

Assessment of Orlistat and Niclosamide Against Isoprenaline-induced Cardiac Fibrosis in Mouse Model

Thesis

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Dated: 01/03/2024

Certificate

*This is to be certified that the research work embodied in this thesis entitled
“Assessment of Orlistat and Niclosamide Against Isoprenaline-induced
Cardiac Fibrosis in Mouse Model” submitted by Dr. Manju Gari, Roll No. P-
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Pharmacology at ICAR-Indian Veterinary Research Institute, Izatnagar, is
the original work carried out by the candidate herself under my supervision
and guidance.*

*It is further certified that Dr. Manju Gari, Roll No. P-2339, has worked
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We the undersigned members of Advisory Committee of **Dr. Manju Gari**, Roll No. P-2339, a candidate for the degree of **Doctor of Philosophy** with the major discipline **Veterinary Pharmacology**, agree that the thesis entitled "**Assessment of Orlistat and Niclosamide Against Isoprenaline-induced Cardiac Fibrosis in Mouse Model**" may be submitted in partial fulfilment of the requirement for the degree.

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Doctor of Philosophy Degree** of this Institute.

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Manju Gari
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ABBREVIATIONS

%	: Per cent
&	: And
$(Ca^{2+})_i$	: Intracellular calcium ions
/	: Per
@	: At the rate
<	: Lesser than
=	: Equal to
>	: Greater than
°C	: Degree centigrade
µg	: Microgram
µl	: Microliter
µM	: Micromole
ALP	: Alkaline phosphatase
ALT	: Alanine transaminase
AMPK	: AMP-activated protein kinase
ANOVA	: Analysis of variance
AP-1	: Activator protein
APS	: Ammonium persulfate
ARE	: Antioxidant response element
AST	: Aspartate aminotransferase
ATP	: Adenosine triphosphate
BCIP	: 5-bromo-4-chloro-3-indolyl-phosphate
BMI	: Body mass index
BMPBI	: BMP and activin membrane-bound inhibitor
BSA	: Bovine serum albumin
BWT/ b.w.	: Body weight
cAMP	: Cyclic adenosine 3',5'-monophosphate
CAT	: Catalase
CHF	: Congestive heart failure
CK	: Creatine kinase
CK-MB	: Creatine kinase-myocardial band
COPD	: Chronic obstructive pulmonary diseases
CRP	: C-reactive Protein
C_T	: Cycle Threshold
CTGF	: Connective tissue growth factor

cTnI	: Cardiac troponin I
cTnT	: Cardiac troponin T
CVDs	: Cardiovascular diseases
DAB	: Diaminobenzidine tetrahydrochloride
DCM	: Dilated cardiomyopathy
DEPC	: Diethyl pyrocarbonate
DF	: Dilution factor
dl	: Deciliter
Dlk-1	: Delta-like homologue-1
DMSO	: Dimethyl Sulfoxide
DTNB	: 5,5'-dithiobis-(2-nitrobenzoic acid)
DW	: Distilled water
E/EC	: Molar extinction coefficient
ECM	: Extracellular matrix
ELISA	: Enzyme linked immunosorbent assay
ERK	: Extracellular regulated protein kinases
et al	: Et alli/alia
etc.	: And so forth
FAS	: Fatty acid synthase
FDA	: Food and Drug Administration
Fig.	: Figure
FLS	: Fibroblast-like synoviocytes
g/gm	: Gram
GAPDH	: Glyceraldehyde-3-phosphate dehydrogenase
GGT	: Gamma-glutamyltransferase
GPx	: Glutathione peroxidase
GR	: Glutathione reductase
GSH	: Reduced glutathione
GST	: Glutathione-S-transferase
H&E	: Hematoxylin and eosin
h/hr	: Hour
H ₂ O ₂	: Hydrogen peroxide
HCl	: Hydrochloric acid
HDL-c	: High-density lipoprotein cholesterol
HO-1	: Heme oxygenase-1
HWT	: Heart weight
i.e.	: That is
I.P.	: Intraperitoneally

IAEC	: Institutional Animal Ethics Committee
IL-6	: Interleukin-6
ISP	: Isoprenaline
Keap 1	: Kelch-like ECH-associated protein 1
Kg	: Kilogram
L/l	: Liter
LDH	: Lactate dehydrogenase
LDL-c	: Low-density lipoprotein cholesterol
LPO	: Lipid peroxidation
M	: Moles
mA	: Milliamperes
MAPK	: Mitogen-activated protein kinases
MDA	: Malondialdehyde
mg	: Milligram
Min	: Minute
ml	: Milliliter
mM	: Millimole
mm	: Millimeter
MMLV	: Moloney Murine Leukemia Virus
mmol	: Millimoles
MMPs	: Matrix metalloproteinases
mRNA	: Messenger Ribonucleic Acid
mTOR	: Mammalian target of rapamycin
MTS	: Masson trichrome stain
MTT	: 3-(4-5 dimethyl-thiazol-2-yl) 2,5diphenyltetrazolium bromide
n	: No. of animals
N	: Normal solution
NAFLD	: Non-alcoholic fatty liver disease
NASH	: Non-alcoholic steatohepatitis
NBT	: Nitro blue tetrazolium
NEN	: Niclosamide ethanolamine
NFW	: Nuclease free water
NF- κ β	: Nuclear factor -kappa β
ng	: Nanogram
nm	: Nanometer
nmol	: Nanomole
NPC1L1	: Niemann-Pick C1-like 1

Nrf2	: Nuclear factor erythroid 2-related factor
O	: Orlistat
OD	: Optical density
OTC	: Over-the-counter
PBS	: Phosphate buffer saline
PCR	: Polymerase chain reaction
pg	: Picogram
pH	: Negative logarithm of hydrogen ion
PI3K	: Phosphatidylinositol-3-kinase
pmol	: picomoles
PVDF	: Polyvinylidene fluoride
ROS	: Reactive oxygen species
rpm	: Revolution per minute
RT-PCR	: Real-Time polymerase chain reaction
S.E.	: Mean standard error
S/C	: Subcutaneously
SABC	: HRP-Streptavidin Conjugate
SBE	: Smad binding element
SDS	: Sodium dodecyl sulfate
Smads	: Sma and Mad-related proteins
SOD	: Superoxide dismutase
SST	: Serum separation tubes
STAT3	: Signal transducers and activators of transcription 3
T2DM	: Diabetes mellitus type 2
TAC	: Total antioxidant capacity
TAK1	: TGF β -activated kinase 1
TBA	: Thiobarbituric acid
TBST	: Tris-buffered saline with Tween-20
TCA	: Trichloroacetic acid
TEMEM	: N,N,N',N'-tetramethylethylenediamine
TGF- β	: Transforming growth factor- β
TIMP	: Tissue inhibitors of matrix metalloproteinases
TL	: Tibial length
TMB	: 3,3',5,5'-tetramethylbenzidine
TNF- α	: Tumor necrosis factor- α
T β RI	: TGF- β receptor I
T β RII	: TGF- β receptor II
U	: Units

V	: Volts
v/v	: Volume per volume
VEGF	: Vascular endothelial growth factor
viz.,	: “that is to say”/namely
VLDL-c	: Very low-density lipoprotein cholesterol
vs	: Versus
WHO	: World Health Organization
α -SMA	: α -smooth muscle actin
Δ OD	: Change in absorbance per minute
μ m	: Micrometer

LIST OF TABLES

Table No.	Title	On/After Page No.
Table 3.1	List of chemicals and reagents used for different experimental protocols	33
Table 3.2	Solutions of isoprenaline, orlistat and niclosamide ethanolamine (NEN) prepared for the administration in experimental mice	36
Table 3.3.1	Phosphate buffer saline (pH-7.4)	37
Table 3.3.2	Phosphate buffer 50 mM (pH-7.0)	37
Table 3.3.3	Acetate citrate buffer (pH -6.5)	37
Table 3.3.4	5X Laemmli sample buffer	37
Table 3.3.5	Running buffer for western blotting and gelatin zymography	38
Table 3.3.6	Transfer buffer for western blotting	38
Table 3.3.7	Developing buffer for nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (pH-9.5)	38
Table 3.3.8	Developing buffer for gelatin zymography (pH-7.4)	38
Table 3.4a	Experimental design followed for orlistat study	39
Table 3.4b	Experimental design followed for niclosamide ethanolamine (3 mg/kg body weight; I.P.) study	40
Table 3.5	Standards preparation for protein estimation by Bradford method	56
Table 3.6	Description of primers used	71
Table 3.7	Composition of RT-PCR mixture	72
Table 3.8	Thermal cycling conditions	72
Table 4.1:	Body weight (g) at day zero and day 15 th in mice of control, orlistat- alone, isoprenaline-induced cardiac injury, and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	75
Table 4.2:	Percentage change in body weights during experimental period of the mice of control, orlistat-alone, isoprenaline-induced cardiac injury, and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	76

Table No.	Title	On/After Page No.
Table 4.3:	Absolute heart weight of the control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	77
Table 4.4:	Heart weight to body weight ratio (HWT/BWT) of the control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	77
Table 4.5:	Heart weight to tibial length ratio (HWT/TL) of the control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	78
Table 4.6:	Serum CK-MB level in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	79
Table 4.7:	Serum LDH level in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	79
Table 4.8:	Serum cholesterol level in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups.	80
Table 4.9:	Serum triglyceride level in control, orlistat alone, isoprenaline alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	81
Table 4.10:	Serum HDL-c level in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	81
Table 4.11:	Serum LDL-c level in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	82
Table 4.12:	Serum HDL-c/LDL-c ratio in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	83
Table 4.13:	Serum CRP level in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	83

Table No.	Title	On/After Page No.
Table 4.14:	Serum SOD activity in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups.	84
Table 4.15:	Serum catalase activity in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	85
Table 4.16:	Fold change of serum GSH in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	86
Table 4.17:	Serum MDA level in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups of mice.	86
Table 4.18:	Heart tissue SOD activity in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	87
Table 4.19:	Catalase activity in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	88
Table 4.20:	Fold change of non-enzymatic antioxidant, GSH in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	89
Table 4.21:	MDA level in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups of mice.	89
Table 4.22:	Hydroxyproline level in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	90
Table 4.23:	Glucosamine level in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	91

Table No.	Title	On/After Page No.
Table 4.24:	Collagen type I content of heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	92
Table 4.25:	CTGF in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups.	92
Table 4.26:	Fold change of MMP-2 activity in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat-administered cardiac injury (orlistat+ISP) groups of mice.	93
Table 4.27:	Fold change of MMP-9 activity in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	94
Table 4.28:	Fold change of Nrf-2 protein expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	94
Table 4.29:	Fold change of NF- κ B protein expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups of mice.	95
Table 4.30:	Comparative fold change in TGF- β mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	96
Table 4.31:	Comparative fold change in Smad-3 mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	97
Table 4.32:	Comparative fold change in α -SMA mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat-administered cardiac injury (orlistat+ISP) groups of mice.	98
Table 4.33:	Comparative fold change in collagen type I mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat-administered cardiac injury (orlistat+ISP) groups of mice.	98

Table No.	Title	On/After Page No.
Table 4.34:	Comparative fold change in TNF- α mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	99
Table 4.35:	Comparative fold change in IL-6 mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	100
Table 4.36:	Comparative fold change in collagen type III mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	101
Table 4.37:	Comparative fold change in CTGF mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	102
Table 4.38:	Total histopathological scoring of inflammation and fibrosis in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.	103
Table 4.39:	Body weight (g) at day zero and day 15 th in mice of control, niclosamide ethanolamine-alone, isoprenaline-induced cardiac injury, and niclosamide ethanolamine administered with isoprenaline (NEN+ISP) groups.	103
Table 4.40:	Absolute heart weight of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	104
Table 4.41:	Heart weight to body weight ratio (HWT/BWT) of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	105
Table 4.42:	Heart weight to tibial length ratio (HWT/TL) of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	106

Table No.	Title	On/After Page No.
Table 4.43:	Serum CK-MB level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	107
Table 4.44:	Serum LDH level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	107
Table 4.45:	Serum cholesterol level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	108
Table 4.46:	Serum triglyceride level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	109
Table 4.47:	Catalase activity in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	110
Table 4.48:	Fold change of non-enzymatic antioxidant, GSH in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	110
Table 4.49:	MDA level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	110
Table 4.50:	Hydroxyproline level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	112
Table 4.51:	Glucosamine level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	113

Table No.	Title	On/After Page No.
Table 4.52:	Body weight (g) at day zero and day 15 th in mice of control, niclosamide ethanolamine (3 mg/kg body weight; I.P)-alone, isoprenaline-induced cardiac injury, and niclosamide ethanolamine (3 mg/kg body weight; I.P.)-administered with isoprenaline (NEN+ISP) groups.	113
Table 4.53:	Percentage change in body weights during experimental period of the mice of control, niclosamide ethanolamine-alone, isoprenaline-induced cardiac injury, and niclosamide ethanolamine-administered with isoprenaline (NEN+ISP) groups of mice.	114
Table 4.54:	Absolute heart weight of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	115
Table 4.55:	Heart weight to body weight ratio (HWT/BWT) of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	116
Table 4.56:	Heart weight to tibial length ratio (HWT/TL) of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	117
Table 4.57:	Serum CK-MB level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	117
Table 4.58:	Serum LDH level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	118
Table 4.59:	Serum cholesterol level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	119

Table No.	Title	On/After Page No.
Table 4.60:	Serum triglyceride level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	120
Table 4.61:	Serum HDL-c level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	120
Table 4.62:	Serum LDL-c level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	121
Table 4.63:	Serum HDL-c/LDL-c ratio in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	122
Table 4.64:	Serum CRP level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	123
Table 4.65:	Serum SOD activity in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	124
Table 4.66:	Serum catalase activity in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	124
Table 4.67:	Fold change of serum GSH in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	125
Table 4.68:	Serum MDA level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	126
Table 4.69:	Heart tissue SOD activity in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	127

Table No.	Title	On/After Page No.
Table 4.70:	Catalase activity in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	128
Table 4.71:	Fold change of non-enzymatic antioxidant, GSH in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	129
Table 4.72:	MDA level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	129
Table 4.73:	Hydroxyproline level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	130
Table 4.74:	Glucosamine level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	131
Table 4.75:	Fold change of collagen type I content of heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	132
Table 4.76:	CTGF in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	132
Table 4.77:	Fold change of MMP-2 activity in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	133

Table No.	Title	On/After Page No.
Table 4.78:	Fold change of MMP-9 activity in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine- administered cardiac injury (NEN+ISP) groups of mice.	134
Table 4.79:	Fold change of Nrf-2 protein expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	135
Table 4.80:	Fold change of NF- κ B protein expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	136
Table 4.81:	Comparative fold change in TGF- β mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	137
Table 4.82:	Comparative fold change in Smad-3 mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	138
Table 4.83:	Comparative fold change in α -SMA mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	139
Table 4.84:	Comparative fold change in TNF- α mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	140
Table 4.85:	Comparative fold change in IL-6 mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	141

Table No.	Title	On/After Page No.
Table 4.86:	Comparative fold change in collagen type III mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	142
Table 4.87:	Total histopathological scoring of inflammation and fibrosis in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice.	143

LIST OF FIGURES

Figure No.	Title	On/After Page No.
Fig. 4.1.	Bar diagram showing the change in body weight on day zero and day 15 th in mice of different groups.	76
Fig. 4.2.	Bar diagram showing the percentage change in body weight in different groups of mice during the experimental period.	76
Fig. 4.3.	Bar diagram showing absolute heart weight of control, orlistat-alone, ISP-alone and orlistat+ISP groups in mice.	78
Fig. 4.4.	Bar diagram showing the heart weight to body weight ratio (HWT/BWT) of control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	78
Fig. 4.5.	Bar diagram showing the heart weight to tibial length (HWT/TL) ratio of control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	80
Fig. 4.6.	Bar diagram showing serum CK-MB level of control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	80
Fig. 4.7.	Bar diagram showing serum LDH level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	80
Fig. 4.8.	Bar diagram showing the serum cholesterol level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	80
Fig. 4.9.	Bar diagram showing the serum triglyceride level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	82
Fig. 4.10.	Bar diagram showing the serum HDL-c in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	82
Fig. 4.11.	Bar diagram showing the serum LDL-c in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	84
Fig. 4.12.	Bar diagram showing the serum HDL-c/LDL-c-ratio in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	84
Fig. 4.13.	Bar diagram showing the serum CRP level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	84

Figure No.	Title	On/After Page No.
Fig. 4.14.	Bar diagram showing the SOD activity in serum in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	84
Fig. 4.15.	Bar diagram showing the serum catalase activity in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	86
Fig. 4.16.	Bar diagram showing the serum reduced glutathione (GSH) level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	86
Fig. 4.17.	Bar diagram showing the serum MDA level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	88
Fig. 4.18.	Bar diagram showing the SOD activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	88
Fig. 4.19.	Bar diagram showing the catalase activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	90
Fig. 4.20.	Bar diagram showing the reduced glutathione (GSH) level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	90
Fig. 4.21.	Bar diagram showing the MDA level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	90
Fig. 4.22.	Bar diagram showing the hydroxyproline level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	90
Fig. 4.23.	Bar diagram showing the fold change in glucosamine level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	92
Fig. 4.24.	Bar diagram showing collagen type I of heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	92
Fig. 4.25.	Bar diagram showing the CTGF level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	92
Fig. 4.26a.	Bar diagram showing the fold change in MMP-2 activity in heart in control, orlistat-alone, ISP alone and orlistat+ISP groups of mice.	94
Fig. 4.26b.	Representative images of gel (i & ii) showing MMP-2 activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	94
Fig. 4.27a.	Bar diagram showing the fold change in MMP-9 activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice	94

Figure No.	Title	On/After Page No.
Fig. 4.27b.	Representative image of gel showing MMP-9 activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	94
Fig. 4.28a.	Bar diagram showing the fold change in Nrf-2 protein expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	94
Fig. 4.28b.	Representative images of blot showing Nrf-2 (i & ii) and GAPDH (iii & iv) protein expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	94
Fig. 4.29a.	Bar diagram showing the fold change in NF-κB protein expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	96
Fig. 4.29b.	Representative images of blot showing NF-κB (i & ii) and GAPDH (iii & iv) protein expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	96
Fig. 4.30.	Bar diagram showing the comparative fold change in TGF-β mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	96
Fig. 4.31.	qRT-PCR melt curve plot (a), amplification plot (b) and agarose gel electrophoresis image of PCR product (c) of GAPDH mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	96
Fig. 4.32	qRT-PCR melt curve plot (a), amplification plot (b) and agarose gel electrophoresis images (c) of TGF-β mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	96
Fig. 4.33.	Bar diagram showing the comparative fold change in Smad-3 mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	98
Fig. 4.34.	qRT-PCR melt curve plot (a), amplification plot (b) and representative image of agarose gel electrophoresis (c) of Smad-3 mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	98
Fig. 4.35.	Bar diagram showing the comparative fold change in α-SMA mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	98

Figure No.	Title	On/After Page No.
Fig. 4.36.	qRT-PCR melt curve plot (a), amplification plot (b) and representative image of agarose gel electrophoresis (c) of α -SMA mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	98
Fig. 4.37.	Bar diagram showing the comparative fold change in collagen type I mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	100
Fig. 4.38.	qRT-PCR melt curve plot (a), amplification plot (b) and representative image of agarose gel electrophoresis (c) of collagen type I mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	100
Fig. 4.39.	Bar diagram showing the comparative fold change in TNF- α mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	100
Fig. 4.40.	qRT-PCR melt curve plot (a) and amplification plot (b) of TNF- α mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	100
Fig. 4.41.	Bar diagram showing the comparative fold change in IL-6 mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	100
Fig. 4.42.	qRT-PCR melt curve plot (a) and amplification plot (b) of IL-6 mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	100
Fig. 4.43.	Bar diagram showing the comparative fold change in collagen type III mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	102
Fig. 4.44.	qRT-PCR melt curve plot (a) and amplification plot (b) of collagen type III mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	102
Fig. 4.45.	Bar diagram showing the comparative fold change in CTGF mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	102
Fig. 4.46.	qRT-PCR melt curve plot (a) and amplification plot (b) of CTGF mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	102

Figure No.	Title	On/After Page No.
Fig. 4.47.	Bar diagram showing the total histopathological scoring of inflammation and fibrosis in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.	104
Fig. 4.48.	Effect of orlistat on histopathology (H & E staining) of heart tissue in ISP-induced cardiac fibrosis in mice.	104
Fig. 4.49.	Effect of orlistat on histopathology (Masson's Trichrome staining) of heart tissue in ISP-induced cardiac fibrosis in mice.	104
Fig. 4.50.	Bar diagram showing the change in body weight on day zero and day 15 th in mice of different groups.	104
Fig. 4.51.	Bar diagram showing absolute heart weight of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	104
Fig. 4.52.	Bar diagram showing the heart weight to body weight ratio (HWT/BWT) of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	106
Fig. 4.53.	Bar diagram showing heart weight to tibial length (HWT/TL) ratio of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	106
Fig. 4.54.	Bar diagram showing serum CK-MB level of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	108
Fig. 4.55.	Bar diagram showing serum LDH level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	108
Fig. 4.56.	Bar diagram showing the serum cholesterol level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	110
Fig. 4.57.	Bar diagram showing the serum triglyceride level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	110
Fig. 4.58.	Bar diagram showing the catalase activity in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	110

Figure No.	Title	On/After Page No.
Fig. 4.59.	Bar diagram showing the reduced glutathione (GSH) level in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	110
Fig. 4.60.	Bar diagram showing the MDA level in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	112
Fig. 4.61.	Bar diagram showing the hydroxyproline level in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	114
Fig. 4.62.	Bar diagram showing the fold change in glucosamine level in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	114
Fig. 4.63.	Bar diagram showing the change in body weight on day zero and day 15 th in mice of different groups.	114
Fig. 4.64.	Bar diagram showing the percentage change in body weight in different groups of mice during the experimental period.	114
Fig. 4.65.	Bar diagram showing absolute heart weight of control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	116
Fig. 4.66.	Bar diagram showing the heart weight to body weight ratio (HWT/BWT) of control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	116
Fig. 4.67.	Bar diagram showing heart weight to tibial length (HWT/TL) ratio of control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	118
Fig. 4.68.	Bar diagram showing serum CK-MB level of control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	118
Fig. 4.69.	Bar diagram showing serum LDH level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	118
Fig. 4.70.	Bar diagram showing the serum cholesterol level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	120
Fig. 4.71.	Bar diagram showing the serum triglyceride level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	120

Figure No.	Title	On/After Page No.
Fig. 4.72.	Bar diagram showing the serum HDL-c in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	122
Fig. 4.73.	Bar diagram showing the serum LDL-c in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	122
Fig. 4.74.	Bar diagram showing the serum HDL-c/LDL-c ratio in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	124
Fig. 4.75.	Bar diagram showing the serum CRP level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	124
Fig. 4.76.	Bar diagram showing the SOD activity in serum in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	124
Fig. 4.77.	Bar diagram showing the serum catalase activity in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	124
Fig. 4.78.	Bar diagram showing the serum reduced glutathione (GSH) level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	126
Fig. 4.79.	Bar diagram showing the serum MDA level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	126
Fig. 4.80.	Bar diagram showing the SOD activity in heart tissue in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	128
Fig. 4.81.	Bar diagram showing the catalase activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	128
Fig. 4.82.	Bar diagram showing the reduced glutathione (GSH) level in heart tissue in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	130
Fig. 4.83.	Bar diagram showing the MDA level in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	130
Fig. 4.84.	Bar diagram showing the hydroxyproline level in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	132
Fig. 4.85.	Bar diagram showing the fold change in glucosamine level in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	132
Fig. 4.86.	Bar diagram showing collagen type I of heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	132

Figure No.	Title	On/After Page No.
Fig. 4.87.	Bar diagram showing the fold change in CTGF level in heart tissue in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	132
Fig. 4.88a.	Bar diagram showing the fold change in MMP-2 activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	134
Fig. 4.88b.	Representative images of gel (i & ii) showing MMP-2 activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	134
Fig. 4.89a.	Bar diagram showing the fold change in MMP-9 activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	134
Fig. 4.89b.	Representative image of gel showing MMP-9 activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	134
Fig. 4.90a.	Bar diagram showing the fold change in Nrf-2 protein expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	136
Fig. 4.90b.	Representative immunoblot images showing Nrf-2 (i & ii) and GAPDH (iii & iv) protein expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	136
Fig. 4.91a.	Bar diagram showing the fold change in NF- κ B protein expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	136
Fig. 4.91b.	Representative image of immunoblot showing NF- κ B (i & ii) and GAPDH (iii & iv) protein expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	136
Fig. 4.92.	Bar diagram showing the comparative fold change in TGF- β mRNA expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	138
Fig. 4.93.	qRT-PCR melt curve plot (a) and amplification plot (b) of GAPDH mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	138
Fig. 4.94.	qRT-PCR melt curve plot (a) and amplification plot (b) of TGF- β mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	138

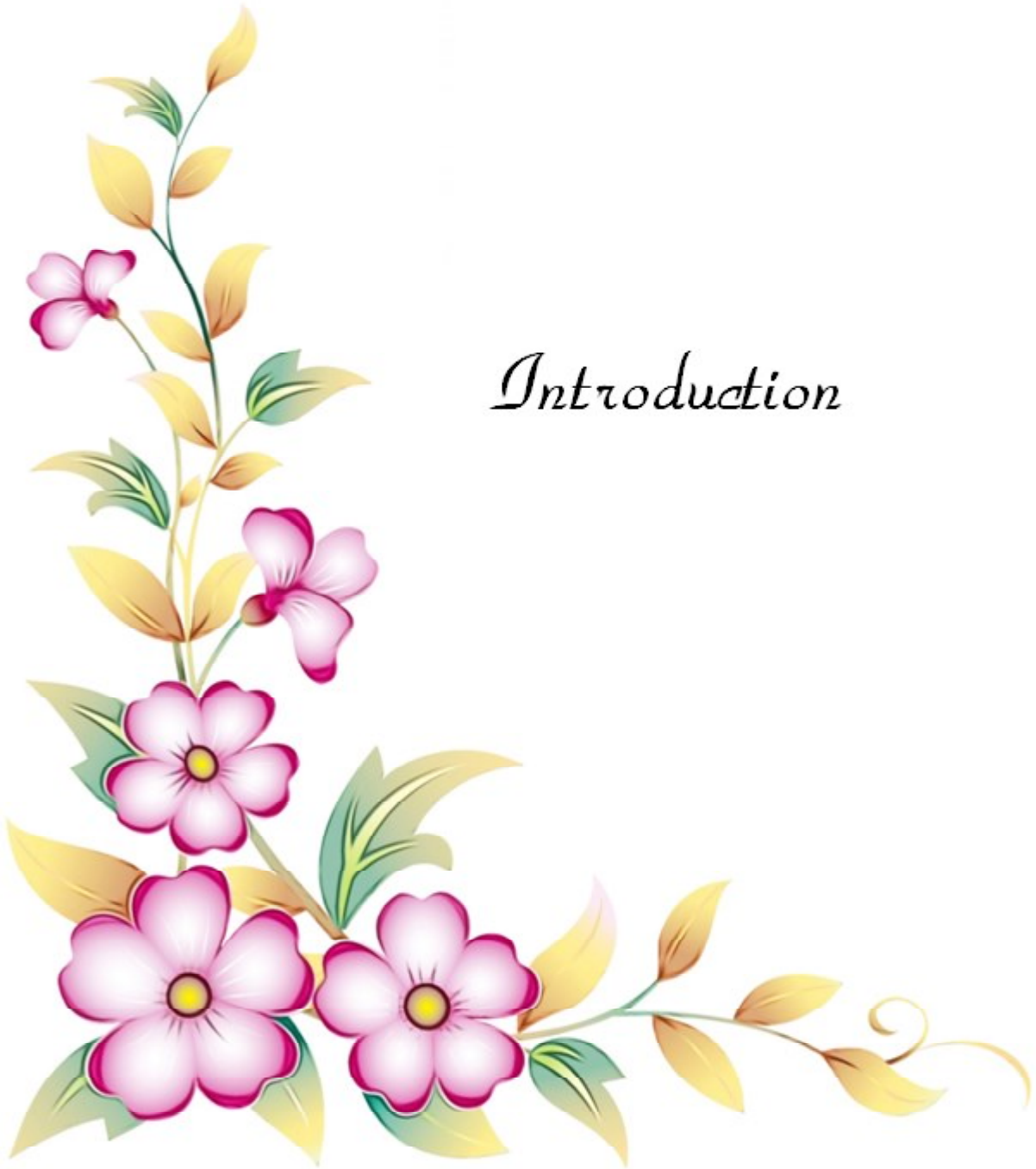
Figure No.	Title	On/After Page No.
Fig. 4.95.	Bar diagram showing the comparative fold change in Smad-3 mRNA expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice.	138
Fig. 4.96.	qRT-PCR melt curve plot (a) and amplification plot (b) of Smad-3 mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	138
Fig. 4.97.	Bar diagram showing the comparative fold change in α -SMA mRNA expression in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	140
Fig. 4.98.	qRT-PCR melt curve plot (a) and amplification plot (b) of α -SMA mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	140
Fig. 4.99.	Bar diagram showing the comparative fold change in TNF- α mRNA expression in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	140
Fig. 4.100.	qRT-PCR melt curve plot (a) and amplification plot (b) of TNF- α mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	140
Fig. 4.101.	Bar diagram showing the comparative fold change in IL-6 mRNA expression in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	143
Fig. 4.102.	qRT-PCR melt curve plot (a) and amplification plot (b) of IL-6 mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	143
Fig. 4.103.	Bar diagram showing the comparative fold change in collagen type III mRNA expression in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	143
Fig. 4.104.	qRT-PCR melt curve plot (a) and amplification plot (b) of collagen type III mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	143
Fig. 4.105.	Bar diagram showing the total histopathological scoring of inflammation and fibrosis in heart in control, niclosamideethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.	143

Figure No.	Title	On/After Page No.
Fig. 4.106.	Effect of niclosamide ethanolamine (NEN) on histopathology (H & E staining) of heart tissue in ISP-induced cardiac fibrosis in mice.	143
Fig. 4.107.	Effect of niclosamide ethanolamine (NEN) on histopathology (Masson's Trichrome staining) of heart tissue in ISP-induced cardiac fibrosis in mice.	143
Fig. 5	Mechanistic signaling of orlistat-treated cardiac fibrosis	167
Fig. 6	Mechanistic signaling of niclosamide ethanolamine (NEN)-treated cardiac fibrosis	167

CONTENTS

Sl. No.	CHAPTER	PAGE NO.
1.	INTRODUCTION	01-09
2.	REVIEW OF LITERATURE	10-32
3.	MATERIALS AND METHODS	33-74
4.	RESULTS	75-143
5.	DISCUSSION	144-158
6.	SUMMARY AND CONCLUSIONS	159-167
7.	MINI ABSTRACT	168
8.	HINDI ABSTRACT	169
9.	REFERENCES	170-201

Introduction



Disease conditions affecting heart and vessels of the body are known as cardiovascular diseases (CVDs). These diseases have emerged as a global concern due to their high prevalence, morbidity, disability, and mortality and causing high economic losses (Mensah *et al.*, 2019). According to Stewart and Co-workers (2017), in developing countries, the sedentary lifestyle has introduced novel risk factors for cardiovascular diseases, resulting in a cardiovascular disease risk boom throughout the developing world. CVDs have an impact on human beings as well as animals and major cardiovascular disease risk factors are age, diet, and pathological conditions in them (Urfer *et al.*, 2017). CVDs caused nearly 17.9 million deaths in 2019, which represents 32% of all fatalities (WHO, 2021). The prevalence of cardiovascular disease in India in 2016 was estimated to be approximately, 54.5 million (Maini *et al.*, 2021). Nowadays, one of every four fatalities in India is due to CVDs. Cardiac ischemia and stroke causes >80% of this burden (Abdul-Aziz *et al.*, 2019).

Cardiac diseases affect 10-15% of all dogs (MacPete, 2018; Hoque *et al.*, 2019). Dilated cardiomyopathy is common cardiac disease in dogs and characterized by dilatation of the heart chamber (atrial and ventricular) and diastolic and systolic myocardial dysfunctions (Borgarelli *et al.*, 2001). In medium and large-size dog breeds, idiopathic dilated cardiomyopathy is the leading cause of congestive heart failure (CHF) and sudden cardiac death. DCM is more prevalent in some breeds of dogs such as Boxer, Cocker Spaniel, Doberman Pinscher, Irish Wolfhound, Great Dane, and Portuguese waterdog (Tidholm *et al.*, 2001; Broschk and Distl, 2005). Hypertrophic cardiomyopathy is often occurring cardiac disease affecting approximately 15% of the general feline population (Fuentes *et al.*, 2020).

According to Alexandersen and co-workers (2003), in cattle cardiac diseases are rare, but myocarditis developed in FMD causes high mortality in affected calves.

According to Berenji and co-workers (2005), cardiac hypertrophy is an important adaptive response of cardiomyocytes to hemodynamic stress and exerts a compensatory role to preserve cardiac functions by enhancing heart performance and reducing the wall tension of ventricles and oxygen consumption. The body's heart reacts to hemodynamic overload by hypertrophy to improve the contractility and lower the stress of the ventricular wall, however, this adaptive hypertrophy eventually results in heart failure due to pathological remodeling (Nakamura and Sadoshima, 2018). Among various cardiovascular conditions, myocardial fibrosis and ischemia are commonly responsible for cardiovascular dysfunctions which lead to mortality in cardiac patients (Allawadhi *et al.*, 2018). Myocardial hypertrophy is a crucial element in the cardiac remodeling process which includes hypertrophic expansion of cardiomyocytes as well as proliferation and activation of interstitial fibroblasts resulting in cardiac fibrosis (Ge *et al.*, 2021).

Cardiac fibrosis is defined as the excessive build-up of extracellular matrix (ECM) proteins such as collagen and fibronectin in the interstitial space of cardiac tissue due to an imbalance between the formation and breakdown of extracellular matrix (Kong *et al.*, 2014). It is mainly characterized by both thickening and scarring of cardiac tissue due to abnormal proliferation and deposition of fibroblast cells of the heart, resulting in a distorted shape and cardiac dysfunctions (Krenning *et al.*, 2010). Reactive fibrosis is caused by a discrepancy in the metabolism of ECM proteins, which results in an excess of fibrillar collagen accumulating in the perivascular, interstitial, and coronary arteries. In reparative fibrosis, the deposition of extracellular fibrotic proteins leads to cardiac stiffness that exerts a negative impact on the pumping action of a hypertrophied heart and is responsible for ventricular dysfunction. Reparative-type fibrosis occurs after the necrosis of cardiomyocytes, which stimulates the growth and proliferation of fibroblasts along with enhancing the synthesis of collagen (Allawadhi *et al.*, 2018).

Type I and type III fibrillar collagens are significant elements of the cardiac interstitium. Collagen type I is made up of thick fibres and provides tensile strength, but collagen type III is

made up of fine reticular fibres networks having more distensibility than type I. Type I is the main cause of enhanced cardiac fibrosis (Krenning *et al.*, 2010; Allawadhi *et al.*, 2018). Various stimuli viz mechanical stretch, inflammatory mediators such as tumor necrosis factor- α , interleukin-6, and growth factors (connective tissue growth factors and transforming growth factors- β) stimulate the fibroblast cells of heart to synthesize type I and III collagen along with other less abundant molecules of ECM such as type IV, V, VI collagen, laminin and elastin (Bosman and Stamenkovic, 2003; Krenning *et al.*, 2010). Additionally, the ECM degradation is also regulated by cardiac fibroblasts through modulating the MMPs (matrix metalloproteinases) and their natural tissue inhibitors expression. Angiotensin II, nor-adrenaline, and endothelin-1 are some important pro-fibrotic factors that derive fibrosis by regulating the synthesis of collagen along with the activity of MMP/TIMP (Tsuruda *et al.*, 2004).

During the cardiac remodeling process, cardiac muscle cell death due to hypoxia has triggered the induction of an inflammatory response (Frangogiannis, 2014). Inflammatory, proliferative, and maturation phases are the overlapping phases in cardiac remodeling and fibrosis (Rai *et al.*, 2017). After an injury to the cardiomyocytes, firstly the blood neutrophils are arrived at the site of injury and secrete chemokines that cause infiltration of monocytes. By phagocytosis, they digest the injured tissue and eliminate the cellular debris (Bujak and Frangogiannis, 2007). Afterward, active suppression of the inflammatory phase is followed by the preparation of a site for the proliferative phase. Various cells viz lymphocytes, endothelial progenitor cells, mast cells, and fibroblasts infiltrate at the site during the proliferative phase. In the proliferative phase, growth factors are secreted by macrophages and mononuclear cells which help in reparative cells like fibroblast and myofibroblast recruitment and activation (Frangogiannis, 2014). Additionally, the ECM is generated by the activated myofibroblasts, which provide support to the newly synthesized micro-vasculature. A scar with cross-linked collagen bundles, myofibroblast stem cells, and newly created vasculature forms during the maturation phase (Bujak and Frangogiannis, 2007; Bujak *et al.*, 2007). Scar formation and maturation impair cardiac contractility, leading to ventricular remodeling and heart failure and also impairment of electrical signaling synchronicity, which may lead to arrhythmias (Miragoli *et al.*, 2007; Czubryt, 2012).

The fibrotic response is triggered when the magnitude of myocardial as well as circulating cytokines and pro-fibrotic growth factors rise in response to stimuli (Kong *et al.*, 2014). After binding with receptors, these pro-fibrotic growth factors as well as cytokines begin the process of activating various signaling pathways and associated transcriptional factors such as Smad, protein kinase B, mitogen-activated protein kinases (MAPK), and nuclear factor -kappa β (NF- $\kappa\beta$). These activated pathways and transcriptional factors cause trans-differentiation of fibroblast cells into myofibroblasts, which express α -SMA (alpha-smooth muscle actin) and produce various metalloproteinases of matrix and their tissue inhibitors in ECM. These growth factors of fibrosis and cytokines also amplify the fibrotic response by positive feedback mechanism to fibroblast and cardiomyocytes (Kong *et al.*, 2014; Frieler and Mortensen, 2015).

Overproduction of reactive oxygen species (ROS) and insufficient endogenous antioxidant defense in response to stimuli may produce oxidative stress. ROS activation is attributed via growth factors and neuro-humoral signals such as angiotensin II, isoprenaline (ISP), aldosterone, and transforming growth factor- β (TGF- β) and produces cardiac fibrosis (Zhao *et al.*, 2008; Lijnen *et al.*, 2012). ROS activation triggers a wide range of fibrogenic signaling such as inflammatory cytokines, stress-responsive protein kinases, and a variety of transcription factors, including nuclear factor-k β (NF-k β) (Siwik and Colucci, 2004).

In response to the ischemic injury, various pro-inflammatory cytokines (tumor necrosis factor- α , and interleukin-6), and pro-fibrotic growth factors (TGF- β , CTGF) are produced by infiltrated monocyte and macrophage cells (Nahrendorf *et al.*, 2007; Kong *et al.*, 2014). Cytokines, that have a direct influence on fibroblasts, activate mononuclear cells, fibroblast progenitors and modulate angiogenesis, and may play a pivotal role in the fibrotic process (Dobaczewski and Frangogiannis, 2009). Interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) are pro-inflammatory cytokines, that play a crucial role in cardiac fibrosis by acting on resident cardiac fibroblasts and matrix protease cascades (Frangogiannis, 2021).

According to Bujak and Frangogiannis (2007), in cardiac fibrosis, one of the best-known fibrogenic growth factors is TGF- β . Activated TGF- β is an important mediator for

alteration in the fibroblast gene expression and phenotype. It enhances the accumulation of ECM proteins by up-regulating the synthesis of collagen and fibronectin and lowering the matrix breakdown by inducing protease inhibitors. Furthermore, CTGF (connective tissue growth factor) is also induced by TGF- β , which enhances cardiac hypertrophy and fibrosis by acting synergistically (Panek *et al.*, 2009).

Three isoforms of TGF- β viz TGF- β 1, TGF- β 2, and TGF- β 3 are present in mammals (Bujak and Frangogiannis, 2007; Dobaczewski *et al.*, 2011). Among these, the most prominent form is TGF- β 1. The binding of TGF- β with TGF- β receptor I (T β RI, sometimes called ALK5) and TGF- β receptor II (T β RII) activates the Smad2/3 signaling, leading to cardiac fibrosis process (Shi and Massague, 2003). Activation of TGF- β receptors by binding with the TGF- β leads to the recruitment and activation of Sma and Mad-related proteins (Smads), especially Smad 2 and Smad 3. These Smad 2 and 3 are released from the receptor and form a complex with Smad 4. Afterward, this complex is moved to the nucleus and modulates the target gene transcription (Ma *et al.*, 2018).

