Original Research Article **Pharmacology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

STUDY OF CARDIOPROTECTIVE ACTIVITY OF NORMOVEDIC IN CCL_4 INDUCED CARDIOTOXICITY IN RATS.

UTTAM PAUL[1] AND SHIVALINGE GOWDA KP*[2]

[1.] M.Pharm Research Scholar, Department of Pharmacology, PES College of Pharmacy, 50 ft Road, Hanumanthanagar, Bengaluru,- 560050 Karnataka, India.
[2]Asso. Prof and HOD, Department of Pharmacology, PES College of Pharmacy, 50 ft Road, Hanumanthanagar, Bengaluru,- 560050 Karnataka, India.

ABSTRACT

The cardioprotective activity of Normovedic was investigated using carbon tetrachloride (CCl_4) induced cardiotoxicity. The rats were divided into 5 groups (n=6). Group I was considered normal. The Normovedic was administered orally at a dose of 39 mg/kg p.o for 8 weeks to the group II, IV. Silymarin (100mg/kg) was used as a standard cardioprotective drug and was administered for 8 weeks to the group V. The CCl_4 1 ml/kg with liquid paraffin (1:1 ratio) i.p twice a week for 4 weeks (5^{th} to 8^{th} week) to the group III,IV and V. After 24h of the last treatment ECG was measured under ketamine and xylazine anesthesia (65, 9mg/kg bw ip) in all the rats. Then the rats were sacrificed by overdose ketamine anesthesia (150mg/kg ip). The blood was withdrawn by retro orbital puncture and processed to serum. The heart was isolated and heart PMS was prepared. The serum biomarkers CK-MB, CK, LDH and cardiac tissue SOD were determined. The CK-MB, CK, LDH levels were significantly decreased, whereas SOD levels were significantly increased in Normovedic treated rats when compared to CCl_4 toxic control rats (group III). The CCl_4 administered rats (Group III) showed alterations in ECG and heart weight, histological examination when compared to normal rats these alterations were significantly recovered in the Group IV & Group V treated rats. From the results of this study it is concluded that the Normovedic (a poly herbal formulation) possesses cardioprotective properties which may help to protect users against complications due to cardiotoxicity.

KEYWORDS: **Carbon tetrachloride; Silymarin; Normovedic; Serum enzymes & antioxidant enzymes**.

SHIVALINGE GOWDA KP
Asso. Prof and HOD, Department of Pharmacology, PES College of Pharmacy, 50 ft Road, Hanumanthanagar, Bengaluru,- 560050 Karnataka, India.

INTRODUCTION

Severe acute toxic insults cause cardiac cell death instantly. In the early response to mild environmental stimuli, biochemical changes such as alterations in calcium homeostasis occur [1]. Cardiotoxicity may occur during or shortly after treatment (within days or weeks), or it may become evident a long period after completion of chemotherapy. Cardiotoxicity can range from asymptomatic subclinical abnormalities, including electrocardiographic changes to life-threatening events such as congestive HF or acute coronary syndromes. It can develop in a subacute, acute, or chronic manner [2-3]. The most widely accepted mechanism of CCl_4 induced cardiotoxicity is the formation of free radicals which is a rate limiting process in tissue peroxidative damage. This free radical and related reactive species may cause oxidative stress, which produces interrelated rearrangements of cellular metabolism, increase in intracellular free calcium, damage to membrane ion transport and permeability, and destruction of the cells by lipid peroxidation. This leads to loss of myocardial structural integrity and depressed cardiac function resulting in cardiotoxicity and congestive cardiac failure [4].The anti-oxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and indirectly glutathione reductase[5].The Normovedic capsules which are available in market and are used for its anti-hypertensive and cardioprotective actions contains *Rauwolifa serpetina, Terminalia chebula, Terminalia belerica, Embilica officinalis, Tribulus terrestris, Terminalia arjuna, Nardostachys jatamansi, Celastrus paniculata* which possesses antihypertensive[6], antioxidant [7], cardioprotective ,hepatoprotective, antispasmodic , antibacterial ,analgesic , anti ulcer [8]etc. Silymarin reacts with the reactive oxygen species (ROS) and converts them into less reactive and toxic compounds. It potentiates the effects of the physiological antioxidants (glutathione, superoxide dismutase) and prevents the reduction of their concentrations, as well as its structural and functional consequences [9]. The main active agent of milk thistle is silymarin, which is a mixture of flavanolignans constituting silydianine, silycristine and silybinin [10]. New applications of silymarin are highlighted by giving the nontraditional uses of this drug in order to protect other organs in addition to liver [11].

