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In Vivo Cardioprotective Effect of With a coagulin Isolated from *Withania coagulans* Fruits on Isoprenaline- Induced Myocardial Injury in Experimental Rats.

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Abstract

Myocardial infarction (MI) (ie, heart attack) is the irreversible death (necrosis) of heart muscles. The possible cardioprotective effect of withacoagulin isolated from the fruits of *Withania coagulans* against isoprenaline-induced myocardial injury has been investigated in rat model in this study.

Albino rats (120 ± 10 g) were divided into five groups (n=6) as follows: Group (1) control, Group (II) Toxic control (ISO 85 mg/kg, s.c.), Group (III) *per se* (Withacoagulin 25 mg/kg, p.o.), Group (IV) Treatment 1 (WC+ ISO) and Group (V) Treatment 2 (Vit E+ ISO). To assess the efficacy of WC treatment against ISO-induced cardiotoxicity, lipid peroxidation, biochemical parameters and histopathological examinations were conducted.

Administration of ISO induced severe myocardial injury and altered lipid peroxidation. However, WC pretreatment secured cardiovascular derangements, and could be correlated by amelioration in lipid profiles such as triglyceride,total cholesterol, lactate dehydrogenase, LDL-cholesterol and HDL-cholesterol. In addition to these, cardiac indexes, atherogenic indexes, and coronary artery indexes were significantly improved in Group (IV) when compared to ISO intoxicated animals. Moreover, WC administration to ISO-treated rats obviously mitigated the malondialdehyde level when compared to Group (II). The histopathological observations also demonstrated that WC pretreatment significantly restored the damage induced by ISO.

These results suggests that WC improved myocardial injury and could be recommended as a potential candidate for the development of novel cardioprotective agents.

Keywords Withacoagulin; *Withania coagulans;* Isoprenaline; Cardioprotective; Vit E; Myocardial injury.

Introduction

Withacoagulin is an isolated natural product obtained from *Withania coagulans* fruits [1]. Antihypertensive effect of withacoagulin has been established in earlier studies [2]. However, cardioprotective effect of withacoagulin have never been established. In this study, protective effect of withacoagulin against isoprenaline- induced oxidative stress and myocardial injury in rats have been undertaken.

Myocardial infarction is highly lethal cardiovascular disorder and has been a topic for intense investigation [3]. Acute myocardial infarction (AMI) is characterized by a focus of necrosis resulting from low tissue perfusion showing coagulated necrosis (White infarct formation), a characteristic of myocardial infarction [4]. The pathological changes in MI are characterized by vacuolization and mycocytolysis followed by necrosis (irreversible cell injury) and edema (Na^+- H_2O entry). Within 1-3 days macrophages causes phagocytosis and yellow tan is observed. Troponin protein starts leaking from cardiac muscles into the blood which can be determined biochemically [5]. The animal model of myocardial infarction (MI) plays an important role in understanding the prevention, diagnosis, and therapy of human myocardial infarction (Heart attack) [6]. Current modern therapy has more side effects, lower survival rate and are costlier. In the present scenario of therapy it has been realized that phytoconstituents can influence the course of cardiovascular diseases and its treatment by providing an integrated structure of nutritional and therapeutic substances which aid in restoring and maintaining homeostasis of the physiological system [7]. Hence evidence based therapies due to phytoconstituents may provide better result in survival rate and undoubtedly are cheaper to treat cardiovascular disorders like myocardial ischemia.

Isoprenaline (ISO), a synthetic catecholamine is a β-adrenergic agonist and develops an infarct-like necrosis in the heart muscle caused by severe oxidative stress in the myocardium. Due to generation of free radicals and stimulation of lipid peroxidation isoprenaline may cause irreversible damage to the myocardial membrane [8]. The isoprenaline treated albino rats have been used to affect the adenylate cyclase cAMP signaling pathway in the myocardium [9]. The albino rats developed myocardial necrosis and a progressive enlargement of the ventricular cavity out of proportion due to isoprenaline, as in humans with discrete myocardial infarction [10]. Isoprenaline develops cardiomyopathy and a functional desensitization of β-adrenoceptor function of heart in albino rats [11].