Beta-adrenoceptor is an essential regulator of heart function. During stressful conditions, the level of endogenous catecholamine is elevated to generate a short-term adaptative response known as a “fight-or-flight response” (Bers and Despa, 2009). Sustained stimulation of adrenoceptors by elevated levels of circulating catecholamine is linked to maladaptive cardiac remodeling, including myocardial hypertrophy, cardiac fibrosis, and apoptosis, which eventually leads to heart failure (Fu *et al.*, 2012). Increased energy and oxygen consumption, as well as oxidative stress-mediated cardiac damage, are caused by chronic activation of the β -adrenoceptors (Meng *et al.*, 2006; Zhu *et al.*, 2003). Isoprenaline (ISP) is a non-selective synthetic β -receptor agonist. Spontaneous auto-oxidation of catecholamine (isoprenaline) forms a toxic compound amino-chrome and causes oxidative stress by generating free radicals, leading to mitochondrial damage, changes in biochemical markers in the heart, and eventually resulting in myocardial injury. Therefore, this property of isoprenaline is employed as an experimental screening paradigm for the research of cardioprotective action of a variety of test compounds (Allawadhi *et al.*, 2018).

Over-production of oxidants and free radicals, exceeds the endogenous antioxidant capability of cells, leading to oxidative stress with intense cytotoxic consequences of oxidative damage in lipids, nucleic acids, and proteins of cells (Liaudet *et al.*, 2009; Pacher *et al.*, 2007). Benjamin and co-workers (1989) reported that isoproterenol administration leads to fibroblast proliferation, cardiac enlargement, connective tissue deposition, fibrosis, and myocyte necrosis. In heart-failing conditions, isoprenaline has emerged as a crucial mediator for the synthesis of collagen fragments and it leads to enhanced expression of matrix metalloproteinases (MMPs). In isoproterenol-induced myocardial injury, the expression of collagen type I, matrix metalloproteinases (MMP-2 & MMP-9), α -smooth muscle actin, and TGF- β is up-regulated (Thangaiyan *et al.*, 2020).

MAPK signaling activates the inflammatory nuclear factor- κ B (NF- κ B), which further moves into the nucleus for target gene transcription. Therefore, activated NF- κ B stimulates the production of various cytokines such as tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) and progresses the myocardial injury (Chunhua *et al.*, 2017). In isoprenaline-induced cardiac fibrosis, the expression of α -smooth muscle actin, collagen I, and collagen III is increased through TGF- β 1/Smad signaling (Yang *et al.*, 2021).

Orlistat (tetrahydro-lipstatin) is a semi-synthetic derivative of lipstatin. *Streptomyces toxytricini* naturally produces lipstatin, an irreversible blocker of pancreatic lipase (Barbier and Schneider, 1987). Orlistat is an over-the-counter (OTC) anti-obesity drug approved by the Food and Drug Administration (FDA) in 1999 (Stephanie, 2007). In the gastrointestinal tract, orlistat blocks gastric and pancreatic lipase enzymes by covalently binding with its active serine site and decreases dietary fat absorption (Borgstrom, 1988; Curran and Scott, 2004; Henness and Perry, 2006). It also inhibits cholesterol absorption by inhibiting the transport protein, Niemann-Pick C1-like 1 (NPC1L1) (Alqahtani *et al.*, 2015). Furthermore, fatty acid synthase (FAS) is also inhibited by orlistat, which is mainly upregulated in several tumor cells (Kridel *et al.*, 2004; Kuhajda, 2006). Several pharmacological activities of orlistat, including anti-oxidant, anti-inflammatory, anti-obesity, and anti-fibrotic have been reported.

Khan and co-workers (2017) reported that orlistat treatment for 16 weeks in non-alcoholic fatty liver obese patients reduced the level of triglyceride, total cholesterol, LDL-C,

serum periostin, and TNF- α along with increased serum adiponectin levels. The orlistat has improved the diastolic and systolic blood pressure, lipid profile, and oral glucose tolerance test (Broom *et al.*, 2002; Kiortsis *et al.*, 2005; Swinburn *et al.*, 2005). In the cardiac ischemia/reperfusion injury model, orlistat administration has reduced the occurrence of ventricular arrhythmia, atrioventricular block, as well as serum creatine kinase levels, showing a cardioprotective function to reduce myocardial damage in acute myocardial infarction (Menezes-Rodrigues *et al.*, 2018).

Hussein and co-workers (2007) reported that the orlistat therapy has reduced the levels of LDL-C, aminotransferases, triglycerides, total cholesterol, malondialdehyde (MDA), and insulin resistance, liver fibrosis score, and inflammation in obese patients with non-alcoholic steatohepatitis (NASH). According to Khan and co-workers (2017), orlistat therapy at therapeutic dose has decreased the levels of serum periostin, and TNF-alpha as well as increased serum adiponectin levels with slightly improved fibrosis scores. According to Mullen and co-workers (2007), the pre-treatment of *Burkholderia strains* with orlistat has reduced the microbial invasion in lung epithelial cells, suggesting the possible role of lipase and its inhibitors in cystic fibrosis.

According to Suleiman and co-workers (2020), orlistat has improved the fertility potential in obesity by targeting oxidative stress and lactate metabolism in the testis. Othman and co-workers (2021) have also reported an improved lipid profile, increased aortic anti-oxidant enzymes, total antioxidant capacity, decreased pro-inflammatory markers expression along with increased anti-inflammatory markers, indicating the anti-atherogenic efficacy of orlistat in obesity-mediated oxidative stress in a rat model.

The anti-helminthic medication, niclosamide is used to treat parasite illness all over the world. In 1953, Bayer's chemotherapeutic research laboratories discovered the niclosamide and offered it as a molluscicide (Andrews *et al.*, 1982). In 1982, the US FDA approved niclosamide for tapeworm infection treatment in humans, and it is also included in the essential medicines list by the World Health Organisation (WHO, 2007). Being an uncoupler of oxidative phosphorylation, it disrupts the helminth's energy metabolism by preventing glucose uptake

and adenosine triphosphate production (Frayha *et al.*, 1997; Swan, 1999; Chen *et al.*, 2018). It has potential in various diseases by targeting nuclear factor- κ B, Wnt/ β -catenin, STAT3, and Notch signaling pathways (Chen *et al.*, 2018).

Niclosamide has exhibited hepatoprotective activity against cholestatic liver fibrosis in rat models (Esmail *et al.*, 2021) and a neuroprotective effect (Cerles *et al.*, 2017), by reducing oxidative stress and inflammation. Niclosamide had anti-inflammatory activity in rheumatoid arthritis (Liang *et al.*, 2015), and inflammatory bowel disease patients (Shivaji *et al.*, 2019) by decreasing the cytokine levels as well as the TNF- α -induced downstream signaling.

Niclosamide has shown anti-fibrotic activity in the bleomycin-induced pulmonary fibrosis model by decreasing the migration of fibroblast, suppressing fibrotic signaling, and attenuating the epithelial-to-mesenchymal transition (Boyapally *et al.*, 2019). Furthermore, the suppression of TGF- β 1-mediated transition of the epithelium into mesenchyme, collagen, and extracellular matrix deposition by niclosamide have also been reported (Pei *et al.*, 2022). Additionally, niclosamide has exhibited anti-fibrotic activity against renal fibrosis by targeting NF- κ B and Smad signaling pathways (Chang *et al.*, 2017).

Niclosamide has also shown an anti-fibrotic action against pulmonary fibrosis (Zhang *et al.*, 2018) and cystic fibrosis (Costabile *et al.*, 2015). Niclosamide has also suppressed muscle fibrosis by targeting mammalian target of rapamycin (mTOR), and NF- κ B (Milani *et al.*, 2021). According to Fu and co-workers (2021), niclosamide improved cardiac dysfunction, by stimulating the mitochondrial respiration of cardiac cells and decreasing the inflammatory cytokines in transverse aortic constriction-induced heart failure.

The potential role of niclosamide has also been shown against various conditions, including neuropathic pain (Zhang *et al.*, 2013; Ai *et al.*, 2016), endometriosis (Prather *et al.*, 2016), hypertension (Li *et al.*, 2017), restenosis (Xiao *et al.*, 2018), diabetes (Li *et al.*, 2013; Engin *et al.*, 2021), Parkinson's disease (Barini *et al.*, 2018), and neoplasia (Chen *et al.*, 2018).

Various activities such as anti-oxidant, anti-inflammatory, anti-neoplastic, anti-diabetic, and anti-hyperlipidaemic have been reported for both orlistat and niclosamide. However, to

the best of our knowledge, the effect of orlistat and niclosamide on isoprenaline-induced cardiac fibrosis is not reported. In view of the existing knowledge gap, the present study was designed with the following objectives:

- **To evaluate the cardioprotective potential of orlistat on isoprenaline-induced cardiac fibrosis in a mouse model.**
- **To assess the cardioprotective potential of niclosamide on isoprenaline-induced cardiac fibrosis in a mouse model.**



*Review
of
Literature*



Cardiovascular diseases (CVDs) are still one of the major causes of death globally. These diseases are of global issues due to high prevalence, morbidity, mortality, disability and high economic losses (Mensah *et al.*, 2019). Cardiovascular diseases include cardiomyopathy, coronary heart disease, hypertension, stroke, angina, congestive heart failure, myocardial ischemia, atherosclerosis, cardiac fibrosis, which have unique cellular and molecular aspects (Gotlieb, 2018). The important risk factors for development of cardiovascular diseases are age, diet, sedentary lifestyle, alcohol consumption, sex, and stress (Chiuve *et al.*, 2014). Changed lifestyle experience in developing countries introduces crucial risk factors for the cardiovascular diseases, resulting in cardiovascular diseases risk boom in whole developing world (Stewart *et al.*, 2017). Cardiovascular illness is also caused by a variety of co-morbidities such as diabetes and chronic renal disease. Among different cardiovascular conditions, myocardial ischemia and cardiac fibrosis are mostly responsible for mortalities (Allawadhi *et al.*, 2018). Cardiac failure is a complex condition with disturbed myocardial functioning, ventricular remodeling, neuro-humoral activation, hemodynamics alteration, cytokine overexpression, endothelial and vascular remodeling (Rai *et al.*, 2017).

Epidemiology of Cardiovascular Diseases (CVDs)

According to World Health Organization (WHO), approximately 17.9 million people were died due to CVDs in 2019, which represents 32% of total global deaths. In low- and middle-income nations, more than 75% of all CVD deaths have been documented. In 2019, non-communicable diseases caused 17 million deaths under the age of 70 years and

cardiovascular diseases were accounting for 38% of these deaths (WHO, 2021). Globally, almost 23 million fatalities are predicted to be caused by CVDs by 2030 (Lozano *et al.*, 2012).

According to the India-state-level Disease Burden Initiative CVD Collaborators (2018), the prevalence of cardiovascular disease in India in 2016 was estimated to be 54.5 million. Nowadays, one of every four deaths in India are due to CVDs with ischemic heart disease and stroke responsible for >80% of this burden (Abdul-Aziz *et al.*, 2019). In 2016, in India, contribution of cardiovascular diseases was 28.1 % of total deaths and 14.1 % of total disability-adjusted life years (Ruhil, 2018).

Animals are also affected with cardiovascular diseases. Major risk factor in dogs and human for heart disease is age (Urfer *et al.*, 2017). At the age of 5-8 years, approximately 10%; at 9-12 years age 20-25%; at more than 13 years age 30-35% and above 16 years 75% of dogs are affected with cardiac diseases (Sisson, 2002; Guglielmini, 2003). In dogs, the most prevalent acquired cardiac disease is mitral valve disease, comprising up to 75%, followed by dilated cardiomyopathy (DCM) which is a common cause of morbidity in larger breed and having a higher prevalence, accounting 58% in Doberman pinschers (Baisan *et al.*, 2021). Hypertrophic cardiomyopathy is most common cardiac disease affecting about 15% of general feline population (Fuentes *et al.*, 2020). In equines, cardiac diseases like murmur, arrhythmias, pulmonary hypertension, congestive heart failure are responsible for decreased exercise performance, reduced lifespan and also safety concern for horses (Bonagura, 2019). In cattle, cardiac disease manifestations are rare and mostly involved pericardium, valve structures, myocardium or blood vessels. Early diagnosis of usual cardiac diseases in cattle like infectious pericarditis, endocarditis, neoplasm, DCM and congenital heart diseases can help to avoid animal suffering (Buczinski *et al.*, 2010).

Cardiac fibrosis

Excessive deposition of extra-cellular matrix (ECM) proteins viz collagens, matrix metalloproteinases (MMPs), fibronectins and tissue inhibitors of matrix metalloproteinases (TIMPs) in heart is known as cardiac fibrosis; which is crucial element of cardiac remodeling

(Kong *et al.*, 2014). The extracellular matrix is an organised network of collagen fibers, fibronectin, elastic fibers, laminins, proteoglycans, glycosaminoglycans, glycoproteins and soluble factors surrounding cardiomyocytes (Frantz *et al.*, 2010; Schaefer and. Schaefer, 2010). ECM serves as scaffolding for the various components of cell as well as helps to maintain homeostasis, coordinates contractile function and electrical coupling between cardiomyocytes (Martin and Blaxall, 2012). Under normal condition, cardiac fibroblasts produce ECM components, most commonly collagen type I and III and it is continuous process of newer collagen being synthesized and older being degraded. Fibroblasts maintain this balance in collagen level by the production of cytokines, growth factors and MMPs (Baum and Duffy, 2011). Adult human cardiac muscle is composed of approximately 85% of collagen type I and 11% of collagen type III (Hinderer and Scheneke-Layland, 2019). Initially, it is an adaptive process, however, over the time it progresses to ventricular remodeling irreversibly and impaired cardiac function responsible for number of cardiac conditions. Fibrosis plays a crucial role in several cardiovascular diseases, out of which myocardial infarction and heart failure are common (Murtha *et al.*, 2017).

Cardiac fibrosis is classified into three types such as replacement, reactive interstitial and infiltrative interstitial fibrosis. Reactive interstitial type fibrosis is characterized by an enhanced deposition of ECM without a considerable loss of cardiomyocytes, leading to a pressure-overload condition and cardiomyopathies. Infiltrative interstitial fibrosis results in glycolipid accumulation in cardiac cells. It is observed in a genetic origin rare Fabry disease due to sphingolipids metabolism dysfunction. Cardiac injury such as myocardial infarction results in replacement fibrosis, which is characterized by cardiac cell damage, replacement of dead cells and predominantly collagen type I containing scar formation (Disertori *et al.*, 2017; Graham-Brown *et al.*, 2017).

Cardiac fibrosis has three phases: inflammatory phase, proliferative and maturation phase. Sudden death of cardiac-myocytes after cardiac injury results in myocardial necrosis and initiates the inflammatory phase of fibrosis. This phase leads to stimulation of innate immune mechanisms, recruitment of leucocytes to the site of infarction and activation of neutrophils (Zymek *et al.*, 2006; Frangogiannis, 2014). Afterwards, active suppression of inflammatory

phase is followed by preparation of site for proliferative phase. In proliferative phase, growth factors are secreted by macrophage and mononuclear cells which help in reparative cells like fibroblast and myofibroblast recruitment and activation (Frangogiannis, 2014). Accumulation of these reparative cells in granulation tissue leads to deposition of ECM proteins and contributes in tissue structural integrity preservation and formation of scar, followed by reparative cells apoptosis. At that point, the phase of inflammation is started to resolve and the maturation phase transitions of wound begins to take place. Vascular granular tissue is replaced by a collagen scar as a result of resolution of the phase of inflammation (Zymek *et al.*, 2006; Frangogiannis, 2014). Maturation of scar results in significant cross-linking of collagen and increases its tensile strength (Czubryt, 2012). Scar formation and maturation impair the cardiac contractility, resulting in the remodeling of ventricles and heart failure (Miragoli *et al.*, 2007; Czubryt, 2012).

Role of oxidative stress

Overproduction of the reactive oxygen species (ROS) and insufficient endogenous antioxidant defense in response to stimuli may produce oxidative stress. ROS activation has been attributed by growth factors and neurohumoral signals viz angiotensin II, isoprenaline (ISP), aldosterone and transforming growth factor- β (TGF- β), that activate cardiac fibrosis (Zhao *et al.*, 2008; Lijnen *et al.*, 2012). ROS activation triggers various fibrogenic signaling such as inflammatory cytokines, stress-responsive protein kinases and variety of transcription factors, including nuclear factor (NF- κ B) and activator protein (AP-1) (Siwik and Colucci, 2004). Cytokine-induced NADPH oxidase plays a significant role in cardiac fibrosis mediated by ROS (Sirker *et al.*, 2007).

Role of inflammation and cytokines

Cardiac insult leads to recruitment of immune and inflammatory cells viz macrophage, mast cells, granulocytes, dendritic cells, monocytes, B and T-lymphocytes to injury site (Murtha *et al.*, 2017). Several chemokines and cytokines are responsible for remodeling and fibrosis after cardiac injury. Cytokines regulate fibrotic process by directly affecting resident cardiac fibroblasts, mononuclear cells and fibroblast-progenitor cells recruitment and activation and

angiogenesis modulation (Rai *et al.*, 2017). IL-6 and TNF- α are pro-inflammatory cytokines, play a crucial role in cardiac fibrosis by acting on resident cardiac fibroblasts, matrix protease cascades driven and by enhancing ECM deposition (Frangogiannis, 2021). According to Sivakumar and co-workers (2008), expression of TNF- α and IL-6 were increased in DCM patients with the transcription of matrix proteases; meanwhile, collagen turnover association with these has been demonstrated by Timonen and co-workers (2008) and proposed a relation in ECM remodeling and cytokine activation. TNF- α plays a novel role in cardiac fibrogenesis by acting on TNF receptor 1 and enhances the monocyte fibroblast precursors generation in angiotensin-II-induced myocardial fibrosis (Duerrschmid *et al.*, 2013).

Role of pro-fibrotic growth factors

TGF- β

In cardiac fibrosis, one of the well-known growth factors for fibrosis is TGF- β . Three isoforms of TGF- β viz TGF- β 1, TGF- β 2 and TGF- β 3 are present in mammals (Bujak and Frangogiannis, 2007; Dobaczewski *et al.*, 2011). Among these, most prominent form is TGF- β 1. Binding of TGF- β with TGF- β receptor I (T β RI, sometimes called ALK5) and TGF- β receptor II (T β RII) activates Smad2/3 signaling and leads to cardiac fibrosis process (Shi and Massague, 2003). This pleiotropic mediator TGF- β has diverse effect on different cells in cardiac fibrosis. Over-production of ROS, increased proteases and several other factors in cardiac injury, lead to TGF- β 1 activation and ultimately cause downstream target activity and pro-fibrotic actions in myocardium. Cardiac fibroblasts, cardiomyocytes, macrophages vascular cells and lymphocytes are mainly involved in TGF- β 1-induced cardiac fibrosis (Dobaczewski *et al.*, 2011).

Several studies reported a possible link between over-expression of TGF- β 1 and enhanced fibrogenesis response and collagen deposition in myocardial fibrosis model. TGF- β 1 effects are mediated by Smad2/3- dependent and smad-independent downstream signaling. In cardiac fibroblast cells, TGF- β activates matrix synthesizing myofibroblasts and upregulates ECM proteins through Smad3 pathway and preserves the structural integrity of infarct area of ventricle via an integrin-mediated axis (Khalil *et al.*, 2017). In Smad-independent pathway, activation of p38MAPK in cardiac fibroblasts is carried out by TGF- β and calcineurin transcriptional signals, leading to stimulation of fibroblast differentiation (Molkentin *et al.*, 2017).

Several endogenous signaling also play important role to restrain cardiac fibrosis through TGF- β 1 down-regulation and act as negative regulator for TGF- β -induced fibrosis. BMPBI (BMP and activin membrane-bound inhibitor) and endoglin are major negative regulators, which act as TGF- β pseudo-receptor (Villar *et al.*, 2013) and by releasing a soluble TGF- β accessory receptor, (Kapur *et al.*, 2012) respectively. Recently, the delta-like homologue-1 (Dlk-1), an unorthodox (non-canonical) notch ligand has been suggested to involve in inhibition of trans-differentiation of fibroblast to myofibroblast by restraining of TGF- β /Smad3 signaling (Rodriguez *et al.*, 2019). Therefore, in cardiac injury, the activated TGF- β helps in modulation of gene expression and fibroblast phenotype, leading to collagen deposition and up-regulation of fibronectin synthesis. Further, the decrease of matrix degradation via protease-inhibitors induction ultimately results into enhanced ECM deposition by TGF- β . TGF- β is also involved in reduced inflammatory mediator production from macrophages by down regulation of inflammatory gene synthesis, while concurrently enhances trans-differentiation of myofibroblast and extra cellular matrix deposition via Smad3-dependent signaling (Bujak *et al.*, 2007; Biernacka *et al.*, 2015).

Connective tissue growth factor (CTGF)

Connective tissue growth factor is a 36-38 kDa extracellular matrix protein high in cysteine and belongs to CCN family. It is also called as CCN2. Although, the fetal myocardium is rich in CTGF expression, while in adult heart, its expression is confined to large blood vessels and the atria (Chuva *et al.*, 2004; Friedrichsen *et al.*, 2005). Koitabashi and co-workers (2007) reported that enhanced level of pro-fibrotic cytokine CTGF in cardiac samples from heart failure patients suggests a possible correlation between the areas of myocardial fibrotic and CTGF-stained. In fibroblast cells of heart and cardiomyocytes, TGF- β and Ang II are also responsible for induction of CTGF expression (Zhang *et al.*, 2011). Several studies suggested the potential role of CTGF in myocardial fibrosis through stimulation of fibroblasts proliferation, trans-differentiation of myofibroblasts and excessive buildup of extracellular matrix (Daniels *et al.*, 2003; Lam *et al.*, 2003; Grotendorst *et al.*, 2004).

Panek and co-workers (2009) reported that transgenic mice heart's having overexpression of cardiac-specific CTGF did not induce fibrosis, suggesting that CTGF itself

is unable to induce cardiac fibrosis rather it has pro-hypertrophic function. CTGF helps to establish a fibrotic environment and growth factors activity modulation in extracellular matrix (Liu *et al.*, 2011). According to Dorn and co-workers (2018), the TGF β -stimulated production of collagen is effectively inhibited by deletion of CTGF in embryonic fibroblast of mice. It has also been reported that CTGF-antibody administration significantly improved left ventricular function as well as cardiac fibrosis in the mouse model (Koshman *et al.*, 2015). CTGF plays a pivotal role in cardiac fibrotic response modulation in cardiac remodeling, therefore it becomes a possible cardiac fibrosis biomarker as well as a potential target for mitigation of cardiac fibrosis (Daniels *et al.*, 2009).

Role of matrix metalloproteinase

Cardiac fibroblast cells are responsible for ECM homeostasis. Besides extracellular matrix protein production, cardiac fibroblast cells also produce the metalloproteinases of matrix (MMPs), and their inhibitors viz tissue inhibitors of metalloproteinases (TIMPs). Therefore, well balanced functioning of MMPs and their inhibitor TIMPs is necessary for ECM homeostasis (Moore *et al.*, 2012). Matrix metalloproteinases are Zn²⁺-activated proteases and responsible for ECM protein degradation. Therefore, MMPs play an important role in cardiac fibrosis development. Family of MMPs consists collagenase (MMP-1 & MMP-13), stromelysin (MMP-3), gelatinase (MMP-2 and MMP-9) and membranous type MMP (MT1-MMP) (Tsuruda *et al.*, 2004). Spinale and co-workers (2013) suggested that in experimental animal models, different MMPs are induced according to etiology of heart failure. Myocardial biopsies of aortic stenosis and left ventricular pressure overload models have shown an increased level of MMP-2 and MMP-14, while in end-stage dilated cardiomyopathy, enhanced level of MMP-9 and others have been reported. Furthermore, in early stage of cardiac failure, MMP-2 level is higher than MMP-9, whereas in end-stage condition of heart failure, the level of MMP-9 is exceeded than MMP-2. MMP-2 and MMP-9 are mainly responsible for degradation of different collagens such as type I, IV, V, while in addition MMP-2 also cleaves type III collagen (Steffensen *et al.*, 1995). According to Munch and co-workers (2016), serum MMP-9 level is an important biomarker for different cardiac events and myocardial fibrosis in hypertrophic cardiomyopathic females, while, MMP-3 level has been related to high incidence of fibrosis and sex-independent cardiac events.

Signaling pathways of Cardiac Fibrosis

TGF- β -mediated Smad-dependent pathway

In cardiac fibrosis, TGF- β mediates its effects by Smad-dependent as well as smad-independent signaling pathways. Binding of TGF- β with its receptor type II phosphorylates the type-I receptor, leading to the Sma and Mad-related proteins (Smads) recruitment and activation especially Smad2 and Smad3. Afterwards, these activated Smad2 and Smad3 are released from the receptors and interacts with Smad4 to produce a heteromeric complex. This heteromeric complex is moved to the nucleus and control the target gene transcription by recruitment of co-activators. Transcription of Smad2 and Smad3 are competitively inhibited by inhibitory Smads viz Smad6 and Smad7, which interact as well as increase type I receptor degradation (Ma *et al.*, 2018). Bujak and co-workers (2007) reported the decreased fibrotic remodeling following myocardial infarction in Smad-3 deficient mice. However, in Smad3 knockout mice, Smad-3 dependent anti-proliferative effect on cardiac fibroblast has also suspended, resulting in surprisingly increased myofibroblast density.

MAPKs, extracellular regulated protein kinases (ERK) and phosphatidylinositol-3-kinase (PI3K) and protein kinase B (AKT) pathways are the Smad-independent (non-canonical) pathways of TGF- β . Binding and activation of type II receptors by TGF- β leads to the TAK1 (TGF β -activated kinase 1) activation, which further phosphorylates p38 or JNK by acting on MKK4 or MMK3/6, respectively (Wang *et al.*, 1997). TGF- β is also responsible for extracellular regulated protein kinases (ERK) activation either by direct ShcA phosphorylation or through the small GTPase Ras (Lee *et al.*, 2007).

In addition to the TGF- β -mediated canonical (Smad-dependent) and non-canonical (Smad-independent) pathways of cardiac fibrosis, AMP-activated protein kinase pathway α and Wnt/ β -catenin pathways are also important signaling pathways in cardiac fibrosis. In cardiac fibroblasts, AMPK α mediates the cross-talk with ERK, leading in attenuation of cardiac fibrosis (Du *et al.*, 2008). Binding of Wnt ligand with Fz receptor and LRP5/6 co-receptor leads to the stabilization of cytoplasmic β -Catenin. Afterwards, the β -catenin translocates to the nucleus and control Wnt-signaling related genes through interaction with factor for T-cell (TCF) (Logan and Nusse, 2004; Naito *et al.*, 2010; Rao and Kühl, 2010).

Transcription factors activated in the fibrosis

Smad

Binding of TGF- β to the receptors leads to the receptor activation. The activated TGF- β receptors phosphorylate the Smad2 and Smad3, which after interaction with Smad4 moves into the nucleus and regulate the gene expression. Smad is associated with co-activators and co-repressors to precisely regulate the transcription (Ma *et al.*, 2018). Binding of the complex of Smad to the specific DNA sequence is mainly in enhancers or promoters of target genes element for Smad binding (SBE), is firstly identified in the promoter PAI-1, which binds the Smad complexes through MH1 domains (Dennler *et al.*, 1998; Zawel *et al.*, 1998).

Nuclear factor- kappa B (NF- κ B)

Nuclear factor-kappa B is one of the well-known transcription factors that regulates mainly inflammation-related genes. NF- κ B is mainly a heterodimer and regulates the $\kappa\beta$ binding sites containing genes transcription. Generally, in the absence of activating stimuli, transcription factor NF- κ B is mainly bound with inhibitors of κ B (I- κ B) family protein and present in the cytoplasm as an inactive dimer. In cells, the proinflammatory factors rapidly induce the ability of NF- κ B to bind DNA (Karin and Lin, 2002). NF- κ B is activated by several pro-fibrotic stimuli, producing the ROS, growth factors, cytokines and hypoxia. Then, activated NF- κ B is moved into the nucleus and binds with κ B binding site in DNA-sequence and directly regulates the fibrosis-related genes expression such as PAI-1, MMPs and fibronectin (Ma *et al.*, 2018). Levels of pro-fibrotic cytokines such as TNF- α and IL-6 are also regulated by NF- κ B (Valen *et al.*, 2001). Further activation of NF- κ B by these factors lead to the fibrotic response amplification.

Nuclear factor erythroid 2-related factor (Nrf2)

Nrf2 is a crucial transcription factor for antioxidant response. In normal cell condition, Cullin 3 and Kelch-like- ECH- associated protein 1 (Keap1) suppress the Nrf2 and remain in cytoplasm as an inactive form (Itoh *et al.*, 1999). Nrf2 acts as a substrate for ubiquitination by Cullin3. Keap1 is a substrate adaptor, which helps in Cullin3-mediated ubiquitination of Nrf2. Therefore, under normal condition the half-life ($t_{1/2}$) of Nrf2 is short that lasts for 20 min

(Kobayashi *et al.*, 2004). Oxidative stress disrupts the ubiquitination system of Keap1-Cullin3 by destroying the critical cysteine residue of Keap1. Yamamoto and co-workers (2008) reported that in absence of ubiquitination, Nrf2 level is increased in cytoplasm and leads to nuclear translocation of it. In the nucleus, Nrf2 creates a heterodimer by combining with small protein known as Maf and initiates the transcription of several antioxidant genes, including heme oxygenase-1 (HO-1), catalase (CAT), superoxide dismutase (SODs), by binding with antioxidant response element (ARE) (Itoh *et al.*, 1997; Howden, 2013).

Xing and co-workers (2012) reported the protective action of dihydro-CDDO-trifluoroethylamine (dh404), an analog of Nrf2 inducer CDDO, against pathological cardiac remodeling, fibrosis and dysfunction by activating the Nrf2 in transverse aortic constriction mouse model. Isoproterenol-induced cardiac fibrosis was attenuated by microRNA-26b through activation of Nrf2/Keap1 signaling pathway (Xiang *et al.*, 2020). Cai and co-workers (2018) suggested the preventive effect of puerarin in cardiac fibrosis, which is due to the downregulation of Keap1.

Isoprenaline-induced cardiac fibrosis

Sustained activation of sympathetic system is a crucial factor in the advancement of myocardial hypertrophy (Tank and Wong, 2015). The overall functioning of heart is mainly regulated by β -adrenoceptors; therefore, these play a key role in cardiac disease, especially heart failure (Wachter and Gilbert, 2012). Chronic stimulation of adrenoceptors by elevated level of circulating catecholamine is responsible for maladaptive cardiac remodeling, including myocardial hypertrophy, cardiac fibrosis and apoptosis, which may lead to the heart failure (Fu *et al.*, 2012). Sustained stimulation of β 1-adrenoceptors is responsible for the increased energy and oxygen consumption along with progression of oxidative stress-mediated cardiac toxicity (Meng *et al.*, 2006; Zhu *et al.*, 2003).

Isoprenaline (ISP) is a synthetic non-selective β -receptor agonist. Isoprenaline causes oxidative stress through free radical generation, leading to the mitochondrial damage and alteration in biochemical parameters in heart and ultimately result in cardiac injury. Therefore, this property of isoprenaline (ISP) has been used as experimental screening model for study of cardioprotective activity of various test substances (Allawadhi *et al.*, 2018).

Several studies suggested that isoprenaline (ISP) is used as a standard agent to study the preventive and protective effects of various test substances against myocardial infarction, myocardial ischemia and cardiac fibrosis. Isoprenaline model is relatively non-invasive, corresponding to the disease process and have lower mortality rate in experimental animals (Benjamin *et al.*, 1989; Nagano *et al.*, 1992; Brooks and Conrad, 2009; Samuel *et al.*, 2014). Benjamin and co-workers (1989) suggested that ISP administration leads to fibroblast proliferation, cardiac hypertrophy, accumulation of connective tissue, fibrosis and myocyte necrosis. In heart failing condition, isoprenaline has been emerged as a crucial mediator for synthesis of collagen fragments and it leads to enhanced expression of matrix metalloproteinases (MMPs). In isoprenaline-mediated myocardial infarction rat model, the expression of collagen type I, matrix metalloproteinase 2 (MMP-2), MMP-9, α -smooth muscle actin, collagen type I and TGF- β are upregulated (Thangaiyan *et al.*, 2020).

Mechanism of isoprenaline-induced cardiac fibrosis

During stressful condition, the level of endogenous catecholamine is increased to provide a short-period adaptative response known as “fight-or-flight response” (Bers and Despa, 2009). However, sustained elevated level of catecholamine in body exerts the deleterious effect on the cardiovascular system and initiates cardiotoxicity, as evident in chronic heart failure (Floras, 2009) and stress-induced cardiomyopathy (Nef *et al.*, 2010). Cardiac injury leads to apoptosis and necrosis of cardiomyocytes, mononuclear leukocytes and polymorphonuclear cells infiltration in myocardium, subepicardial and subendocardial haemorrhages, interstitial edema and progressive cardiac fibrosis (Rona, 1985; Costa *et al.*, 2011).

Catecholamine-mediated cardiotoxicity is a multifactorial phenomenon. The sustained overstimulation of receptors by elevated level of catecholamine increases the heart rate and contractility, myocardial oxygen demand and out-balance the supply of oxygen and leads functional hypoxic condition in heart. Further, the vasoconstriction of coronary micro- and macro-circulation aggravates the hypoxia and decreases the high-energy phosphate supply (Costa *et al.*, 2011). Chronically elevated level of catecholamines also lead metabolic

alterations. In cardiomyocytes, the increased lipolysis along with neutral lipid droplets deposition leads to the uncoupling of oxidative phosphorylation and exacerbates the condition (Jodalén and Neely, 1991; Behonick *et al.*, 2001). Moreover, electrolyte imbalance such as hypokalemia, hypomagnesemia is also occurred due to change in cell membrane permeability, which disturb cellular homeostasis and favours the myocardial toxicity (Borkowski *et al.*, 2011).

Despite the earlier processes, there are considerable evidence to implicate the intracellular calcium overload, mitochondrial dysfunction and oxidative stress as the prime mechanism considering for the catecholamine-mediated cardiotoxicity. β -adrenoceptors stimulation by catecholamine lead to the activation of protein kinase A, which results into the phosphorylation of several Ca^{2+} -cycling proteins such as L-type calcium-channels of sarcolemma, ryanodine receptor-mediated Ca^{2+} -release channel of the sarcoplasmic reticulum, myofilament proteins in cardiomyocytes and increase the intracellular calcium in cytosol and mitochondria (Bers and Despa, 2009). Moreover, it is followed by the mitochondrial matrix swelling, loss of cell respiration control and ROS generation, which augments the calcium-induced mitochondrial dysfunction (Khan *et al.*, 2012).

Spontaneous auto-oxidation of catecholamine forms a toxic compound amino-chrome. Presence of free radicals, oxidants, redox metals viz iron and copper, and enzymatic catalysis (mainly myeloperoxidase, xanthine oxidase and cytochrome oxidase) accelerate the amino-chrome formation (Remiao *et al.*, 2001; Remiao *et al.*, 2003; Haskova *et al.*, 2011). Several studies reported that amino-chromes exert toxic effects by directly acting on the coronary arteries and myocardium, resulting in vasoconstriction of coronary arteries, oxidative phosphorylation inhibition and depletion of calcium binding, followed by decreased contractile force and extensive necrotic damage (Karmazyn *et al.*, 1981; Yates *et al.*, 1981; Taam *et al.*, 1986). Apart from direct action, amino-chromes also stimulate the abundant formation of ROS, primarily through mitochondrial redox cycling process (Bindoli *et al.*, 1990).

Over-production of oxidants and free radical exceeds the endogenous antioxidant capacity of cells and lead to the oxidative stress with intense cytotoxic consequences of damage

in nucleic acid, lipids and proteins of cells (Pacher *et al.*, 2007; Liaudet *et al.*, 2009). Several studies have reported usefulness of the antioxidant therapies viz ascorbic acid (Buttros *et al.*, 2009), melatonin (Patel *et al.*, 2010), N-acetylcysteine (NagoorMeeran *et al.*, 2012) and quercetin (Panda *et al.*, 2012) in catecholamine-induced cardiotoxicity.

Several studies reported the contribution of MAPK pathway in organ fibrosis by regulation of cellular response to the growth, apoptosis and stress signals (Griendling *et al.*, 2000; Ma *et al.*, 2014). MAPK pathway is stimulated by enhanced level of free radicals. Activated MAPK signaling leads to activation and nuclear translocation of inflammatory nuclear factor- κ B (NF- κ B). Activated NF- κ B stimulates the inflammatory cytokines viz TNF- α , and IL-6 and progresses myocardial injury (Chunhua *et al.*, 2017). Molecular signals like nuclear factor- κ B stimulates the MMPs synthesis and downregulates the TIMPs, leading the cardiac fibrosis (Thangaiyan *et al.*, 2020). Expression of MMP-9 is upregulated in myocardial infarction model (Podesser *et al.*, 2001).

Biochemical and pathophysiological changes in isoprenaline-induced cardiac fibrosis

Isoprenaline-mediated cardiac myopathy has shown a complex pattern of biochemical changes in enzymatic markers of cardiac injury, non-enzymatic and enzymatic antioxidant levels, lipid metabolizing enzymes, lipid profile, decrease in ATP store along with myocardial tissue changes (Fleckenstein *et al.*, 1974; Lehr, 1972). The morphometric markers like heart weight, heart weight/ body weight ratio and heart weight/tibial length are increased in isoprenaline-induced cardiac hypertrophy (Li *et al.*, 2012; Al-rasheed *et al.*, 2015). Increased heart weight in isoprenaline-induced cardiac hypertrophy may be contributed by increased water content, edema of intramuscular space and inflammatory cells infiltration followed by necrosis of cardiomyocytes (Patel *et al.*, 2010).

Modification in sarcoplasmic reticulum, sarcolemma, mitochondria are primarily mediated by the oxidative stress and results in transport system and enzyme activity alteration, which ultimately cause cellular homeostasis disturbance (Takeo *et al.*, 1980). Moreover, lipolysis is also an important causal factor for isoprenaline-mediated myocardial injury. The activity of adenyl cyclase is enhanced by isoprenaline, leading to the increased cAMP formation

and followed by increased myocardial lipid accumulation (Dhawan *et al.*, 1978). Therefore, isoprenaline-induced lipid metabolism disturbance is reflected as increased level of cholesterol, triglycerides, free fatty acids, LDL-C, VLDL-C along with decreased level of high-density lipoprotein cholesterol (HDL-C) in serum (Rajadurai and Prince, 2005; Stanely and Karthick, 2007).

As isoprenaline causes incomplete glucose and oxygen supply to cardiomyocytes, resulting in cardiac cell injury and elevates level of cardiac marker enzymes in serum (Derbali *et al.*, 2015). Several studies have demonstrated the increased serum enzymatic activity of CK, CK-MB, LDH, cTnI and cTnT in isoprenaline-treated experimental animals (Li *et al.*, 2012; Thangaiyan *et al.*, 2020). Isoprenaline-mediated oxidative stress is mainly characterized by elevated level of several markers of oxidative stress such as MDA, with simultaneously decrease in endogenous antioxidant reservoir of cell, including SOD, glutathione and catalase (Sumi *et al.*, 2019; Ulla *et al.*, 2017).

Collagen accumulation in extracellular matrix is a key contributor of myocardial hypertrophy and fibrosis (Borbély *et al.*, 2009). Ischemic injury leads to the abnormal synthesis and degradation of extracellular matrix and leads to deposition of collagen I. Hydroxyproline is a post-translational proline hydroxylation product, which is considered to be chiefly due to collagen (Reddy and Enwemeka, 1996). Several studies have demonstrated the increased hydroxyproline content in isoproterenol-mediated cardiac fibrosis models (Liu *et al.*, 2015; Wang *et al.*, 2019; Jin *et al.*, 2020). Thangaiyan and co-workers (2020) reported the increased expression of collagen type I, collagen type III, MMP-2, MMP-9, fibronectin, TGF- β 1, α -smooth muscle actin, Smad-2 and Smad-3 in isoproterenol induced rat cardiac fibrosis model.