MATERIALS AND METHODS

Animals

Albino Wistar rats (150-200g) were procured from Venkateshwara Enterprises, Bengaluru. The animals were kept in the animal house of PES College of Pharmacy, Bengaluru for experimental use. All the animals were acclimatized for 7 days under standard husbandry conditions, i.e.; room temperature of 18-29^{0}C; relative humidity 45- 55% and a 12:12h light/ dark cycle. The animals had free access to the standard rat pellet and water. Animals were habituated to laboratory conditions for 7 days prior to experimental protocol to minimize if any of non-specific stress. The approval of the Institutional Animal Ethical Committee (PESCP/IAEC/08/2014, Dated: 13-12-2014, CPCSEA Reg.No 600/PO/Ere/S /02/CPCSEA) was taken prior to the experiments. All the experiments were conducted in strict compliance according to ethical principles and guidelines provided by CPCSEA.

Experimental design

The rats were randomly divided into 5 groups consists of 6 animals each. Wistar rats weighing (180-200g) either sex were selected under healthy conditions for experimental purpose. Group I was considered as normal control received purified water (vehicle) for 8 weeks. Group II served as standard control received 39 mg/kg of Normovedic for 8 weeks orally. Group III was treated as toxic control received CCl_4 at a dose of 1 ml/kg on 5th -8th weeks (twice a week), i.p route. Group IV served as pre-treatment group received 39 mg/kg of Normovedic for 8 weeks orally and CCl_4 at a dose of 1 ml/kg on 5th -8th weeks (twice a week), i.p route. Group V Served as pre-treatment group received 100mg/ kg of Silymarin orally for 8weeks and CCl_4 at a dose of 1 ml/kg on 5th -8th weeks twice a week , i.p route.

Evaluation of ECG alteration

The CCl_4 treated rats were subjected to evaluate heart functions; the rats were anesthetized with Ketamine 65 mg/kg and Xylazine 9 mg/kg, i.p route and, diluted with normal saline. The standard ECG was measured for 10 min using computer based software i.e. Lab chart software employing subcutaneous needle electrodes. The recordings were stored in the computer software for future analysis (Lab chart) [12, 13].

Collection of blood samples and separation of serum

The animals were subjected to light ketamine anesthesia. The blood was collected from retro-orbital plexus in each rat and collected in centrifuge tubes and allowed to coagulate for 30 min at 37ºC. The coagulated blood was centrifuged in micro-centrifuge at 2500 rpm for 10 min. The freshly prepared serum is used for assay.

Observation of physical parameters and histopathological study.

In the present study the weight of wet heart is considered as physical parameter. Animals were euthanized with an over dose of ketamine overdose. The heart in each animal was dissected out; washed in normal saline and blotted with filter paper and weighed immediately and stored in 10% ice cold phosphate buffer and used for various biochemical estimations. Portions of heart from all the experimental groups were fixed in 10% neutral formalin and preceded for histopathology examination [14].

Preparation of tissue homogenate

The isolated heart was rinsed in ice-cold normal saline to remove excess of blood and a 10% w/v homogenate was prepared in 10% chilled phosphate buffer (pH 7.0) (1g of tissue with 10ml of PBS). The tissue was chopped and minced with Teflon homogenizer (on ice) at 3000 rpm for 15 minutes at 4ºC to separate the debris. The collected supernatant was again centrifuged at 5000 rpm for 20 minutes at 4ºC to further break the cell

membranes. The obtained supernatant was used for assay or stored at ≤- 20ºC [15].

Biochemical Estimation

The serum LDH, CK& CK-MB were estimated spectrophometrically using kits from Trans Asia Bio-Medical Ltd, (ERBA). Following, the animal was sacrificed by ketamine overdose and tissue biochemical estimation superoxide dismutase (SOD) was estimated by the method described by Kakkar *et al.* [16] and expressed as Units mg protein^{-1}.

Statistical evaluation

The entire data were expressed as Mean ± SD of six animals in an each group. Statistical comparisons were performed by one way ANOVA followed by Bonferroni multiple comparison tests using Graph Pad Prism software, version 5.0. ^{b}*P<0.05, ^{b}**P<0.001, ^{b}***P<0.0001 was considered as significant when compared to toxic group. ^{a}*P<0.05, ^{a}**P<0.001, ^{a}***P<0.0001, was considered as significant when compared to the normal group. ^{a}*compared with normal group. ^{b}*compared with CCl_4 induced group.