Withania coagulans Dunal (family *Solanaceae*) has gained popularity in indigenous system of medicine. It is distributed in the east of the Mediterranean region extending to South Asia including northern and western India [12]. The drug has been reported to possess anti-inflammatory [13], cardiotonic activities [14], hepatoprotective [15], antifungal [16], free radical scavenging activity [17], hypoglycemic [18], hypolipidemic [19], wound healing activity [20] and useful in diabetic nephropathy [17]. The aqueous extract of *W. coagulans* fruits is diuretic which may be associated with the presence of the active principles of polar nature where withanolides are the main chemical protagonist of this activity [21]. *W. coagulans* has a wide range of active phytoconstituents mainly withanolides (steroidal lactones), withaferin A, and coagulins [22,23,24]. Withacoagulins are steroidal lactones isolated from *Withania coagulans* fruits [1].

By virtue of its antioxidant activity the present study was aimed to evaluate the cardioprotective effect of one of the isolated phytoconstituents of *Withania coagulans* fruit.

Materials and Methods

Chemicals and preparation of withacoagulin oral formulation: Isoprenaline (Sigma Chemical Company) was a generous gift from Department of Pharmacology, AIIMS, New Delhi. All other chemicals and assay kits were purchased from Sigma Chemical Company. Withacoagulin was isolated from the fresh, shade dried fruits of *Withania coagulans.*

Isolation scheme of withacoagulin from Withania coagulans berries [24]

W. coagulans fruits were purchased from Khari Bowli spice market in Old Delhi. The fruits were identified and authenticated by National Herbarium of cultivated plants (NHCP), New Delhi and the specimen voucher (NHCP/NBPGR/2014-09) has been retained for future reference.

