myocardial SOD activity was significantly lower in GII group (1.31±0.16) than that in control group (2.91±0.14). Myocardial SOD levels showed no significant change in the GIII groups ($1.47±0.18^{ns}$) in comparison to GII group.

However, the myocardial SOD level was significantly higher in the GIV AND GV group (2.78 ± 0.18^{b} and 3.86 ± 0.09^{c} respectively) in comparison to GII group (**Table 1**).

The blood SOD levels of the ISO treated animals showed a significant decrease (105.74±2.07) as compared to normal control group (139.86±0.6). Here the dose of MEGB 500mg/kg (120.12±1.80) and 1000 mg/kg provided a highly significant ($p < 0.001$) increase (136.3±1.28) in blood SOD as compared to Group II (**Table 2**).

Myocardial catalase: Myocardial catalase was significantly lower in the GII group (27.9±1.48) in comparison to that of the control group (49.65±0.81). There was slight increase in myocardial catalase levels in the GIII group (35.0 ± 0.92^{a}) and the GIV group (40.15 ± 0.63^{b}) groups, whereas in GV group myocardial catalase was significantly higher (48.25 ± 1.20^{c}) in comparison to the control group (**Table 1**).

The blood CAT level of the ISO treated animals showed a significant decrease (2.28±0.19) as compared to normal control group (12.13±0.37). MEGB at doses 500 mg/kg provided a highly significant increase (4.05±0.11 and 7.16±0.10 respectively) in blood CAT level as compared to Group II (**Table 2**).

TABLE 1: EFFECT OF MEGB ON TBARS, GSH, SOD AND CAT IN RAT HEART

Parameters	GI	GII	GIII	GIV	GV
TBARS	44.82±1.54	68.65±1.28	59.25 ± 1.46^{a}	53.76 ± 2.46^{b}	44.45 ± 1.95^{c}
GSH	353.85±3.69	217.66±5.81	304.5 ± 7.57^{b}	312.58 ± 6.31^{b}	330.4 ± 4.83^{c}
SOD	2.91±0.14	1.31±0.16	1.47 ± 0.18^{ns}	2.78 ± 0.18^{b}	3.86 ± 0.09^{c}
CAT	49.65±0.81	27.9±1.48	35.0 ± 0.92^{a}	40.15 ± 0.63^{b}	48.25 ± 1.20^{c}

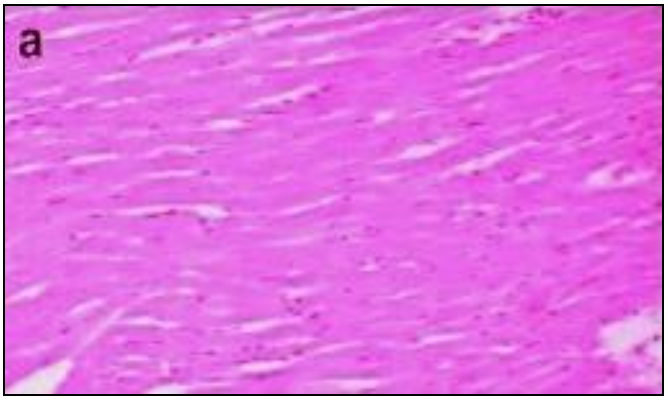
Values are mean as ± SEM (N=6). Values in the same row with different alphabet superscripts are significantly different at a < 0.05, b < 0.01, c < 0.001. Values are obtained by one way ANOVA followed by Dunnett multiple comparison test.

TABLE 2: EFFECT OF MEGB ON TBARS, GSH, SOD AND CAT LEVEL IN SERUM

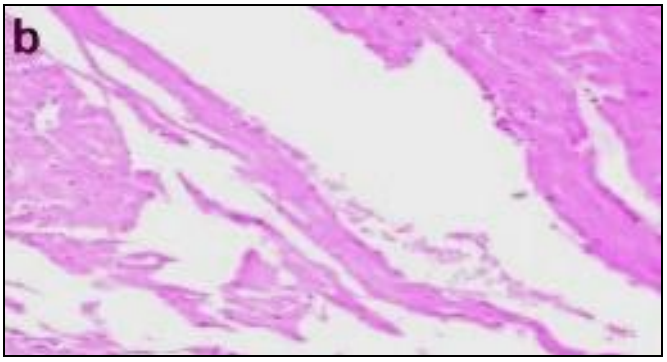
Parameters	GI	GII	GIII	GIV	GV
GSH	73.16±1.29	64.33±1.11	65.85 ± 1.02^{ns}	66.75 ± 1.01^{b}	71.8 ± 1.14^{c}
SOD	139.86±0.6	105.74±2.07	108.44 ± 2.80^{ns}	120.12 ± 1.80^{c}	136.3 ± 1.28^{c}
CAT	12.13±0.37	2.28±0.19	3.06 ± 0.15^{b}	4.05 ± 0.11^{c}	7.16 ± 0.10^{c}