RESULTS AND DISCUSSION

Effect of Normovedic on heart weight in CCl_4 induced cardiotoxicity

Table 1
Effect of Normovedic on heart weight in CCl_4 induced cardiotoxicity

Treatment	Dose (mg/kg)	Route	Heart weight in gm
Normal	-----	po	0.6673±0.005
Normovedic treated	39	po	0.6800±0.004
CCl_4 treated	1	ip	0.7673±0.008 ^{a}***
Normovedic + CCl_4 treated	39+1	po + ip	0.6713±0.0055^{b}***
Silymarin + CCl_4 treated	100+1	po + ip	0.6790±0.0059^{b}***

Each value is expressed as Mean ± SD for groups of 6 animals in each group.

The cardiotoxic rats (CCl_4 alone treated) group III showed increase in heart weight (P<0.0001) as compared to normal group (0.6673 ± 0.005). Normovedic (39 mg/kg) (0.6713 ± 0.0055) & Silymarin (100 mg/kg) (0.6790 ± 0.0059) with a significance of (P<0.0001) showed decrease in the heart, weight when compared to toxic group (0.7673 ± 0.008).

Effect on ECG alteration

Table 2
Effect of Normovedic on ECG

Treatment	Dose (mg/kg)	P wave (sec)	QRS complex(sec)	T wave(sec)	Heart rate (bpm)
Normal	-----	0.0350±0.0014	0.0330±0.0014	0.0215±0.0007071	115.0±2.610
Normovedic	39	0.0375±0.0007	0.0385±0.0049	0.0235±0.00070	116.9±3.517
CCl_4 treated	1	0.0465±0.0021^{a}**	0.0660±0.0028^{a}***	0.0325±0.00070 ^{a}***	138.1±1.572^{a}***
Normovedic + CCl_4	39+1	0.0345±0.0007^{b}***	0.0250±0.0014 ^{b}***	0.0275±0.00070 ^{b}**	126.6±2.066^{b}***
Silymarin + CCl_4	100+1	0.0275±0.0007^{b}***	0.0330±0.0014 ^{b}***	0.0285±0.00070 ^{b}**	120.0±1.801^{b}***

Each value is expressed as Mean ± SD for groups of 6 animals in each group.

The ECG of different groups was compared. Normal rats showed a standard ECG pattern, whereas animals treated with CCl_4 alone showed significant elevation in P wave (0.0465 ± 0.0021), QRS complex (0.0660 ± 0.0028) and T wave (0.0325 ± 0.00070). The elevation of P wave may be due to enlarged atria. The heart rate was increased (138.1 ± 1.572), widening of QRS complex and increased T wave was observed when compared to normal control rats. Rats pretreated with Normovedic at dose of (39 mg/kg) and Silymarin (100 mg/kg) followed by CCl_4 resorted the elevated levels of P wave, QRS complex and T wave, & heart rate near to the normal.