Studies have also demonstrated that isoprenaline upregulates the proinflammatory and inflammatory cytokines such as TNF- α , Interleukin-6 (IL-6), Stat-3 and NF- κ B expression in the experimental animal heart (Hamid *et al.*, 2011; Thangaiyan *et al.*, 2020). Moreover, as the Nrf2 is a main contributor of oxidative stress pathways (Zhou *et al.*, 2014). Levels of Nrf2 and HO-1 are reduced by isoprenaline in experimental animal models (Hasan *et al.*, 2020; Abdelzaher *et al.*, 2021). Higher level of isoproterenol also causes histological changes in

heart, including inflammatory cells accumulation, myocardial fibres degeneration and necrosis, lipid droplets, interstitial edema and endocardial haemorrhage (Lehr, 1972).

Orlistat

Orlistat is a semisynthetic derivative of lipstatin and also known as tetrahydrolipstatin. Lipstatin, an irreversible blocker of pancreatic lipase, is naturally obtained from *Streptomyces toxytricini* (Barbier and Schneider, 1987). Orlistat is an over-the-counter anti-obesity drug approved by the FDA (Food and Drug Administration) in 1999 (Stephanie, 2007). In overweight patients having body mass index (BMI) ≥ 28 kg/m² and in obese patients having BMI ≥ 30 kg/m², a dose of 120 mg three times per day of anti-obesity drug orlistat along with a mild hypocaloric diet is recommended (Dahlin and Beermann, 2007). Obesity is an important risk factor for the several chronic diseases viz non-alcoholic fatty liver disease (Huh *et al.*, 2017), cardiovascular disease (Wormser *et al.*, 2011), diabetes mellitus type 2 (T2DM) (Graessler *et al.*, 2013), musculoskeletal disorders primarily osteoarthritis (Jiang *et al.*, 2011; Jiang *et al.*, 2012) and cancers (Lauby-Secretan *et al.*, 2016). Therefore, orlistat is currently used as auxiliary treatment for obesity related cardiovascular diseases and type II diabetes mellitus (Qi, 2018).

Mechanism of action

Orlistat is a selective inhibitor of gastric and pancreatic lipase enzymes (Borgstrom, 1988; Hadvary *et al.*, 1991). These enzymes play an important role in digestion and absorption of dietary fat. Triacylglycerols are hydrolysed into monoacylglycerols and free fatty acids by lipase enzymes and absorbed (Curran and Scott, 2004; Henness and Perry, 2006). Although, orlistat exerts little or no action against other enzymes, including trypsin, amylase, phospholipase and chymotrypsin. It's main site of action is gastrointestinal tract (Alqahtani *et al.*, 2015). In gastrointestinal tract, it covalently binds with the serine active site of lipase enzyme and leads to the inhibition of enzymatic hydrolysis of triacylglycerol into the cell absorbable free fatty acids forms and excretion of undigested form of triacylglycerols in faeces (Curran and Scott, 2004; Henness and Perry, 2006). Therefore, lipase inhibition by orlistat reduces the dietary fat absorption and leads to decreased caloric intake.

According to Alqahtani and co-workers (2015), Niemann-Pick C1-like 1 (NPC1L1), a cholesterol transport protein is also inhibited by orlistat, therefore, restricts the absorption of cholesterol. Furthermore, fatty acid synthase (FAS) enzyme is inhibited by orlistat, which is mainly upregulated in several tumor cells (Kridel *et al.*, 2004; Kuhajda, 2006). Several studies suggested that blocking of FAS (fatty acid synthase) by orlistat elicits the endoplasmic reticulum stress and inhibition of tumor growth (Kridel *et al.*, 2004; Little *et al.*, 2007). According to the crystalline structure of fatty acid synthase complex of human, orlistat acts as a thioesterase domain substrate containing two subdomains (Pemble *et al.*, 2007).

Pharmacological activity of orlistat

Anti-oxidant and anti-inflammatory activity

It has been reported that obesity is an important contributor of systemic oxidative stress development in animal studies as well as in human (Furukawa *et al.*, 2004; Wonisch *et al.*, 2012). Several factors such as modification in nutritional status, hyperlipidemia, hyperglycaemia and chronic inflammation trigger oxidative stress in obesity (Manna and Jain, 2015).

Suleiman and co-worker (2020) reported that orlistat has increased catalase (CAT), glutathione-S-transferase (GST), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) activities, and total antioxidant capacity (TAC), along with decreased malondialdehyde level in the penile tissue, suggesting the role of orlistat in improvement of fertility potential in obesity by targeting the penile oxidative stress in testis. Khan and co-workers (2017) demonstrated that orlistat treatment in non-alcoholic fatty liver obese patients reduced the level of triglyceride, total cholesterol, LDL-C, serum periostin and TNF-alpha along with increased serum adiponectin levels.

Othman and co-workers (2021) reported that orlistat improves the lipid profile in obese rats, as evident by decreased level of triglycerides, total cholesterol, LDL, and increased level of high-density cholesterol (HDL) as compared to obese rat group. Furthermore, they have also found that daily administration of orlistat for 6 weeks significantly increased the aortic anti-oxidant enzymes such as catalase, SOD, GST, GR, GPx activities and total anti-

oxidant capacity along with decreased MDA level in aorta than the obese group. The expression of pro-inflammatory markers viz TNF- α is decreased along with increased level of an anti-inflammatory marker (IL-10) in orlistat-treated group than the obese group, suggesting the anti-atherogenic activity of orlistat on obesity-mediated oxidative stress in rat model (Othman *et al.*, 2021). Moreover, the inhibition of the intestinal lipase enzyme by orlistat (@16 mg/kg body weight and @32 mg/kg body weight) has increased the immune enzyme activities, anti-inflammatory cytokines gene expression and antioxidant activity, along with decreased pro-inflammatory cytokines gene expression in Nile tilapia fish model (Zhang *et al.*, 2020).

Anti-obesity

Orlistat is a currently marketed and FDA approved drug for weight loss. Orlistat at therapeutic dose along with mildly hypocaloric diet is inhibited the absorption of around 30 % of fat intake (Hadvary *et al.*, 1991; Alqahtani *et al.*, 2015). Several studies reported the greater weight loss along with improved lipid profile in orlistat therapy along with hypocaloric diet than placebo therapy, indicating the anti-obesity activity and reduction of cardiovascular risk in orlistat treated obese patients (Zavoral, 1998; Davidson *et al.*, 1999; Leblanc *et al.*, 2011; Yanovski and Yanovski, 2014; Khera *et al.*, 2016). Abdel-Baky and Abdel-Rahman (2021) reported that orlistat has improved the high fat diet-stimulated serum triglycerides, LDL-C and total cholesterol level in obese rat model.

Anti-cancer activity

Orlistat inhibits the thio-esterase domain of fatty acid synthase enzyme and leads to the endoplasmic reticulum stress, inhibition of tumour cell proliferation, initiation of apoptosis in tumor cells. Therefore, it also delays the tumor progression on the several cancer cells such as prostate, ovary, breast and melanoma cancer cells (Kridel *et al.*, 2004; Little *et al.*, 2007; Carvalho *et al.*, 2008). Knowles and co-workers (2004) reported that FAS inhibition by orlistat is responsible for decreased DNA synthesis, cell cycle arrest and apoptotic cell death in cultured cancer cells. The anti-tumor activity of orlistat has also been reported for suppression of growth of melanoma tumor, gastric and prostate cancer (Dowling *et al.*, 2009; Martinez-Villaluenga *et al.*, 2010).

In diabetes mellitus

As the obesity is a crucial risk factor for diabetes mellitus type 2. Therefore, weight loss in type 2 diabetic patients is co-related with better glycaemic control along with reduced risk factors for cardiovascular diseases. Several studies have been reported the greater weight loss and improved glycaemic control as well as greater reduction in type 2 diabetes incidence in the clinically representative obese population to orlistat along with lifestyle change as compared to lifestyle change alone (Hollander *et al.*, 1998; Torgerson *et al.*, 2004; Aldekhail *et al.*, 2015).

Cardiovascular risk

Obesity is a crucial health problem with increasing the cardiovascular diseases risk factors. The orlistat has shown beneficial effects by reducing obesity-mediated risk factors for the cardiovascular diseases primarily through improving the diastolic and systolic blood pressure and lipid profile (Broom *et al.*, 2002). Several studies have suggested that orlistat therapy has produced greater improvement in lipid parameters such as reduction in triglycerides, LDL cholesterol, ratio of LDL to HDL cholesterol and total cholesterol as compared to the placebo (Hollander *et al.*, 1998; Bloch *et al.*, 2003; Didangelos *et al.*, 2004; Kiortsis *et al.*, 2005). Orlistat has reduced the occurrence of ventricular arrhythmia, atrioventricular block, mortality as well as serum creatine kinase level in a cardiac ischemia/reperfusion injury model, showing a cardioprotective function to reduce myocardial damage in acute myocardial infarction (Menezes-Rodrigues *et al.*, 2018).

Anti-fibrotic activity

Non-alcoholic fatty liver disease (NAFLD) is a chronic disease that is frequently associated with the insulin resistance by the stimulatory effect of triglycerides accumulation in the liver (Petersen *et al.*, 2005; Greenberg *et al.*, 2011). Further, NAFLD is progressed to the non-alcoholic steatohepatitis (NASH), cirrhosis, fibrosis and even hepatocellular cancer (Bhala *et al.*, 2011). Orlistat therapy has exhibited the beneficial effect in this metabolic syndrome by reducing insulin resistance, fatty liver infiltration and improving the liver fibrosis score (Esmail *et al.*, 2021). According to Hussein and co-workers (2007), the orlistat therapy

at therapeutic dose has decreased the levels of LDL-C, aminotransferases, triglycerides, total cholesterol, the malonyl dialdehyde (MDA) level, insulin resistance, liver fibrosis score and inflammation in obese patients with NASH.

According to Zakaria and co-workers (2021), feeding of high fat diet for 12 weeks to *Sprague-Dawley* rats has increased the body weight, level of serum and liver lipids (triglycerides, total cholesterol), insulin resistance, adiponectin, level of liver enzymes such as AST, ALP and ALT, liver oxidative stress along with decreased antioxidant enzymes (GPx, SOD, CAT, GR, GST) activities and GSH level. High fat diet has also increased the pro-inflammatory markers viz TNF- α , nuclear factor- kappa beta (NF- κ B), while, decreases the level of anti-inflammatory marker (interleukin-10). Furthermore, it has also increased the nuclear expression of Nrf-2, cytoplasmic expression of Keap 1 along with changes such as hepatocyte hypertrophy, steatosis, infiltration of inflammatory cells and collagen deposition in the hepatic tissue histopathology, indicating the NASH and fibrosis. Orlistat (10 mg/kg/day for 12 weeks along with high fat diet) and bee bread (0.5 gm/kg/day for 12 weeks along with high fat diet) attenuated all these high fat diet-induced changes, suggesting their anti-inflammatory, anti-steatotic, anti-oxidant and anti-fibrotic properties (Zakaria *et al.*, 2021).

Niclosamide

The oral antihelminthic drug, niclosamide is used for the therapy of parasitic infection globally. In 1953, Bayer's chemotherapeutic research laboratories discovered the niclosamide. In 1959, it was firstly sold as Bayluscide as a molluscicide to kill snails, which are an intermediate host of schistosomiasis (Andrews *et al.*, 1982). It was shown to be effective against human tapeworm (cestoda) infection by Bayer scientists in 1960, and it was commercialized as Yomesan for human usage in 1962 (Andrews *et al.*, 1982; Pearson and Hewlett., 1985). In 1982, US FDA had approved niclosamide for tapeworm infection treatment in humans, and it is included in essential medicines list by the World Health Organization (WHO, 2007). Structurally, niclosamide is part of a wide family of lipophilic, mildly acidic compounds known as salicylanilides (Kadri *et al.*, 2018).

Mechanism of action

Niclosamide acts as an oxidative phosphorylation uncoupler and interferes with helminth's energy metabolism, potentially by blocking the glucose uptake and production of adenosine triphosphate (ATP) (Frayha *et al.*, 1997; Swan, 1999; Chen *et al.*, 2018). Furthermore, nuclear factor- $\kappa\beta$, Wnt/ β -catenin, STAT3 and Notch pathways are also targeted by niclosamide, suggesting the therapeutic potential of it in several diseases (Chen *et al.*, 2018).

Pharmacological activity of niclosamide

Anti-oxidant activity

According to Esmail and co-workers (2021) the niclosamide has shown the hepatoprotective effect against cholestatic liver fibrosis in rat model as induced through bile duct ligation by decreasing the level of liver enzymes (AST, ALP, ALT and GGT), reducing oxidative stress (by lowering MDA and raising SOD and GSH) and inflammation by lowering IL-6, TNF- α and NF- $\kappa\beta$. Niclosamide has also reported to reduce the elevated level of IL-6 and TNF- α caused by oxaliplatin, suggesting the *in-vivo* and *in-vitro* neuroprotective effect of it by minimizing the oxidative stress and inflammation of neuron caused by oxaliplatin (Cerles *et al.*, 2017). In diabetic rat model, the co-administered linagliptin and niclosamide decreased the level of total cholesterol, triglyceride and serum glucose, LPO level, as well as increased the SOD and CAT levels in the sciatic nerve (Jadhav *et al.*, 2020).

Anti-inflammatory activity

Studies reported the anti-inflammatory activity of anti-helminthic drug, niclosamide. Niclosamide has inhibited the VEGF (vascular endothelial growth factor)-mediated angiogenesis along with inhibition of IKK-mediation NF- κB pathway activation in TNF- α -stimulated endothelial cells (Huang *et al.*, 2015).

Rheumatoid arthritis is a chronic-inflammatory autoimmune disease that causes joint destruction, synovial inflammation, and synovial hyperplasia (Nogueira *et al.*, 2016). According to Liang and colleagues (2015), niclosamide has exhibited the inhibitory effect on the inflammation

and fibroblast-like synoviocytes (FLS) migration in TNF- α -stimulated rheumatoid arthritis (RA). Niclosamide has also decreased the secretion and expression of cytokines such as IL-6 and inhibited the TNF- α -induced downstream signalling such as IKK/ NF- κ B and MAP kinase pathways, indicating the anti-inflammatory effect of niclosamide in RA. Niclosamide had a robust anti-inflammatory role in patients with inflammatory bowel disease by lowering the cytokine levels (Shivaji *et al.*, 2019). Furthermore, orally administered niclosamide has decreased the severity of arthritis in arthritic rat model by reducing the inflammatory indicators such as interleukin-1 and TNF- α (Al-Gareeb *et al.*, 2019). Moreover, inhibition of cytokine and mucus production, relaxation of bronchi and antimicrobial properties make niclosamide a promising treatment option for inflammatory airway illnesses as asthma, cystic fibrosis and COPD (chronic obstructive pulmonary diseases) (Cabrita *et al.*, 2019).

Anti-neoplastic activity

Cancer is the condition that has gotten the greatest attention in the repositioning of niclosamide. Several studies demonstrated the anti-cancer property of niclosamide against adrenocortical carcinoma (Sato *et al.*, 2016), breast cancer (Londoño-Joshi *et al.*, 2014), colon cancer (Osada *et al.*, 2011; Suliman *et al.*, 2016), glioma (Wieland *et al.*, 2013), prostate cancer (Liu *et al.*, 2015), renal cell carcinoma (Zhao *et al.*, 2016), ovarian cancer (King *et al.*, 2015), osteosarcoma (Liao *et al.*, 2015), lung cancer (Li *et al.*, 2013; Lee *et al.*, 2014; You *et al.*, 2014), leukaemia (Wang *et al.*, 2009; Jin *et al.*, 2017), head and neck cancer (Li *et al.*, 2013). The potential of niclosamide to damage tumour cell mitochondria, reduce tumour cell proliferation, induce apoptosis and inhibit numerous aberrant tumour-signalling pathways such as nuclear factor- κ B (NF- κ B), STAT3, Wnt/ β -catenin, mammalian target of rapamycin complex 1 (mTORC1), and the Notch pathway has been related to its anticancer properties (Moskaleva *et al.*, 2015).

Anti-diabetic activity

Niclosamide is an effective mTOR inhibitor that could be used as an anti-diabetic medication (Fonseca *et al.*, 2012; Li *et al.*, 2013). Niclosamide ethanolamine has been reported to be responsible for improvement in diabetic symptoms through induction of uncoupling

of mitochondrial oxidative phosphorylation in db/db mice (Tao *et al.*, 2014). Several studies reported the effectiveness of niclosamide in diabetes and diabetes associated conditions (Han *et al.*, 2018; Cai *et al.*, 2021; Engin *et al.*, 2021).

Anti-fibrotic activity

Niclosamide (low dose @ 3mg/kg body weight and at high dose @ 10mg/kg body weight for 21 days) has the ability to exhibit anti-fibrotic activity in bleomycin-induced model of pulmonary fibrosis by decreasing the migration of fibroblast, suppressing fibrotic signaling, attenuating the epithelial to mesenchymal transition and modulating the Wnt/ β -catenin signaling (Boyapally *et al.*, 2019). Pei and co-workers (2022) reported that niclosamide has suppressed TGF- β 1-stimulated epithelial to mesenchymal transition and collagen deposition, accumulation of extracellular matrix. Inhalable nanosuspension of repurposed niclosamide have also shown to be effective for treating *Pseudomonas aeruginosa* associated lung infection in cystic fibrosis patients (Costabile *et al.*, 2015). Inhibition of S100a4 by niclosamide exerted an anti-fibrotic action and could be a promising treatment option for pulmonary fibrosis (Zhang *et al.*, 2018) and amyotrophic lateral sclerosis (Serrano *et al.*, 2019; Milani *et al.*, 2021).

Moreover, niclosamide has reduced total bilirubin, liver enzymes such as AST and ALT, l-hydroxyproline, β -catenin, l-glutaminase activity along with expression of TGF- β 1 gene and low reactivity of α -SMA in CCl₄-induced liver fibrosis rat model. Therefore, it exerts protective effect in liver fibrosis by inhibiting the Wnt/ β -catenin signaling and glutaminolysis (El-Ashmawy *et al.*, 2020). Additionally, the anti-fibrotic action of niclosamide has also reported against hepatic fibrosis (Esmail *et al.*, 2021), renal fibrosis (Chang *et al.*, 2017), muscle fibrosis (Milani *et al.*, 2021) and systemic sclerosis (Morin *et al.*, 2016), by targeting different pathways such as STAT3, Notch, Wnt/ β -catenin, mTOR, and NF- κ B. Orally administered niclosamide has improved cardiac dysfunction by stimulating the mitochondrial respiration of cardiac cell, decreasing the inflammatory cytokine, interleukin-6 and by reducing the collagen production from fibroblast cells in transverse aortic constriction-induced heart failure in mouse model (Fu *et al.*, 2021).

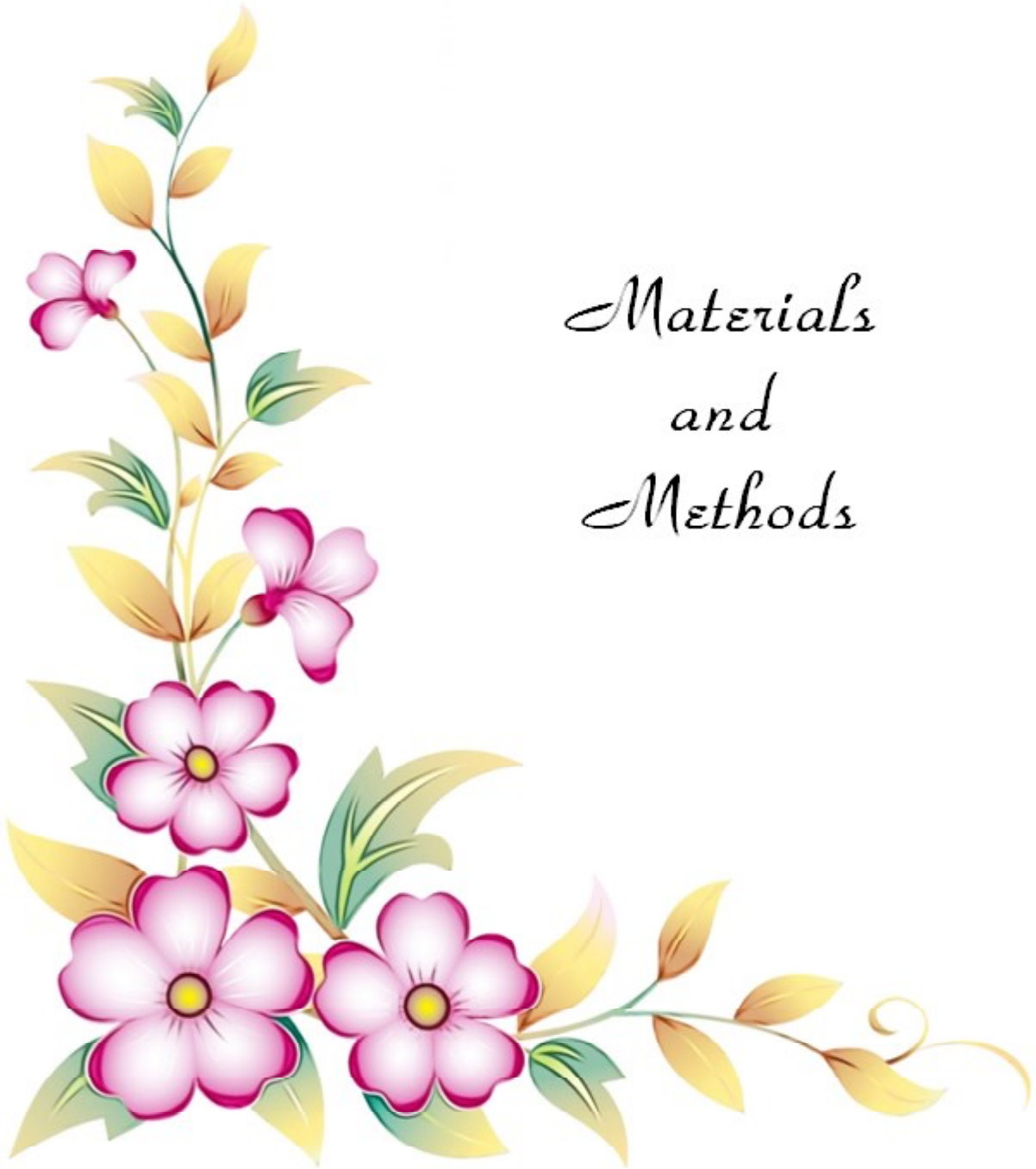
Niclosamide in cardiovascular diseases

Niclosamide has suppressed the proliferation and the migration of smooth muscle cells of vessels, as well as neointimal hyperplasia, in balloon-injured carotid arteries of rat, via a STAT3 inhibitory mechanism, suggesting that niclosamide could be used to treat restenosis (Xiao *et al.*, 2018). Li and co-workers (2017) reported that niclosamide ethanolamine relaxes as well as inhibits the high K⁺(KPSS) and phenylephrine induced vasoconstriction in rat mesenteric arteries. Furthermore, it has lowered the ratio of cellular ATP/ADP as well as enhanced the AMPK activity in smooth muscle cells of vessels and thoracic aorta of rat due to its mitochondrial uncoupling action. They have also reported that in smooth muscle cells of vessels, niclosamide ethanolamine may enhance the cytosolic intracellular calcium (Ca²⁺)_i and cause mitochondrial membrane depolarisation, imply that it could be used to treat hypertension (Li *et al.*, 2017).

Despite the above pharmacological activities, the mitochondrial phosphorylation uncoupler, niclosamide has also shown to be effective in endometriosis (Prather *et al.*, 2016), neuropathic pain (Zhang *et al.*, 2013; Ai *et al.*, 2016), intimal hyperplasia (DiRenzo *et al.*, 2016), neuronal impairment (Pan *et al.*, 2020) and Parkinson's disease (Barini *et al.*, 2018; Kadri *et al.*, 2018).



*Materials
and
Methods*



Experimental materials

3.1 Animals

Apparently healthy adult male albino mice (25-30 g) were procured from the Laboratory Animal Resource Section, ICAR-Indian Veterinary Research Institute, Izatnagar, U.P. Mice were housed in polypropylene cages under 12-12 h dark-light cycle and had free access to feed and drinking water. Animals were acclimatized for a period of one week in the Animal House prior to the start of the study. All the experimental protocols were carried out after approval of the project from the Institutional Animal Ethics Committee (IAEC).

Table 3.1: List of chemicals and reagents used for different experimental protocols

Chemicals/Reagents	Molecular weight	Manufacturer/ Make
Drugs		
Isoprenaline	247.72	Sigma, USA
Orlistat	—	Cayman, USA
Niclosamide (ethanolamine salt)	—	Cayman, USA
Chemicals		
Dimethyl Sulfoxide (DMSO)	78.13	Genetix Biotech Asia, New Delhi, India
Diagnostic kits for Biochemical Studies		
Creatine kinase - myocardial band (CK-MB)		Coral Clinical Systems, Goa, India
Lactate dehydrogenase (LDH)		Coral Clinical Systems, Goa, India
Cholesterol		Coral Clinical Systems, Goa, India
Triglyceride		Coral Clinical Systems, Goa, India
C-reactive Protein (CRP)		Coral Clinical Systems, Goa, India

Table 3.1: Contd...

Chemicals/Reagents	Molecular weight	Manufacturer/ Make
High density lipoprotein (HDL) cholesterol		Coral Clinical Systems, Goa, India
Low density lipoprotein (LDL) cholesterol		Coral Clinical Systems, Goa, India
Chemicals used for oxidative stress parameters		
Pyrogallol	126.11	SRL, New Mumbai, India
MTT	414.32	SRL, New Mumbai, India
Sodium chloride (NaCl)	58.44	Merck, Mumbai, India
Potassium chloride (KCl)	74.55	Merck, Mumbai, India
Potassium dihydrogen phosphate (KH_2PO_4)	136.04	CDH, New Delhi, India
Disodium hydrogen phosphate (Na_2HPO_4)	177.99	SRL, New Mumbai, India
DMSO	78.13	Puregene, Genetix Biotech Asia, New Delhi, India
Hydrogen peroxide (H_2O_2)	34.01	LOBA CHEMIE, Mumbai, India
Thiobarbituric acid (TBA)	144.15	HiMedia, Maharashtra, India
Trichloroacetic acid (TCA)	163.39	SRL, New Mumbai, India
Concentrated hydrochloric acid (HCl)	36.46	LOBA CHEMIE, Mumbai, India
EDTA (Na_2)	372.24	Duchefa Biochemie, Netherlands
Tris base	121.14	MP Biomedicals, Navi Mumbai, India
5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)	396.35	Sigma, USA
Chemicals used for protein estimation		
Bovine serum albumin (BSA)	-	CDH, New Delhi, India
Sodium chloride (NaCl)	58.44	Merck, Mumbai, India
Bradford reagent	-	SRL, Mumbai, India
Chemicals used for tissue fibrotic marker estimation (hydroxyproline and glucosamine)		
6N Hydrochloric acid (HCl)	36.46	SRL, Mumbai, India
L-Hydroxyproline standard	131.13	SRL, Mumbai, India
Sodium acetate trihydrate	136.08	Merck, Mumbai, India
Citric acid monohydrate	210.14	SRL, Mumbai, India
Glacial acetic acid	80.05	Merck, Mumbai, India
Sodium hydroxide	40.00	BDH, New Delhi, India
Chloramine-T	281.69	SRL, Mumbai, India
n-propanol	60.10	SRL, Mumbai, India
Perchloric acid	100.46	Merck, Mumbai, India
p-Dimethylaminobenzaldehyde	149.19	SRL, Mumbai, India
Glucosamine standard	215.64	SRL, Mumbai, India
Acetyl acetone	100.12	SRL, Mumbai, India
Sodium carbonate (Na_2CO_3)	105.99	BDH, New Delhi, India
Absolute alcohol	46.07	Sigma-Aldrich, USA
Concentrated HCl	36.46	LOBA CHEMIE, Mumbai, India

Table 3.1: Contd...

Chemicals/Reagents	Molecular weight	Manufacturer/ Make
Phenolphthalein indicator	318.33	SRL, Mumbai, India
Histopathological Studies		
Formaldehyde	30.03	Thermo Fisher scientific, USA
Hematoxylin	302.28	Sigma, USA
Eosin Y	691.86	Sigma, USA
Molecular Studies		
ELISA kits		
Collagen type I		FineTest, (EM0939) Wuhan, China
Connective tissue growth factor (CTGF)		FineTest, (EM0325) Wuhan, China
Western blotting and gelatin zymography		
RIPA lysis buffer	—	HiMedia, Maharashtra, India
Acrylamide	71.08	MP Biomedicals, Navi Mumbai, India
Bis-acrylamide	154.17	Amresco, Ohio, USA
Tris-base	121.14	MP Biomedicals, Navi Mumbai, India
Sodium dodecyl sulfate (SDS)	288.4	MP Biomedicals, Navi Mumbai, India
Ammonium persulfate	228.20	SRL, Mumbai, India
N,N,N',N'-tetramethylethylenediamine (TEMEM)	116.21	Puregene, Genetix, New Delhi
Glycine	75.07	Amresco, Ohio, USA
Methanol	32.04	Merck, Mumbai, India
Sodium chloride	58.44	Merck, Mumbai, India
Tween-20	—	Amresco, Ohio, USA
Bovine serum albumin	—	CDH, New Delhi, India
Nrf-2 primary antibody	—	Santa Cruz, USA
NF-kB primary antibody	—	Genetex, North America
GAPDH primary antibody	—	Genetex, North America
HRP-conjugated secondary antibody	—	Santa Cruz, USA
ALP-conjugated secondary antibody	—	Elabscience, USA
Diaminobenzidine tetrahydrochloride (DAB) Kit	—	Genei, Bangalore, India
Nitro blue tetrazolium (NBT)	817.65	SRL, Mumbai, India
5-bromo-4-chloro-3-indolyl-phosphate (BCIP)	433.62	SRL, Mumbai, India
Gelatin	—	Amresco, Ohio, USA
Triton X-100	—	LOBA CHEMIE, Mumbai, India
Calcium chloride (CaCl ₂)	147.02	CDH, New Delhi, India
Zinc chloride (ZnCl ₂)	136.29	LOBA CHEMIE, Mumbai, India
Magnesium chloride (MgCl ₂)	203.3	Merck, Mumbai, India
Coomassie Brilliant Blue (G-250)	854.04	Amresco, Ohio, USA
Acetic acid	60.05	Merck, Mumbai, India

Table 3.1: Contd...

Chemicals/Reagents	Molecular weight	Manufacturer/ Make
RNA isolation		
Diethyl pyrocarbonate (DEPC, 0.1%)	162.14	Amersco, Ohio, USA
TRIzol Reagent	-	Ambion, USA
Chloroform	119.38	HiMedia, Maharashtra, India
Isopropyl Alcohol	60.09	Amersco, Ohio, USA
Ethanol	46.07	Sigma-Aldrich, USA
cDNA synthesis		
oligoDT	BCGB100	BR Biochem, NewDelhi, India
RNA protector	BCGB100	BR Biochem, NewDelhi, India
M-MLV RTase	BCGB100	BR Biochem, NewDelhi, India
RT Buffer (contain dNTP mix)	BCGB100	BR Biochem, NewDelhi, India
Polymerase chain reaction (PCR) and qRT-PCR		
PCR master mix (2X)		Thermo Scientific, USA
SYBR Green		Takara, USA
Nuclease free water		Thermo Scientific, USA
Primers		Forward primer Reverse primer Imperial life sciences, Gurugram, Haryana

3.2 Solutions of different drugs

Solutions of isoprenaline, orlistat, and niclosamide ethanolamine were prepared freshly by using the following solvents according to their solubility as mentioned in Table 3.2.

Table 3.2: Solutions of isoprenaline, orlistat and niclosamide ethanolamine (NEN) prepared for the administration in experimental mice

Name of Drug	Strength of drug solution	Solvents used
Isoprenaline	2.5 mg/ml	Distilled water
Orlistat	10 mg of orlistat was dissolved in 50 µl of DMSO. Then, 2 µl of stock solution was further dissolved in 98 µl of peanut oil.	DMSO Further diluted with peanut oil
Niclosamide ethanolamne (NEN)	10 mg of niclosamide was dissolved in 400 µl of DMSO. Then, 2 µl of stock solution was further dissolved in 98 µl of peanut oil.	DMSO Further diluted with peanut oil

3.3 Compositions of buffer solutions used during experimental protocols

3.3.1. Phosphate buffer saline (pH-7.4)

Constituents	mM	g/L
Sodium chloride (NaCl)	137	8.0
Potassium chloride (KCl)	2.7	0.2
Potassium dihydrogen phosphate (KH_2PO_4)	1.4	0.24
Di-sodium hydrogen phosphate (Na_2HPO_4)	10	1.42

3.3.2. Phosphate buffer 50 mM (pH-7.0)

Constituents	mM	g/0.200L
Potassium dihydrogen phosphate (KH_2PO_4)	50	1.37
Di-sodium hydrogen phosphate (Na_2HPO_4)	50	1.42

3.3.3. Acetate citrate buffer (pH -6.5)

Constituents	Strength (g/L)
Sodium acetate trihydrate	120
Citric acid	46
Glacial acetic acid	12 ml
Sodium hydroxide	34

3.3.4. 5X Laemmli sample buffer

Constituents	ml/16 ml
Distilled water	6.8
0.5 M Tris (Ph: 6.8)	2.0
Glycerol	3.2
20 % SDS	1.6
β -mercaptoethanol	0.8
1 % bromophenol blue	1.6

3.3.5. Running buffer for western blotting and gelatin zymography

Constituents	Strength (g/L)
Tris-base	3.02
Glycine	14.4
Sodium Dodecyl Sulfate (SDS)	1

3.3.6. Transfer buffer for western blotting

Constituents	Strength (g/L)
Tris-base	3.02
Glycine	14.4

Methanol was added in transfer buffer at 15 % (for smaller molecular weight protein) and 10 % (for larger molecular weight protein).

3.3.7. Developing buffer for nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (pH-9.5)

Constituents	mM	g/100 ml
Tris-base	100	1.211
Sodium chloride (NaCl)	100	0.5844
Magnesium chloride (MgCl ₂)	5	0.10165

3.3.8. Developing buffer for gelatin zymography (pH-7.4)

Constituents	Molar	500 ml
Tris	50 mM	3.025 g
Calcium chloride (CaCl ₂)	5 mM	0.365 g
Sodium chloride (NaCl)	200 mM	5.84 g
Zinc chloride (ZnCl ₂)	1 µM	5 µl
Triton X-100	1 %	5 ml

3.4 The present study was undertaken according to the following experimental protocols of the individual test substance (orlistat or niclosamide ethanolamine):

a) Experimental design for orlistat studies

After the acclimatization period, mice were divided randomly into four groups for orlistat studies. The drugs were given as per the following schedule mentioned in Table 3.4a in each group.

Table 3.4a Experimental design followed for orlistat study

Group	Treatment	Dose	Route	Period of exposure
Group I	Control (Vehicle)	The same volumes of vehicles were administered according to the body weight.	-	14 days
Group II	Orlistat-alone	30 mg/kg body weight	Intraperitoneal (I.P.)	14 days
Group III	Isoprenaline-alone (Cardiac Injury group)	20 mg/kg body weight	Subcutaneous (SC)	14 days
Group IV	Orlistat with Isoprenaline (Treatment group)	ISP @ 20 mg/kg body weight	SC	14 days
		+Orlistat @ 30 mg/kg body weight	I.P.	14 days

b) Experimental design for niclosamide ethanolamine studies

After the acclimatization period, mice were divided randomly into four groups for niclosamide ethanolamine studies. A pilot study was conducted for niclosamide ethanolamine @ 1 mg/kg body weight dose, intraperitoneally on alternate days for initial eight days of experimental period. However, no significant effect was observed on the various parameters viz., cardiac injury markers, lipid profile, antioxidants and fibrotic markers. The results of NEN @ 1 mg/kg body weight were also presented under results. Therefore, the dose of niclosamide ethanolamine was increased to 3 mg/kg body weight and drugs were given as per the following schedule mentioned in Table 3.4b in each group.

Table 3.4b: Experimental design followed for niclosamide ethanolamine (3 mg/kg body weight; I.P.) study

Group	Treatment	Dose	Route	Period of exposure
Group I	Control (Vehicle)	The same volumes of vehicles were administered according to the body weight.	-	14 days
Group II	Niclosamide ethanolamine-alone	3 mg/kg body weight	Intraperitoneal (I.P.)	On alternate days for 14 days period
Group III	Isoprenaline-alone (Cardiac Injury group)	20 mg/kg body weight	Subcutaneous (SC)	14 days
Group IV	Niclosamide ethanolamine with Isoprenaline (Treatment group)	ISP @ 20 mg/kg body weight + Niclosamide ethanolamine @ 3 mg/kg body weight	SC	14 days
			I.P.	On alternate days for 14 days period

3.5.1 Induction of Cardiac Fibrosis

Isoprenaline was used for the induction of cardiac fibrosis in mice. Experimental cardiac fibrosis was induced in mice by administering isoprenaline subcutaneously at a dose of 20 mg per kg body weight daily for 14 consecutive days with a 24-hour interval between each dose.

3.5.2 Administration of test substance (orlistat and niclosamide ethanolamine)

The test substances orlistat and niclosamide ethanolamine were administered as per the schedule mentioned in the Table 3.4a and 3.4b respectively.

3.6 Collection of blood and tissues

A day after the last dose of isoprenaline (on the day 15th), mice in each group were weighed using an electronic balance. Following this, the mice were anesthetized with urethane @ 1.2 g per kg body weight (I.P.). Blood samples were obtained from heart by the cardiac puncture. Afterward, the mice were sacrificed, and their hearts were collected by opening the abdomen and thoracic cavity. Both blood and hearts from all groups were utilized for further analysis of different parameters.

3.7 Experimental protocols

3.7.1 Evaluation of morphometric parameters

a) Assessment of body weights of mice

The body weights of mice of each group were assessed using a weighing balance both before the administration of orlistat, niclosamide, and isoprenaline (day zero) and on the day of sacrifice (day 15th).

b) Estimation of heart weights of mice

On the day 15th, after proper anesthesia the abdomen and thoracic cavity of mice of all groups were opened and hearts were collected. The adhering tissues and fats were carefully removed and cleaned hearts were weighed by using a high-precision digital weighing balance.

c) Estimation of tibial length of mice

The tibial lengths of mice of all groups were measured on sacrifice day by using the vernier calipers.

d) Evaluation of heart weight to body weight ratio

The heart weights of the mice were divided by their respective body weights for all groups to calculate the heart weight to body weight ratio or relative heart weight.

e) Estimation of heart weight to tibial length index

A more precise measurement of cardiac hypertrophy was determined by comparing the heart weight of mice to their respective tibial length, following the method described by Helms and co-workers (2010).

3.7.2 Serum biochemical parameter evaluation

Whole blood samples were collected from all groups of mice in serum separation tubes (SST). SST tubes were kept for 1 hour undisturbed at room temperature to allow clot formation. Then, the tubes were centrifuged at 3000 rpm for 10 minutes at 4°C by using a refrigerated centrifuge. The clear supernatants (serum) from SST tubes were transferred into

sterile 2 ml tubes. The 100 µl aliquots from each sample were prepared to avoid repeated freeze and thaw cycles during the estimation of various parameters. Serum samples were stored at -80°C. Several biochemical parameters, including cardiac injury markers (CK-MB; LDH), lipid profile (cholesterol, triglyceride, HDL cholesterol, LDL cholesterol), and C-reactive proteins were estimated in serum samples by using the commercially available biochemical kits. Furthermore, serum samples were also used for antioxidants and lipid peroxidation estimations.

A) Estimation of cardiac injury markers

i) CK-MB enzyme activity in serum

The CK-MB (NAC act.) kit was used to estimate the CK-MB enzyme activity in the serum samples of mice of all groups.

Principle

The CK-MB kit utilizes an immunoinhibition/ modified IFCC approach to measure CK-MB activity in a sample. Initially, an anti-CK-M agglutinating sera in the reagent completely inhibits the CK-M fractions of CK-MM and CK-MB present in the sample. Subsequently, the remaining activity, attributed to the CK-B fraction is measured using the CK (NAC act.) method. The CK-MB activity is then calculated by multiplying the measured CK-B activity by two. This method enables the quantification of CK-MB activity, a vital marker for cardiac injury (CK-MB kit; Coral Clinical system).

Procedure

The CK-MB activity was determined following the protocol provided in the kit. A working reagent was prepared by mixing the enzyme reagent (L1) and the starter reagent (L2) in a 4:1 ratio. Subsequently, 500 µl of the prepared working reagent was dispensed into labeled clean, dry tubes. To this, 25 µl of the serum sample was added and thoroughly mixed. The spectrophotometer was calibrated to zero using distilled water. After, 10 minutes, the initial absorbance (A_0) was measured at a wavelength of 340 nm. Then, three consecutive absorbance readings were taken at one-minute intervals (1, 2, and 3 minutes). The average absorbance change per minute ($\Delta A/\text{min}$) was calculated for each sample.