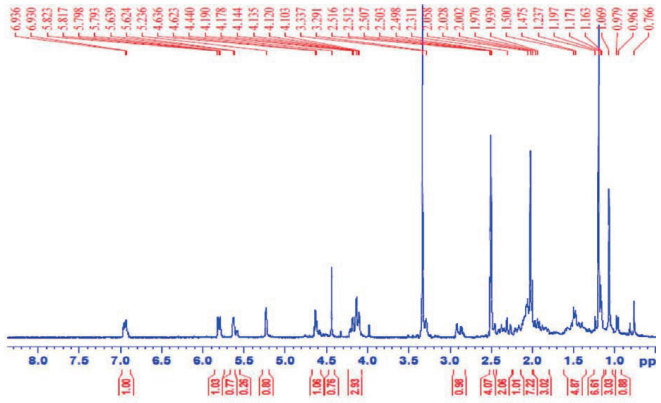

Fig. 1: 1H NMR spectra (400 MHz, in DMSO-d6) of Withacoagulin

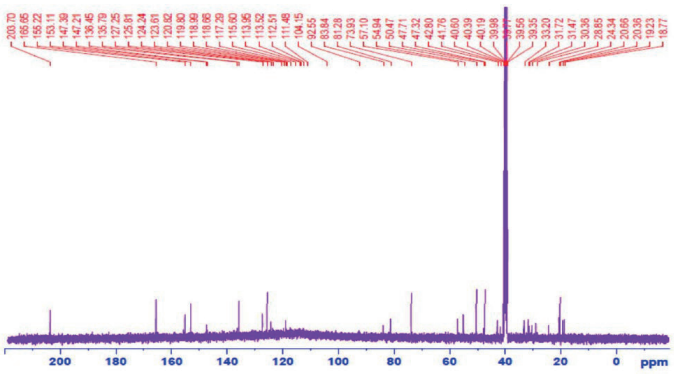

Fig. 2: 13C NMR spectra (400 MHz, in DMSO-d6) of Withacoagulin

Shade dried and crushed berries of *Withania coagulans* were macerated in a mixture of chloroform and ethanol (1:1) for three days by occasional shaking followed by filtration. The filtrate was dried in rotary evaporator. A dark brown colored semisolid mass so obtained was fractionated by solvent-solvent extraction. Crude extract so obtained was suspended in hot water and extracted thrice using a separating funnel with n-hexane. Aqueous layer was separated and extracted with ethyl acetate. The organic portion was separated and dried in rotary evaporator at 35 °C to obtain ethyl acetate fraction (WCE). WCE was subjected to normal phase column chromatography (silica gel 60, 230-400 mesh) using n-Hex: EA/5:1-0:1 as mobile phase. Multiple fractions (100ml each) so obtained were combined followed by column chromatography (sephadex LH20) using ethanol as mobile phase. Few fractions were combined and submitted to normal phase column chromatography (silica gel 60, 5-40 µm) using n-Hex: EA/5:1-0:1 as mobile phase. Finally, few more fractions were collected and subjected to RP-MPLC (Bondesil-C18, 40µm) by using the gradient mobile phase EtOH:H_2O/30:70. The collected fractions were dried and confirmed by NMR spectroscopy as withacoagulin (Fig.1 and Fig.2).

Isoprenaline-induced cardiotoxicity in vivo

Experimental Animals

Albino wistar rats (120 ± 10 g, 8 weeks old) were procured from National Institute of Biologicals, Sec-62, Noida, India as per the protocol (RIVTE/ IAEC/16/02) approved by IAEC in Ram-Eesh Institute of vocational and technical education, Greater Noida, India.

After quarantine period of two days healthy rats were kept in polypropylene rat cages group wise under standard laboratory conditions. The animals were allowed free access on standard pellet diet (Hindustan Lever Limited, Chandigarh) and drinking water *ad libitum*. Experimental procedures were performed as per CPCSEA guidelines for laboratory animal use.

Formulation and administration of withacoagulin

Withacoagulin was suspended in 0.5% carboxy methylcellulose (CMC). Each animal from two different groups (Gp III and Gp IV) received 1.0 ml of withacoagulin suspension (25 mg/kg; p.o.) daily.

Experimental Setup

After treatment of 28 days to the animals of withacoagulin and vitamin E group, the albino rats in the toxic control and withacoagulin/ vitamin E treatment groups were given isoprenaline (85 mg/kg, s.c.), on day 29th and day 30th , at an interval of 24 hours.

Treatment protocol

30 rats were divided into five groups (n=6) with treatment schedule as follows: Group I (Normal Control) rats were given 1.0 ml of 0.5% carboxy methyl cellulose (CMC) p.o. every day. Group II rats were given isoprenaline (85 mg/kg, s.c.) on day 29th and 30th at an interval of 24 hours. The rats of Group III were given withacoagulin (50 mg/kg; p.o.) for a period of 30 days. Group IV and Group V rats were given withacoagulin and Vitamin E respectively for 30 days. Towards the end of the experimental period on 29th and 30th days the rats were given isoprenaline (85 mg/kg body weight) injections twice at an interval of 24 hours through subcutaneous route. Twenty four hours after the last treatment animals were sacrificed. Blood samples for biochemical estimation were collected from the left ventricle of heart. The hearts were removed, weighed and dissected into two halves, one for histopathological analysis and the other for biochemical assays. The dissected and sliced heart tissues were stored in liquid nitrogen until further analysis.

Body weight and heart weight

The body weights of rats were measured at the beginning of the treatment and on the day of sacrifice. The difference (%) of body weight was calculated. The heart of animals were removed and

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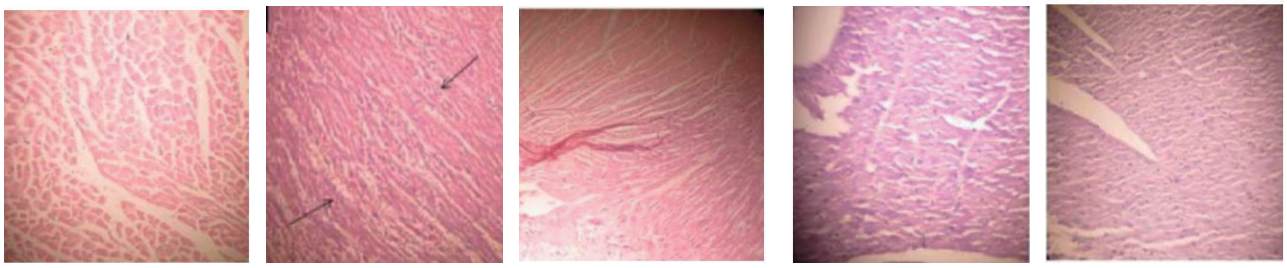