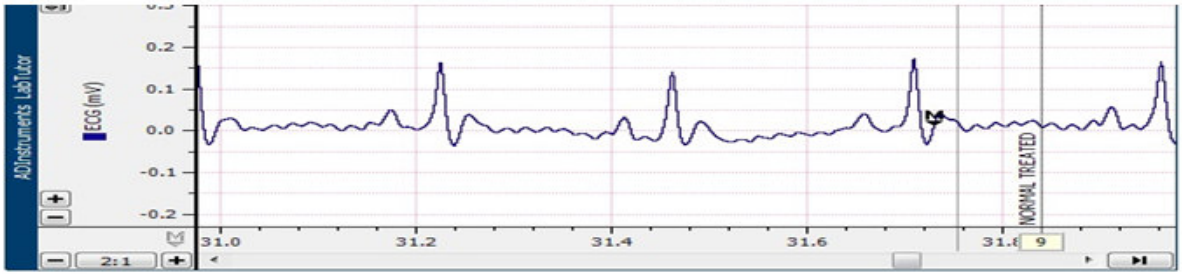

Figure 1
Effect of vehicle on rats ECG

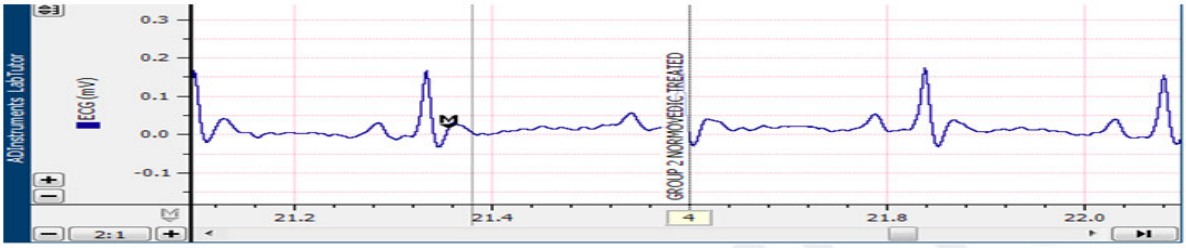

Figure 2
Effect of Normovedic on rats ECG

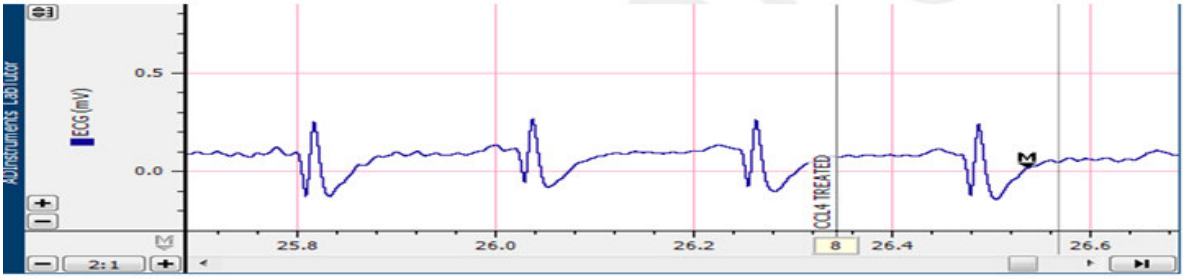

Figure 3
Effect of CCl_4 on rats ECG

Figure 4
Effect of Normovedic + CCl_4 on rats ECG

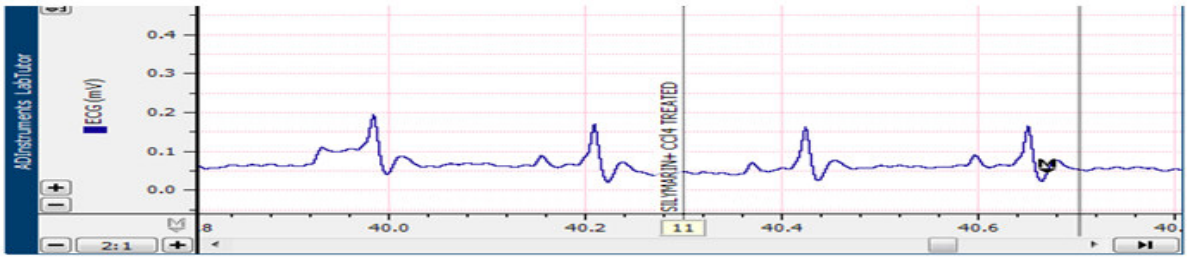

Figure 5
Effect of Silymarin + CCl_4 on rats ECG

Effect on CK-MB, CK, LDH & SOD

Table 3
Effect on biochemical markers CK, CK-MB, LDH and SOD

Treatment	Dose (mg/kg)	CK-MB (IU/L)	CK (IU/L)	LDH (IU/L)	SOD Units/mg of protein
Normal	-----	201.4±55.03	123.1±15.96	41.82±13.94	37.49±1.291
Normovedic treated	39	240.2±59.14	147.2±19.65	70.16±22.70	$31.12\pm0.045^{a***}$
CCl_4 treated	1	$962.9\pm81.83^{a***}$	$455.3\pm23.30^{a***}$	$693.5\pm54.10^{a***}$	$24.40\pm0.095^{a***}$
Normovedic + CCl_4 treated	39+1	$504.9\pm40.01^{b***}$	$294.4\pm44.96^{b***}$	$325.1\pm54.28^{b***}$	$34.91\pm1.057^{b***}$
Silymarin + CCl_4 treated	100+1	$378.3\pm37.58^{b***}$	$205.7\pm30.03^{b***}$	$205.1\pm87.14^{b***}$	$42.13\pm2.616^{b***}$

Each value is expressed as Mean ± SD for groups of 6 animals in each group.