Calculation

CK-MB activity in samples at 37°C was calculated by the following formula.

$$\text{CK-MB activity (U/L)} = \Delta A/\text{min} \times 6666$$

ii) Serum Lactate dehydrogenase activity

To estimate the LDH activity in serum samples of different groups, an LDH kit was used.

Principle

The lactate dehydrogenase (LDH) enzyme facilitates the reduction of pyruvate with NADH, leading to the formation of lactate and NAD. The oxidation rate of NADH to NAD is quantified by measuring a decrease in absorbance. This decrease in absorbance is directly proportional to the LDH activity present in the sample (LDH kit; Coral Clinical system).

Procedure

The protocol given with the kit was followed to measure the LDH activity in serum samples. The working reagent was prepared according to the protocol by mixing buffer reagent (L1) and starter reagent (L2) in a 4:1 ratio. The 25 µl of serum sample was thoroughly mixed in 500 µl of this prepared working reagent. Following a one-minute incubation at 37 °C, the initial absorbance (A_0) was taken. Subsequently, absorbance readings were taken at one-minute intervals over a span of 3 minutes. The mean absorbance change per minute ($\Delta A/\text{min}$) was calculated.

Calculation

The lactate dehydrogenase enzymatic activity at 37°C was calculated by the following formula.

$$\text{LDH activity (U/L)} = \Delta A/\text{min} \times 8095$$

B) Evaluation of lipid profile**i) Serum cholesterol level**

Cholesterol levels in serum samples of all groups were estimated by using the commercially available diagnostic biochemical kit that was based on the CHOD/PAP method.

Principle

Cholesterol esterase breaks down esterified cholesterol into free cholesterol. The free cholesterol undergoes oxidation, producing hydrogen peroxide. This hydrogen peroxide reacts with phenol and 4-amino antipyrine in the presence of peroxidase, leading to the formation of a red-colored quinone imine dye complex. The intensity of this red color directly correlates with the amount of cholesterol in the sample (Cholesterol kit; Coral Clinical system).

Procedure

The cholesterol measurement was conducted following the prescribed protocol of the kit.

- The working reagent for cholesterol was prepared by combining 4 parts of enzyme reagent 1 (L1) with 1 part of enzyme reagent 2 (L2).
- Test tubes were labeled as blank (B), standard (S), and test (T), and ensured to be clean and dry.
- Each tube was filled with 1000 µl of the prepared working reagent.
- Distilled water, cholesterol standard, and test serum samples (10 µl each) were added to the blank, standard, and test (T) tubes, respectively.
- The contents of the tubes were thoroughly mixed and then incubated at 37°C for 5 minutes.
- The absorbance of both the standard and serum test samples was measured against the blank at a wavelength of 505 nm after incubation.

Calculation

The cholesterol levels in serum samples of all the groups were calculated by using the following formula.

$$\text{Cholesterol (mg/dl)} = \frac{\text{Absorbance of Test (serum sample)}}{\text{Absorbance of standard}} \times 200$$

ii) Serum triglyceride levels

The triglyceride level in serum samples was assessed by a biochemical diagnostic kit based on the GPO/PAP method.

Principle

Lipoprotein lipase acts on triglycerides, breaking them down into glycerol and free fatty acids. Glycerol, in the presence of ATP and glycerol kinase, converts into glycerol 3-phosphate. Glycerol 3-phosphate is then oxidized by the enzyme glycerol phosphate oxidase, producing hydrogen peroxide. This hydrogen peroxide reacts with a phenolic compound and 4-amino antipyrine under the catalytic action of peroxidase, forming a red-colored quinoneimine dye complex. The intensity of the resulting color is directly proportional to the amounts of triglyceride present in the sample (Triglyceride kit; Coral Clinical system).

Procedure

The prescribed protocol given with the kit was followed to estimate the triglyceride levels in serum samples.

- A working reagent of the triglyceride was prepared by mixing the enzyme reagent 1 (L1) and enzyme reagent 2 (L2) in 4:1 ratio.
- 1000 µl of the prepared working reagent was added in each tube labeled as blank, standard, and test serum samples.
- 10 µl of distilled water, triglyceride standard, and serum samples were added in respective tubes.
- After thoroughly mixing the content, tubes were incubated at 37°C for 5 minutes.
- The absorbance of both standard and serum samples was taken at 505 nm wavelength against the blank.

Calculation

The triglyceride levels in serum samples of all the groups were calculated by using the following formula.

$$\text{Triglyceride (mg/dl)} = \frac{\text{Absorbance of Test (serum sample)}}{\text{Absorbance of standard}} \times 200$$

iii) High-density lipoprotein cholesterol (HDL cholesterol) levels

A commercially available HDL-D cholesterol kit based on the direct enzymatic method was used for the determination of the HDL cholesterol in serum.

Principle

The serum HDL cholesterol levels can be directly determined through an enzymatic method that eliminates the need for any pre-treatment or centrifugation of the sample. This method relies on a specific detergent that solubilizes only HDL, enabling the release of HDL cholesterol. This released HDL cholesterol reacts with cholesterol esterase, cholesterol oxidase, and chromogens, resulting in the formation of a colored product. Other lipoproteins such as LDL, VLDL, and chylomicrons are prevented from reacting with the enzymes due to the absorbance of detergent on their surfaces. The intensity of the colour formed is directly proportional to the concentration of HDL cholesterol in the samples (HDL-c kit; Coral Clinical system).

Procedure

Clean and dry 2 ml tubes were labeled as blank (B), calibrator (C), and test (T). In each tube, 450 µl of HDL-D reagent 1 (L1) was added. Afterward, 6 µl of serum calibrator and test samples were added to their respective tubes. The contents were mixed well and incubated for 5 minutes at 37°C. Subsequently, 150 µl of HDL-D reagent 2 (L2) was added to all tubes, including the blank. After thorough mixing, the tubes were incubated again for 5 minutes at 37°C. The absorbance of the calibrator and test samples against the blank was measured at 578 nm.

Calculation

The serum HDL cholesterol (HDL-c) was calculated by the following formula

$$\text{HDL-c (mg/dl)} = \frac{\text{Absorbance of Test (serum sample)}}{\text{Absorbance of calibrator}} \times \text{Conc. of calibrator}$$

iv) Serum Low-density lipoprotein cholesterol (LDL-c)

LDL-D cholesterol kit was used for the estimation of LDL cholesterol in serum.

Principle

The LDL-c in serum is determined by the direct enzymatic method without pre-treatment or centrifugation. This process involves two steps. In the first step, non-LDL cholesterol is

removed. Then, in the second step, the remaining LDL cholesterol is directly measured. The concentration of LDL cholesterol is determined by the intensity of the colour formed during the reaction (LDL-c kit; Coral Clinical system).

Procedure

The tubes were designated as blank (B), calibrator (C), and test (T). In each tube, 375 µl of LDL-D reagent 1 (L1) was dispensed. Subsequently, 5 µl of serum sample was added to the respective test tube and 5 µl of calibrator was added to the calibrator tube. The contents of each tube were thoroughly mixed and then incubated at 37°C for 5 minutes. After the incubation period, 125 µl of LDL-D reagent 2 (L2) was added to each tube. The contents were mixed again and incubated for an additional 5 minutes at 37°C. Following the incubation, the absorbance of the calibrator and test samples against the blank was measured at a wavelength of 546 nm.

Calculation

The LDL cholesterol levels in the serum samples of all groups were determined using the following formula.

$$\text{LDLc (mg/dl)} = \frac{\text{Absorbance of Test (serum sample)}}{\text{Absorbance of calibrator}} \times \text{Conc. of calibrator}$$

v) Serum HDL to LDL ratio (HDL/LDL ratio)

The HDL and LDL cholesterol values were calculated as mentioned above. Then, the HDL cholesterol values were divided by the respective animal's LDL cholesterol values to get the ratio of HDL/LDL.

C) Serum C-reactive protein estimation

Serum C reactive protein was estimated by the TURBILYTE-CRP kit.

Principle:

TURBILYTE-CRP is a turbidimetric immunoassay designed to measure C-reactive protein levels in test samples viz., serum. This assay relies on the principle of agglutination

reaction. In this test, the sample mixes with activation buffer (R1) and TURBILYTE-CRP latex reagent, leading to a reaction. If C-reactive protein is present in the sample, it forms an insoluble complex, causing turbidity. The turbidity level, indicating the concentration of CRP in the sample is measured at a wavelength of 630 nm (CRP kit; Coral Clinical system).

Procedure

- Serum samples were evaluated for C-reactive protein levels following the TURBILYTE-CRP kit instructions.
- Reagents and samples were equilibrated to room temperature before use.
- The calibrator was reconstituted with 1 ml of distilled water and gently swirled for homogeneity after 10 minutes.
- Tubes labeled as calibrator and test sample received 450 µl of R1 and 50 µl of R2 reagents.
- After thorough mixing, the mixture was incubated for 5 minutes.
- 5 µl of the calibrator and serum sample were added to their respective tubes.
- The contents were mixed again, and absorbance was measured initially (A1) at 10 seconds and then after (A2) at 2-minute intervals.
- The change in the absorbance per minute was measured.

Calculation

The concentration of CRP in serum samples was calculated by the following formula.

$$\text{Conc. of CRP (mg/l)} = \frac{\Delta \text{ Absorbance of Test (serum sample)}}{\Delta \text{ Absorbance of calibrator}} \times \text{Conc. of calibrator}$$

3.7.3 Estimation of antioxidant parameters in heart tissue and serum

Levels of different oxidative stress parameters including enzymatic antioxidants (superoxide dismutase and catalase), non-enzymatic antioxidants (reduced glutathione; GSH), and lipid peroxidation (LPO) in heart tissue were estimated by employing different assay procedures and using UV-visible spectrophotometer.

Preparation of tissue homogenate

For the estimation of SOD, catalase, and LPO, heart tissues were weighed and homogenized in phosphate buffer saline (PBS; pH 7.4) to prepare 10 % of tissue homogenate. Furthermore, 10% of tissue homogenate was prepared separately in 0.02 M EDTA (Na₂) for GSH.

a) Superoxide dismutase

Superoxide dismutase (SOD) activity in heart tissue was assessed following the protocol outlined by Madesh and Balsubramaniam (1998).

Principle

This method is based on measuring the ability of SOD to impede the autoxidation of pyrogallol and the subsequent production of superoxide radicals. During this procedure, superoxide radicals generated during the autoxidation of pyrogallol reduce a tetrazolium dye viz., MTT [3-(4-5 dimethyl-thiazol-2-yl)2,5diphenyltetrazolium bromide] to its formazan derivative. The SOD enzyme inhibits the reduction of MTT dye by serving as a scavenger of these superoxide radicals. The reaction is terminated by adding dimethyl sulfoxide (DMSO), which also aids in solubilizing the formazan formed during the reaction. The color of formazan produced during the dye reduction is measured at 570 nm by using a spectrophotometer. The level of inhibition was expressed in terms of SOD units. One unit of SOD is defined as the quantity of enzyme required to suppress the MTT reduction by 50%.

Reagents

- a) Pyrogallol (100 mM): 6.3 mg of pyrogallol was dissolved in 5 ml of distilled water to prepare the stock solution of pyrogallol. For working solution of pyrogallol, 0.1 ml of stock solution was added to the 9.9 ml of distilled water.
- b) MTT (1.25 mM): 2.58 mg MTT was dissolved in 5 ml of distilled water.
- c) Phosphate buffer saline (PBS): 800 mg of sodium chloride (NaCl), 20 mg of potassium chloride (KCl), 24 mg of potassium dihydrogen phosphate (KH₂PO₄), and 141.96

mg of disodium hydrogen phosphate (Na_2HPO_4) were dissolved in 80 ml of distilled water. Then, the pH was adjusted to 7.4 and the final volume was made up to 100 ml by adding distilled water.

d) Dimethyl sulfoxide (DMSO)

Procedure

All the reagents were prepared freshly and the following steps were followed to assess SOD activity in heart tissues.

- Clean and dry tubes were labeled as control and test samples.
- 650 μl of PBS was taken in each tube.
- Then, 30 μl of MTT dye was added to control as well as test sample tubes.
- 10 μl of tissue homogenate was added to respective test sample tubes.
- 75 μl of pyrogallol working solution was added in both control and test sample tubes.
- The reaction mixtures were incubated for 5 minutes at room temperature.
- Then, to stop the reaction and solubilize the formazan, 750 μl of DMSO was added in each and every tube.
- In the control tube, 10 μl of distilled water was added.
- Then, the absorbances of control and test samples were taken against the blank (DW) at 570 nm.

Calculation

Superoxide dismutase activity in heart tissue homogenates was calculated by following the formula and expressed in SOD units/mg of proteins.

$$Y\% = \text{OD of Test samples} / \text{OD of control} \times 100$$

$$\text{SOD (Units)} = 1/Y\% \times 50 \times \text{Dilution Factor}$$

$$\text{SOD (Units/mg of proteins)} = \text{SOD (Units)} / \text{mg of proteins}$$

Where,

$Y\% = \% \text{ inhibition of MTT reduction by SOD protein;}$

$\text{Dilution factor} = \text{Total volume of reaction mixture} / \text{Total volume of sample}$

$$\text{DF} = 1515/10 = 151.5$$

One unit of SOD is the amount of protein required to inhibit the 50% reduction of MTT dye. Therefore, the multiplication factor 50 was added for one unit of SOD.

b) Catalase

Catalase activity was determined using a spectrophotometric method following the procedure outlined by Aebi in 1983.

Principle

This method is based on measuring the decomposition of hydrogen peroxide (H_2O_2) added into the sample by the catalase enzyme present in the sample. Hydrogen peroxide is catalyzed into oxygen and water by the catalase enzyme. The rate of the decrease in the absorbance is directly proportional to the catalase activity that is measured spectrophotometrically at a specific wavelength over time.

Reagents

- 50 mM phosphate buffer (pH 7.0)
 - i) A 50 mM solution of KH_2PO_4 was prepared by dissolving 1.37 gm of KH_2PO_4 in 200 ml of distilled water.
 - ii) A 50 mM solution of Na_2HPO_4 was prepared by dissolving 1.42 gm of Na_2HPO_4 in 200 ml of distilled water.

50 mM phosphate buffer was prepared by mixing the solution (i) and solution (ii) in a 1:1.5 ratio. Then, the pH was adjusted to 7.0.
- 10 mM Hydrogen peroxide (H_2O_2) solution: 10 mM Hydrogen peroxide (H_2O_2) solution was prepared by adding 100 μl of 30% H_2O_2 in 100 ml of phosphate buffer.

Procedure

For the estimation of catalase activity in heart tissue, 0.1 ml of 10 % tissue homogenate in PBS was mixed with 1.9 ml of phosphate buffer (pH 7.0) and transferred to the quartz cuvette. Then, 10 mM hydrogen peroxide (H_2O_2) solution was added directly into the cuvette as the reaction started immediately after adding hydrogen peroxide. A decrease in the absorbance was measured every 15 seconds for the 1-minute duration at 240 nm of wavelength against distilled water as a blank.

Calculation

Catalase activity in heart tissue homogenates was calculated by following formula and expressed in mmol H₂O₂ utilized/min/mg of proteins.

$$\text{Catalase activity} = \frac{\Delta\text{OD}}{E} \times \text{DF} \times \frac{1}{\text{mg of proteins}}$$

Where,

ΔOD = Change in absorbance per minute

E (Extinction coefficient) = 0.071 mmol cm⁻¹

DF (Dilution factor) = total volume of reaction mixture/ sample volume

DF = 3000/100 = 30

c) Lipid peroxidation (LPO)

Heart tissue lipid peroxidation was assessed colorimetrically using the Thiobarbituric Acid Reactive Substances (TBARS) method developed by Beuge and Aust in 1978.

Principle

This method is based on the principle of measuring the reaction between malondialdehyde (MDA), a product of lipid peroxidation, and thiobarbituric acid (TBA) under acidic conditions. MDA forms a colored complex with TBA and the intensity of this color is directly proportional to the MDA level in the samples which is quantified spectrophotometrically at a specific wavelength to determine the extent of lipid peroxidation in biological samples.

Reagents

- i) 0.37 % thiobarbituric acid (TBA) solution: 0.37 gm of TBA was dissolved in 100 ml of distilled water. The solution was warmed to properly dissolve the TBA.
- ii) 15 % trichloroacetic acid (TCA) solution: 15 gm of TCA was dissolved in 100 ml of distilled water.
- iii) 0.25 N hydrochloric acid solution (HCl): This solution was prepared by diluting the 6N aqueous HCl solution.

TCA-TBA-HCl reagent: This reagent was prepared by mixing 0.37% TBA, 15 % TCA, and 0.25 N HCl solutions in a 1:1:1 ratio.

Procedure

All the reagents were prepared freshly and the following steps were used to assess MDA level in heart tissues.

- 0.1 ml of tissue homogenate was mixed in 2 ml of TCA-TBA-HCl reagent.
- For blank, the buffer used for homogenization of tissue samples was mixed in 2 ml of TCA-TBA-HCl reagent.
- Then, the solutions were heated in a boiling water bath for 15 minutes.
- After cooling, the reaction mixtures were centrifuged at 1000 g for 10 minutes to remove the flocculent precipitate from the solutions.
- The absorbance of clear supernatant was measured at 535 nm wavelength against the blank.

Calculation

- The concentration of MDA in heart tissue was calculated using the following formula and expressed in nmol/mg of proteins.

$$\text{MDA (nmol/mg of proteins)} = \frac{\text{OD}}{\text{EC}} \times \text{DF} \times \frac{1}{\text{mg of proteins}}$$

Where,

OD= Absorbance,

EC (Extinction coefficient) = $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$

Dilution factor = total volume of reaction mixture/sample volume

DF= $2100/100 = 21$

d) Reduced glutathione (GSH)

The non-enzymatic antioxidant reduced glutathione (GSH) in heart tissue was determined using the method developed by Sedlak and Lindsay (1968).

Principle

This method of glutathione estimation relies on the interaction between reduced glutathione (GSH) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), also known as Ellman's reagent. In this process, GSH undergoes oxidation by DTNB, resulting in the formation of a yellow-colored compound called 5-thio-2-nitrobenzoic acid. The intensity of this yellow color can be quantified through spectrophotometry at wavelength of 412 nm.

Reagents

- i) 0.01 M Ellman's reagent solution: 99 milligrams of Ellman's reagent was dissolved in 25 ml of absolute methanol.
- ii) 1 M solution of Tris Buffer (pH 8.9): 12.1 gm of tris base was dissolved in distilled water. Then, pH of the prepared solution was adjusted to 8.9 and distilled water was added to make final volume 100 ml.
- iii) 50 % solution of trichloroacetic acid (TCA) solution: 50 gm of TCA was weighed and dissolved in distilled water to make 100 ml solution.

Procedure

1 ml of supernatant from tissue homogenate was mixed with 0.8 ml of distilled water and 0.2 ml of 50 % TCA solution. The reaction mixture was allowed to incubate at room temperature for 15 minutes. Afterward, the solution was centrifuged at 3000 rpm for 15 minutes. The 0.4 ml of clear supernatant was transferred to new tube and 0.8 ml of tris buffer was added. To this, 0.2 ml of Ellman's reagent was mixed and absorbance of the yellow-colored solution was measured at 412 nm wavelength within a 5 minute. The reagent blank was prepared having all components except sample homogenate.

Calculation

The tissue reduced glutathione (GSH) was calculated by using following formula.

$$\text{GSH} = \frac{\text{OD}}{\text{EC}} \times \text{DF} \times 1000$$

Where,

OD= Absorbance,

E (Extinction coefficient) = $13600 \text{ M}^{-1} \text{ cm}^{-1}$

Dilution factor (DF)= total volume of reaction mixture/sample volume

Serum antioxidant activity (SOD, catalase and reduced GSH) and lipid peroxidation were also assessed by following the above-mentioned protocols.

3.7.4 Estimation of protein in tissue homogenate

Proteins in tissue homogenate was estimated by Bradford's method.

Principle

This method involves the interaction between dye known as coomassie brilliant blue G-250 and proteins in samples. This dye undergoes a color change and shifting in the absorbance maximum from around 465 nm to 595 nm in the presence of proteins. The binding of the dye to proteins forms a complex that has a characteristic absorption peak at 595 nm. The intensity of this color peak is directly proportional to the protein concentration in the sample.

Reagents

- i) **Standard of bovine serum albumin:** 5 mg of bovine serum albumin was dissolved in 1 ml of distilled water to prepare 5 mg/ml strength solution. This solution was further diluted with distilled water to prepare 0.5 mg/ml strength stock solution.
- ii) **0.15 M sodium chloride solution:** 0.09 gm of sodium chloride was dissolved in 10 ml distilled water to prepare 0.9 % NaCl solution having 0.15 M concentration.

Procedure

Different solutions of bovine serum albumin standards were prepared as per following table 3.5.

Standards	Volume of 0.5 mg/ml BSA (in μ l)	Volume of 0.15 M of NaCl	BSA (μ g)
Blank	0	100	0
S1	5	95	2.5
S2	10	90	5
S3	15	85	7.5
S4	20	80	10
S5	25	75	12.5

- 5 μ l of homogenate supernatant from test samples were taken in tubes.
- Upto 1 ml of Bradford reagent was added in each tube of standard and test samples.
- The reaction mixtures were incubated at room temperature for 5 minutes.
- Absorbances were taken at 595 nm wavelength using spectrophotometer.
- The standard calibration curve of absorbance vs concentration of standards was plotted to determine the protein concentration in test samples.
- Similarly, protein in serum samples were also estimated.

3.6.5 Determination of fibrotic markers in heart tissue

To assess fibrosis markers, 50 mg of heart tissues were placed in tubes containing 2 ml of 6N hydrochloric acid (HCl). The tubes were properly sealed and subjected to autoclaving at 15 pounds of pressure for a duration of 3 hours. The resulting hydrolysate was utilized for the quantification of fibrosis markers viz., hydroxyproline and glucosamine in the heart tissue.

A) Tissue hydroxyproline estimation

The hydroxyproline in heart tissues was estimated by method developed by Reddy and Enwemka (1996).

Principle

The method relies on determining the liberated hydroxyproline in the hydrolysate following the hydrolysis process. A pyrrole is produced due to the oxidation of the released hydroxyproline by the chloramine T. A chromophore forms upon the addition of Ehrlich's

reagent. The intensity of the chromophore can be measured spectrophotometrically at 550 nm wavelength to quantify hydroxyproline in tissue.

Reagents

- i) Hydroxyproline standard stock solution: 1 mg of hydroxyproline standard was dissolved in 1 ml of distilled water to prepare stock solution of 1 mg/ml strength.
- ii) Phenolphthalein indicator: 1% of phenolphthalein solution was prepared in absolute alcohol.
- iii) Acetate citrate buffer (p^H 6.5): 12 gm of sodium acetate trihydrate, 4.6 gm of citric acid, 1.2 ml of glacial acetic acid and 3.4 gm of sodium hydroxide were dissolved in distilled water. After thorough mixing, p^H of the solution was adjusted to the 6.5 and distilled water was added to make the final volume up to 100 ml.
- iv) Chloramine-T (0.056 M) reagent solution: Firstly 50 % solution of the n-propanol was prepared by mixing n-propanol and distilled water in 1:1 ratio. 1.27 gm of chloramine-T was weighted and dissolved in 20 ml of 50 % n-propanol solution. Then acetate citrate buffer was added to make the final volume of solution up to 100 ml.
- v) Ehrlich's reagent: Ehrlich's reagent is not stable therefore it was prepared freshly immediately before use. n-propanol and perchloric acid were mixed in 2:1 v/v ratio. Then, 15 gm of p-dimethylaminobenzaldehyde was dissolved in n-propanol/perchloric acid solution and brought to 100 ml.

Procedure

- The hydroxylate was neutralized by using sodium hydroxide.
- Different concentrations of hydroxyproline standards (0.625 μ g/ml to 20 μ g/ml) were prepared.
- 50 μ l of different standards and test samples were taken in tubes.
- 450 μ l of chloramine T solution was added in each tube and mixed gently.
- Tubes were kept at room temperature for 25 minutes to allow the oxidation of released hydroxyproline by chloramine T.
- Then, 500 μ l of Ehrlich's reagent was added in each tube and after mixing solutions were incubated at 65 °C for 20 minutes in water bath. During this incubation, chromophore was developed.

- Absorbances of standards and test samples were measured at 550 nm wavelength using spectrophotometer.
- The standard curve of concentrations of hydroxyproline standards vs absorbance was plotted to determine the hydroxyproline in test samples.

Calculation

Hydroxyproline in heart tissues was calculated from the standard curve of concentration vs absorbance and expressed as μg of hydroxyproline/mg of tissue.

B) Tissue glucosamine estimation

Glucosamine in heart tissue was assessed by protocol described by Randle and Morgan (1955) in acid hydrolyzed hydrolysate.

Principle

This method is based on the measuring the color intensity generated during the reaction. Amino sugars viz., glucosamine undergo a reaction with acetylacetone in alkaline solution at 100°C and forms chromogenic material. Upon combining this resultant solution with an acidic solution of Ehrlich's reagent in the presence of ethanol, a chromophore is produced, giving rise to a colored solution.

Reagents

- i) Preparation of glucosamine standard solution: 1 mg of glucosamine standard was dissolved in 1 ml of distilled water to prepare stock solution with a strength of 1 mg/ml.
- ii) Acetyl acetone solution: 0.5 N solution of sodium carbonate was prepared by dissolving 5.3 mg of its in 50 ml of distilled water. 1 ml of acetylacetone was added in 49 ml of 0.5 N sodium carbonate solution.
- iii) Ehrlich's reagents: 0.8 gm of p-dimethylaminobenzaldehyde was weighed and mixed with 30 ml of absolute alcohol. Then, 30 ml of concentrated HCl was added to prepare Ehrlich's reagent.

Procedure

- Different concentrations of glucosamine (ranging from 3.125 $\mu\text{g}/\mu\text{l}$ to 200 $\mu\text{g}/\mu\text{l}$) were prepared from the stock solution standard.
- 1 ml of standards of different concentration and neutralized tissue hydrolysate samples were taken into 15 ml tubes.
- 1 ml of acetylacetone solution was added in each tube.
- After gentle shaken, 1 ml of distilled water was added along the side wall of tubes.
- Then, tubes were properly sealed and incubated in boiling water bath for 20 minutes.
- After incubation, tubes were removed and cooled under running tap water.
- 0.5 ml of absolute alcohol was added in each tube.
- 1 ml of Ehrlich's reagent was added and final volume was adjusted to the 10 ml with absolute alcohol.
- The contents were mixed thoroughly and incubated at 65 °C for 10 minutes in water bath.
- After cooling the mixture to room temperature, mixed thoroughly and absorbance was measured at 535 nm wavelength by spectrophotometer.
- The standard curve of absorbance vs concentrations of glucosamine standards was plotted to determine the glucosamine in test samples.

Calculation

Glucosamine in heart tissues was calculated from the standard curve of concentration vs absorbance and expressed as μg of glucosamine/mg of tissue.

3.7.6 Estimation of collagen I by ELISA method

Collagen type I in heart tissue was determined by using sandwich enzyme linked immunosorbent assay (ELISA) kit from FineTest (Cat. No. EM0939; Wuhan, China).

Preparation of samples

Heart tissue was collected and residual blood was removed by washing tissue with the pre-cooled 0.01 M PBS buffer (pH 7.4). Heart tissue was weighed and homogenized in

PBS containing 1X protease inhibitor to prepare 5 % homogenate. Samples were subjected to three cycles of freeze-thaw to further cells breakdown. Then, homogenate was centrifuged at 5000 g for 5 minutes. The clear supernatant was collected in new tubes.

Sample dilution

Test samples were diluted 5 times with sample dilution buffer.

Principle

The Collagen type I kit is based on sandwich ELISA technology. The ELISA plate is precoated with a captured antibody that binds to collagen antigen in standards and samples. Biotin conjugated antibody is used as detection antibodies. After washing away unbound components, a horseradish peroxidase enzyme conjugated streptavidin is added which amplify the signals and produce a blue color by enzymatic reaction on substrate viz., 3,3',5,5'-tetramethylbenzidine (TMB). The blue color is changed into yellow color after adding acidic stop solution in wells. The intensity of yellow color is proportional to the collagen type I antigen amount in the samples. Absorbance is taken at 450 nm in a microplate reader and then the target antigen collagen type I can be quantified (Mouse Collagen type I ELISA Kit; FineTest, Wuhan, China).

Reagents

All the reagents of ELISA kit brought to room temperature before use.

- 1) **Wash buffer (25 X):** Wash buffer solution was diluted with autoclaved distilled water to prepare 1 X solution. The 1 ml of wash buffer was dissolved in 24 ml of distilled water.
- 2) **Preparation of collagen type I standards:** Standard given in kit was dissolved in 1 ml of sample dilution buffer and kept at room temperature for 10 minutes and mixed thoroughly. This tube was labeled as zero tube. Seven 2 ml tubes were taken and labeled as 1st to 6th tube having concentration of standards as 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank, respectively. 0.3 ml of sample dilution buffer was added in each tube. 0.3 ml standard solution of zero tube was transferred to 1st tube and mixed

properly. Then, 0.3 ml of solution of 1st tube was transferred to the 2nd tube and mixed thoroughly and so on to prepare different concentration of collagen type I standards. For blank control, sample dilution buffer was used.

- 3) **Preparation of working solution of Biotin- labeled antibody:** 100X solution of biotin labeled antibody was provided with kit. To prepare 1 X working solution, required volume of biotin labeled antibody solution was calculated by the formula (required total volume= 0.1 ml/well × quantity of wells) and diluted with antibody dilution buffer at 1:100 and mixed thoroughly.
- 4) **Preparation of working solution of HRP-Streptavidin Conjugate (SABC):** 100X solution of SABC was provided with kit. Required volume of SABC solution was calculated by the formula (required total volume= 0.1 ml/well × quantity of wells). Then, 1X working SABC solution was prepared by diluting 1 µl of SABC stock solution with 99 µl of SABC dilution buffer.
- 5) TMB substrate solution
- 6) Stop solution

Procedure

Assay procedure provided with ELISA kit was followed for the estimation of collagen type I in heart tissue.

- The required number of wells were taken and washed with wash buffer before adding blank, standards and tissue samples.
- Wells for the blank, standards and samples were decided and numbered respectively.
- 100 µl of sample dilution buffer, standards of different concentration (zero tube to 6th tube) and test samples were added in respective wells of ELISA plate.
- Then, plate was sealed properly with the plate sealer and incubated at 37 °C for 90 minutes.
- After incubation, contents of wells were discarded and 2 times washed with wash buffer.
- Subsequently, 100 µl of working solution of biotin-labeled antibody was added into each well.

- Plate wells were covered with sealer and incubated at 37 °C for 60 minutes.
- After discarding the contents, plate wells were washed 3 times with wash buffer and 100 µl of working solution of HRP-streptavidin was added in each well and incubated at 37 °C for 30 minutes.
- After 5 times plate washing with wash buffer, 90 µl TMB substrate (equilibrated at 37 °C for 30 minutes) was added in each well and incubated 37 °C for 20 minutes in dark.
- After incubation, 50 µl of stop solution was added in each well.
- The color was turned into yellow. Then, the absorbance was taken in microplate reader at 450 nm immediately after adding stop solution.

Calculation

- The relative optical density (OD) of standards and samples was calculated by subtracting the blank from OD of standards and samples.
- The standard curve of relative O.D. and respective concentration of standards was plotted.
- The concentration of collagen type I in tissue homogenate was calculated by using CurveExpert 1.4 software.

3.7.7 Estimation of Connective Tissue Growth Factor (CTGF) by ELISA method

CTGF in heart tissue was determined by using sandwich enzyme linked immunosorbent assay (ELISA) kit from FineTest (Cat. No. EM0325; Wuhan, China).

Preparation of samples

Heart tissue was collected and residual blood was removed by washing tissue with the pre-cooled 0.01 M PBS buffer (pH 7.4). Heart tissue was weighed and homogenized in PBS containing 1X protease inhibitor to prepare 5 % homogenate. Samples were subjected to three cycles of freeze-thaw to further cells breakdown. Then, homogenate was centrifuged at 5000 g for 5 minutes. The clear supernatant was collected in new tubes to estimate CTGF.

Sample dilution

Test samples were diluted 2 times with sample dilution buffer.

Principle

The CTGF kit is based on sandwich ELISA technology, in which an ELISA plate is pre-coated with a capture antibody specific to CTGF antigen in standards and samples. Biotin conjugated antibody serve as detection antibodies. After washing away unbound components, a horseradish peroxidase enzyme conjugated streptavidin is added which produces a blue color through enzymatic reaction with substrate viz., 3,3',5,5'-tetramethylbenzidine (TMB). The blue color is transformed into yellow color upon the addition of acidic stop solution in wells. The intensity of yellow color is directly proportional to the CTGF antigen level in the samples. Measurement of the absorbance at 450 nm using a microplate reader allows for the quantification of the CTGF antigen in the samples. (Mouse CTGF ELISA Kit; FineTest, Wuhan, China).

Reagents

All the reagents of ELISA kit brought to room temperature before use.

- a) **Wash buffer (25 X):** Wash buffer solution was diluted with autoclaved distilled water to prepare 1 X solution. The 1 ml of wash buffer was dissolved in 24 ml of distilled water.
- b) **Preparation of CTGF standards:** Standard given in kit was dissolved in 1 ml of sample dilution buffer and kept at room temperature for 10 minutes and mixed thoroughly. This tube was labeled as zero tube. Seven 2 ml tubes were taken and labeled as 1st to 6th tube having concentration of standards as 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank, respectively. 0.3 ml of sample dilution buffer was added in each tube. 0.3 ml standard solution of zero tube was transferred to 1st tube and mixed properly. Then, 0.3 ml of solution of 1st tube was transferred to the 2nd tube and mixed thoroughly and so on to prepare different concentration of CTGF standards. For blank control, sample dilution buffer was used.

- c) **Preparation of working solution of Biotin- labeled antibody:** 100X solution of biotin labeled antibody was provided with kit. To prepare 1 X working solution, required volume of biotin labeled antibody solution was calculated by the formula (required total volume= 0.1 ml/well × quantity of wells) and diluted with antibody dilution buffer at 1:100 and mixed thoroughly.
- d) **Preparation of working solution of HRP-Streptavidin Conjugate (SABC):** 100X solution of SABC was provided with kit. Required volume of SABC solution was calculated by the formula (required total volume= 0.1 ml/well × quantity of wells). Then, 1X working SABC solution was prepared by diluting 1 µl of SABC stock solution with 99 µl of SABC dilution buffer.
- e) TMB substrate solution
- f) Stop solution

Procedure

Assay procedure provided with ELISA kit was followed for the estimation of CTGF in heart tissue.

- The requisite wells were taken and washed with wash buffer before adding blank, standards and tissue samples.
- Wells were assigned numbers for the blank, standards and samples, respectively.
- In each respective well of the ELISA plate, 100 µl of sample dilution buffer, standards of various concentration (zero tube to 6th tube) and test samples were added.
- The plate was properly sealed properly and incubated at 37 °C for 90 minutes.
- After incubation, well contents were discarded and the wells were washed twice with wash buffer.
- Subsequently, 100 µl of working solution of biotin-labeled antibody was added into each well.
- The plate wells were covered with sealer and incubated at 37 °C for 60 minutes.
- After discarding the contents, plate wells were washed three times with wash buffer and 100 µl of working solution of HRP-streptavidin was added in each well and incubated at 37 °C for 30 minutes.

- After washing the plate five times with wash buffer, 90 µl TMB substrate (equilibrated at 37 °C for 30 minutes) was added in each well and incubated 37 °C for 20 minutes in dark.
- Post incubation, 50 µl of stop solution was added in each well, turning the color yellow.
- Then, the absorbance was taken in microplate reader at 450 nm immediately after adding stop solution.

Calculation

- The relative optical density (OD) of standards and samples was determined by subtracting the blank from OD of standards and samples.
- The standard curve correlating relative O.D. and respective concentration of standards was plotted.
- Using CurveExpert 1.4 software, the concentration of CTGF in tissue homogenate was calculated.

3.7.8 Western blotting for protein expression studies

Expression patterns of proteins viz., Nrf-2 and NF-kB were determined by the western blotting.

Preparation of samples: Heart tissue was homogenized in ice-cooled lysis buffer to prepare 5 % homogenate and centrifuged at 12000 g for 10 minutes at 4°C to obtain clear supernatant. Proteins of homogenate supernatants were estimated by Bradford method. Then, aliquots of samples were stored at -80°C until further use. Protein containing samples were mixed with required amount of 5X Laemmli sample buffer. Then, the mixture was boiled in water bath (100 °C for 5 minutes) and immediately cooled down to the 4 °C. Then, equal amount of protein containing samples were used for SDS-PAGE and western blotting.

Principle

Western blotting helps to separate and identify the specific protein in a complex mixture. This technique involves the separation of individual proteins in complex mixture by polyacrylamide gel electrophoresis based on their molecular weights and then transferring them to a membrane. Then, the primary antibody binds with the target protein. After washing,

secondary antibody with labelled with probes viz., enzymes react with specific substrate and produce signals. These signals appear as specific bands which can be quantified by densitometry for quantitative comparison of protein expression (Gavini and Parameshwaran, 2023).

Reagents

30 % acrylamide -bis-acrylamide solution (29:1): Acrylamide-bis-acrylamide solution was prepared by dissolving 29 gm of acrylamide and 1 gm of bis-acrylamide in distilled water. The solution was heated at 37 °C to dissolve acrylamide completely. Then, the final volume was made 100 ml with distilled water. The solution was filtered with filter paper and stored in amber colored bottle at 4 °C.

1.5 M Tris (pH 8.8): 18.165 gm of Tris-base was dissolved in distilled water and pH^H was adjusted to 8.8. Then, distilled water was added to prepare final volume 100 ml and stored at 4 °C.

0.5 M Tris (pH 6.8): 6.05 gm of Tris-base was dissolved in distilled water and pH was adjusted to 6.8. Then, distilled water was added to make final volume 100 ml and stored at 4 °C.

10 % Sodium dodecyl-sulfate (SDS): 10 gm of SDS was dissolved in distilled water and final volume was made 100 ml.

10 % Ammonium persulfate (APS): 100 mg of APS was dissolved in 1 ml (final volume) of distilled water.

N, N, N₂, N₂ -tetramethylethylenediamine (TEMED): Commercially available TEMED was used.

Running buffer: Running buffer was prepared by dissolving 3.02 gm of Tris-base, 14.4 gm of glycine and 1 gm of SDS in distilled water and final volume of solution was made 1 liter.

Transfer buffer: 3.02 gm of Tris-base, 14.4 gm of glycine were dissolved in 1 liter of distilled water to prepare transfer buffer. During transfer of smaller molecular weight proteins,

15 % of methanol was added in transfer buffer. For larger molecular weight transfer, methanol was reduced to 10 % in transfer buffer.

Tris-buffered saline with Tween-20 (TBST) solution (pH 7.4): TBST solution was used for washing of the membrane. 6.057 gm of Tris-base, 8.76 gm of sodium chloride and 1 ml of Tween-20 were added in distilled water. The pH was adjusted to 7.4 and distilled water added to make 1 liter solution.

5 % Bovine serum albumin (BSA): The blocking solution of BSA was prepared by dissolving 2.5 gm of BSA in 50 ml of TBST solution. Then, solution was filtered by syringe filter before use and stored at 4 °C.

Procedure

- After sealing the bottom of gel casting glass plates with agarose, 6-10 % of separating gel (pH 8.8) was prepared in between the glass plates using the Genei gel casting assembly spacers of 1-1.5 mm thickness.
- The 5% stacking gel in Tris-buffer of pH 6.8 was poured over it and comb was placed according to the thickness of spacer. After polymerization, gel was transferred to the electrophoretic apparatus (Genei) and buffer chambers were filled with gel running buffer.
- Equal amount of protein samples was loaded into different wells of gel.
- Pre-stained protein marker of predetermined molecular weights was added in one of the wells.
- Then, the gel was subjected for electrophoresis at 80 V till the tracking dye reached at the bottom of the separating gel.
- After SDS-PAGE, gel was removed from apparatus and washed with transfer buffer.
- The polyvinylidene fluoride (PVDF; 0.45 µm) membrane of appropriate was cut and merged in absolute methanol for 3 minutes to activate it. Then, PVDF was equilibrated to transfer buffer.
- In semi-dry transfer blotting, from anode to cathode the various layers are arranged sequentially as foam pad, Whatman filter paper, activated PVDF membrane, gel, filter

paper and lastly foam pad soaked in the transfer buffer. The air bubbles and excessive buffer were removed by rolling the glass rod over it.