Gp I **Gp II** **Gp III** **Gp IV** **Gp V**

*Arrows indicate infiltration and necrosis of the myocardium.
Fig. 3: Histopathology of myocardial tissues

Groups	Body weight (BW)			Heart weight (HW)	
	Initial (g)	Final (g)	% Gain in weight	Absolute wt (g)	Relative wt (g) (g/100g BW)
Gp I Normal Control	125 ± 2	138 ± 2	11 ± 3	0.316 ± 0.043	0.831±0.049
Gp II ISO (85 mg/kg)	126 ± 1	129 ± 2***	2.38 ± 1.6***	0.287 ± 0.037***	0.814±0.041*
Gp III WC 25 mg/kg *Per se*	125 ± 2	140 ± 2	12.45 ± 2	0.310 ± 0.039	0.811±0.044
Gp IV WC (25 mg/kg) + ISO	122 ± 2	145 ± 1###	16.3 ±2.15###	0.302 ± 0.036###	0.784±0.037##
Gp V Vit E + ISO	125 ± 1	142 ± 1###	12.87 ± 3.7###	0.294 ± 0.045###	0.797±0.046###

Table 1. Effects of WC treatment on body and heart weight of albino rats
All the datas are expressed as SD means for six albino rats in each group.
*Significant difference at p<0.05 and *** p<0.001 between Normal Control and ISO treated group.
#Significant difference at p<0.05 and ### p<0.001 between ISO treated group and WC + ISO treated group.

Groups	GSH (µmol GSH/ mg prot)	GPx (nmol CDNB/ minute/ mg prot)	GR (nmol NADPH/ minute/ mg prot)
Gp I Normal Control	1.37 ± 0.13	5.67 ± 0.31	6.37 ± 0.29
Gp II ISO (85 mg/kg)	0.21 ± 0.18	1.79 ± 0.21	1.31 ± 0.18
Gp III WC 25 mg/kg *per se*	1.22 ± 0.15	5.30 ± 0.29	5.14 ± 0.53
Gp IV WC (25 mg/kg) + ISO	1.08 ± 0.10	3.87 ± 0.25	3.81 ± 0.32
Gp V Vit E + ISO	0.98 ± 0.07	3.34 ± 0.18	3.57 ± 0.45

Table: 2 Serum endogenous glutathione level (Antioxidant parameter)

Groups	SOD (Unit/g of tissue)	Catalase (µmol of H_2O_2 consumed/min/g of tissue)	TBARS (nmol MDA/g of tissue)
Gp I Normal Control	13.55 ± 0.62	2.66 ± 0.24	1.65 ± 0.12
Gp II ISO (85 mg/kg)	5.38 ± 0.31	0.54 ± 0.02	3.82 ± 0.21
Gp III WC 25 mg/kg *Per se*	12.56 ± 0.61	1.87 ± 0.37	2.23 ± 0.16
Gp IV WC (25 mg/kg) + ISO	12.14 ± 0.33	2.09 ± 0.32	2.41 ± 0.21
Gp V Vit E + ISO	11.16 ± 0.31	2.22 ± 0.35	1.53 ± 0.21