CK-MB

The level of CK-MB in the CCl_4 alone treated animals with a dose of 1 mg/kg showed a significant raise in the CK-MB levels with mean ± SD (962.9 ± 81.83) & significance of P<0.0001 when compared to normal (201.4 ± 55.03).The pre-treated animals with Normovedic (39 mg/kg) (504.9 ± 40.01) & Silymarin (100 mg/kg) with(378.3 ± 37.58) with P<0.0001 respectively showed a significant decrease in the levels of CK-MB when compared to CCl_4 treated group.

Creatinine kinase

The level of Creatinine kinase in the CCl_4 alone treated rats showed a significant raise in the CK levels with mean ± SD (455.3 ± 23.30) & significance of P<0.0001 when compared to normal (123.1 ± 15.96). Pre-treated animals with Normovedic (39 mg/kg) & Silymarin (100 mg/kg) showed a significant decrease in the levels of CK (294.4 ± 44.96) & (205.7 ± 30.03) with P<0.0001, when compared to CCl_4 treated group.

LDH

The level of LDH in the CCl_4 alone treated rats showed a elevated levels of LDH with mean ± SD (693.5 ± 54.10) & significance of P<0.001 when compared to normal (41.82 ± 13.94). Pre-treated animals with Normovedic (39 mg/kg) & Silymarin (100 mg/kg) showed a significant decrease in the levels of LDH (325.1 ± 54.28) & (205.1 ± 87.14) with P<0.0001 respectively when compared to CCl_4 treated group.

SOD

The level of SOD in the CCl_4 alone treated rats with a dose of 1 mg/kg showed a significant decrease in the SOD levels (24.40 ± 0.095) with mean ± SD & significance of p<0.0001 when compared to normal (37.49 ± 1.291). Pre-treated animals with Normovedic (39 mg/kg) & Silymarin showed a raise in the levels of SOD (34.91 ± 1.057) & (42.13 ± 2.616) respectively with P<0.0001 and when compared to CCl_4 group.