- Electrodes were fixed and transfer process was done at 20V, 230 mA for 45 to 60 minutes.
- After transfer the PVDF membrane was removed carefully and placed in 5 % BSA solution for 2-3 hours to block non-specific sites at room temperature.
- Then, membrane was washed thrice with TBST solution for 5 minute each.
- Then, membrane was incubated with target specific primary antibodies viz., Nrf-2 (1:250), NF-kB (1:2000) and GAPDH (1:5000) antibody for overnight at 4 °C.
- After washing with TBST (3 times for 5 minutes each), secondary antibody conjugated with HRP (orlistat; 1:2000) or ALP (niclosamide ethanolamine; 1:2000) was added and incubated for 2 hours at room temperature.
- Blot was developed by using diaminobenzidine tetrahydrochloride (DAB) or nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP).
- Image J software was used for the quantification of band intensities.

3.7.9 Gelatin zymography

The gelatinolytic activity of matrix metalloproteinases (MMPs), including MMP-2 and MMP-9 was determined by the gelatin zymography (Chew *et al.*, 2004).

Preparation of samples: Heart tissue was homogenized in lysis buffer to prepare 5 % homogenate and centrifuged to obtain clear supernatant. Proteins of homogenate supernatants were estimated by Bradford method.

Principle: Gelatin zymography is based on the incorporation of gelatin into electrophoresis gel to assess the gelatinolytic activity of MMPs. Electrophoresis is carried out under non-reducing condition to maintain enzymatic activity of samples. Washing with Triton X-100 removes SDS from gel. When the gel incubated with the buffer containing calcium, partial renaturation of enzymes occur which degrade the gelatin and leaves a cleared white zone after staining and destaining of gel. These clear zone in gel indicates the substrate digestion areas, reflecting the enzymatic activity (Toth and Fridman, 2012).

Reagents

Gelatin: 20 mg of gelatin was dissolved in 1 ml of distilled water and heated at 65 °C for 15-20 minutes (till gelatin completely dissolved). Then, solution was cooled down to room temperature. The solution was prepared freshly.

2.5 % Triton X-100 solution: 2.5 ml of Triton X-100 was dissolved in 97.5 ml distilled water to make final volume 100 ml.

Developing buffer: To prepare the developing buffer, 50 mM Tris HCl, 5 mM CaCl₂, 200 mM NaCl, 1 μM ZnCl₂, 1% Triton X 100 were dissolved in distilled water and p^H was adjusted to 7.4.

Coomassie Brilliant Blue (G-250): 0.05 % solution of Coomassie Brilliant Blue was prepared in a mixture of methanol-acetic acid-water (2.5:1:6.5 v/v).

Destaining solution: Aqueous solution containing 4 % methanol-8% acetic acid (v/v) was prepared.

Procedure:

- For this, heart tissue homogenate samples were prepared by mixing with non-reducing zymography buffer.
- Then, these un-boiled samples were used to separate the proteins by SDS-PAGE, gel (7 % for MMP-2 and 6 % for MMP-9) co-polymerized with gelatin.
- Gel was washed twice (for 15 minute each) with Triton X-100 solution to remove SDS from gel.
- Developing buffer was added in gel and after 10 minutes, it was replaced with newer developing buffer and incubated for 16-24 hours at room temperature.
- Then, gel was removed from developing buffer and after washing stained with Coomassie Brilliant blue stain for 2-3 hours.
- Then, gel was destained with destaining solution until clear white bands appears against blue background.
- Image J software was used for semi-quantification of band intensities to assess the gelatinolytic activity.

3.7.10 mRNA expression studies

A) Isolation of total RNA

Heart tissue was collected in 0.1% diethylpyrocarbonate (DEPC)-treated tubes in RNA later and stored at -80 °C. Total RNA was isolated by TRIzol method (Chomczynski and Mackey, 1995). 50 mg of tissue was homogenized in 1 ml of TRIzol. Then, 0.2 ml of chloroform was added in each ml of TRIzol homogenate, mixed and incubated for 2-3 minute at room temperature and followed by centrifugation at 10,000 rpm for 10 minutes. The aqueous phase was collected carefully without disturbing the interphase in new 1.5 ml tube. Subsequently, equal amount of absolute isopropyl alcohol was added and incubated for 10 minutes at room temperature. Then, centrifuged at 10,000 rpm for 10 minutes and supernatant was discarded. The pellet was washed with 75% ethanol twice by vortexing and centrifugation at 10,000 rpm for 10 minutes. Then the pellet was air dried for 5-10 minutes. Nuclease free water was added to resuspend the RNA pellet and dissolved properly by frequent pipetting. The purity of RNA was checked by nanodrop and quantified as follow:

$$1\text{OD}_{260} = 40\ \mu\text{g/ml}$$

The RNA samples showing A_{260}/A_{280} ratio 1.8-2.0 and A_{260}/A_{230} ratio >1.6 were used for cDNA synthesis.

B) cDNA synthesis

cDNA from total RNA was synthesized using cDNA synthesis kit (Cat: BCGB100) as per manufacture's instructions. Briefly, 400 ng of total RNA was taken and 250 ng of oligo dT for each μg of RNA was added. Then, 3 μl of 5X RNA protector was added and nuclease free water (NFW) was added to make final volume upto 15 μl . Reaction mixture was incubated at 65 °C for 10 minutes in thermocycler. Subsequently, the reaction was kept at room temperature for 10-15 minutes. To this, 1 μl of Moloney murine leukemia virus (MMLV) reverse transcriptase and 4 μl of 5X RT buffer were mixed. Afterwards, the tubes were incubated at 42 °C for 60 min and 70 °C for 10 min in a thermocycler. Then, cDNA was stored at -20 °C till further use.

C) Primer validation

Primer sequences were obtained from the previous publications and respective gene sequences were checked from NCBI nucleotide database. The cDNA was subjected to amplification with the respective primers at various annealing temperatures to optimize the PCR conditions. Real time RT-PCR analysis was carried out after checking each primer for its specificity.

Table 3.6: Description of primers used

Gene	Primer sequences	Amplicon size(bp)
GAPDH	F: ACTCCACTCACGGCAAAT TC R: TCTCCATGGTGGTGAAGACA	170
SMAD-3	F:GAATTTGCTGCCCTCCTAGCTC R: CTCCCCAGCCTTTGACGAAG	105
α SMA	F:GGCTCTGGGCTCTGTAAGG R: CTCTTGCTCTGGGCTTCATC	148
TGF-β	F: AGCCTGGACACACAGTACAGC R: CGACCCACGTAGTAGACGATG	125
CTGF	F: CTCCACCCGAGTTACCAATGACAA R: CCAGAAAGCTCAAACCTTGACAGGC	164
IL-6	F: TGTATGAACAACGATGATGCAC R: TGGTACTCCAGAAGACCAGAGG	137
TNF-α	F: CGTCAGCCGATTTGCTATCT R: CGGACTCCGCAAAGTCTAAG	208
Collagen III	F: ACGTAGATGAATTGGGATGCAG R: GGGTTGGGGCAGTCTAGT	154

D) Reverse transcriptase Polymerase chain reaction (RT-PCR)

The cDNA samples were checked for quality by performing PCR with standard GAPDH primers. The polymerase chain reaction was standardized for each gene using cDNA from mouse heart. The reaction was carried out at different annealing temperatures and the optimum annealing temperatures for different genes were found to be 56 °C (TGF- β , Collagen type I & GAPDH), 60 °C (SMAD-3, α -SMA & TNF- α), 53 °C (Collagen III), 55 °C (IL-6 & CTGF).

Table 3.7: Composition of RT-PCR mixture

Composition	Volume
PCR master mix (2X)	5 µl
Forward primer (10 pmol/µl)	0.5 µl
Reverse primer (10 pmol/µl)	0.5 µl
Template (20 ng/µl)	1 µl
Nuclease free water	up to 10 µl

Table 3.8: Thermal cycling conditions

Sl. No.	Steps	Temperature and duration
1.	Initial denaturation	94 °C for 10 min.
2.	Denaturation	94 °C for 15 sec.
3.	Annealing	Based on gene for 30 sec
4.	Extension	72 °C for 30 sec.
5.	Step 2-4 repetition	40 cycles
6.	Final extension	72°C for 10 min
7.	Held at	4°C

E) Agarose gel electrophoresis

DNA amplified by PCR was subjected to agarose gel electrophoresis as described by Sambrook and Russel (2006). 2.5 % of agarose was used. 0.5 gm of agarose was dissolved in 20 ml of distilled water and 400 µl of 50X TAE buffer by heating and allowed to cool to 50 °C. To this, 2 µl ethidium bromide was added and mixed thoroughly. The mixture was poured onto a gel-casting tray fitted with acrylic comb and allowed to solidify. Once the gel had formed, the gel was immersed in an electrophoresis tank containing 1X TAE buffer. One µl of 6X DNA loading dye was mixed with 5 µl of DNA samples and loaded into the wells. Electrophoresis was carried out at 100 volts until the tracking dye reached the end of the gel. The DNA samples were visualized as bands under UV-illumination and documented. The size of the PCR products amplified was calculated from the standard 100 bp DNA ladder.

F) Quantitative Real-Time RT-PCR reaction conditions

Real-Time RT-PCR was performed using SYBR Green master mix. Each sample was run in duplicate in 10 µl reaction. The 10 µl reaction mixture consisted of 5 µl SYBR Green master mix, 0.25 µl from 10 pmol/µl stock solution of each of the gene-specific forward and reverse primers, and 1 µl of cDNA and volume was made up to 10 µl with nuclease free water (NFW). The real-time PCR reaction was started with initial incubation at 95 °C for 30 second followed by 40 cycles of amplification with denaturation at 95 °C for 5 second, annealing (temperature as mentioned above for gene specific primer pairs) for 30 sec and extension at 72 °C for 30 sec each. To assess the specificity of the amplified product, dissociation curve was generated at temperature of 60 °C through 95 °C. The results were expressed as threshold cycle values (C_T). This value is the cycle number when the fluorescence of the reporter dye is appreciably higher than the background fluorescence. The threshold, automatically adjusted by the instrument, was used for the generation of C_T values.

To study the relative change in gene expression, the $2^{-\Delta\Delta C_T}$ method was used as described previously by Livak and Schmittgen (2001). The formula used to calculate the fold change in gene expression was “fold change = $2^{-\Delta\Delta C_T}$ ” [where $-\Delta C_T = (C_{T,\text{target gene}} - C_{T,\text{GAPDH}})_{\text{treatment}} - (C_{T,\text{target gene}} - C_{T,\text{GAPDH}})_{\text{control}}$]. The gene-specific amplification was corrected for the difference in input of RNA by taking housekeeping gene GAPDH to account. The results were analyzed in comparison with the C_T (minimum threshold of amplification) value of the target gene and the reference gene (GAPDH).

3.7.11 Histopathological examination

The heart tissues from various experimental groups underwent fixation in 10% formalin for 24 hours. Subsequently, the tissues were dehydrated using an ascending alcohol series and then embedded in paraffin wax. Thin sagittal sections of approximately 4-5 µm were prepared from the paraffin-embedded tissues and subjected to staining using the hematoxylin and eosin (H&E) technique (Luna, 1968) as well as Masson trichrome stain (MST) (Lillie, 1940) for the microscopic assessment of lesions.

Statistical analysis

Graph pad prism 5.03 was used to analyze data. Results of the different experiments are expressed as mean $\pm$ SEM with 'n' equal to number of animals. The mean values from different groups were analyzed by One way ANOVA and *Tukey's Post-hoc* test was used for multiple comparison using Graph Pad Prism Version 5.03 software. To study the relative change in gene expression, the $2^{-\Delta\Delta C_T}$ method was used as described previously by Livak and Schmittgen (2001). The formula used to calculate the fold change in gene expression was "fold change = $2^{-\Delta\Delta C_T}$ " [where $-\Delta C_T = (C_{T, \text{target gene}} - C_{T, \text{GAPDH}})_{\text{treatment}} - (C_{T, \text{target gene}} - C_{T, \text{GAPDH}})_{\text{control}}$]. The gene-specific amplification was corrected for the difference in input of RNA by taking housekeeping gene GAPDH to account. The results were analyzed in comparison with the C_T (minimum threshold of amplification) value of the target gene and the reference gene (GAPDH).



Results



A. Orlistat study

4.1 Body weight at day zero and day 15th in different groups of mice

Body weights of mice at day zero and day 15th of the experiment in control, orlistat alone, isoprenaline-induced cardiac injury and orlistat-treated cardiac injury groups are summarized in Table 4.1 and illustrated in Figure 4.1. A significant ($p<0.0001$) increase in body weight on day 15th in comparison to day zero was observed in control group (33.49 ± 1.17 g; $n=18$ vs 28.34 ± 1.35 g; $n=18$), orlistat-alone group (31.17 ± 0.94 g; $n=15$ vs 26.07 ± 0.96 g; $n=15$), and orlistat-treated cardiac injury group (29.90 ± 1.30 g; $n=15$ vs 25.45 ± 0.89 g; $n=15$). In isoprenaline-alone administered cardiac injury group also on day 15th a significantly ($p<0.001$) higher body weight was noted than zero day (32.33 ± 1.40 g; $n=15$ vs 30.20 ± 1.25 g; $n=15$).

Table 4.1: Body weight (g) at day zero and day 15th in mice of control, orlistat- alone, isoprenaline-induced cardiac injury, and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Body weight (g) at zero day	Body weight (g) at 15 th day
	Mean $\pm$ S.E.	Mean $\pm$ S.E.
Control (n=18)	28.34 $\pm$ 1.35	33.49 $\pm$ 1.17****
Orlistat alone (n=15)	26.07 $\pm$ 0.96	31.17 $\pm$ 0.94****
Isoprenaline-alone (n=15)	30.20 $\pm$ 1.25	32.33 $\pm$ 1.40***
Orlistat+ISP (n=15)	25.45 $\pm$ 0.89	29.90 $\pm$ 1.30****

Data are presented as mean $\pm$ S.E.

**** $p<0.0001$ & *** $p<0.001$ vs 15th day.

4.2. Percentage change in body weight from day zero to day 15th of mice

Percentage change in body weight of mice from day zero to day 15th in control, orlistat-alone, isoprenaline-induced cardiac injury group and orlistat-treated cardiac injury group are presented in Table 4.2 and shown in Figure 4.2. Data revealed that in cardiac injury group, the percentage (7.13 ± 1.75 %; $n=15$) increase in the body weight during experiment period was least and significantly ($p<0.01$) lower than the control group (19.76 ± 2.44 %; $n=18$). A significant ($p<0.05$) higher percentage change was observed in orlistat-treated group (17.21 ± 2.51 %; $n=15$) than isoprenaline-alone administered cardiac injury group (7.13 ± 1.75 %; $n=15$). However, percentage change in body weight between orlistat-alone (20.39 ± 2.73 %; $n=15$) and control (19.76 ± 2.44 %; $n=18$) groups of mice were almost comparable.

Table 4.2: Percentage change in body weights during experimental period of the mice of control, orlistat-alone, isoprenaline-induced cardiac injury, and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Percentage change in body weight (%)
Control (n=18)	19.76 ± 2.44
Orlistat-alone (n=15)	20.39 ± 2.73
Isoprenaline-alone (n=15)	$7.13 \pm 1.75^{**}$
Orlistat+ISP (n=15)	$17.21 \pm 2.51^{\#}$

Data are presented as mean $\pm$ S.E.

$^{**}p<0.01$ in comparison to control; $^{\#}p<0.05$ vs isoprenaline-alone administered group.

4.3 Effect of orlistat on heart weight of mice in ISP-induced cardiac injury

The absolute heart weights in different groups of mice are summarized in the Table 4.3 and illustrated in Figure 4.3. The data shown in Table 4.3 revealed that a significant ($p<0.001$) increase was noted in absolute heart weight in ISP-alone cardiac injury group (173.8 ± 5.88 mg; $n=15$) than the control group (136.1 ± 3.91 mg; $n=18$). Orlistat co-administration with ISP significantly ($p<0.001$) decreased the absolute heart weight (141.9 ± 4.05 mg; $n=15$) compared to the isoprenaline-alone administered group (173.8 ± 5.88 mg; $n=15$). Almost comparable

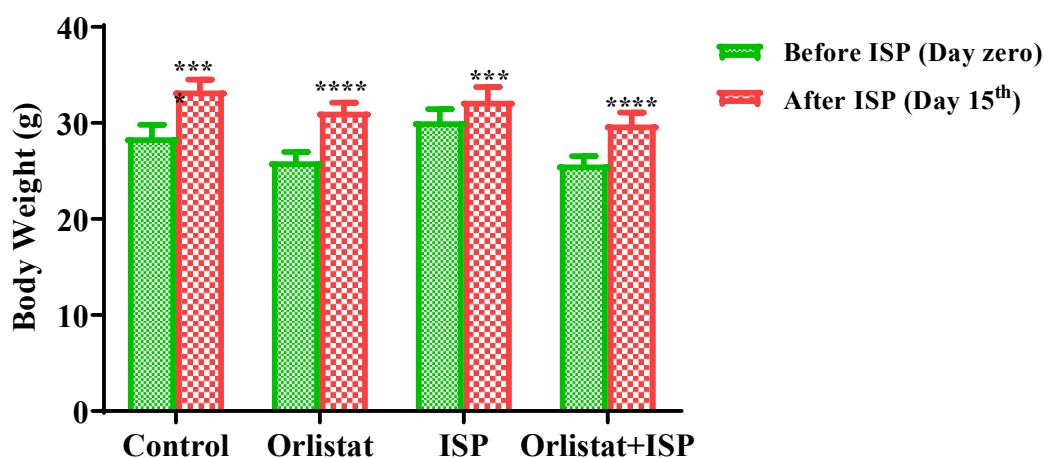


Fig. 4.1: Bar diagram showing the change in body weight on day zero and day 15th in mice of different groups. Data are analyzed by paired *t*-test. Different superscripts on the bars present significant ($p<0.001$ - 0.0001) difference between day zero and day 15th of experiment within groups.

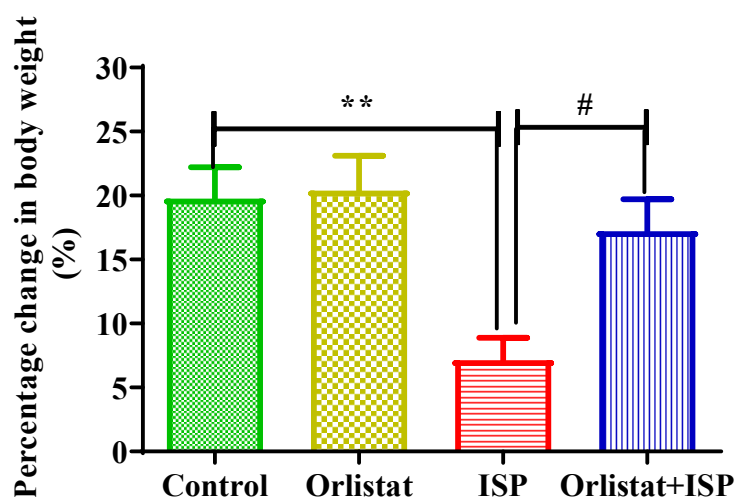


Fig. 4.2: Bar diagram showing the percentage change in body weight in different groups of mice during the experimental period. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (**, #) on the bars present significant ($p<0.01$ - 0.05) difference between groups.

heart weights were observed in orlistat-alone (130.4 ± 2.82 mg; $n=15$) and control (136.1 ± 3.91 mg; $n=18$) groups of mice.

Table 4.3: Absolute heart weight of the control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Absolute heart weight (mg)
Control (n=18)	136.1 ± 3.91
Orlistat-alone (n=15)	130.4 ± 2.82
ISP-alone (n=15)	$173.8 \pm 5.88^{***}$
Orlistat+ISP (n=15)	$141.9 \pm 4.05^{###}$

Data are presented as mean $\pm$ S.E.

*** $p < 0.001$ vs control; ### $p < 0.001$ vs isoprenaline-alone group.

4.4. Effect of orlistat on heart weight to body weight ratio in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.4 and illustrated in Figure 4.4 show the heart weight to body weight (HWT/BWT) ratio of mice in different groups viz., control, orlistat-alone, ISP-alone and orlistat+ISP. Heart weight to body weight ratio in isoprenaline-alone administered group (5.45 ± 0.18 mg/g; $n=15$) was significantly ($p < 0.001$) increased than the control group (4.24 ± 0.12 mg/g; $n=18$). However, a significant ($p < 0.01$) reduction was observed in orlistat-treated cardiac injury group (4.69 ± 0.08 mg/g; $n=15$) as compared to the ISP-alone administered

Table 4.4: Heart weight to body weight ratio (HWT/BWT) of the control, orlistat alone, isoprenaline alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	HWT/BWT ratio (mg/g)
Control (n=18)	4.24 ± 0.12
Orlistat-alone (n=15)	4.22 ± 0.11
Isoprenaline (ISP) -alone (n=15)	$5.45 \pm 0.18^{***}$
Orlistat+ISP (n=15)	$4.69 \pm 0.08^{##}$

Data are presented as mean $\pm$ S.E.

*** $p < 0.001$ vs control; ## $p < 0.01$ vs isoprenaline-alone administered group.

group (5.45 ± 0.18 mg/g; $n=15$). No significant change in HWT/BWT was observed in orlistat-alone (4.22 ± 0.11 mg/g; $n=15$) and control (4.24 ± 0.12 mg/g; $n=18$) groups of mice.

4.5. Effect of orlistat on heart weight to tibial length (HWT/TL) ratio in mice

Table 4.5 and Figure 4.5 depict HWT/TL ratio in different groups viz., control, orlistat-alone, ISP-alone and orlistat with ISP of mice. A significantly ($p < 0.001$) higher HWT/TL was observed in isoprenaline-alone administered group (8.38 ± 0.24 mg/mm; $n=15$) when compared to control (6.29 ± 0.16 mg/mm; $n=18$). Orlistat-treated cardiac injury group (6.78 ± 0.18 mg/mm; $n=15$) showed a significantly ($p < 0.001$) lower HWT/TL than ISP-alone administered group (8.38 ± 0.24 mg/mm; $n=15$). HWT/TL ratio was almost comparable between orlistat-alone (6.18 ± 0.14 mg/mm; $n=15$) and control (6.29 ± 0.16 mg/mm; $n=18$) groups of mice.

Table 4.5: Heart weight to tibial length ratio (HWT/TL) of the control, orlistat alone, isoprenaline alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	HWT/TL ratio (mg/mm)
Control ($n=18$)	6.29 ± 0.16
Orlistat-alone ($n=15$)	6.18 ± 0.14
Isoprenaline-alone ($n=15$)	$8.38 \pm 0.24^{***}$
Orlistat+ISP ($n=15$)	$6.78 \pm 0.18^{###}$

Data are presented as mean $\pm$ S.E.

$^{***}p < 0.001$ vs control; $^{###}p < 0.001$ vs isoprenaline-alone group.

4.6. Effect of orlistat on serum CK-MB in isoprenaline-induced cardiac injury in mice

As summarized in Table 4.6 and illustrated in Figure 4.6, a significant ($p < 0.01$) elevation in CK-MB level was observed in ISP-alone administered group (240.8 ± 32.86 U/L; $n=8$) than the control group mice (116.2 ± 10.77 U/L; $n=7$). However, in orlistat-treated cardiac injury group significantly ($p < 0.05$) lower CK-MB level (147.1 ± 14.04 U/L; $n=7$) was observed in comparison to the ISP-alone group (240.8 ± 32.86 U/L; $n=8$). No significant alteration in CK-MB was observed between orlistat-alone (124.0 ± 27.04 U/L; $n=5$) and control (116.2 ± 10.77 U/L; $n=7$) groups of mice.

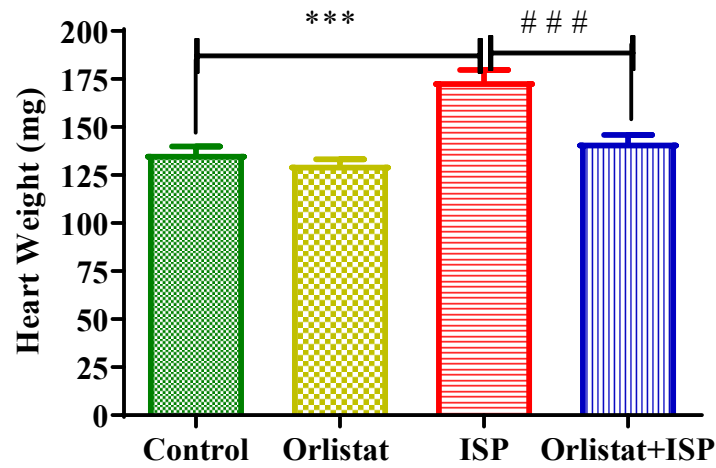


Fig. 4.3: Bar diagram showing absolute heart weight of control, orlistat-alone, ISP-alone and orlistat+ISP groups in mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, ###) on the bars present significant ($p < 0.001$) difference between groups.

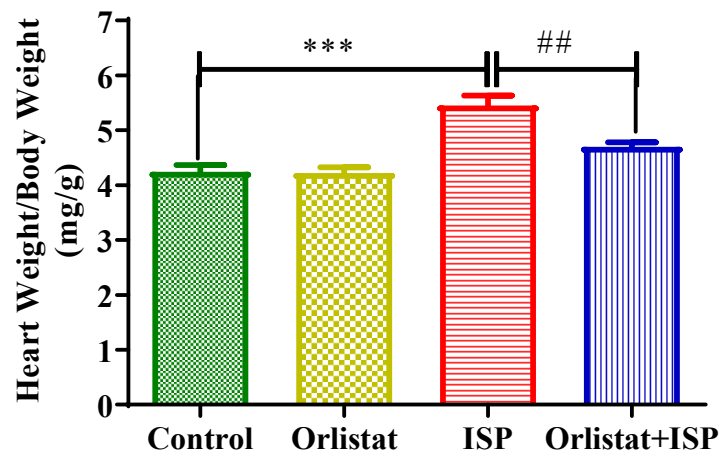


Fig. 4.4: Bar diagram showing the heart weight to body weight ratio (HWT/BWT) of control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, ##) on the bars present significant ($p < 0.001-0.01$) difference between groups.

Table 4.6: Serum CK-MB level in control, orlistat alone, isoprenaline alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	CK-MB (U/L)
Control (n=7)	116.2±10.77
Orlistat-alone (n=5)	124.0±27.04
Isoprenaline-alone (n=8)	240.8±32.86**
Orlistat+ISP (n=7)	147.1±14.04 [#]

Data are presented as mean±S.E.

** $p<0.01$ vs control; [#] $p<0.05$ vs isoprenaline-alone group.

4.7. Effect of orlistat on serum LDH level in isoprenaline-induced cardiac injury in mice

The serum LDH level of different groups of mice are summarized in Table 4.7 and illustrated in Fig. 4.7. Data revealed that LDH level was significantly ($p<0.05$) increased in ISP-alone group (1449±93.94 U/L; n=8) than the control group (1113±91.32 U/L; n=8). A significantly ($p<0.001$) decreased serum LDH level was observed in orlistat-treated cardiac injury group (853.0±78.23 U/L; n=8) in comparison to the ISP-alone group (1449±93.94 U/L; n=8). LDH level was found almost comparable in orlistat-alone (918.7±80.64 U/L; n=8) and control (1113±91.32 U/L; n=8) groups of mice.

Table 4.7: Serum LDH level in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	LDH (U/L)
Control (n=8)	1113±91.32
Orlistat-alone (n=8)	918.7±80.64
Isoprenaline-alone (n=8)	1449±93.94*
Orlistat+ISP (n=8)	853.0±78.23 ^{###}

Data are presented as mean±S.E of 8 animals.

* $p<0.05$ vs control; ^{###} $p<0.001$ vs isoprenaline-alone group.

4.8. Effect of orlistat on serum cholesterol level in ISP-induced cardiac injury in mice

Data summarized in Table 4.8 and illustrated in Figure 4.8 show the serum cholesterol level in control, orlistat-alone, isoprenaline-alone and orlistat-treated cardiac injury groups of mice. A significant ($p<0.05$) elevation in cholesterol level was observed in ISP-alone administered group (75.29 ± 4.59 mg/dl; $n=8$) in comparison to control group (52.96 ± 3.39 mg/dl; $n=8$). However, orlistat administration moderately improved the cholesterol level (57.83 ± 5.56 mg/dl; $n=8$) in cardiac injury caused by ISP. No significant alteration was observed in cholesterol level between orlistat-alone (50.41 ± 4.86 mg/dl; $n=8$) and control (52.96 ± 3.39 mg/dl; $n=8$) groups of mice.

Table 4.8: Serum cholesterol level in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups

Groups (mice)	Cholesterol (mg/dl)
Control (n=8)	52.96 ± 3.39
Orlistat-alone (n=8)	50.41 ± 4.86
Isoprenaline-alone (n=8)	$75.29\pm4.59^*$
Orlistat+ISP (n=8)	57.83 ± 5.56

Data are presented as mean $\pm$ S.E of 8 animals.

* $p<0.05$ vs control group.

4.9. Effect of orlistat on serum triglyceride level in ISP-induced cardiac injury in mice

Table 4.9 and Figure 4.9 depict data of serum triglyceride level in control, orlistat-alone, isoprenaline-alone and orlistat-treated cardiac injury group of mice. A significantly ($p<0.05$) higher level of serum triglyceride was noted in ISP-alone administered group (77.11 ± 9.28 mg/dl; $n=8$) in comparison to control group (48.96 ± 5.69 mg/dl; $n=8$). Orlistat treatment significantly ($p<0.01$) reduced triglyceride level (40.00 ± 9.05 mg/dl; $n=8$) in cardiac injury group when compared to ISP-alone group (77.11 ± 9.28 mg/dl; $n=8$). Almost comparable triglyceride level was observed in orlistat-alone (28.72 ± 3.34 mg/dl; $n=8$) and control (48.96 ± 5.69 mg/dl; $n=8$) groups of mice.

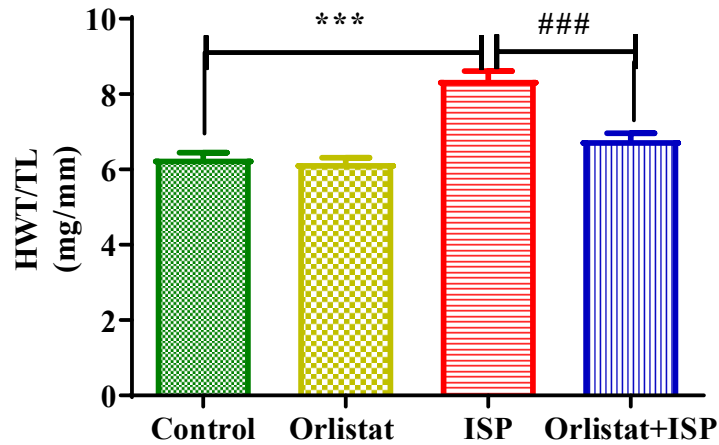


Fig. 4.5: Bar diagram showing the heart weight to tibial length (HWT/TL) ratio of control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, ###) on the bars present significant ($p<0.001$) difference between groups.

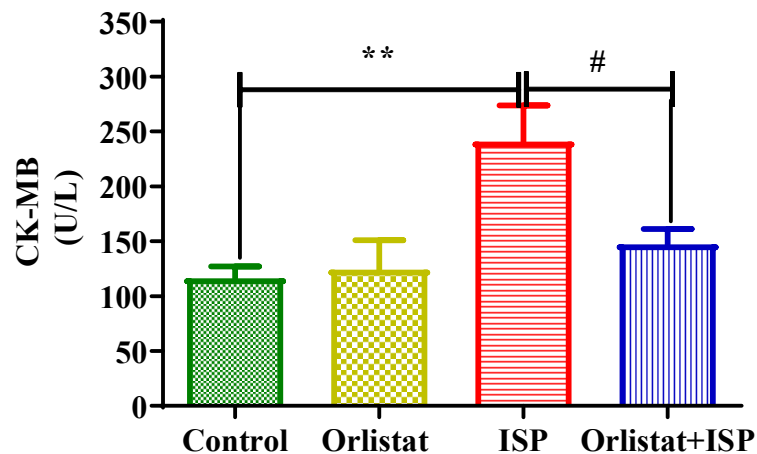


Fig. 4.6: Bar diagram showing serum CK-MB level of control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (**, #) on the bars present significant ($p<0.01-0.05$) difference between groups.

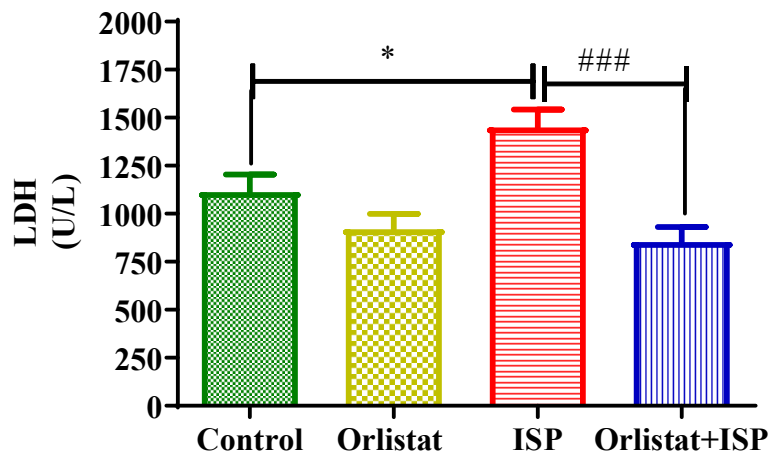


Fig. 4.7: Bar diagram showing serum LDH level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, ###) on the bars present significant ($p<0.05$ - 0.001) difference between groups.

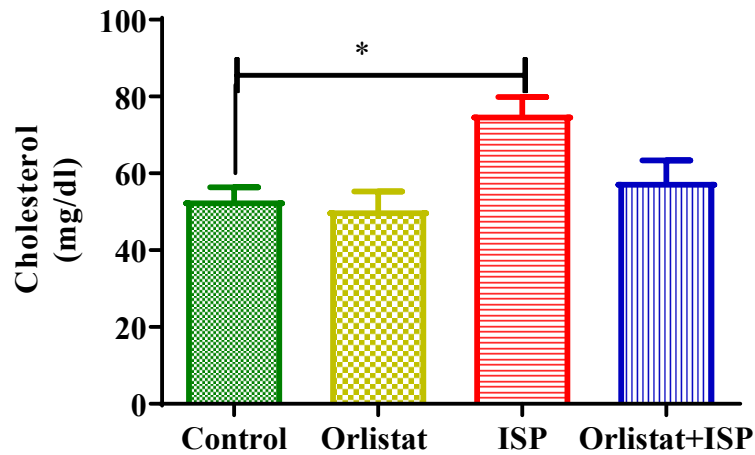


Fig. 4.8: Bar diagram showing the serum cholesterol level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between ISP-alone and control groups.

Table 4.9: Serum triglyceride level in control, orlistat alone, isoprenaline alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Triglyceride (mg/dl)
Control (n=8)	48.96±5.69
Orlistat-alone (n=8)	28.72±3.34
Isoprenaline-alone (n=8)	77.11±9.28*
Orlistat+ISP (n=8)	40.00±9.05 ^{##}

Data are presented as mean±S.E of 8 animals.

* $p<0.05$ vs control; ^{##} $p<0.01$ vs isoprenaline-alone group.

4.10 Effect of orlistat on high-density lipoprotein (HDL) cholesterol level in ISP-induced cardiac injury in mice

Data summarized in Table 4.10 and illustrated in Figure 4.10 revealed that ISP-alone administration significantly ($p<0.05$) decreased the HDL-c level (76.05±5.17 mg/dl; n=6) in comparison to control group (92.33±3.88 mg/dl; n=6). A significantly ($p<0.01$) higher HDL-c level was observed in orlistat-treated cardiac injury group (98.71±2.36 mg/dl; n=6) compared to ISP-alone administered group (76.05±5.17 mg/dl; n=6). No significant alteration in HDL-c level was observed between orlistat-alone (90.13±3.88 mg/dl; n=5) and control (92.33±4.44 mg/dl; n=6) groups of mice.

Table 4.10: Serum HDL-c level in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	HDL-c (mg/dl)
Control (n=6)	92.33±4.44
Orlistat-alone (n=5)	90.13±3.88
Isoprenaline-alone (n=6)	76.05±5.17*
Orlistat+ISP (n=6)	98.71±2.36 ^{##}

Data are presented as mean±S.E.

* $p<0.05$ vs control; ^{##} $p<0.01$ vs isoprenaline-alone group.

4.11. Effect of orlistat on low-density lipoprotein cholesterol (LDL-c) level in ISP-induced cardiac injury in mice

Data in Table 4.11 and in Figure 4.11 depict the serum LDL-c level in different groups viz., control, orlistat-alone, ISP-alone and orlistat with ISP groups. Data revealed that a significantly ($p<0.01$) higher level of LDL-c was noted in ISP-alone administered group (98.66 ± 15.58 mg/dl; $n=6$) in comparison to control group (44.70 ± 6.61 mg/dl; $n=6$). However, no significant improvement was observed in LDL-c level in orlistat-treated (69.98 ± 7.22 mg/dl; $n=6$) cardiac injury group in comparison to ISP-alone administered group (98.66 ± 15.58 mg/dl; $n=6$). LDL-c level was almost comparable between orlistat-alone (41.39 ± 3.29 mg/dl; $n=5$) and control (44.70 ± 6.61 mg/dl; $n=6$) groups of mice.

Table 4.11: Serum LDL-c level in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	LDL-c (mg/dl)
Control (n=6)	44.70 ± 6.61
Orlistat-alone (n=5)	41.39 ± 3.29
Isoprenaline-alone (n=6)	$98.66\pm15.58^{**}$
Orlistat+ISP (n=6)	69.98 ± 7.22

Data are presented as mean $\pm$ S.E.

$^{**}p<0.01$ vs control.

4.12. Effect of orlistat on HDL-c to LDL-c ratio (HDL-c/LDL-c) in ISP-induced cardiac injury in mice

As summarized in Table 4.12 and illustrated in Figure 4.12, the HDL-c/LDL-c ratio was noted significantly ($p<0.01$) lower in isoprenaline-induced cardiac injury group (0.82 ± 0.07 ; $n=6$) compared to control group (2.34 ± 0.42 ; $n=6$). No significant improvement in HDL-c/LDL-c was observed in orlistat-treated cardiac injury group (1.52 ± 0.22 ; $n=6$) than ISP-alone administered group (0.82 ± 0.07 ; $n=6$). HDL-c/LDL-c ratio was almost comparable between orlistat-alone (2.21 ± 0.12 ; $n=5$) and control (2.34 ± 0.42 ; $n=6$) groups of mice.

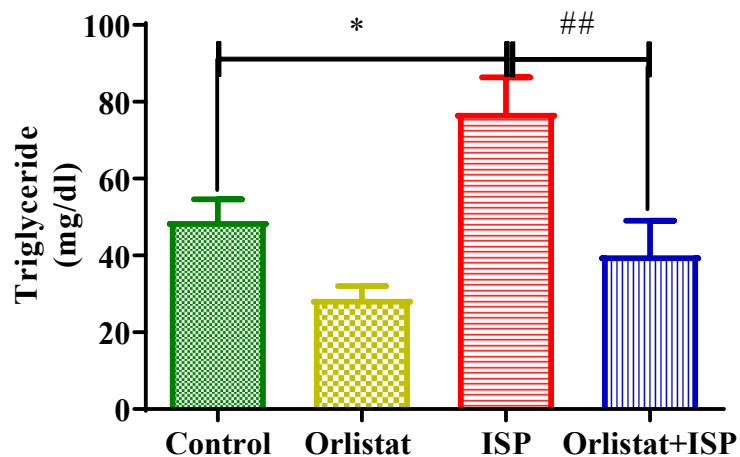


Fig. 4.9: Bar diagram showing the serum triglyceride level in control, orlistat alone, ISP alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, ##) on the bars present significant ($p<0.05-0.01$) difference between groups.