Values are as mean ± SEM (N=6). Values in the same row with different alphabet superscripts are significantly different at b < 0.01, c < 0.001. Values are obtained by one way ANOVA followed by Dunnett multiple comparison test.

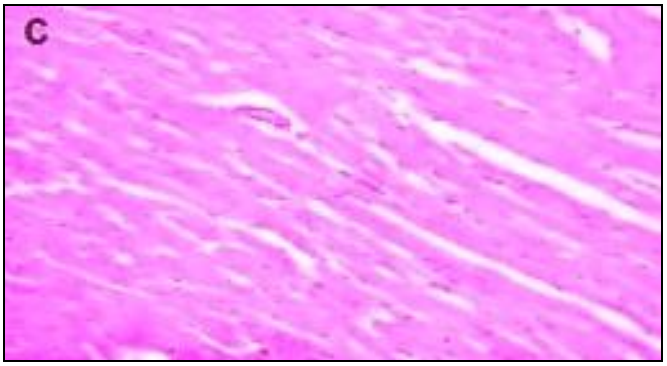
Histopathological finding: Histopathological finding showed the effect of plant *Ginkgo biloba* on myocardial tissues of the ISO induced rats. Histopathological finding of the ISO induced myocardium showed infracted zone with oedema and inflammatory cells and separation of cardiac muscle fibers. Oral pretreatment with *Ginkgo biloba* 125 mg/kg showed myocardium with decreased area of infarction with coagulative necrosis and inflammatory cells with moderate oedema. Oral pretreatment with *Ginkgo biloba* 250 mg/kg showed myocardium with moderate oedema and inflammatory cells with decreased area of coagulative necrosis of myocardial fibers and finally treatment *Ginkgo biloba* 500 mg/kg showed mild myocardium with mild oedema but no infarction and inflammatory cells and the cardiac fibers were within the normal limits. For all the parameters oral pretreatment of *Ginkgo biloba* (125, 250 and 500 mg/kg) to ISO induced rats showed a significance improvement in the myocardial infarction and indicates the prophylactic cardio protective effect of *Ginkgo biloba.*



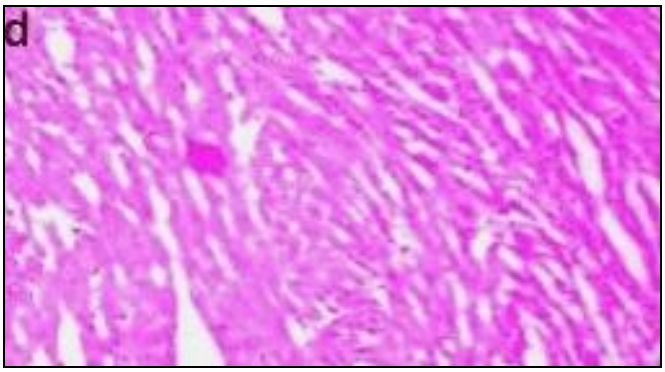
a) Group GI: saline treated rat's shows normal architecture of myocardium



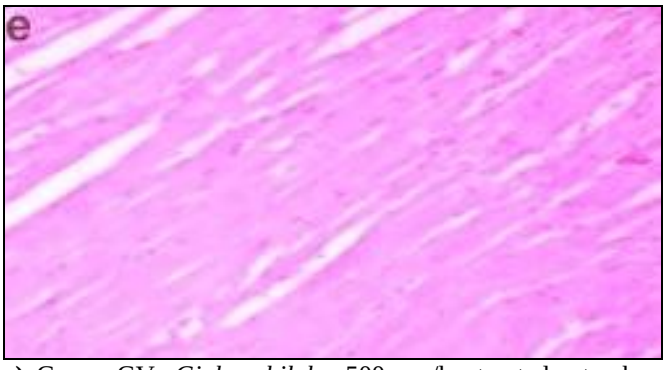
b) Group GII: ISO treated rats' shows infracted zone with oedema and inflammatory cells



c) Group GIII: *Ginkgo biloba* 125 mg/kg treated rats shows well preserved myocardium.



d) Group GIV: *Ginkgo biloba* 250 mg/kg treated rats shows moderate oedema and inflammatory cells with decreased area of coagulative necrosis of myocardial fibers.



e) Group GV: *Ginkgo biloba* 500 mg/kg treated rats show mild oedema but no infarction and inflammatory cells.