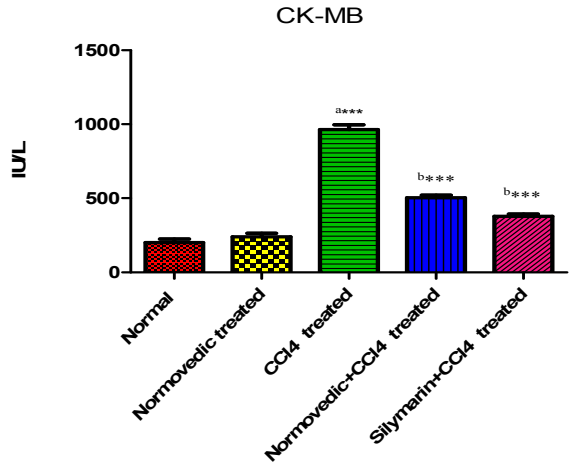

Figure 6
Effect on CK-MB

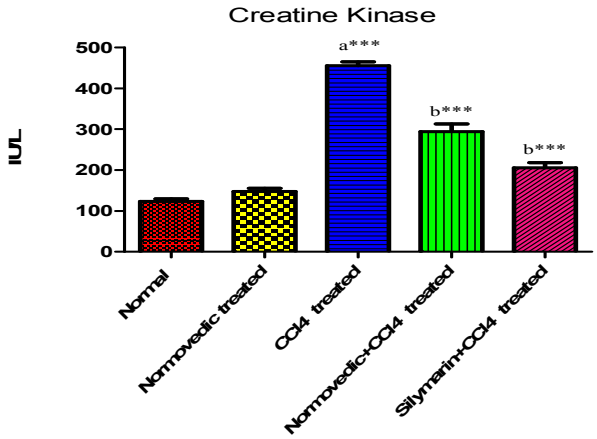

Figure 7
Effect on CK

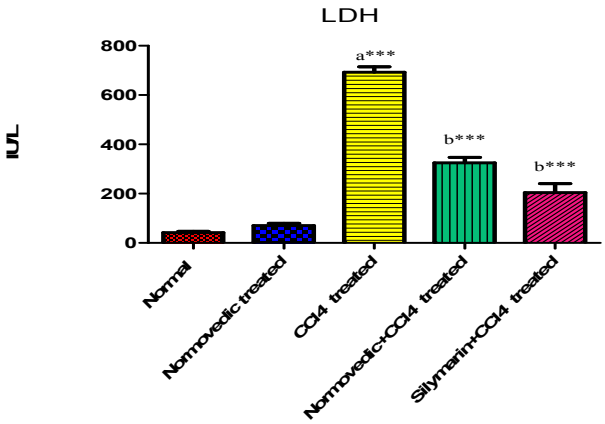

Figure 8
Effect on LDH

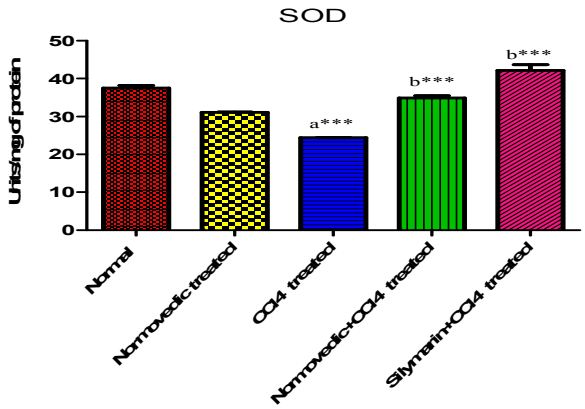

Figure 9
Effect on SOD

Histopathology of rat heart

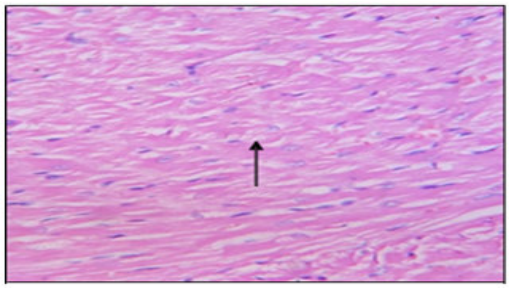

Fig 10
Normal treated

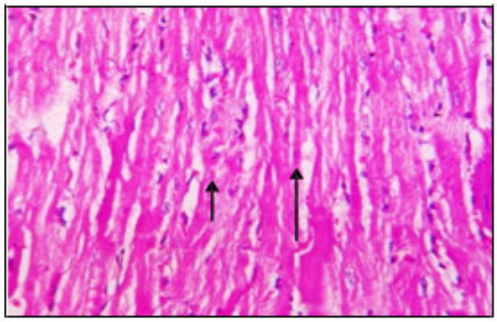

Fig 11
Normovedic treated

Fig No 10 Normal: myocardium shows intact arrangement of the cardiac muscle fibers.
Fig No 11 Normovedic treated: cardiac muscle fibers show intact integrity of myocardial cell membrane, myofibrillar structure with striations and continuity with adjacent myofibrils.

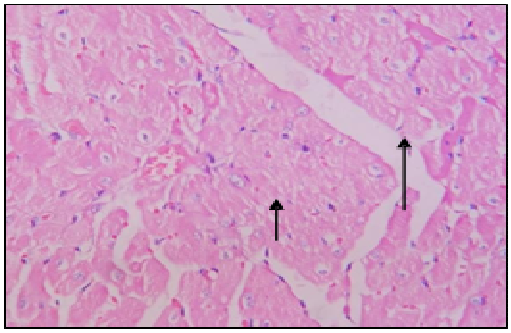

Figure 12
CCl_4 treated

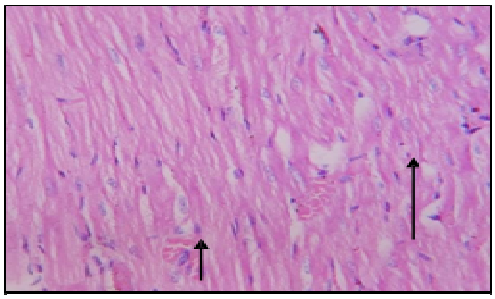

Fig 13
Normovedic + CCl_4 treated

Fig No 12 CCl_4 treated: myocardium shows loss of arrangement of the cardiac muscle fibers, myofibrillar structure with loss of striations and loss of continuity with adjacent myofibrils, The interstitial space at focal areas appears increased.
Fig No 13- Normovedic + CCl_4 treated: myocardium shows intact arrangement of the cardiac muscle fibers, The interstitial space appears mildly increased. Some of the vascular spaces appear congested.
Fig No 14 Silymarin + CCl_4 treated: the myocardium shows intact arrangement of the cardiac muscle fibers, the interstitial space appears mildly increased. Some of the vascular spaces appear congested amidst these cardiac muscle fibers.