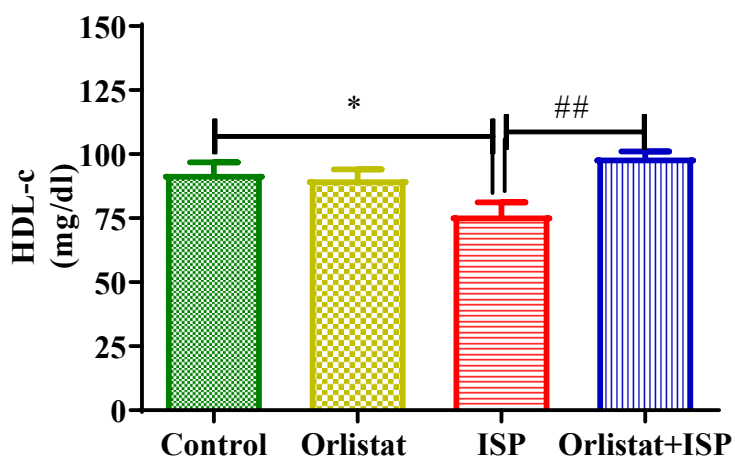


Fig. 4.10: Bar diagram showing the serum HDL-c in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, ##) on the bars present significant ($p<0.05-0.01$) difference between groups.

Table 4.12: Serum HDL-c/LDL-c ratio in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups	HDL-c/LDL-c ratio
Control (n=6)	2.34±0.42
Orlistat-alone (n=5)	2.21±0.12
Isoprenaline-alone (n=6)	0.82±0.07**
Orlistat+ISP (n=6)	1.52±0.22

Data are presented as mean±S.E.

** $p < 0.01$ vs control group.

4.13. Effect of orlistat on serum c-reactive protein (CRP) level in ISP-induced cardiac injury in mice

Data summarized in Table 4.13 and illustrated in Figure 4.13 revealed that isoprenaline-alone administration significantly ($p < 0.05$) increased CRP level (4.62 ± 0.56 mg/l; $n=7$) in comparison to the control group (2.53 ± 0.59 mg/l; $n=7$) mice. No significant improvement was observed in CRP level in orlistat-treated cardiac injury group (3.56 ± 0.44 mg/l; $n=7$) than ISP-alone administrated group (4.62 ± 0.56 mg/l; $n=7$). Furthermore, almost comparable CRP level was noted in orlistat-alone (2.44 ± 0.55 mg/l; $n=6$) and control (2.53 ± 0.59 mg/l; $n=7$) groups of mice.

Table 4.13: Serum CRP level in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	CRP (mg/l)
Control (n=7)	2.53±0.59
Orlistat-alone (n=6)	2.44±0.55
Isoprenaline-alone (n=7)	4.62±0.56*
Orlistat+ISP (n=7)	3.56±0.44

Data are presented as mean±S.E.

* $p < 0.05$ vs control group.

4.14. Effect of orlistat on serum superoxide dismutase (SOD) activity in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.14 and illustrated in Figure 4.14 depict SOD activity in serum of different groups of mice. A significant ($p<0.05$) decrease in SOD activity was observed in ISP-alone administered group (4.43 ± 0.19 units/mg of protein; $n=7$) in comparison to control group (5.38 ± 0.32 units/mg of proteins; $n=6$). A significant ($p<0.05$) increase in the SOD activity was observed in orlistat-treated cardiac injury group (5.27 ± 0.17 units/mg of proteins; $n=7$) compared to ISP-alone administered group (4.43 ± 0.19 units/mg of protein; $n=7$) of mice. Furthermore, no significant difference was found in SOD activity in orlistat-alone (5.43 ± 0.14 units/mg of proteins; $n=6$) and control (5.38 ± 0.32 units/mg of proteins; $n=6$) groups of mice.

Table 4.14: Serum SOD activity in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups

Groups (mice)	SOD (units/mg of proteins)
Control (n=6)	5.38 ± 0.32
Orlistat-alone (n=6)	5.43 ± 0.14
Isoprenaline-alone (n=7)	$4.43\pm0.19^*$
Orlistat+ISP (n=7)	$5.27\pm0.17^\#$

Data are presented as mean $\pm$ S.E.

* $p<0.05$ vs control; $^\#p<0.05$ vs ISP-alone administered group.

4.15. Effect of orlistat on serum catalase activity in ISP-induced cardiac fibrosis in mice

Data in Table 4.15 and Figure 4.15 depict catalase activity in serum samples of control, orlistat-alone, ISP-alone and orlistat-treated cardiac injury groups of mice. Data revealed that ISP-administration significantly ($p<0.05$) reduced the catalase activity (3.78 ± 0.46 mmol H_2O_2 utilized/min/mg of proteins; $n=7$) in comparison to the control group (7.98 ± 1.12 mmol H_2O_2 utilized/min/mg of proteins; $n=6$). A significant ($p<0.05$) elevation in catalase activity was observed in orlistat-treated cardiac injury group (7.38 ± 0.81 mmol H_2O_2 utilized/min/mg of

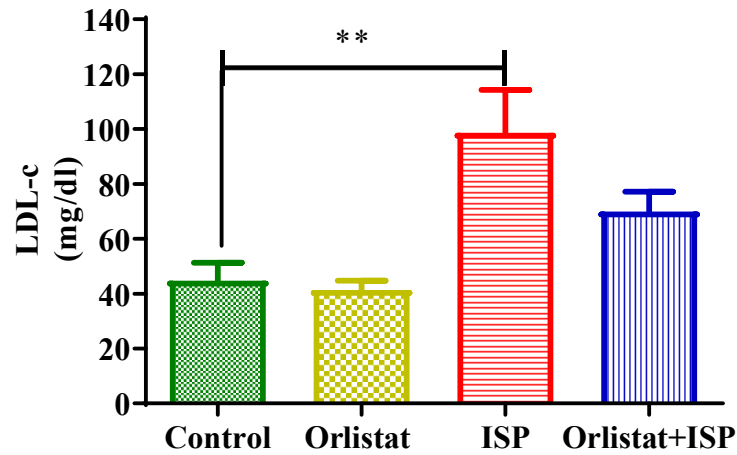


Fig. 4.11: Bar diagram showing the serum LDL-c in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscripts (**) on the bars present significant ($p<0.01$) difference between control and ISP-alone administered groups.

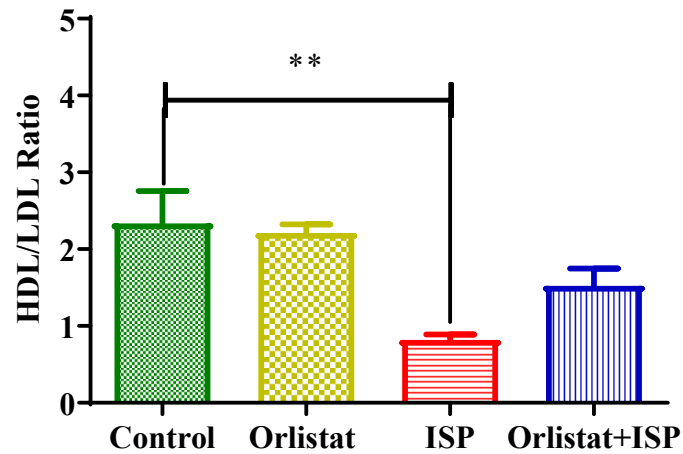


Fig. 4.12: Bar diagram showing the serum HDL-c/LDL-c ratio in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscripts (**) on the bars present significant ($p<0.01$) difference between control and ISP-alone administered group.

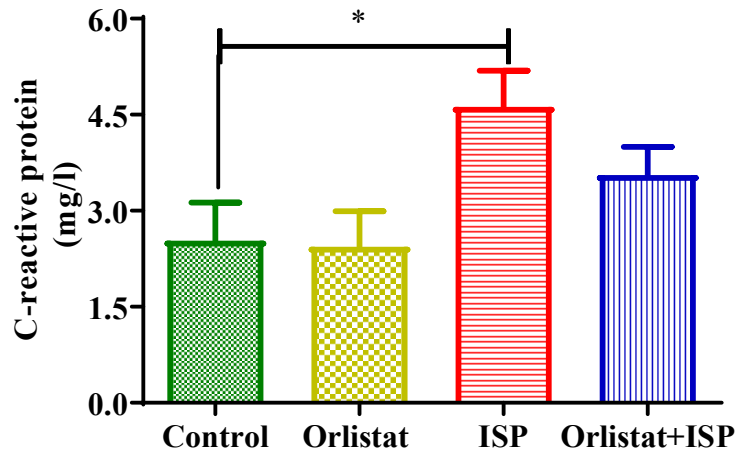


Fig. 4.13: Bar diagram showing the serum CRP level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p < 0.05$) difference between control and ISP-alone administered groups.

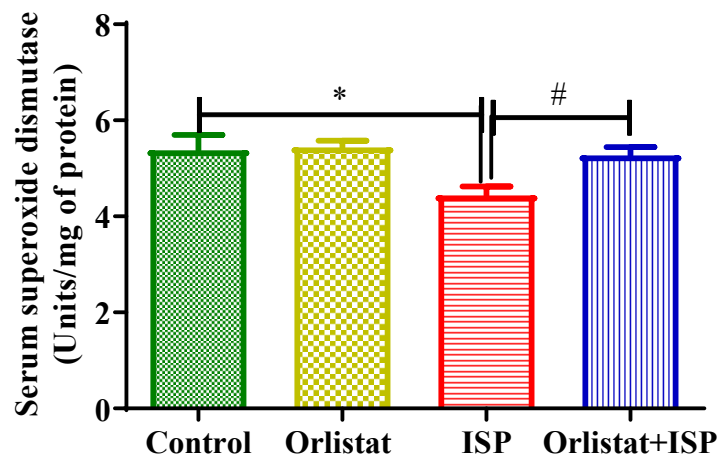


Fig. 4.14: Bar diagram showing the SOD activity in serum in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

proteins; n=7) compared to the ISP-alone administered group (3.78 ± 0.46 mmol H_2O_2 utilized/min/mg of proteins; n=7). No significant difference in serum catalase activities was noted in orlistat-alone (8.22 ± 1.25 mmol H_2O_2 utilized/min/mg of proteins; n=6) and control (7.98 ± 1.12 mmol H_2O_2 utilized/min/mg of proteins; n=6) groups of mice.

Table 4.15: Serum catalase activity in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Catalase (mmol H_2O_2 utilized/min/mg of proteins)
Control (n=6)	7.98 ± 1.12
Orlistat-alone (n=6)	8.22 ± 1.25
Isoprenaline-alone (n=7)	$3.78 \pm 0.46^*$
Orlistat+ISP (n=7)	$7.38 \pm 0.81^\#$

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control; $^\#p < 0.05$ vs isoprenaline-alone group.

4.16. Effect of orlistat on serum non-enzymatic antioxidant reduced glutathione (GSH) in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.16 and illustrated Figure 4.16 depict the fold change in serum reduced glutathione in control, orlistat-alone, ISP-alone and orlistat-treated cardiac injury groups of mice. A significant ($p < 0.05$) reduction was observed in GSH level in ISP-induced cardiac injury group (0.58 ± 0.11 fold change; n=7) in comparison to the control group (1.00 ± 0.08 fold change; n=6). Serum GSH level was significantly ($p < 0.05$) increased in orlistat-treated cardiac injury group (0.98 ± 0.07 fold change; n=7) compared to ISP-alone administered group (0.58 ± 0.11 fold change; n=7). Almost comparable level of serum GSH was seen between orlistat-alone (1.04 ± 0.13 fold change; n=6) and control (1.00 ± 0.08 fold change; n=6) groups of mice.

Table 4.16: Fold change of serum GSH in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Serum GSH (Fold change)
Control (n=6)	1.00±0.08
Orlistat-alone (n=6)	1.04±0.13
Isoprenaline-alone (n=7)	0.58±0.11*
Orlistat+ISP (n=7)	0.98±0.07 [#]

Data are presented as mean±S.E.

* $p<0.05$ vs control; [#] $p<0.05$ vs isoprenaline-alone group.

4.17. Effect of orlistat on serum lipid peroxidation (LPO) in ISP-induced cardiac fibrosis in mice

Level of malondialdehyde is generally used to assess lipid peroxidation. Table 4.17 and Figure 4.17 depict the serum MDA of control, orlistat-alone, ISP-alone and orlistat-treated cardiac injury group of mice. A significant ($p<0.05$) increase in MDA level was noted in ISP-alone administered group (6.17 ± 0.66 nmol/ml of serum; $n=7$) in comparison to control group (3.86 ± 0.46 nmol/ml of serum; $n=6$). Orlistat treatment significantly ($p<0.05$) reduced the MDA level (4.02 ± 0.36 nmol/ml of serum; $n=7$), compared to the ISP-alone administered group (6.17 ± 0.66 nmol/ml of serum; $n=7$). No significant change was observed in MDA level between orlistat-alone (3.77 ± 0.66 nmol/ml of serum; $n=6$) and control (3.86 ± 0.46 nmol/ml of serum; $n=6$) groups of mice.

Table 4.17: Serum MDA level in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups of mice.

Groups (mice)	MDA (nmol/ml of serum)
Control (n=6)	3.86±0.46
Orlistat-alone (n=6)	3.77±0.66
Isoprenaline-alone (n=7)	6.17±0.66*
Orlistat+ISP (n=7)	4.02±0.36 [#]

Data are presented as mean±S.E.

* $p<0.05$ vs control group; [#] $p<0.05$ vs ISP-alone administered group.

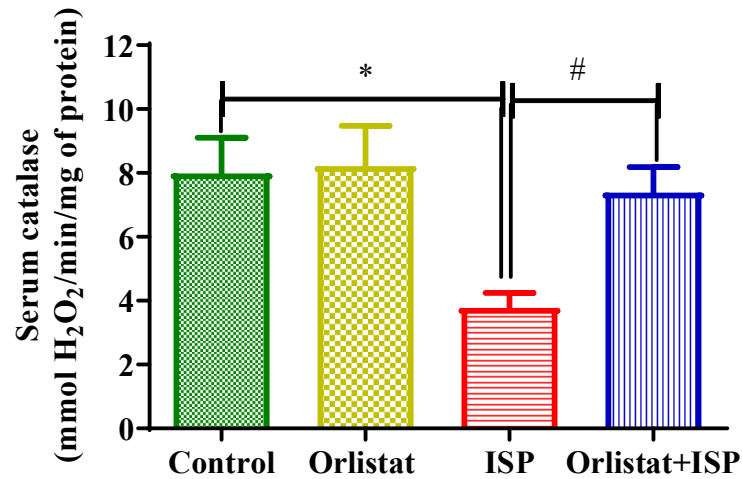


Fig. 4.15: Bar diagram showing the serum catalase activity in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

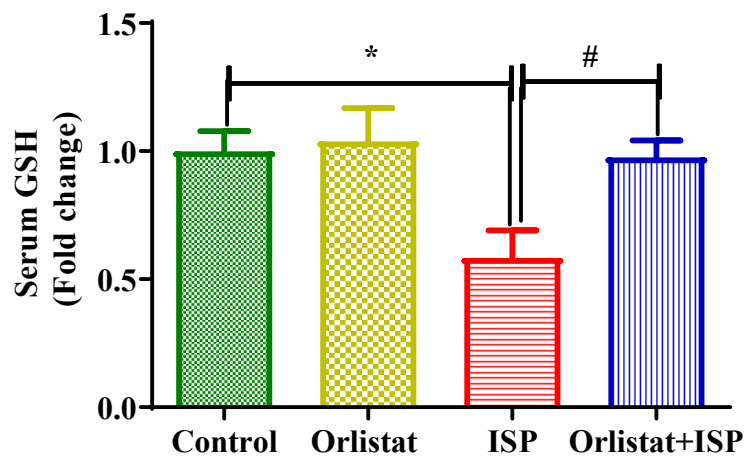


Fig. 4.16: Bar diagram showing the serum reduced glutathione (GSH) level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

4.18. Effect of orlistat on tissue superoxide dismutase (SOD) activity in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.18 and illustrated in Figure 4.18 depict SOD activity in heart tissue of different groups of mice. A significant ($p<0.05$) decrease in SOD activity was observed in ISP-alone administered group (20.80 ± 1.74 units/mg of protein; $n=10$) in comparison to control group (47.74 ± 8.33 units/mg of proteins; $n=11$). Orlistat treatment did not show significant improvement in SOD activity in cardiac injury group (34.30 ± 3.26 units/mg of proteins; $n=11$) compared to ISP-alone administered group (20.80 ± 1.74 units/mg of protein; $n=10$). Furthermore, no significant difference was noted in SOD activity in orlistat-alone (45.22 ± 7.49 units/mg of proteins; $n=11$) and control (47.74 ± 8.33 units/mg of proteins; $n=11$) groups of mice.

Table 4.18: Heart tissue SOD activity in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	SOD (units/mg of proteins)
Control ($n=11$)	47.74 ± 8.33
Orlistat-alone ($n=11$)	45.22 ± 7.49
Isoprenaline-alone ($n=10$)	$20.80\pm1.74^*$
Orlistat+ISP ($n=11$)	34.30 ± 3.26

Data are presented as mean $\pm$ S.E.

* $p<0.05$ vs control group.

4.19. Effect of orlistat on tissue catalase activity in ISP-induced cardiac fibrosis in mice

Data in Table 4.19 and Figure 4.19 depict catalase activity in heart tissue of control, orlistat-alone, ISP-alone and orlistat with ISP groups of mice. Data revealed that ISP-administration significantly ($p<0.001$) reduced the catalase activity (0.85 ± 0.12 mmol H_2O_2 utilized/min/mg of proteins; $n=9$) in heart in comparison to the control group (2.38 ± 0.16 mmol H_2O_2 utilized/min/mg of proteins; $n=11$). A significant ($p<0.05$) elevation in catalase activity was observed in orlistat-treated cardiac injury group (1.92 ± 0.37 mmol H_2O_2 utilized/min/mg of proteins; $n=9$) compared to the ISP-alone administered group (0.85 ± 0.12 mmol H_2O_2

utilized/min/mg of proteins; n=9). Catalase activities in orlistat-alone (2.28 ± 0.29 mmol H_2O_2 utilized/min/mg of proteins; n=8) and control (2.38 ± 0.16 mmol H_2O_2 utilized/min/mg of proteins; n=11) groups of mice were almost comparable to each other.

Table 4.19: Catalase activity in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Catalase (mmol H_2O_2 utilized/min/mg of proteins)
Control (n=11)	2.38 ± 0.16
Orlistat-alone (n=8)	2.28 ± 0.29
Isoprenaline-alone (n=9)	$0.85 \pm 0.12^{***}$
Orlistat+ISP (n=9)	$1.92 \pm 0.37^{\#}$

Data are presented as mean $\pm$ S.E.

*** $p < 0.001$ vs control; $^{\#}p < 0.05$ vs isoprenaline-alone group.

4.20. Effect of orlistat on tissue non-enzymatic antioxidant reduced glutathione (GSH) in mice

Data summarized in Table 4.20 and illustrated in Figure 4.20 show the fold change in GSH in control, orlistat-alone, ISP-alone and orlistat-treated cardiac injury groups of mice. Data revealed that a significant ($p < 0.05$) reduction was observed in GSH level in ISP-induced cardiac injury group (0.81 ± 0.03 fold change; n=9) in comparison to the control group (1.00 ± 0.07 fold change; n=10). Orlistat treatment significantly ($p < 0.05$) increased GSH in cardiac injury group (0.99 ± 0.03 fold change; n=9) compared to ISP-alone administered group (0.81 ± 0.03 fold change; n=9). GSH level was seen almost comparable between orlistat-alone (0.94 ± 0.02 fold change; n=9) and control (1.00 ± 0.07 fold change; n=10) groups of mice.

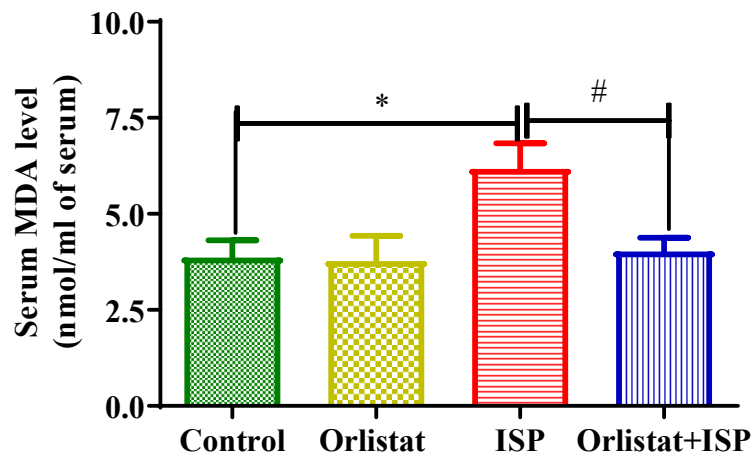


Fig. 4.17: Bar diagram showing the serum MDA level in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

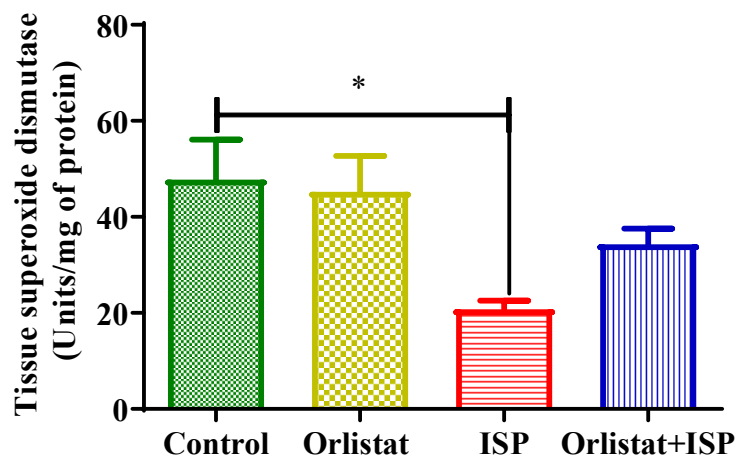


Fig. 4.18: Bar diagram showing the SOD activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between control and ISP-alone administered groups.

Table 4.20: Fold change of non-enzymatic antioxidant, GSH in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	GSH (Fold change)
Control (n=10)	1.00±0.07
Orlistat-alone (n=9)	0.94±0.02
Isoprenaline-alone (n=9)	0.81±0.03*
Orlistat+ISP (n=9)	0.99±0.03 [#]

Data are presented as mean±S.E.

* $p < 0.05$ vs control; [#] $p < 0.05$ vs isoprenaline-alone group.

4.21. Effect of orlistat on tissue lipid peroxidation (LPO) in ISP-induced cardiac fibrosis in mice

Lipid peroxidation was assessed by measuring the malondialdehyde (MDA) level in heart. Table 4.21 and Figure 4.21 depict the MDA data in heart tissue of control, orlistat-alone, ISP-alone and orlistat-treated cardiac injury group of mice. A significant ($p < 0.05$) increase in MDA level was noted in ISP-alone administered group (7.25 ± 0.94 nmol/mg of protein; $n=9$) in comparison to control group (4.88 ± 0.49 nmol/mg of protein; $n=11$). Orlistat treatment significantly ($p < 0.05$) reduced the MDA level (4.58 ± 0.28 nmol/mg of protein; $n=9$) compared to the ISP-alone administered group (7.25 ± 0.94 nmol/mg of protein; $n=9$). No significant change was observed in MDA level between orlistat-alone (4.46 ± 0.19 nmol/mg of protein; $n=6$) and control (4.88 ± 0.49 nmol/mg of protein; $n=11$) groups of mice.

Table 4.21: MDA level in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups of mice.

Groups (mice)	MDA (nmol/mg of protein)
Control (n=11)	4.88±0.49
Orlistat-alone (n=6)	4.46±0.19
Isoprenaline-alone (n=9)	7.25±0.94*
Orlistat+ISP (n=9)	4.58±0.28 [#]

Data presented are mean±S.E.

* $p < 0.05$ vs control group; [#] $p < 0.05$ vs ISP-alone administered group.

4.22 Effect of orlistat on tissue hydroxyproline level in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.22 and illustrated in Figure 4.22 show the hydroxyproline level in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis group of mice. Data revealed that a significant ($p<0.001$) elevation in hydroxyproline level was observed in ISP-alone administered cardiac fibrosis group (11.35 ± 2.11 $\mu\text{g}/\text{mg}$ of tissue; $n=9$) in comparison to control group (2.58 ± 0.47 $\mu\text{g}/\text{mg}$ of tissue; $n=10$). However, orlistat treatment significantly ($p<0.05$) reduced the hydroxyproline level in cardiac fibrosis (6.48 ± 0.81 $\mu\text{g}/\text{mg}$ of tissue; $n=9$) compared to the ISP-alone administered group (11.35 ± 2.11 $\mu\text{g}/\text{mg}$ of tissue; $n=9$). No significant changes were observed in hydroxyproline level between orlistat-alone (2.97 ± 0.34 $\mu\text{g}/\text{mg}$ of tissue; $n=9$) and control (2.58 ± 0.47 $\mu\text{g}/\text{mg}$ of tissue; $n=10$) groups of mice

Table 4.22: Hydroxyproline level in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Tissue hydroxyproline ($\mu\text{g}/\text{mg}$ of tissue)
Control ($n=10$)	2.58 ± 0.47
Orlistat-alone ($n=9$)	2.97 ± 0.34
Isoprenaline-alone ($n=9$)	$11.35\pm2.11^{***}$
Orlistat+ISP ($n=9$)	$6.48\pm0.81^{\#}$

Data are presented as mean $\pm$ S.E.

*** $p<0.001$ vs control group; $^{\#}p<0.05$ vs ISP-alone administered group.

4.23. Effect of orlistat on tissue glucosamine level in ISP-induced cardiac fibrosis in mice

Table 4.23 and Figure 4.23 show the fold change in glucosamine level in different groups viz., control, orlistat-alone, ISP-alone and orlistat-treated cardiac injury group of mice. A significant ($p<0.05$) increase in glucosamine level was noted in ISP-alone administered cardiac fibrosis group (2.03 ± 0.15 fold change; $n=9$) compared to control group (1.00 ± 0.24 fold change; $n=10$). Orlistat treatment showed a significant ($p<0.05$) reduction in glucosamine level (1.07 ± 0.26 fold change; $n=9$) in comparison to ISP-alone administered cardiac fibrosis

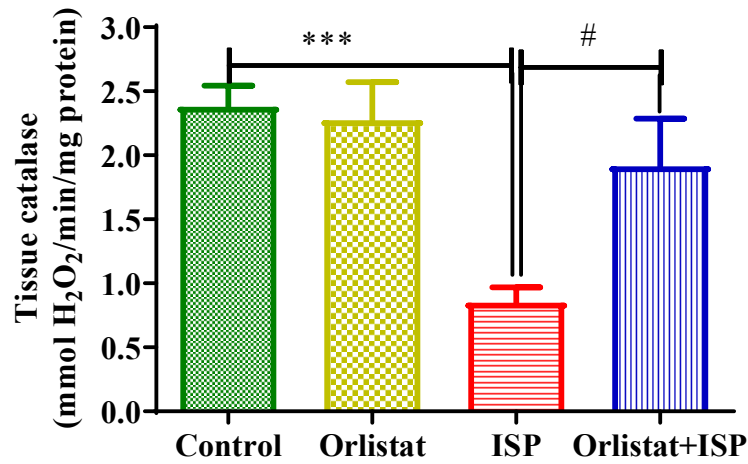


Fig. 4.19: Bar diagram showing the catalase activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, #) on the bars present significant ($p<0.001-0.05$) difference between groups.

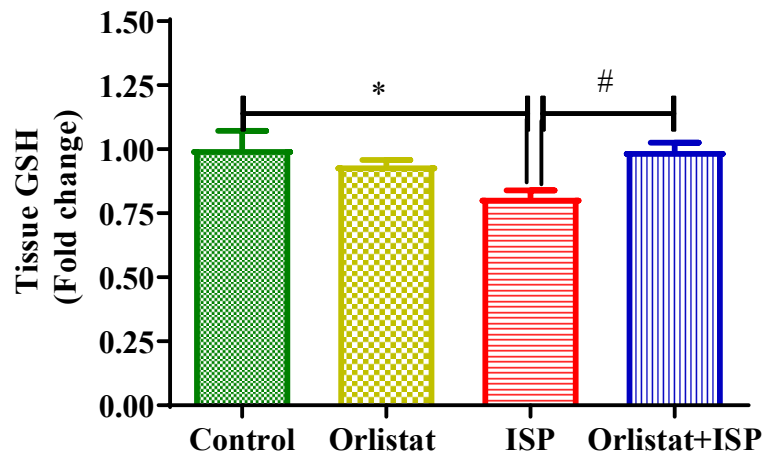


Fig. 4.20: Bar diagram showing the reduced glutathione (GSH) level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

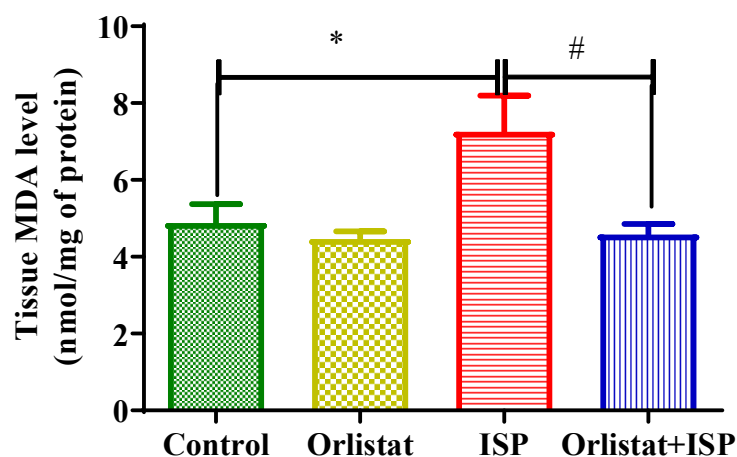


Fig. 4.21: Bar diagram showing the MDA level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

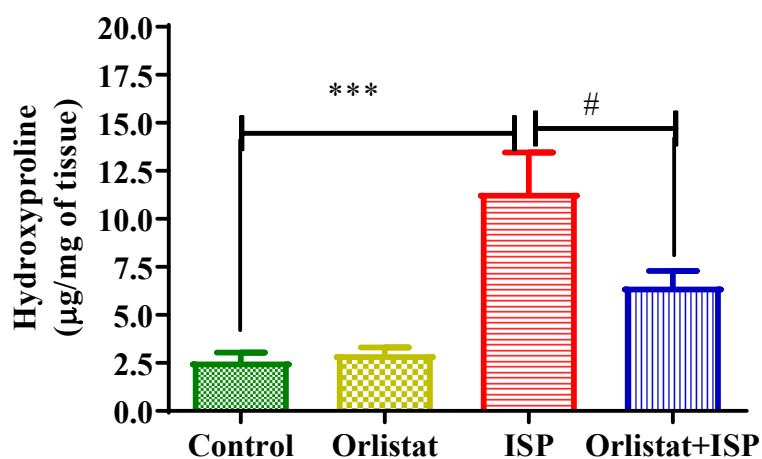


Fig. 4.22: Bar diagram showing the hydroxyproline level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, #) on the bars present significant ($p < 0.001-0.05$) difference between groups.

group (2.03 ± 0.15 fold change; $n=9$). Glucosamine level in orlistat-alone (1.07 ± 0.27 fold change; $n=9$) and control (1.00 ± 0.24 fold change; $n=10$) groups of mice were observed almost comparable.

Table 4.23: Glucosamine level in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Tissue glucosamine (Fold change)
Control ($n=10$)	1.00 ± 0.24
Orlistat-alone ($n=9$)	1.07 ± 0.27
Isoprenaline-alone ($n=9$)	$2.03 \pm 0.15^*$
Orlistat+ISP ($n=9$)	$1.07 \pm 0.26^\#$

Data presented are mean $\pm$ S.E.

* $p < 0.05$ vs control group; $^\#p < 0.05$ vs ISP-alone administered group.

4.24 Effect of orlistat on collagen type I in heart tissue in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.24 and illustrated in Figure 4.24 show the collagen type I content of heart tissue in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis groups of mice. A significant ($p < 0.05$) increase in collagen type I was observed in ISP-alone administered group (31.02 ± 7.19 ng/mg of protein; $n=8$) in comparison to control group (12.46 ± 3.15 ng/mg of protein; $n=8$). However, orlistat treatment moderately improved the collagen type I content (17.77 ± 4.15 ng/mg of protein; $n=7$) compared to ISP-alone administered group (31.02 ± 7.19 ng/mg of protein; $n=8$). Almost comparable collagen type I content was seen in orlistat-alone (13.53 ± 2.79 ng/mg of protein; $n=8$) and control (12.46 ± 3.15 ng/mg of protein; $n=8$) groups of mice.

Table 4.24: Collagen type I content of heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Collagen type I (ng/mg of protein)
Control (n=8)	12.46±3.15
Orlistat-alone (n=8)	13.53±2.79
Isoprenaline-alone (n=8)	31.02±7.19*
Orlistat+ISP (n=7)	17.77±4.15

Data are presented as mean±S.E.

* $p < 0.05$ vs control group.

4.25. Effect of orlistat on connective tissue growth factor (CTGF) in heart tissue in ISP-induced cardiac fibrosis in mice

Table 4.25 and Figure 4.25 depict the CTGF level of heart tissue in control, orlistat-alone, ISP-alone and orlistat along with ISP groups of mice. No significant difference was observed in CTGF level in control (423.3±178.5 pg/mg of protein; n=8), orlistat-alone (240.5±69.61 pg/mg of protein; n=8), isoprenaline-alone (981.1±417.9 pg/mg of protein; n=8) and orlistat-treated cardiac injury (674.0±280.4 pg/mg of protein; n=7) groups. However, an increased (non-significant) CTGF level was observed in ISP-alone administered group (981.1±417.9 pg/mg of protein; n=8) in comparison to control group (423.3±178.5 pg/mg of protein; n=8). Furthermore, orlistat treatment partially improved CTGF level (674.0±280.4 pg/mg of protein; n=7) in cardiac injury group, compared to ISP-alone administered group (981.1±417.9 pg/mg of protein; n=8).

Table 4.25: CTGF in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups.

Groups (mice)	CTGF (pg/mg of protein)
Control (n=8)	423.3±178.5
Orlistat-alone (n=8)	240.5±69.61
Isoprenaline-alone (n=8)	981.1±417.9
Orlistat+ISP (n=7)	674.0±280.4

Data are presented as mean±S.E.

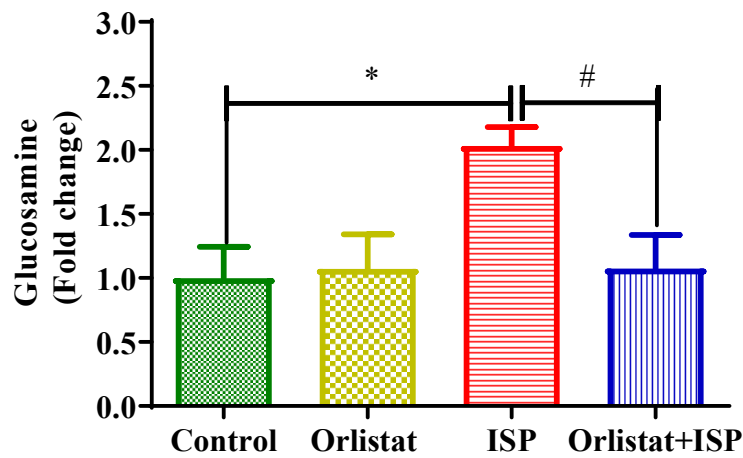


Fig. 4.23: Bar diagram showing the fold change in glucosamine level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

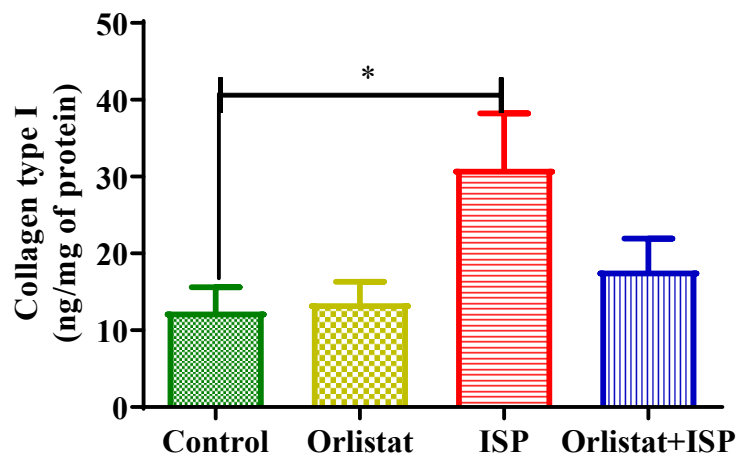


Fig. 4.24: Bar diagram showing collagen type I of heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between control and ISP-alone administered groups.

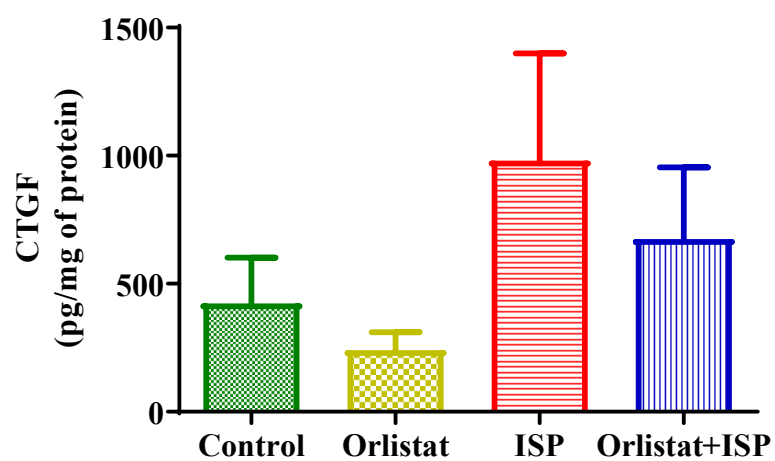


Fig. 4.25: Bar diagram showing the CTGF level in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test.

4.26. Effect of orlistat on matrix metalloprotease-2 (MMP-2) activity in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.26 and illustrated in Figure 4.26a depict fold change in MMP-2 activity in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis groups of mice. Figure 4.26b illustrates a representative gel image showing MMP-2 activity. Data revealed that administration of ISP, significantly ($p<0.001$) increased the MMP-2 activity in cardiac fibrosis (3.22 ± 0.48 fold change; $n=4$) compared to the control group (1.00 ± 0.00 fold change; $n=4$). A significant ($p<0.01$) reduction was observed in MMP-2 activity in orlistat-treated cardiac fibrotic group (1.38 ± 0.16 fold change; $n=4$) in comparison to ISP-alone administered group (3.22 ± 0.48 fold change; $n=4$). No significant difference was noted in MMP-2 activity in orlistat-alone (0.91 ± 0.07 fold change; $n=4$) and control (1.00 ± 0.00 fold change; $n=4$) groups of mice.

Table 4.26: Fold change of MMP-2 activity in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat-administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	MMP-2 (fold change)
Control (n=4)	1.00±0.00
Orlistat-alone (n=4)	0.91±0.07
Isoprenaline-alone (n=4)	3.22±0.48***
Orlistat+ISP (n=4)	1.38±0.16##

Data are presented as mean±S.E.

*** $p<0.001$ vs control group; ## $p<0.01$ vs ISP-alone administered group.

4.27. Effect of orlistat on MMP-9 activity in ISP-induced cardiac fibrosis in mice

Table 4.27 and Figure 4.27a show fold change in MMP-9 activity in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis groups of mice. Figure 4.27b illustrates the representative image of MMP-9 activity in different groups. No significant change was observed in MMP-9 activity in control (1.00 ± 0.00 fold change; $n=2$), ISP-alone administered (1.05 ± 0.05 fold change; $n=2$), orlistat-alone (1.33 ± 0.57 fold change; $n=2$) and orlistat-treated cardiac fibrosis (1.13 ± 0.02 fold change; $n=2$) groups of mice.

Table 4.27: Fold change of MMP-9 activity in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	MMP-9 (fold change)
Control (n=2)	1.00±0.00
Orlistat-alone (n=2)	1.33±0.57
Isoprenaline-alone (n=2)	1.05±0.05
Orlistat+ISP (n=2)	1.13±0.02

Data are presented as mean±S.E.

4.28. Effect of orlistat on Nrf-2 protein expression in heart tissue in ISP-induced cardiac fibrosis in mice

The data of fold changes in Nrf-2 protein expression of heart tissue in control, orlistat-alone, ISP-alone and orlistat-treated cardiac injury groups of mice are summarized in Table 4.28 and illustrated in Figure 4.28a. The representative blot images of Nrf-2 and GAPDH are illustrated in Figure 4.28b. A significant ($p<0.05$) reduction in Nrf-2 protein expression was observed in ISP-alone administered group (0.57 ± 0.09 fold change; $n=5$) in comparison to control group (1.00 ± 0.00 fold change; $n=5$). Orlistat treatment significantly ($p<0.01$) increased the Nrf-2 protein expression (1.11 ± 0.15 fold change; $n=5$) in cardiac injury group compared to ISP-alone administered group (0.57 ± 0.09 fold change; $n=5$). The Nrf-2 protein expression level of control (1.00 ± 0.00 fold change; $n=5$) and orlistat-alone (0.88 ± 0.11 fold change; $n=5$) groups of mice were almost comparable to each other.