Table: 3 Antioxidant enzyme activity

Groups	LDH (IU/I)	CK-MB (IU/I)	AST (IU/I)	ALT (IU/I)	Troponin-I (ng/ml)
Gp I Normal Control	363.12 ± 32.61	483.33 ± 36.19	249.45 ± 7.21	77.14 ± 2.93	0.16 ± 0.04
Gp II Isoprenaline (85 mg/kg)	1315 ± 43.22	1923.57 ± 46.09	308.31 ± 7.42	176.85 ± 3.14	1.34 ± 0.22
Gp III WC 25 mg/kg *Per se*	389.27 ± 40.89	523.54 ± 45.60	264.39 ± 7.11	88.12 ± 2.96	0.26 ± 0.06
Gp IV WC (25 mg/kg) + ISO	967 ± 45.87	1376.87 ± 42.34	279.81 ± 7.57	1.30 ± 3.02	0.87 ± 0.13
Gp V Vit E + ISO	1077 ± 48.45	1658.74 ± 45.10	285.41 ± 8.22	132.47 ± 2.80	1.05 ± 0.11

Table: 4 Cardiac Injury Markers

Groups	Oedema	Infiltration	Necrosis
Gp I Normal Control	0 ± 0	0 ± 0	0 ± 0
Gp II Isoprenaline (85 mg/kg)	2.46 ± 0.47	2.47 ± 0.49	2.82 ± 0.31
Gp III WC 25 mg/kg *Per se*	0.31 ± 0.18	0.23 ± 0.14	0.50 ± 0.10
Gp IV WC (25 mg/kg) + ISO	0.81 ± 0.46	0.86 ± 0.21	0.87 ± 0.24
Gp V Vit E + ISO	0.45 ± 0.21	0.32 ± 0.25	0.53 ± 0.21

Table 5: Oxidative stress markers

cleaned. Hearts were weighed to determine the absolute and relative weights.

Biochemical estimation of cardiac biomarkers

A 10% homogenate of myocardial tissue was prepared in 50 mM phosphate buffer, pH 7.4, and an aliquot was used for lipid peroxidation (LPO) assay [25]. The LPO in tissue homogenates was evaluated by homogenizing the heart tissue. The homogenate was centrifuged at 7000 rpm for 15 minutes and the supernatant was used for the estimation of biochemical parameters: LPO, lactate dehydrogenase [26], glutathione, glutathione peroxidase [27], superoxide dismutase [28], catalase [28,29] and protein [30]. Creatinine phosphokinase was estimated spectrophotometrically using a kit from Randox Laboratories, USA [31]. Lipid profile for the estimation of triglyceride (TG), total cholesterol, LDL and HDL-cholesterol was also done using serum sample [32].

While performing LPO, the absorbance of each tested group was recorded at 532 nm and the results were expressed as the malonyldialdehyde (MDA) content [33]. Cardiac index (CI), atherogenic index (AI) and coronary artery index (CAI) were calculated using following formulas:

Histopathological studies

The hearts were removed and sliced for immediate fixation in 10% buffered neutral formalin solution. The fixed tissues were embedded into paraffin and serial sections were cut and stained with hematoxylin-eosin to observe under a light microscope.

Statistical analysis

Descriptive statistics mean and standard deviation were calculated for all variables of each group. All data were analyzed using a one-way Analysis of Variance (ANOVA) followed by Tukey's test. *P value <0.05and **P<0.001 has been considered as statistical significance and highly significant value.

Results

Effects of WC treatment on body and heart weight

The effects of WC on ISO-treated rats are summarized in Table 1. No changes were observed in the body and heart weights of rats treated with normal saline solution and WC *per se*. However, these parameters in ISO- treated rats were significantly decreased compared to the normal control animals. Similarly, the body weight and the relative heart weight were significantly reduced in ISO-treated rats as compared to the control group. A significant protection was recorded in the WC+ ISO treated group compared with the ISO-treated group.

Effects of WC treatment on biochemical parameters

A significant increase and restoration in the glutathione (GSH) level (*P<0.05) was observed in withacoagulin (25 mg/kg) treated groups as compared to isoprenaline (ISO) toxic group (Table 1). Withacoagulin (25 mg/kg) treatment *per se* has highly significant augmentation on endogenous antioxidant enzymes superoxide dismutase (**P<0.01, Table 2) and glutathione peroxidase (**P<0.01). Along with this, a significant increase in the myocardial catalase activity in withacoagulin (25 mg/kg) (*P<0.05) groups were also observed (Table 2). Isoprenaline-induced myocardial necrosis resulted in a significant depletion of antioxidant enzymes: catalase (*P<0.05) Table 2), superoxide dismutase (*P<0.05, Table 2) compared to normal control.