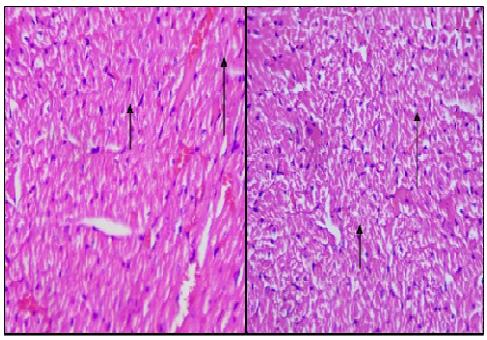

Fig 14
Silymarin + CCl_4 treated

DISCUSSION

CCl_4 induced cardiotoxicity has been predictable in the experimental animals. In the present study screening of the protective role of Normovedic in CCl_4 cardiotoxicity was investigated. Carbon tetrachloride a lipid soluble, heavy, and nonflammable liquid is most widely used for experimental induction of hepatotoxicity. CCl_4 is an organic compound widely used as a dry cleaning solvent until it was recognized as a potent carcinogen. Liver is not the only target organ of CCl_4 but it also affects several organs of the body, such as lungs, hearts, testes, kidneys and brain[5].CCl_4 alone treated groups showed increase in heart weight (showed fatty pattern, swelling & necrosis resulting in increased organ mass) were ($P<0.0001$) as compared to the normal group. Normovedic (39mg/kg) & Silymarin (100 mg/kg) showed decrease in the heart weight when compared to toxic group. The most widely accepted mechanism of CCl_4 induced cardiotoxicity is the formation of free radicals, which is a rate limiting process in tissue peroxidative damage. This free radical and related reactive species may cause oxidative stress, which produces major interrelated rearrangements of cellular metabolism, increase in intracellular free calcium, damage to membrane ion transport and permeability. As cardiac tissue has affinity for CCl_4 due to cytochrome P450. So, oxidative damage to lipids and proteins of heart tissues probably occurred due to CCl_4 intoxication [4].The ECG of different groups was compared. Normal rats showed a standard ECG pattern, whereas animals treated with CCl_4 alone showed significant ($P<0.0001$) elevation in P wave, QRS complex and T wave. The heart rate was increased due to an elevation in P wave, widening of QRS complex and increased T wave was observed

when compared to normal control rats. These changes may be due to the damage of myocardium [17]. Rats pretreated with Normovedic at a dose of (39 mg/kg) and Silymarin (100 mg/kg) followed by CCl_4 resorted the elevated levels of P wave, QRS complex and T wave, & heart rate near to the normal with a significance value of ($P<0.0001$) for P wave and QRS complex whereas for T wave and heart rate showed ($P<0.001$). Injection of CCl_4 to rats induced oxidative heart tissue damage which is proved by an increase in the LDH and CK, CK-MB (enzymes responsible for ATP regeneration) and a decrease in the antioxidant enzymes, SOD, (antioxidant defence system) in hearts of CCl_4-treated rats when compared with normal rats, indicating that the heart is one of the target organs affected by CCl_4 toxicity.The level of CK-MB, CK and LDH in the CCl_4 alone treated animals with a dose of 1mg/kg showed a significant raise in the CK-MB levels with significance of $P<0.0001$ when compared to normal. This may be due to significant increase in the glycolytic enzymes and may be attributed to a generalized increase in membrane permeability and free radical generation activates cardiac myocytes disruption and peroxidation of membrane, which boosted the levels of these enzymes in serum. Superoxide dismutase is found in the cytosol as a zinc/copper-containing enzyme and in mitochondria as a manganese-containing enzyme. It dismutates the superoxide anion by combining it with protons to form hydrogen peroxide and oxygen. Rats pre-treated with Normovedic with a dose of 39 mg/kg significantly resorted to the normal levels with significance value $P<0.0001$ and $P<0.05$ in CK-MB values. The enzyme levels of the standard drug Silymarin (100mg/kg) also showed a normal value ($P<0.0001$) which indicates the protective effect [4, 17].Histological examinations of the heart sections revels that animals treated with CCl_4 1ml/kg shows show loss of integrity of myocardial cell membrane, myofibrillar structure with loss of striations and loss of continuity when compared to the normal animals. Administration and pretreatment with Normovedic (39mg/kg) showed a significant recovery of the above complications. The normal tissue architecture was retained as well as of that of the Silymarin (100 mg/kg) which confirmed the protective effect.