Table 4.28: Fold change of Nrf-2 protein expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice.

Groups (mice)	Nrf-2 protein expression (fold change)
Control (n=5)	1.00±0.00
Orlistat-alone (n=5)	0.88±0.11
Isoprenaline-alone (n=5)	0.57±0.09*
Orlistat+ISP (n=5)	1.11±0.15 ^{##}

Data are presented as mean±S.E.

* $p<0.05$ vs control group; ^{##} $p<0.01$ vs ISP-alone administered group.

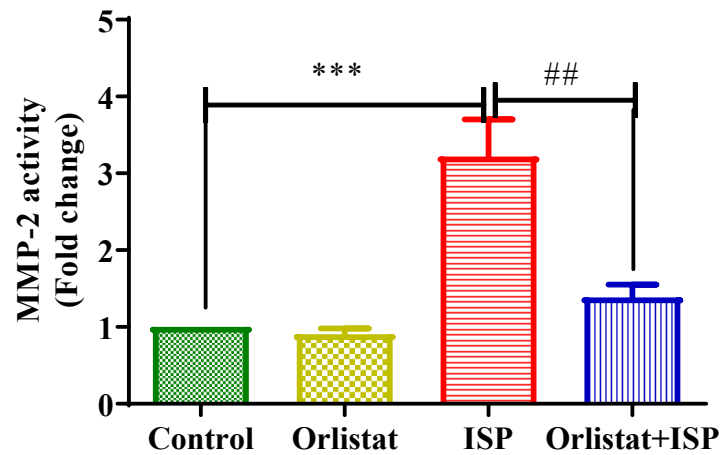


Fig. 4.26a: Bar diagram showing the fold change in MMP-2 activity in heart in control, orlistat-alone, ISP alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, ##) on the bars present significant ($p < 0.001-0.05$) difference between groups.

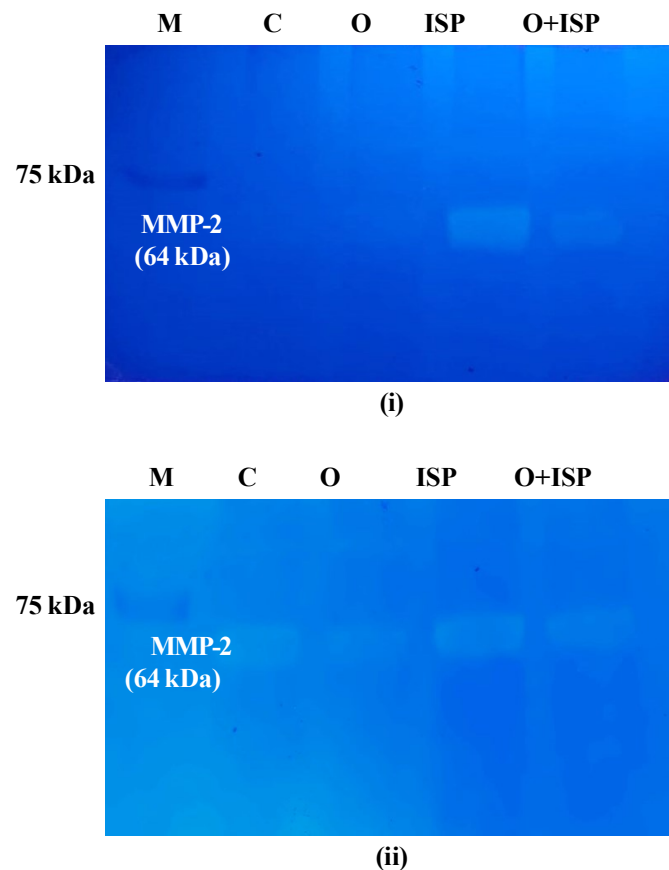


Fig. 4.26b: Representative images of gel (i & ii) showing MMP-2 activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. M-marker; C-control group; O-orlistat alone group; ISP-isoprenaline alone group; O+ISP- orlistat+isoprenaline group.

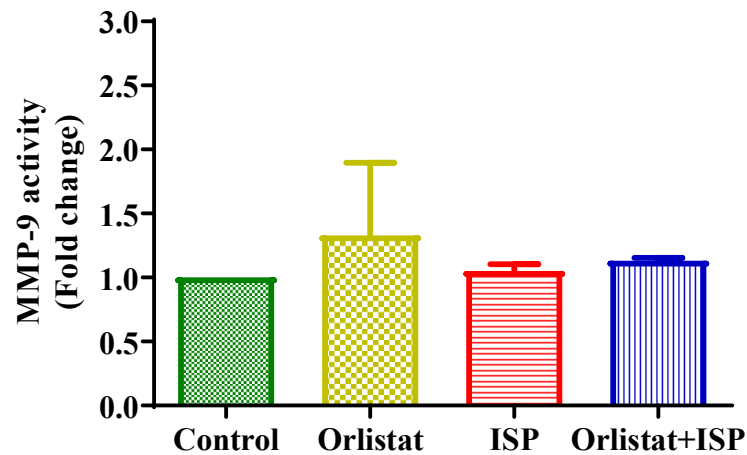


Fig. 4.27a: Bar diagram showing the fold change in MMP-9 activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test.

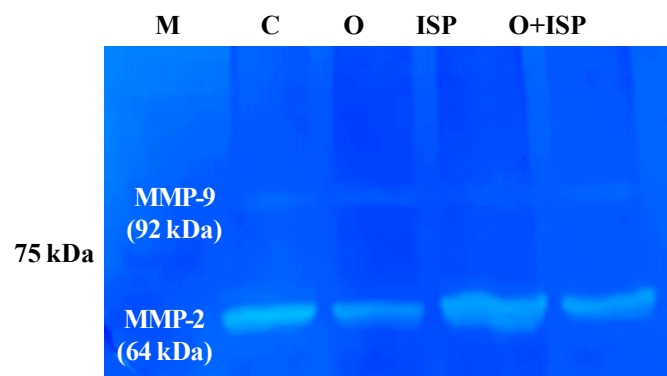


Fig. 4.27b: Representative image of gel showing MMP-9 activity in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. M-marker; C-control group; O-orlistat alone group; ISP-isoprenaline alone group; O+ISP- orlistat+isoprenaline group.

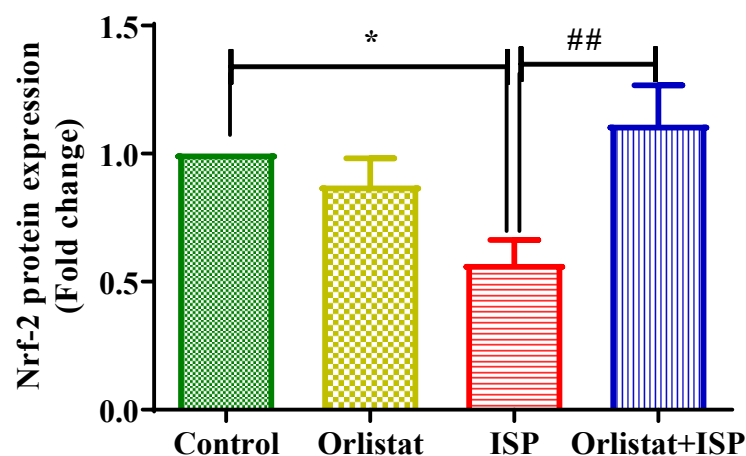


Fig. 4.28a: Bar diagram showing the fold change in Nrf-2 protein expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, ##) on the bars present significant ($p < 0.05$ -0.01) difference between groups.

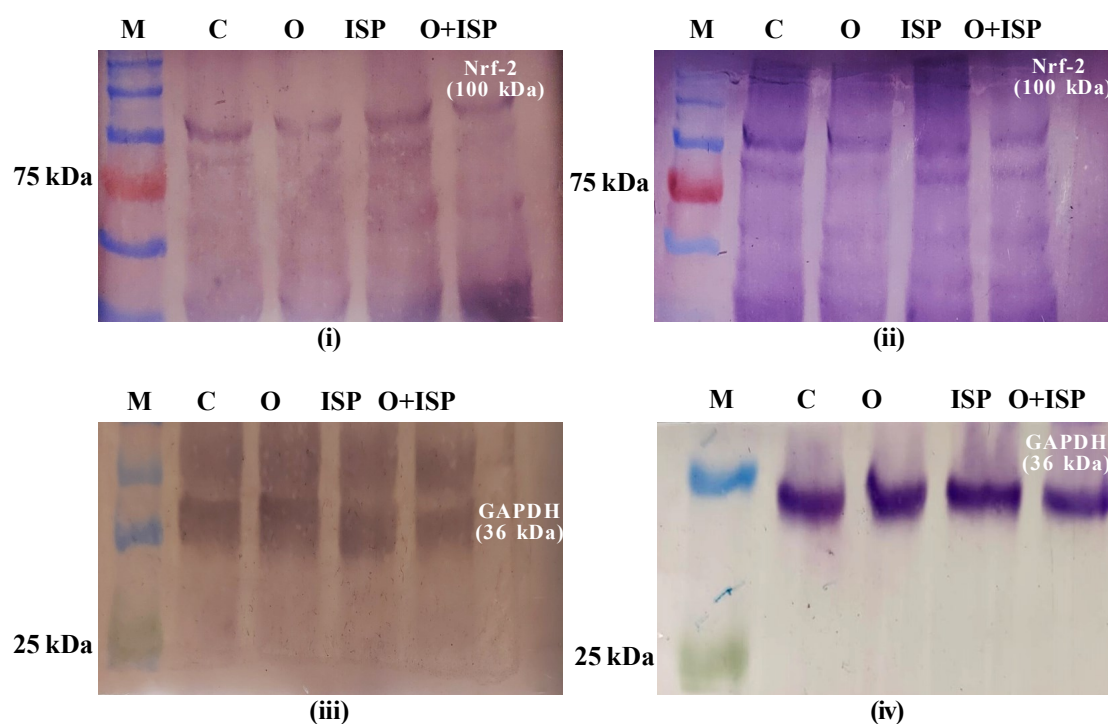


Fig. 4.28b: Representative images of blot showing Nrf-2 (i & ii) and GAPDH (iii & iv) protein expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. M-marker; C-control group; O-orlistat alone group; ISP-isoprenaline- alone group; O+ISP- Orlistat+ISP group.

4.29. Effect of orlistat on NF- κ B protein expression in heart tissue in ISP-induced cardiac fibrosis in mice

Table 4.29 and Figure 4.29 a depict the NF- κ B protein expression in heart tissue of control, ISP-alone, orlistat-alone and orlistat-treated cardiac injury groups of mice. Figure 4.29b illustrates the representative blot images of NF- κ B protein expression. A significant ($p<0.05$) increase in NF- κ B protein expression was observed in ISP-alone administered group (1.66 ± 0.24 fold change; $n=3$) in comparison to control group (1.00 ± 0.00 fold change; $n=3$). Orlistat treatment significantly ($p<0.05$) reduced the NF- κ B protein expression (0.85 ± 0.04 fold change; $n=3$) compared to ISP-alone administered group (1.66 ± 0.24 fold change; $n=3$). No significant difference was observed in orlistat-alone (0.88 ± 0.16 fold change; $n=3$) and control (1.00 ± 0.00 fold change; $n=3$) groups of mice.

Table 4.29: Fold change of NF- κ B protein expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat co-administered cardiac injury (orlistat+ISP) groups of mice.

Groups (mice)	NF- κ B (fold change)
Control (n=3)	1.00 ± 0.00
Orlistat-alone (n=3)	0.88 ± 0.16
Isoprenaline-alone (n=3)	$1.66\pm0.24^*$
Orlistat+ISP (n=3)	$0.85\pm0.04^\#$

Data are presented as mean $\pm$ S.E.

* $p<0.05$ vs control group; $^\#p<0.05$ vs ISP-alone administered group.

4.30. Effect of orlistat on transforming growth factor- β (TGF- β) mRNA expression in heart in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.30 and illustrated in Figure 4.30 depict fold change in comparative mRNA expression of TGF- β in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis groups of mice. The TGF- β and other mRNA expressions in different groups were normalized to corresponding GAPDH mRNA expression in heart tissue from different groups of mice. Figure 4.31 a, b & c illustrate real time PCR dissociation curve,

amplification plot and agarose gel electrophoresis image of GAPDH in heart tissue of different groups of mice. The mRNA expression of TGF- β was significantly ($p<0.05$) increased in isoprenaline-alone administered group (2.38 ± 0.38 fold change; $n=5$) in comparison to control group (1.00 ± 0.00 fold change; $n=5$). However, the TGF- β mRNA expression was significantly ($p<0.01$) reduced in orlistat-treated cardiac injury group (0.88 ± 0.36 fold change; $n=5$) compared to isoprenaline-alone group (2.38 ± 0.38 fold change; $n=5$). No significant difference in TGF- β mRNA expression was observed in orlistat-alone (0.41 ± 0.17 fold change; $n=5$) and control (1.00 ± 0.00 fold change; $n=5$) groups of mice. Figure 4.32 a, b & c illustrate real time PCR dissociation curve, amplification plot and agarose gel electrophoresis image of TGF- β in heart tissue of different groups of mice.

Table 4.30: Comparative fold change in TGF- β mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	TGF- β mRNA expression (fold change)
Control (n=5)	1.00 $\pm$ 0.00
Orlistat-alone (n=5)	0.41 $\pm$ 0.17
Isoprenaline-alone (n=5)	2.38 $\pm$ 0.38*
Orlistat+ISP (n=5)	0.88 $\pm$ 0.36 ^{##}

Data are presented as mean $\pm$ S.E.

* $p<0.05$ vs control group; ^{##} $p<0.01$ vs ISP-alone administered group.

4.31. Effect of orlistat on Smad-3 mRNA expression in heart in ISP-induced cardiac fibrosis in mice

Table 4.31 and Figure 4.33 show the comparative Smad-3 mRNA expression in control, orlistat-alone, isoprenaline-alone and orlistat-treated cardiac injury groups of mice. Figure 4.34 a, b and c illustrate real time PCR dissociation curve, amplification plot and representative image of agarose gel electrophoresis of Smad-3 in heart tissue of different groups of mice. A significant ($p<0.05$) increase in the mRNA expression of Smad-3 was observed in isoprenaline-alone administered group (7.64 ± 2.52 fold change; $n=4$) in comparison to control group (1.00 ± 0.00 fold change; $n=4$). Orlistat-treatment significantly ($p<0.05$)

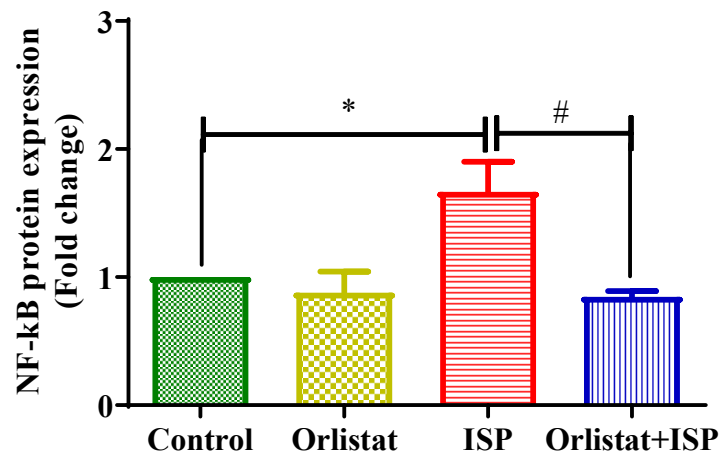


Fig. 4.29a: Bar diagram showing the fold change in NF- κ B protein expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

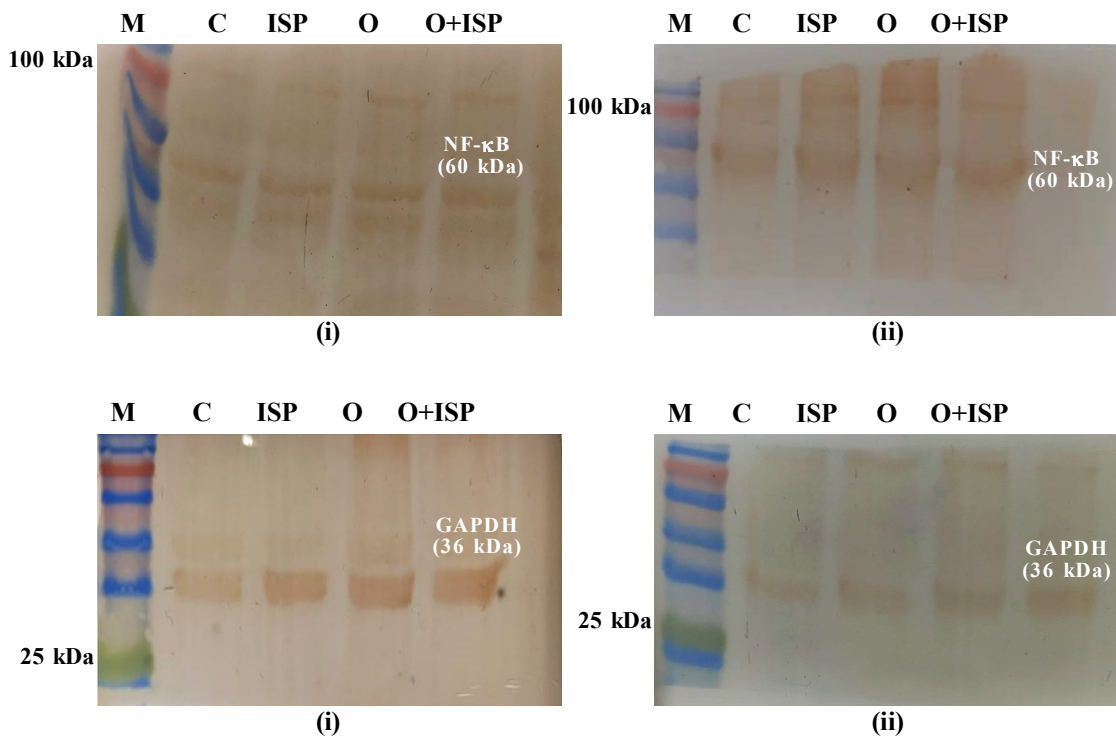


Fig. 4.29b: Representative images of blot showing NF- κ B (i & ii) and GAPDH (iii & iv) protein expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. M-marker; C-control group; O-orlistat-alone group; ISP-isoprenaline-alone group; O+ISP- orlistat+ISP group.

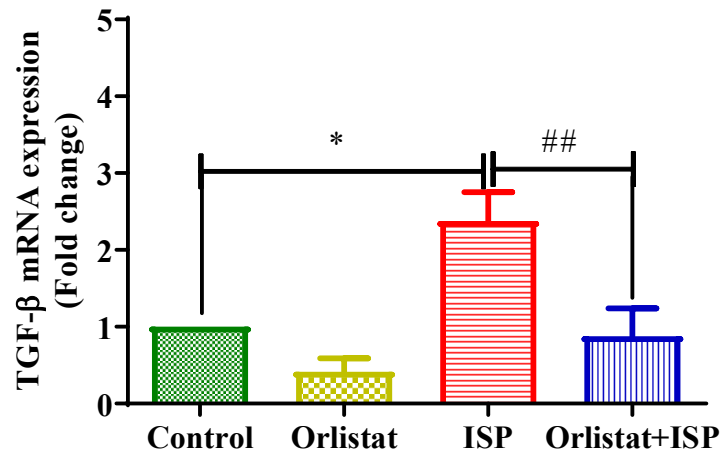


Fig. 4.30: Bar diagram showing the comparative fold change in TGF- β mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, ##) on the bars present significant ($p < 0.05-0.01$) difference between groups.

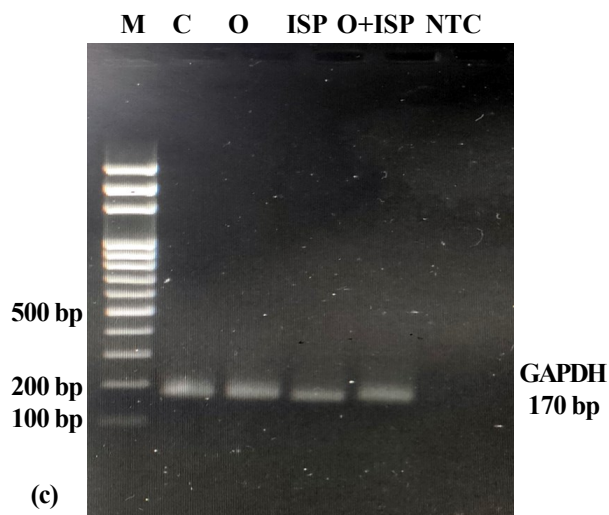
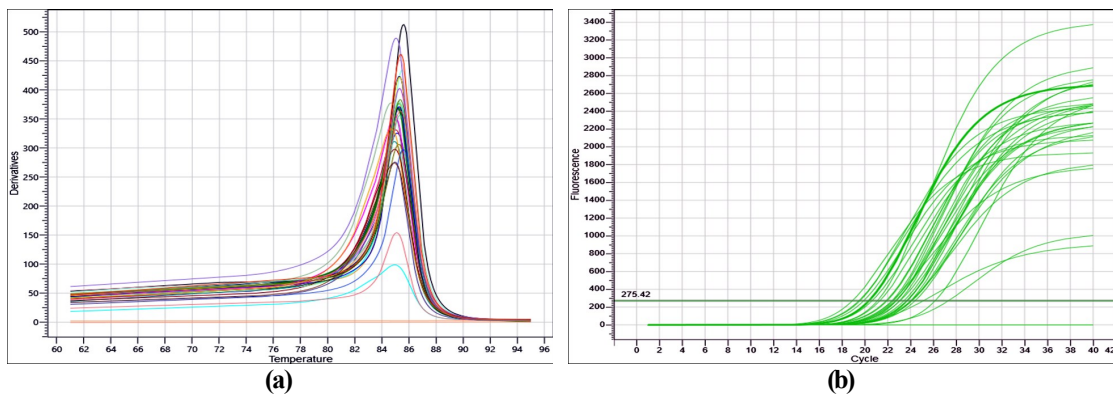


Fig. 4.31: qRT-PCR melt curve plot (a), amplification plot (b) and agarose gel electrophoresis image of PCR product (c) of GAPDH mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. M-marker; C-control group; O-orlistat-alone group; ISP-isoprenaline alone group; O+ISP- Orlistat+ISP group.

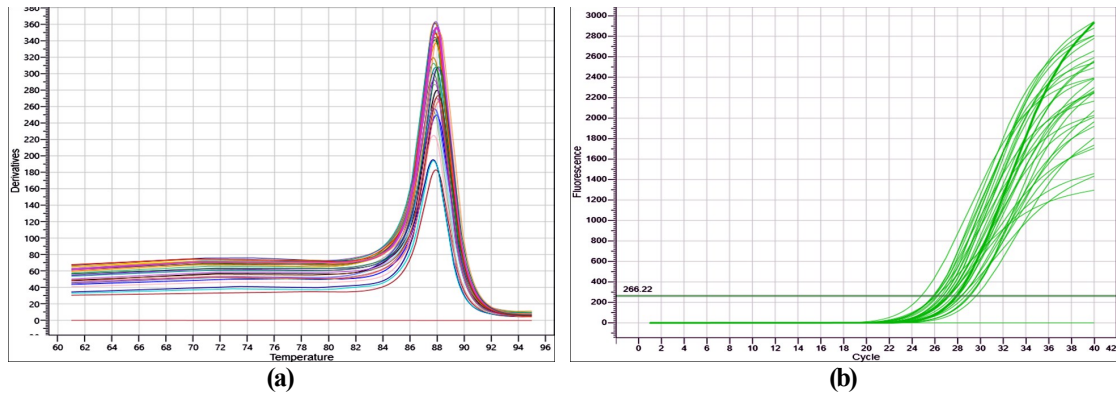


Fig. 4.32: qRT-PCR melt curve plot (a), amplification plot (b) and agarose gel electrophoresis images (c) of TGF- β mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. M-marker; C-control group; O-orlistat-alone group; ISP-isoprenaline alone group; O+ISP-Orlistat+ISP group.

reduced the Smad-3 mRNA expression in cardiac injury group (1.34 ± 0.43 fold change; $n=4$) compared to isoprenaline-alone administered group (7.64 ± 2.52 fold change; $n=4$). The Smad-3 mRNA expression in orlistat-alone (1.11 ± 0.49 fold change; $n=4$) and control (1.00 ± 0.00 fold change; $n=4$) groups of mice were almost comparable.

Table 4.31: Comparative fold change in Smad-3 mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Smad-3 mRNA expression (fold change)
Control (n=4)	1.00 ± 0.00
Orlistat-alone (n=4)	1.11 ± 0.49
Isoprenaline-alone (n=4)	$7.64 \pm 2.52^*$
Orlistat+ISP (n=4)	$1.34 \pm 0.43^\#$

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control group; $^\#p < 0.05$ vs ISP-alone administered group.

4.32. Effect of orlistat on α -SMA mRNA expression in heart in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.32 and illustrated in Figure 4.35 depict fold change in comparative mRNA expression of α -SMA in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis groups of mice. Figure 4.36 a, b & c illustrate real time PCR dissociation curve, amplification plot and representative image of agarose gel electrophoresis of α -SMA in heart tissue of different groups of mice. The mRNA expression of α -SMA was significantly ($p < 0.05$) increased in isoprenaline-alone administered group (2.86 ± 0.84 fold change; $n=4$) in comparison to control group (1.00 ± 0.00 fold change; $n=4$). The α -SMA mRNA expression was significantly ($p < 0.05$) decreased in orlistat-treated cardiac injury group (0.83 ± 0.24 fold change; $n=4$) compared to isoprenaline-alone group (2.86 ± 0.84 fold change; $n=4$). No significant difference in α -SMA mRNA expression was observed in orlistat-alone (0.61 ± 0.11

fold change; n=4) and control (1.00±0.00 fold change; n=4) groups of mice.

Table 4.32: Comparative fold change in α -SMA mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat-administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	α -SMA mRNA expression (fold change)
Control (n=4)	1.00±0.00
Orlistat-alone (n=4)	0.61±0.11
Isoprenaline-alone (n=4)	2.86±0.84*
Orlistat+ISP (n=4)	0.83±0.24 [#]

Data are presented as mean±S.E.

* $p<0.05$ vs control group; [#] $p<0.05$ vs ISP-alone administered group.

4.33. Effect of orlistat on collagen type I mRNA expression in heart in ISP-induced cardiac fibrosis in mice

The comparative fold change in mRNA expression of collagen type I in heart of control, orlistat-alone, isoprenaline-alone and orlistat-treated cardiac injury groups of mice are summarized in Table 4.33 and illustrated in Figure 4.37. Figure 4.38 a, b & c illustrate the dissociation curve, amplification curve and representative image of agarose gel electrophoresis of real time PCR of collagen type I in heart tissue of different groups of mice. A significant ($p<0.05$) increase in the mRNA expression of collagen type I was observed in isoprenaline-

Table 4.33: Comparative fold change in collagen type I mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat-administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Collagen type I mRNA expression (fold change)
Control (n=6)	1.00±0.00
Orlistat-alone (n=7)	1.51±0.48
Isoprenaline-alone (n=7)	4.47±1.49*
Orlistat+ISP (n=7)	1.23±0.31 [#]

Data are presented as mean±S.E.

* $p<0.05$ vs control group; [#] $p<0.05$ vs ISP-alone administered group.

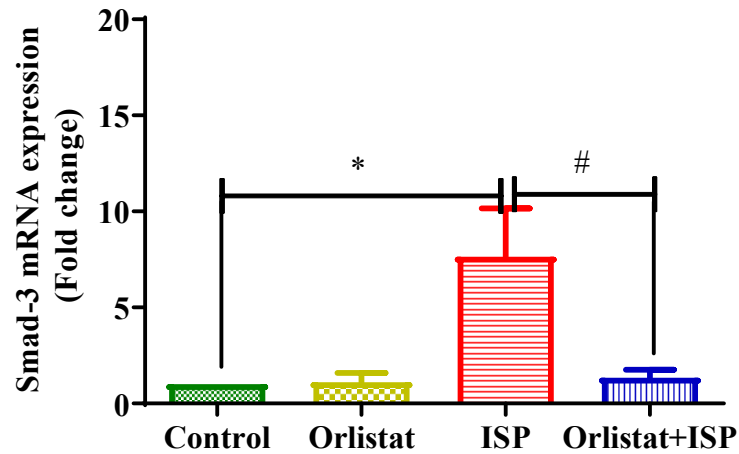


Fig. 4.33: Bar diagram showing the comparative fold change in Smad-3 mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

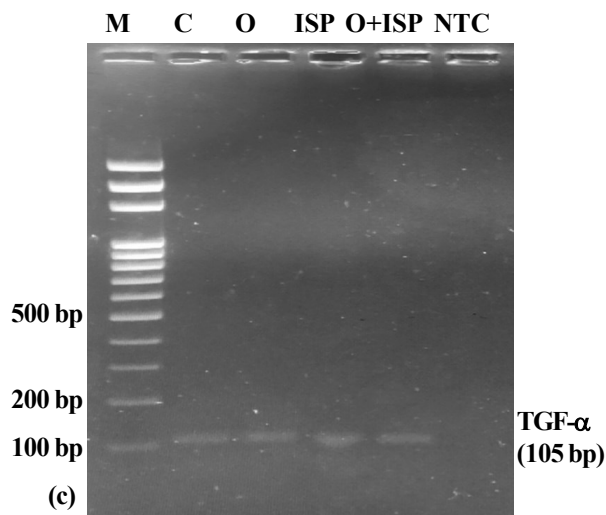
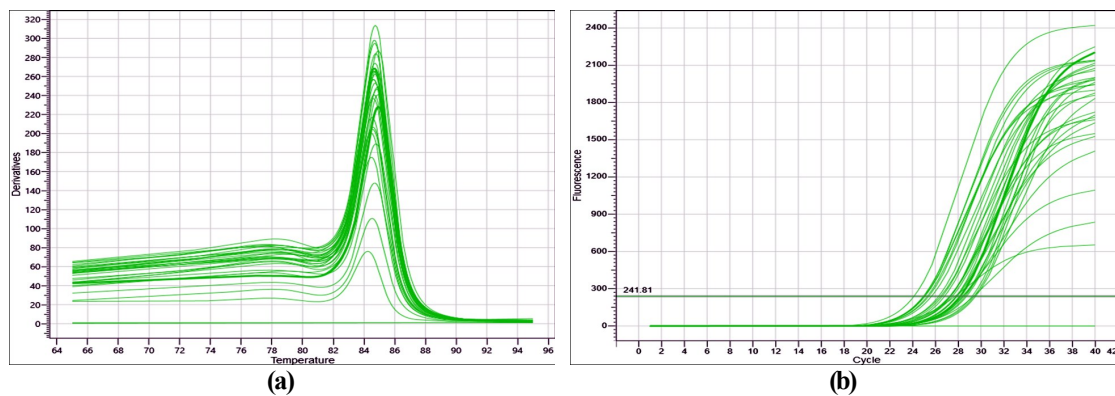


Fig. 4.34: qRT-PCR melt curve plot (a), amplification plot (b) and representative image of agarose gel electrophoresis (c) of Smad-3 mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. M-marker; C-control group; O-orlistat-alone group; ISP-isoprenaline-alone group; O+ISP- Orlistat+ISP group.

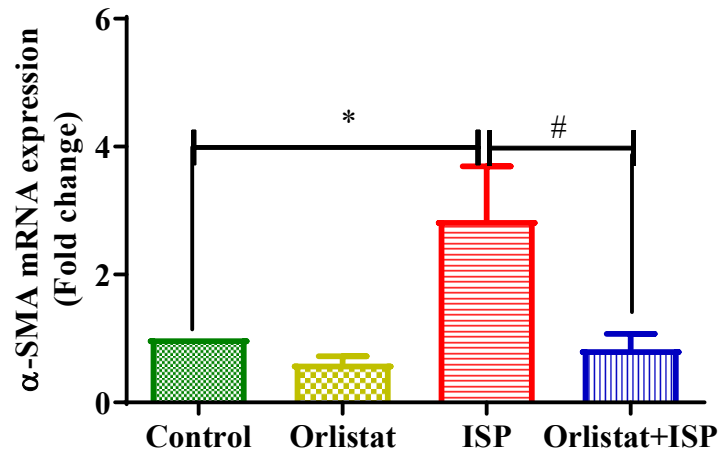


Fig. 4.35: Bar diagram showing the comparative fold change in α -SMA mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

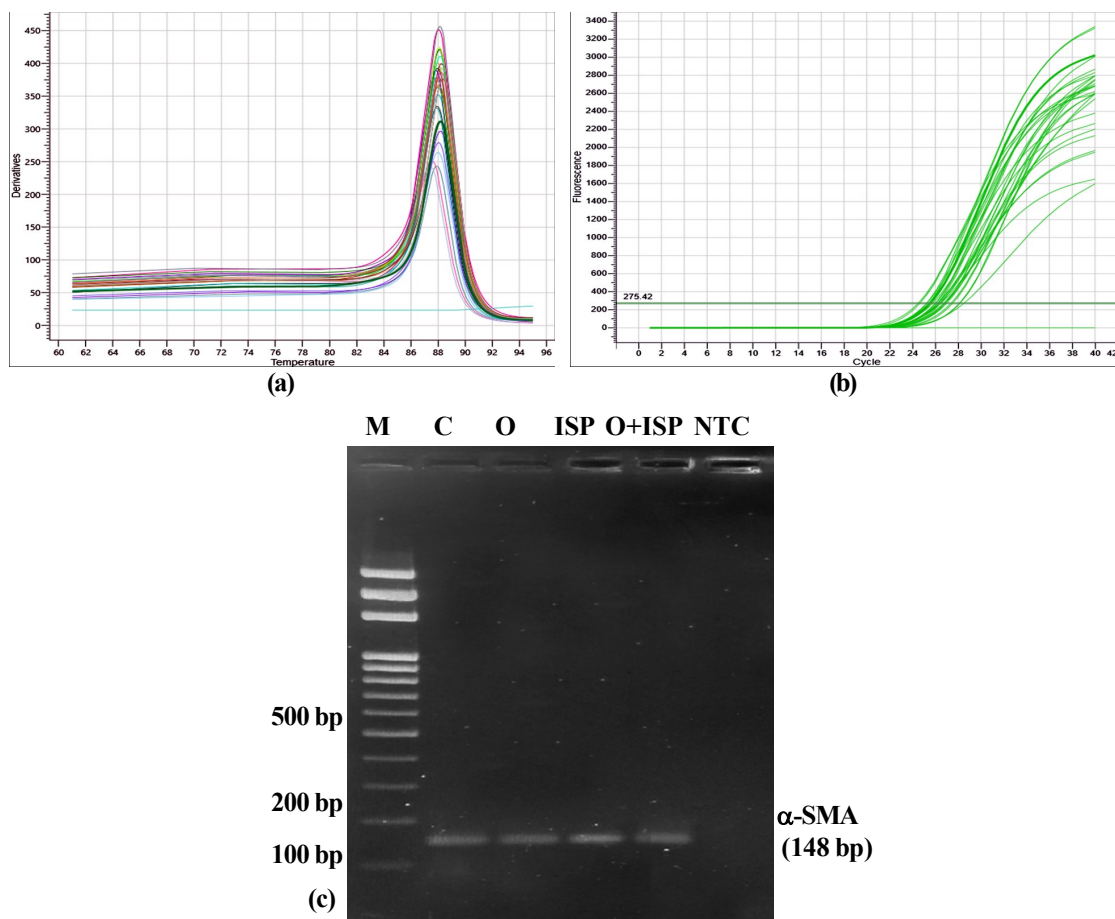


Fig. 4.36: qRT-PCR melt curve plot (a), amplification plot (b) and representative image of agarose gel electrophoresis (c) of α -SMA mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. M-marker; C-control group; O-orlistat-alone group; ISP-isoprenaline-alone group; O+ISP- Orlistat+ISP group.

alone administered group (4.47 ± 1.49 fold change; $n=7$) in comparison to control group (1.00 ± 0.00 fold change; $n=6$). Orlistat-treatment significantly ($p < 0.05$) reduced the collagen type I mRNA expression in cardiac injury group (1.23 ± 0.31 fold change; $n=7$) compared to isoprenaline-alone administered group (4.47 ± 1.49 fold change; $n=7$). The collagen type I mRNA expression in orlistat-alone (1.51 ± 0.48 fold change; $n=7$) and control (1.00 ± 0.00 fold change; $n=7$) groups of mice were almost comparable.

4.34. Effect of orlistat on TNF- α mRNA expression in heart in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.34 and illustrated in Figure 4.39 depict fold change in comparative mRNA expression of TNF- α in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis groups of mice. Figure 4.40 a & b qualitative real time PCR dissociation curve and amplification plot of TNF- α in heart tissue of different groups of mice. The mRNA expression of TNF- α was significantly ($p < 0.05$) increased in isoprenaline-alone administered group (14.36 ± 4.88 fold change; $n=4$) in comparison to control group (1.00 ± 0.00 fold change; $n=4$). The TNF- α mRNA expression was significantly ($p < 0.05$) decreased in orlistat-treated cardiac injury group (2.44 ± 2.09 fold change; $n=4$) compared to isoprenaline-alone group (14.36 ± 4.88 fold change; $n=4$). Almost comparable IL-6 mRNA expression was observed in orlistat-alone (2.19 ± 1.79 fold change; $n=4$) and control (1.00 ± 0.00 fold change; $n=4$) groups of mice.

Table 4.34: Comparative fold change in TNF- α mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	TNF- α mRNA expression (fold change)
Control (n=4)	1.00 ± 0.00
Orlistat-alone (n=4)	2.19 ± 1.79
Isoprenaline-alone (n=4)	$14.36 \pm 4.88^*$
Orlistat+ISP (n=4)	$2.44 \pm 2.09^\#$

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control group; $^\#p < 0.05$ vs ISP-alone administered group.

4.35. Effect of orlistat on IL-6 mRNA expression in heart of mice

Table 4.35 and Figure 4.41 depict fold change in comparative mRNA expression of IL-6 in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis groups of mice. Figure 4.42 a & b illustrate real time PCR dissociation curve and amplification plot of IL-6 in heart tissue of different groups of mice. The mRNA expression of IL-6 did not significantly differ in isoprenaline-alone administered group (1.96 ± 0.49 fold change; $n=4$) in comparison to control group (1.00 ± 0.00 fold change; $n=4$). No significant difference was observed in the IL-6 mRNA expression in orlistat-treated cardiac injury group (0.92 ± 0.24 fold change; $n=4$) compared to isoprenaline-alone group (1.96 ± 0.49 fold change; $n=4$). Almost comparable TNF- α mRNA expression was observed in orlistat-alone (0.92 ± 0.14 fold change; $n=4$) and control (1.00 ± 0.00 fold change; $n=4$) groups of mice.

Table 4.35: Comparative fold change in IL-6 mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	IL-6 mRNA expression (fold change)
Control (n=4)	1.00 ± 0.00
Orlistat-alone (n=4)	0.92 ± 0.14
Isoprenaline-alone (n=4)	1.96 ± 0.49
Orlistat+ISP (n=4)	0.92 ± 0.24

Data are presented as mean $\pm$ S.E.