We observed that the malonyldialdehyde levels were significantly elevated (*P<0.05) in the isoprenaline control group (Table 2). Withacoagulin (25 mg/kg) treatment significantly inhibited lipid peroxidation (*P<0.05) and preserved membrane integrity. A fall in myocardial enzymes creatinine phosphokinase (**P<0.01, Table 3), lactate dehydrogenase (**P<0.01, Table 3) was observed in the withacoagulin group compared to ISO toxic control group.

The WC treatment (25 mg/kg) decreased the levels of TG, Total cholesterol and LDL cholesterol and LDH. HDL level was improved.

Effects of WC treatment on heart histopathological tisuue

Histopathological examination showed a significant myocardial membrane damage and infiltration of inflammatory cells in the isoprenaline control group as compared to nomal control group. Moreover extensive myonecrosis with fibroblastic proliferation and presence of chronic inflammatory cells were observed in the isoprenaline control group compared to that of normal. There is highly significant percentage myofiber loss due to necrosis in the isoprenaline control group was (**P<0.01, Table 4) as compared to normal control. In the present study, *withacoagulin* (25 mg/kg) and vitamin E (100 mg/kg) treatment significantly prevented myonecrosis as indicated by significant reduction in the infiltration of inflammatory cells, vacuolar changes as well as oedema as compared to the isoprenaline control group. There was significantly less % fiber loss in the vitamin E-treated group (*P<0.05). But it was shown that there is highly significant decrease (**P<0.01) in the % fibre loss in the *withacoagulin* (25 mg/kg) groups as compared to isoprenaline control.

Discussion

The present study was designed to evaluate the efficacy of withacoagulin isolated from *Withania coagulans* fruits in isoprenaline-induced myonecrosis in albino rats. The effect of withacoagulin on modulation of biochemical parameters like endogenous antioxidant glutathione, antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, lipid peroxidation product malonyldialdehyde and myocardial enzymes lactate dehydrogenase and creatinine phosphokinase have been studied. Increase in malonyldialdehyde level was observed in the heart tissue after isoprenaline administration. In addition, isoprenaline administration decreases the reduced glutathione content as well as the antioxidant enzyme activity (superoxide dismutase, catalase and glutathione peroxidase) in cardiac tissues. A significant depletion of lactate dehydrogenase and cretanine phosphokinase, an important marker of myocardial injury, in the isoprenaline group was observed. The observation that vitamin E and withacoagulin treatment significantly restored lactate dehydrogenase and creatine phosphokinase activity compared to the isoprenaline control group was suggestive of their cardioprotective effect. Both these drug treatments restored the myocardial antioxidant status and maintained membrane integrity as evidenced by a decline in malonyldialdehyde levels. Furthermore, histopathological examination confirmed the cardioprotective effect of withacoagulin.

On histopathological examination, the presence of focal myonecrosis with myophagocytosis and lymphocytic infiltration (myocarditis) in the subendocardial region was observed.

Conclusion:

The present study showed that WC improved myocardial histoarchitecture against ISO-induced myocardial injury in rats. The effect is associated with a significant restoration in MDA level, improvement in cardiac and atherogenic indexes and significant enhancement in biochemical parameters. In summary, the present study strongly suggests that multiple mechanisms may be responsible for the cardioprotective effect of withacoagulin.

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Disclosure of conflict of interest

The author(s) have no conflicts of interest to disclose with anyone else.

Statement of ethical approval

The study was approved by the Institutional Animal Ethics Committee (RIVTE/ IAEC/16/02) of Ram-Eesh Institute of Vocational and Technical Education, Greater Noida, Uttar Pradesh (Animal House registration number: 385/PO/Re/S/01/CPCSEA).

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