CONCLUSION

As per the obtained results, it is reported that the drug Normovedic has proven cardioprotective, activity against carbon tetrachloride induced cardiotoxicity in rats. The results in this study conclude that rats induced with carbon tetrachloride (1ml/kg) showed a significant attenuation of the free radical scavenging & oxidative stress damage at a dose of 39 mg/kg of the drug Normovedic with comparison to the standard drug Silymarin. This protective effect of Normovedic may act by preventing the uptake & accumulation of CCl_4 in myocardial cells and also may be due to the antioxidant property. Considering the improvement in the serum marker levels, antioxidant enzymes levels & histopathological studies, the Normovedic showed cardioprotective activity.

ACKNOWLEDGEMENT

We thank Prof. Dr. J. Saravanan, Principal of PES College of Pharmacy, and Prof. Dr. S. Mohan, Director of PES College of Pharmacy for their guidance and support.

CONFLICT OF INTEREST

None.

REFERENCES

1. Kang YJ. Molecular and Cellular Mechanisms of Cardiotoxicity. Environmental Health Perspectives. 2001;109(1):27-34.
2. Berardi R, Caramanti M, Savini A. State of the art for cardiotoxicity due to chemotherapy and to targeted therapies: A literature review. Critical reviews in oncology/hematology. 2013;88:75-86.
3. Hirakawa B, Jessen BA, Illanes O. Toxicogenomic analysis of cardiotoxicity in rats. Genomics Insights. 2008;1:3–12.
4. Eshaghi M, Zare S, Banihabib N, Nejati V, Farokhi F, Mikaili P. Cardioprotective effect of *Cornus mas* fruit extract against carbon tetrachloride induced-cardiotoxicity in albino rats. J. Basic. Appl. Sci. Res. 2012;2(11):11106-14.
5. Karthikeyan R, Anantharaman P, Chidambaram N, Balasubramaniam T, Somasundaram ST. *Padina boergessenii* ameliorates carbon tetrachloride induced nephrotoxicity in Wistar rats. Journal of King Saud University-Sciences. 2012; 24: 227-32.
6. Kokate CK, Purohit AP, Gokhale HB. In: Pharmacognosy. 31st ed. Nirali Publication Pune.2005. pp. 13.26, 8.67.
7. Sabu MC, Kuttan R. Antidiabetic activity of medicinal plant and its relationship with antioxidant property. J Ethnopharmacol 2002;81:155-60.
8. Duke JA, Godwin MJB, Ducellier J, Duke PAK. In: Handbook of medicinal herb. 2nd ed. CRC press. Florida: 2002;5:36-37
9. Raskovic A, Stilinovic N, Kolarovic J, Vasovic V, Vukmirovic S, Mikov M. The Protective effects of silymarin against doxorubicin-induced cardiotoxicity and hepatotoxicity in rats. Molecules. 2011; 16 : 8601-13.
10. Morales-González JA, Gayosso-Islas E, Sánchez-Moreno C, et al. Protective effect of silymarin on liver damage by xenobiotics.
11. Pradhan SC, Girish C. Hepatoprotective herbal drug, Silymarin from experimental pharmacology to clinical medicine. Indian J Med Res. 2006;124 (5):491-504.
12. Rade Injac, Martina Perse, Manica Cerne, Nejka Potocnik, Natasa Radic. Protective effects of fullerenol C60 (OH)24 against doxorubicin-induced cardiotoxicity and hepatotoxicity in rats

with colorectal cancer. Biomaterials. 2009; 30; 1184–96.
13. Ketamine and xylazine for surgical anesthesia for rats. J. American Veterinary Medical Association. 1997; 171(9): 842-4.
14. Arthur C. Guyton, John E. Hall. Text book of medical Physiology, Unit 3, 11th ed.2006
15. D. Bhattacharyya, S. Pandit, R. Mukherjee, N. Das, T. K. Sur. Hepatoprotective effect of Himoliv®, a polyherbal formulation in rats. Indian J Physiol Pharmacol 2003; 47 (4): 435–40.
16. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. J Biochem Biophy 1984, 21: 130-32.
17. Sahreen S, Khan MR, Khan RA, Alkreathy HM. Cardioprotective role of leaves extracts of *Carissa opaca* against CCl_4 induced toxicity in rats. Bio Med Central. 2014;7(224).