FIG. 1: EFFECT OF TREATMENT ON HISTOPATHOLOGY OF HEART (10X)

DISCUSSION: The effect of ISO on heart is mediated through beta receptors. Both adrenoceptors mediate the positive inotropic and chronotropic effects to beta adrenoceptor agonist. It has been reported to cause severe stress in the myocardium resulting in infarct like necrosis of the heart muscle. [5] ISO-induced myocardial infarction serves as a well-standardized model to study the beneficial effects of many drugs and cardiac function. [30] It is also well known to generate free radicals and to stimulate lipid peroxidation, which may be a causative factor for irreversible damage to the myocardial membrane. [31] Extract from the leaves of *Ginkgo biloba* extract, EGb 761 (commercial name), contains 22-27% flavonoids (ginkgo-flavone glycosides) and 5-7% terpenoids (ginkgolides and bilobalides), which are the most important active substances in the extract. The most important flavonoids are glycosides of kaempferol, quercetin, and isorhamnetin with glucose or rhamnose. *Ginkgo biloba* extract is well known for its antioxidant property, which may result from its ability to scavenge free radicals, and to neutralize ferryl ion-induced peroxidation. [32] Several studies have reported that the antioxidant activity of *Ginkgo biloba* extract could be helpful in the prevention and therapy of diseases and degenerative processes associated with oxidative stress. [32-36]

However, there have been very limited studies on the cardio protective activity of *Ginkgo biloba* extract. It is reasonably hypothesized that *Ginkgo biloba* extract may be helpful for the therapy of heart failure. Therefore, the purpose of this study was to investigate the cardioprotective effect of *Ginkgo biloba* methanolic extract in rats. Extensive literature survey has shown that there are no scientific reports available on the effect of MEGB in cardiotoxicity induced by isoproterinol. In our laboratory, we observed the preventive effect of MEGB on cardiac marker enzymes, TBARS, GSH, SOD and CAT in ISO-induced myocardial infarction in rats.

In the present study chronic i.p. administration of *Ginkgo biloba* leaves powder caused significant rise in myocardial endogenous antioxidants (SOD, GSH and Catalase) in the 250 and 500 mg/kg treated groups but not with other baseline treated groups.

The increase in TBARS is indicative of an enhanced oxidative stress, which in the absence of any evidence of cellular injury (as evidenced by histological studies), may be considered as non-lethal. It is, therefore possible that the increase in oxidative stress was nonlethal and might be responsible for cellular adaptive mechanisms. The principal finding of the present study is that cardiotoxicity was associated with oxidative stress, as evidenced by increase in myocardial TBARS and depletion of myocardial endogenous antioxidant status (SOD, GSH and Catalase).

Similar observations were made earlier by other studies. [37-39] chronic oral administration of Gingko biloba extract prevents the oxidative stress and the structural changes associated with oxidative stress. The mechanism of such protection of chronic oral administration of *Gingko biloba* extract may be due to myocardial adaptation, oxidative stress is mediated through augmentation of cellular antioxidants such as GSH, SOD, CAT (Das et al, 1995). Protection against oxidative stress through this mechanism may be one of the effective therapeutic approaches.

Histological examination of heart tissue of group 2 rats showed myocardial necrosis and separation of myocardial fibers with inflammatory mononuclear infiltrate whereas the examination of heart tissue of *Gingko biloba* pretreated group (500 mg/kg) showed maximum protective effect by reduced histological changes as compared to ISO myocardial infarcted rats. The protection might have been mediated through extract of *Gingko biloba* induced increase in basal myocardium antioxidant enzyme activities.

CONCLUSION: In this respect, the present study showed for the first time that the leaves of *Ginkgo biloba* are particularly useful agents, as they could enhance myocardial and blood endogenous antioxidants level without producing any cytotoxic effects. Therefore, the protection against myocardial injury in the treated rats is attributed to enhanced endogenous antioxidant activity. So I concluded that this study of Ginkgo *biloba* can help for further research area in cardiovascular and another disease which caused due to oxidative stress.

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