4.36. Effect of orlistat on collagen type III mRNA expression in heart in ISP-induced cardiac fibrosis in mice

The comparative fold change of mRNA expression of collagen type III in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis groups of mice are summarized in Table 4.36 and illustrated in Figure 4.43. Figure 4.44 a & b show real time PCR dissociation curve and amplification plot of collagen type III in heart tissue of different groups of mice. No significant difference was observed in the mRNA expression of collagen type III in isoprenaline-alone administered (12.28 ± 5.75 fold change; $n=4$) and control groups (1.00 ± 0.00 fold change;

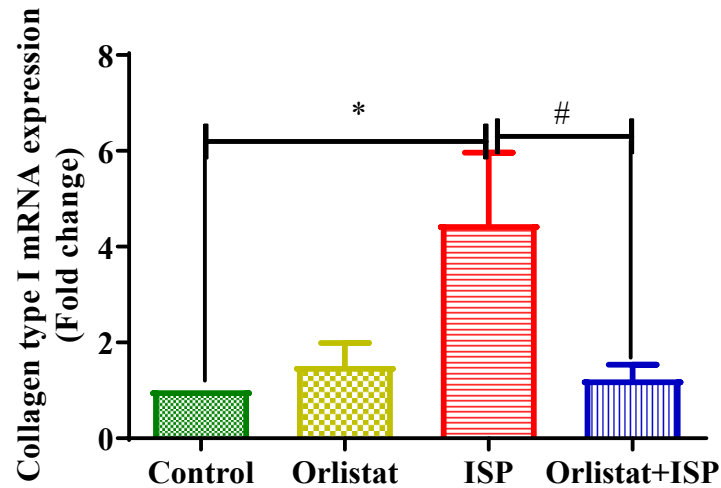


Fig. 4.37: Bar diagram showing the comparative fold change in collagen type I mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

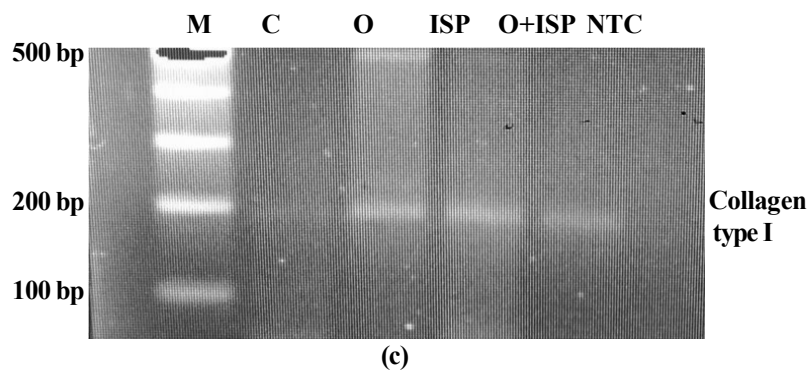
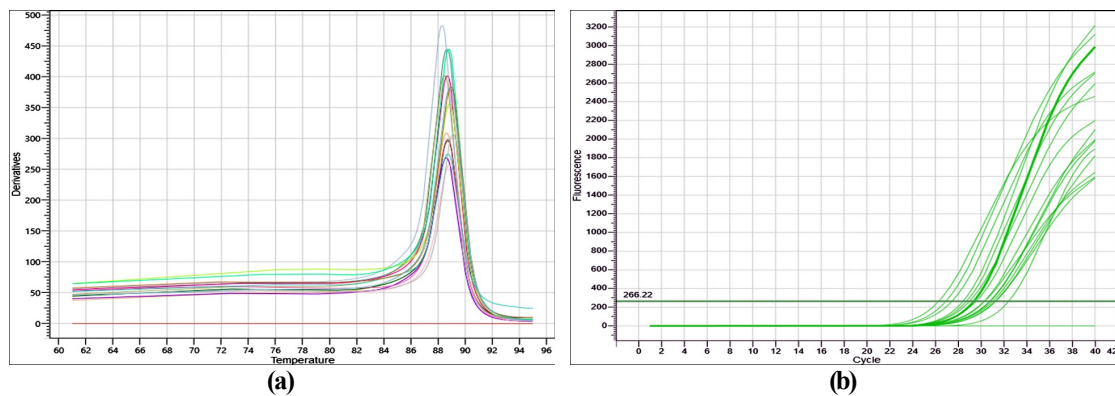


Fig. 4.38: qRT-PCR melt curve plot (a), amplification plot (b) and representative image of agarose gel electrophoresis (c) of collagen type I mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. M-marker; C-control group; O-orlistat-alone group; ISP-isoprenaline-alone group; O+ISP- Orlistat+ISP group.

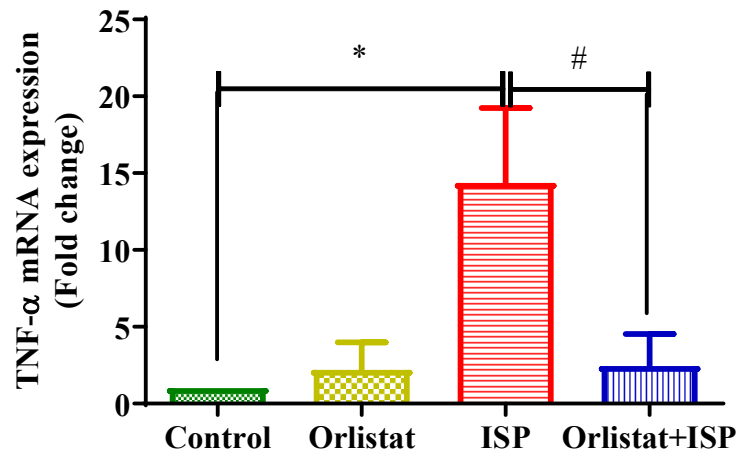


Fig. 4.39: Bar diagram showing the comparative fold change in TNF- α mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

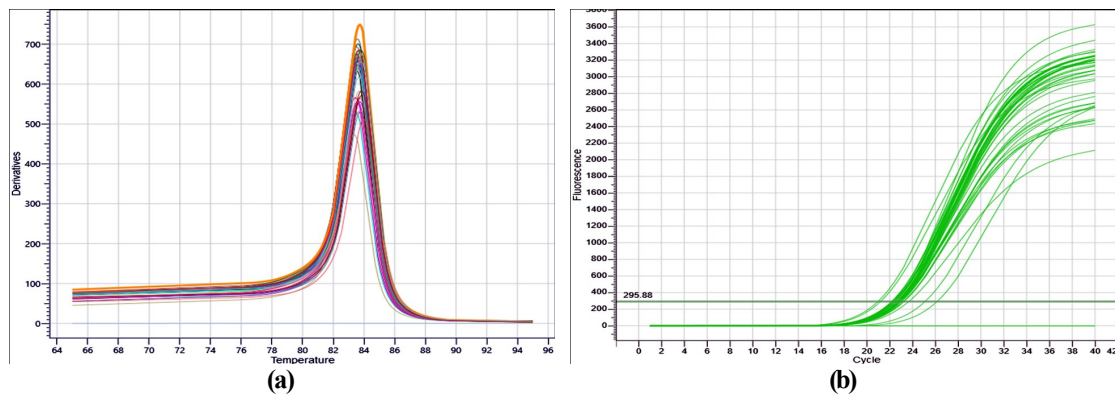


Fig. 4.40: qRT-PCR melt curve plot (a) and amplification plot (b) of TNF- α mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.

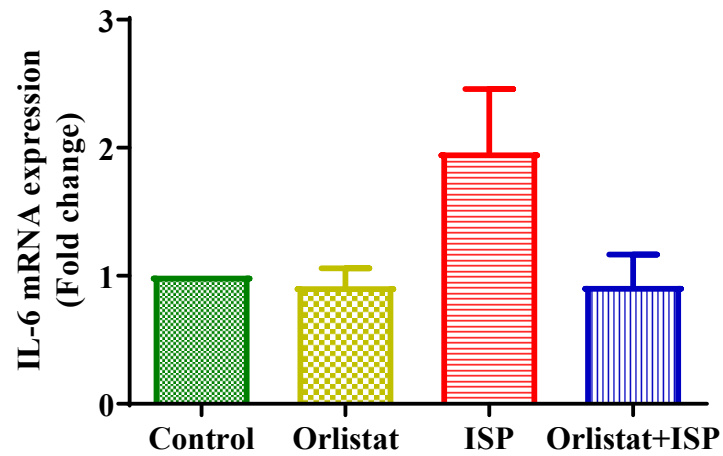


Fig. 4.41: Bar diagram showing the comparative fold change in IL-6 mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test.

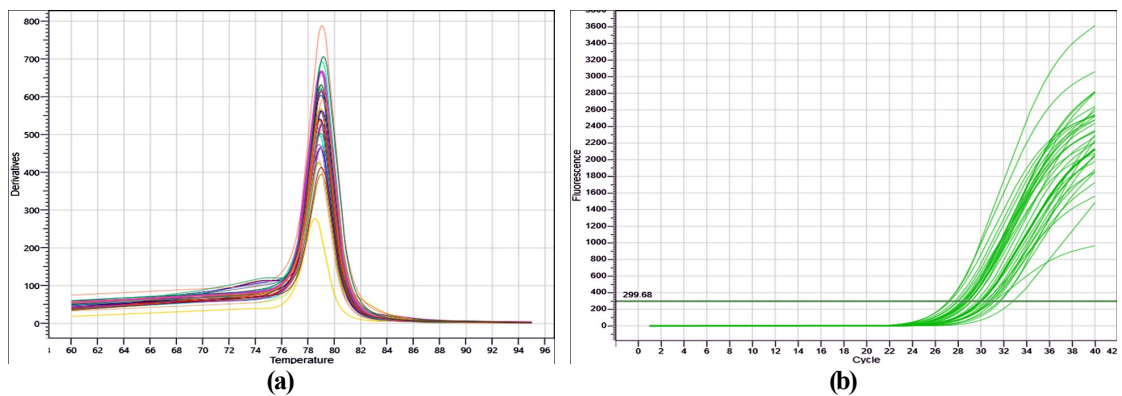


Fig. 4.42: qRT-PCR melt curve plot (a) and amplification plot (b) of IL-6 mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.

n=4). Orlistat treatment did not significantly improve the collagen type III mRNA expression (9.07 ± 3.35 fold change; n=4) compared to isoprenaline-alone group (12.28 ± 5.75 fold change; n=4). Almost comparable collagen type III mRNA expression was observed in orlistat-alone (2.14 ± 1.15 fold change; n=4) and control (1.00 ± 0.00 fold change; n=4) groups of mice.

Table 4.36: Comparative fold change in collagen type III mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Collagen type III mRNA expression (fold change)
Control (n=4)	1.00 ± 0.00
Orlistat-alone (n=4)	2.14 ± 1.15
Isoprenaline-alone (n=4)	12.28 ± 5.75
Orlistat+ISP (n=4)	9.07 ± 3.35

Data are presented as mean $\pm$ S.E.

4.37. Effect of orlistat on CTGF mRNA expression in heart in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.37 and illustrated in Figure 4.45 depict fold change in comparative mRNA expression of CTGF in control, orlistat-alone, ISP-alone and orlistat-treated cardiac fibrosis groups of mice. Figure 4.46 a & b illustrate real time PCR dissociation curve and amplification plot of CTGF in heart tissue of different groups of mice. The mRNA expression of CTGF did not significantly differ in isoprenaline-alone administered group (1.69 ± 0.24 fold change; n=5) in comparison to control group (1.00 ± 0.00 fold change; n=5). No significant difference was observed in the CTGF mRNA expression in orlistat-treated cardiac injury group (0.98 ± 0.36 fold change; n=4) compared to isoprenaline-alone group (1.69 ± 0.24 fold change; n=5). Almost comparable CTGF mRNA expression was observed in orlistat-alone (1.69 ± 0.24 fold change; n=4) and control (1.00 ± 0.00 fold change; n=5) groups of mice.

Table 4.37: Comparative fold change in CTGF mRNA expression in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	CTGF mRNA expression (fold change)
Control (n=5)	1.00±0.00
Orlistat-alone (n=4)	1.11±0.11
Isoprenaline-alone (n=5)	1.69±0.24
Orlistat+ISP (n=4)	0.98±0.36

Data are presented as mean±S.E.

4.38 Histopathological examination of heart tissue in orlistat study

Hematoxylin and eosin (H & E) staining and Masson's Trichrome (MTS) staining of heart were done to identify the alterations and collagen deposition in heart of different group. Table 4.38 and Figure 4.47 depict the total scoring of inflammation and fibrosis of histopathological examination of heart of control, orlistat-alone, ISP-alone administered and orlistat-treated cardiac injury groups. H & E staining showed the normal architecture of cardiomyocytes, normal striation of muscles and fibroblast cells in control and orlistat-alone administrated groups. Extensive infiltration of mononuclear cells along with the proliferation of fibroblast cells was observed in cardiac injury group, which was improved in orlistat-treated cardiac injury groups. MTS staining showed the normal striation of cardiac muscles with minimal collagen content (blue colored) in control and orlistat groups. ISP-induced cardiac injury group showed proliferation of fibrous tissue along with extensive collagen deposition in heart, which was improved in orlistat-treated cardiac injury group. A significantly ($p<0.05$) higher total scoring of inflammatory infiltration and fibrosis was observed in cardiac injury group (5.50 ± 0.50 score; $n=2$) compared to control group (0.50 ± 0.50 score; $n=2$). Orlistat treatment moderately improved the total scoring of inflammation and fibrosis (3.67 ± 0.88 score; $n=3$) in cardiac injury compared to ISP-alone administered (5.50 ± 0.50 score; $n=2$) group. No significant difference was observed in total scoring of control (0.50 ± 0.50 score; $n=2$) and orlistat-alone administered (1.00 ± 0.00 score; $n=2$) groups. Figure 4.48 (a, b, c, & d) and Figure 4.49 (a, b, c, d) illustrate the microphotographs of H & E staining and MTS staining of different groups of mice.

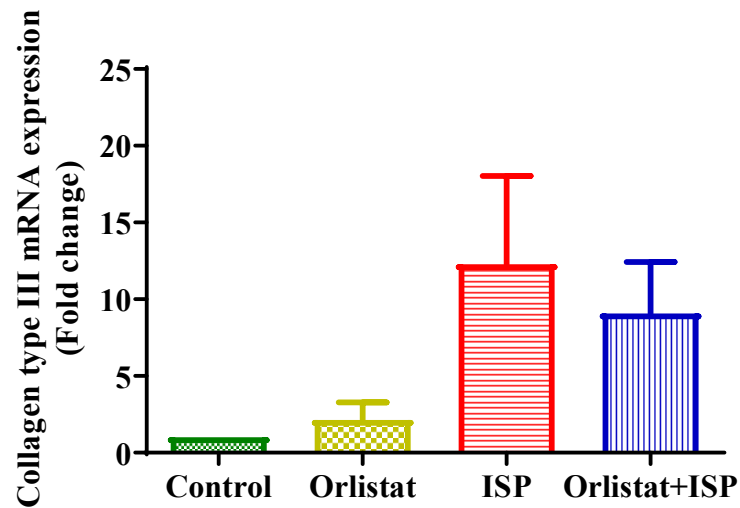


Fig. 4.43: Bar diagram showing the comparative fold change in collagen type III mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test.

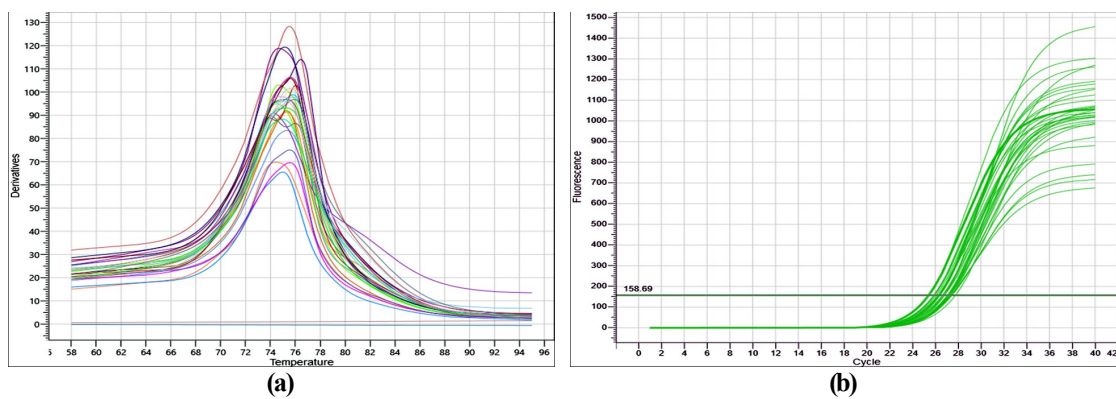


Fig. 4.44: qRT-PCR melt curve plot (a) and amplification plot (b) of collagen type III mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.

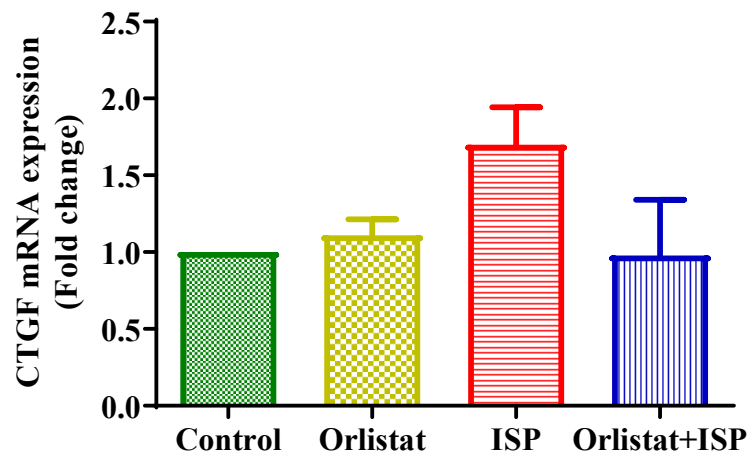


Fig. 4.45: Bar diagram showing the comparative fold change in CTGF mRNA expression in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test.

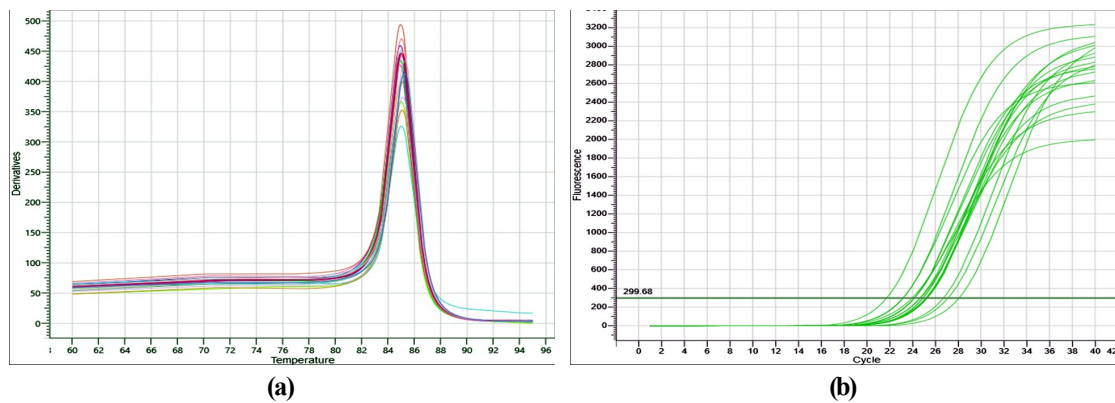


Fig. 4.46: qRT-PCR melt curve plot (a) and amplification plot (b) of CTGF mRNA expression in the control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice.

Table 4.38: Total histopathological scoring of inflammation and fibrosis in heart tissue in control, orlistat-alone, isoprenaline-alone and orlistat administered cardiac injury (orlistat+ISP) groups of mice

Groups (mice)	Histopathological Scoring
Control (n=2)	0.50±0.50
Orlistat-alone (n=2)	1.00±0.00
Isoprenaline-alone (n=2)	5.50±0.50*
Orlistat+ISP (n=3)	3.67±0.88

Data are presented as mean±S.E.

* $p < 0.05$ vs control group.

B) Niclosamide ethanolamine @ 1 mg/kg body weight study

4.39 Effect of niclosamide ethanolamine (@ 1 mg/ kg body weight; I.P.) on body weight at day zero and day 15th in different groups of mice

Body weights of mice at day zero and day 15th of the experiment in control, niclosamide ethanolamine-alone, isoprenaline-induced cardiac injury and niclosamide ethanolamine-treated cardiac injury groups are summarized in Table 4.39 and illustrated in Figure 4.50. A significant ($p < 0.05$) increase in body weight on day 15th in comparison to day zero was observed in control (24.87±2.77 g; n=6 vs 20.78±2.12 g; n=6). In isoprenaline-alone administered group

Table 4.39: Body weight (g) at day zero and day 15th in mice of control, niclosamide ethanolamine-alone, isoprenaline-induced cardiac injury, and niclosamide ethanolamine administered with isoprenaline (NEN+ISP) groups

Groups (mice)	Body weight (g) at zero day Mean±S.E.	Body weight (g) at 15 th day Mean±S.E.
Control (n=6)	20.78±2.12	24.87±2.77*
Niclosamide ethanolamine (1 mg/kg b.w.)-alone (n=4)	15.90±0.46	16.92±1.91
Isoprenaline-alone (n=6)	23.49±2.15	18.99±2.31*
Niclosamide ethanolamine (1 mg/kg b.w.)+ISP (n=4)	17.53±0.84	16.20±0.68

Data are presented as mean±S.E.

* $p < 0.05$ vs day zero.

the body weight on day 15th was significantly ($p<0.05$) decreased compared to day zero (18.99 ± 2.31 g; $n=6$ vs 23.49 ± 2.15 g; $n=6$). No significant difference in body weight on day 15th compared to day zero were observed in niclosamide ethanolamine-alone (16.92 ± 1.91 g; $n=4$ vs 15.90 ± 0.46 g; $n=4$) and niclosamide ethanolamine-treated cardiac injury (16.20 ± 0.68 g; $n=4$ vs 17.53 ± 0.84 g; $n=4$) groups.

4.40 Effect of niclosamide ethanolamine (@ 1 mg/ kg body weight; I.P.) on absolute heart weight in ISP-induced cardiac injury in mice

Table 4.40 and Figure 4.51 depict the absolute heart weight in different groups viz., control, niclosamide ethanolamine-alone, ISP-induced cardiac injury, and niclosamide ethanolamine-treated cardiac injury groups of mice. Data revealed that a significant ($p<0.05$) increase in absolute heart weight was observed in cardiac injury group (185.0 ± 15.86 mg; $n=6$) than the control group (125.0 ± 3.42 mg; $n=6$). Niclosamide ethanolamine treatment significantly ($p<0.01$) reduced the absolute heart weight (102.8 ± 8.96 mg; $n=4$) compared to the isoprenaline-alone administered group (185.0 ± 15.86 mg; $n=6$). No significant difference was noted in absolute heart weight in niclosamide ethanolamine-alone (107.0 ± 18.70 mg; $n=4$) and control (125.0 ± 3.42 mg; $n=6$) groups of mice.

Table 4.40: Absolute heart weight of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Absolute heart weight (mg)
Control (n=6)	125.0 ± 3.42
Niclosamide ethanolamine-alone (n=4)	107.0 ± 18.70
Isoprenaline-alone (n=6)	$185.0\pm15.86^*$
Niclosamide ethanolamine+ISP (n=4)	$102.8\pm8.96^{##}$

Data are presented as mean $\pm$ S.E.

* $p<0.05$ vs control; $^{##}p<0.01$ vs isoprenaline-alone group.

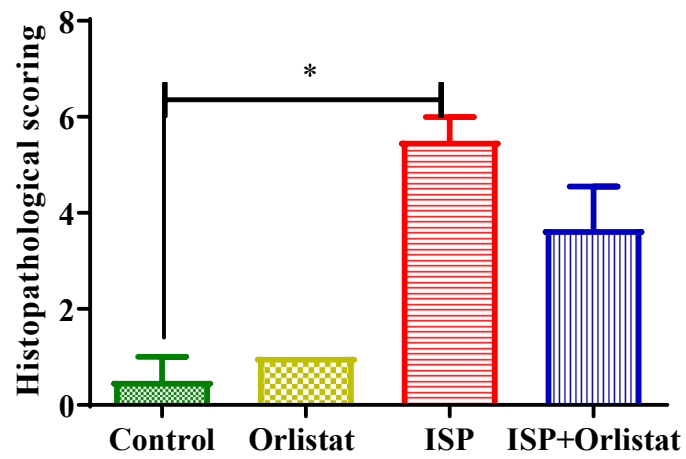


Fig. 4.47: Bar diagram showing the total histopathological scoring of inflammation and fibrosis in heart in control, orlistat-alone, ISP-alone and orlistat+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significantly ($p < 0.05$) difference between control and isoprenaline-alone groups.

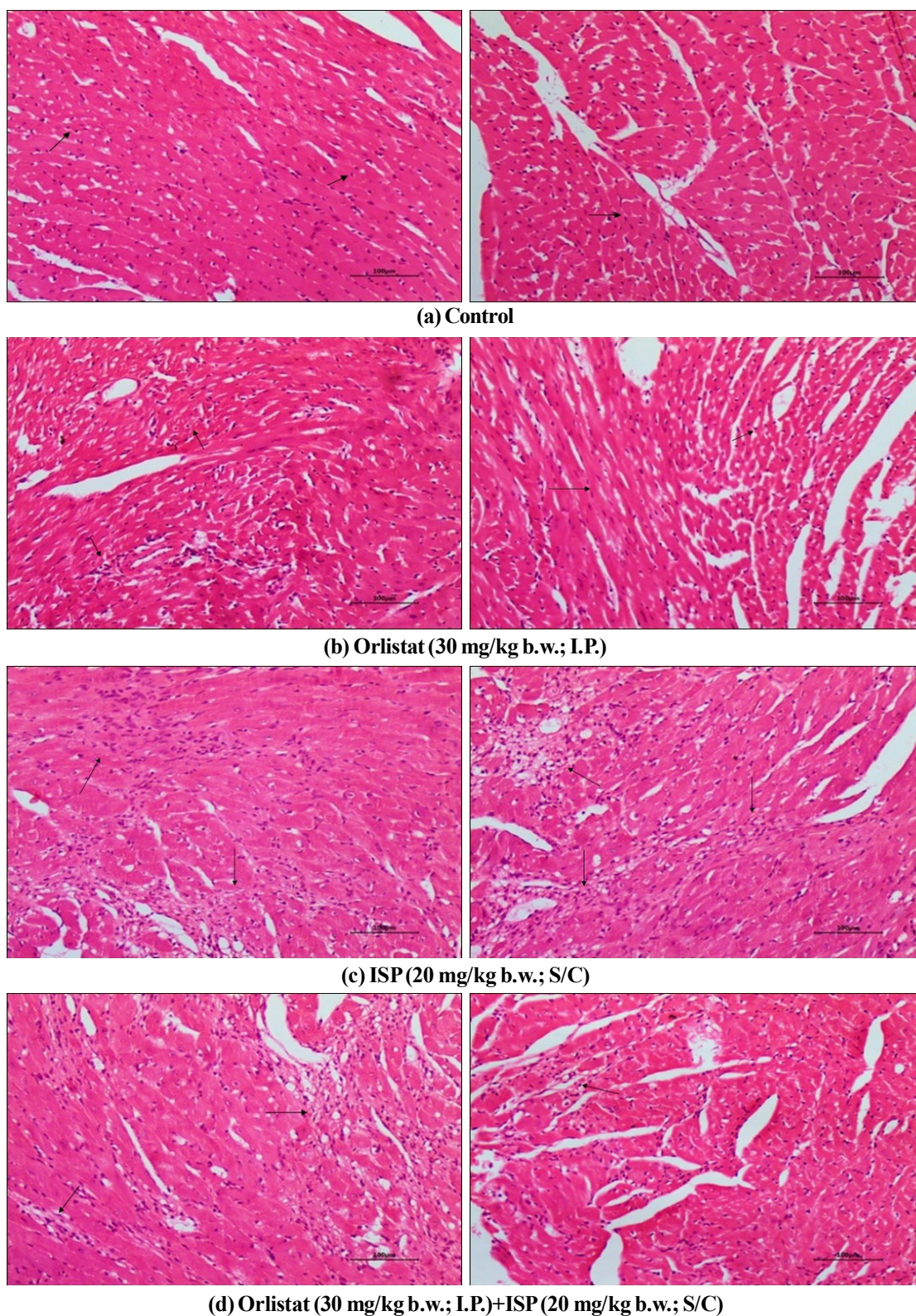


Fig. 4.48: Effect of orlistat on histopathology (H & E staining) of heart tissue in ISP-induced cardiac fibrosis in mice. a) Control mice showing the normal histoarchitecture of cardiomyocytes (arrow) (H & E, 100X); b) Heart tissue of orlistat group showing normal cardiomyocytes with minimal inflammatory cell infiltration (arrow) (H & E, 100X); c) Heart tissue of ISP-induced cardiac injury showing increased proliferation of fibroblasts in multifocal areas along with extensive infiltration of mononuclear cells (arrow) (H & E, 100X); d) orlistat-treated mice heart showing moderate proliferation of fibroblast and inflammatory cell infiltration (arrow) (H & E, 100X).

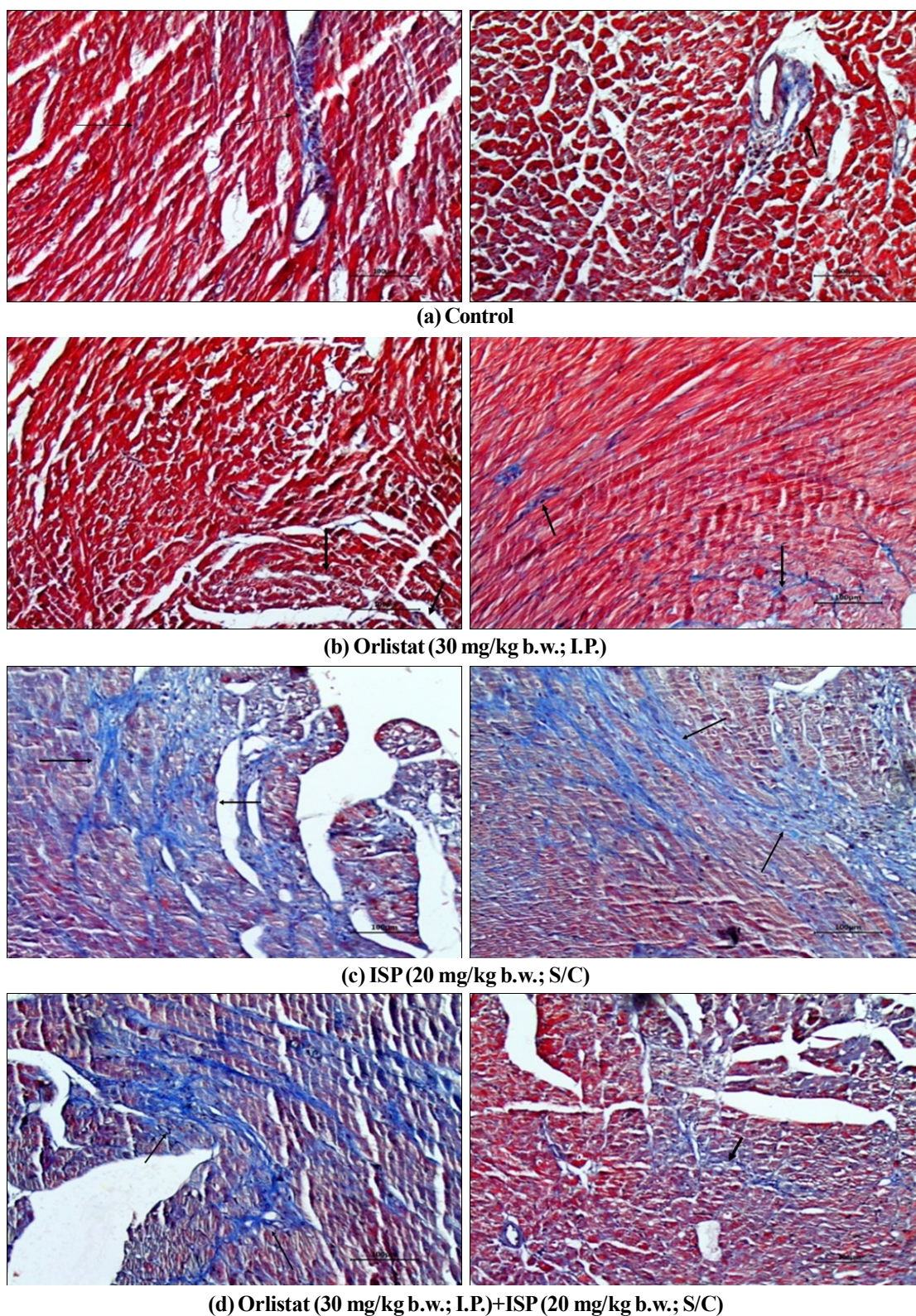


Fig. 4.49: Effect of orlistat on histopathology (Masson's Trichrome staining) of heart tissue in ISP-induced cardiac fibrosis in mice. a) Control mice showing mild proliferation of fibrotic cells and collagen deposition (arrow) (MTS; 100X); b) Heart tissue of orlistat-alone group showing mild proliferation of fibrotic cells with minimal collagen deposition (arrow) (MTS, 100X); c) Heart tissue of ISP-induced cardia injury showing increased proliferated of fibroblasts in multifocal areas along with extensive collagen deposition (arrow) (MTS; 100X); d) Orlistat-treated mice heart showing moderate proliferation of fibroblast and moderate collagen deposition (arrow) (MTS, 100X).

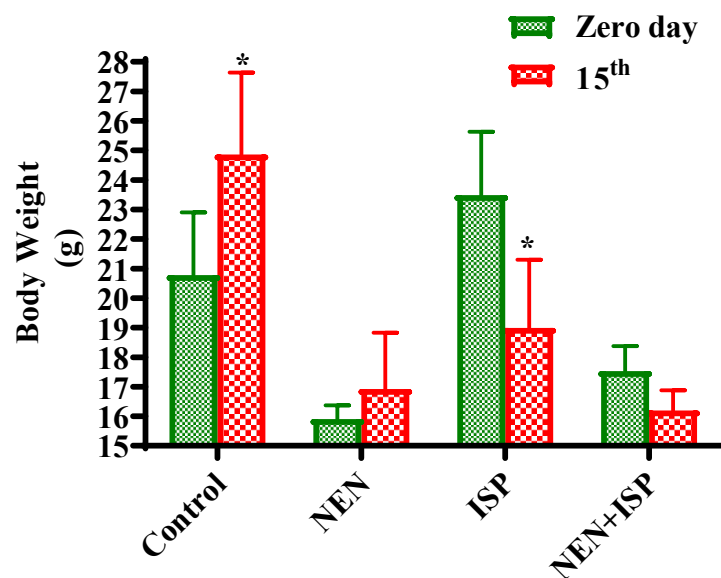


Fig. 4.50: Bar diagram showing the change in body weight on day zero and day 15th in mice of different groups. Data are analyzed by paired *t*-test. Different superscript (*) on the bars present significantly ($p < 0.05$) difference between day zero and day 15th within the groups.

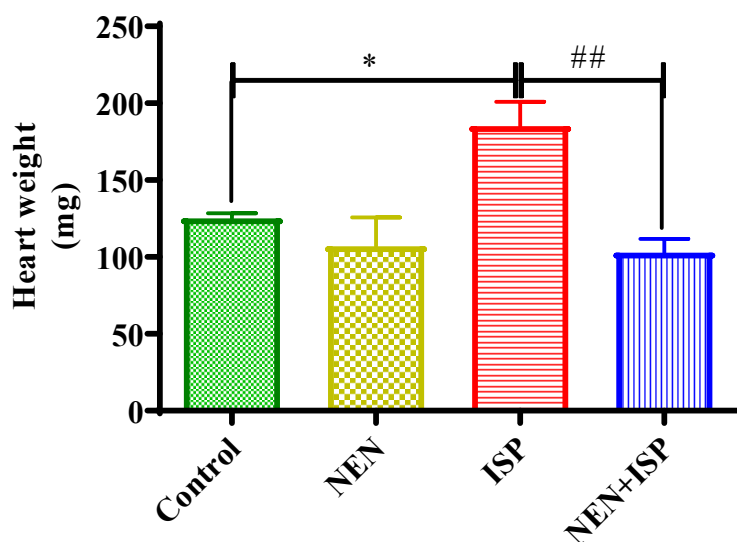


Fig. 4.51: Bar diagram showing absolute heart weight of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, ##) on the bars present significant ($p < 0.05-0.01$) difference between groups.

4.41 Effect of niclosamide ethanolamine (@ 1 mg/ kg body weight; I.P.) on heart weight to body weight (HWT/BWT) ratio in ISP-induced cardiac injury in mice

The HWT/BWT ratio of control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.41 and illustrated in Figure 4.52. Isoprenaline administration significantly ($p < 0.05$) increased the HWT/BWT ratio (9.29 ± 1.54 mg/g; $n=6$) compared to control group (5.49 ± 0.54 mg/g; $n=6$). Niclosamide ethanolamine (@ 1 mg/kg body weight) did not improve the HWT/BWT ratio (6.34 ± 0.43 mg/g; $n=4$) in cardiac injury in comparison to ISP-alone administered group (9.29 ± 1.54 mg/g; $n=6$). HWT/BWT ratio was almost comparable between niclosamide ethanolamine-alone (6.21 ± 0.39 mg/g; $n=4$) and control (5.49 ± 0.54 mg/g; $n=6$) groups of mice.

Table 4.41: Heart weight to body weight ratio (HWT/BWT) of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	HWT/BWT ratio (mg/g)
Control (n=6)	5.49 ± 0.54
Niclosamide ethanolamine-alone (n=4)	6.21 ± 0.39
Isoprenaline-alone (n=6)	$9.29 \pm 1.54^*$
Niclosamide ethanolamine+ISP (n=4)	6.34 ± 0.43

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control group.

4.42 Effect of niclosamide ethanolamine (@ 1 mg/ kg body weight; I.P.) on heart weight to tibial length (HWT/TL) ratio in ISP-induced cardiac injury in mice

The HWT/TL ratio in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.42 and illustrated in Figure 4.53. A significant ($p < 0.01$) increase in HWT/TL was observed in isoprenaline-alone administered group (9.89 ± 0.98 mg/mm; $n=6$) compared to control group

(6.08 ± 0.29 mg/mm; $n=6$). Niclosamide ethanolamine-treated cardiac injury group showed a significant ($p < 0.01$) reduction in HWT/TL (5.80 ± 0.51 mg/mm; $n=4$) than ISP-alone administered group (9.89 ± 0.98 mg/mm; $n=6$). No significant difference was noted in HWT/TL ratio between niclosamide ethanolamine-alone (5.96 ± 0.80 mg/mm; $n=4$) and control (6.08 ± 0.29 mg/mm; $n=6$) groups of mice.

Table 4.42: Heart weight to tibial length ratio (HWT/TL) of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	HWT/TL ratio (mg/mm)
Control ($n=6$)	6.08 ± 0.29
Niclosamide ethanolamine-alone ($n=4$)	5.96 ± 0.80
Isoprenaline-alone ($n=6$)	$9.89 \pm 0.98^{**}$
Niclosamide ethanolamine+ISP ($n=4$)	$5.80 \pm 0.51^{##}$

Data are presented as mean $\pm$ S.E.

$^{**}p < 0.01$ vs control; $^{##}p < 0.01$ vs isoprenaline-alone administered group.

4.43 Effect of niclosamide ethanolamine (@ 1 mg/ kg body weight; I.P.) on serum CK-MB in isoprenaline-induced cardiac injury in mice

Data summarized in Table 4.43 and illustrated in Figure 4.54 depict CK-MB levels in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significant ($p < 0.05$) increase in CK-MB level was observed in ISP-alone group (135.5 ± 11.88 U/L; $n=6$) than the control group (54.44 ± 4.69 U/L; $n=6$). Niclosamide ethanolamine (@ 1 mg/kg body weight) treatment did not improve serum CK-MB level in cardiac injury group (178.3 ± 38.43 U/L; $n=4$) compared to isoprenaline-alone administered group (135.5 ± 11.88 U/L; $n=6$). Almost comparable CK-MB level was observed in niclosamide ethanolamine-alone (68.33 ± 8.77 U/L; $n=4$) and control (54.44 ± 4.69 U/L; $n=6$) groups of mice.

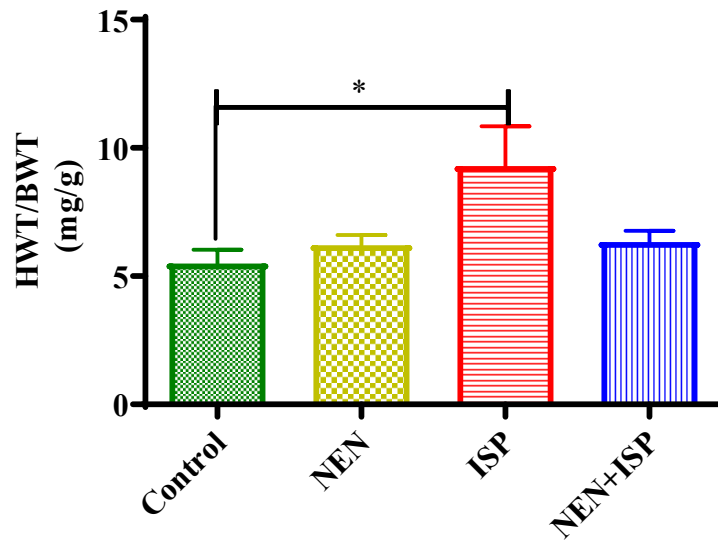


Fig. 4.52: Bar diagram showing the heart weight to body weight ratio (HWT/BWT) of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between groups.

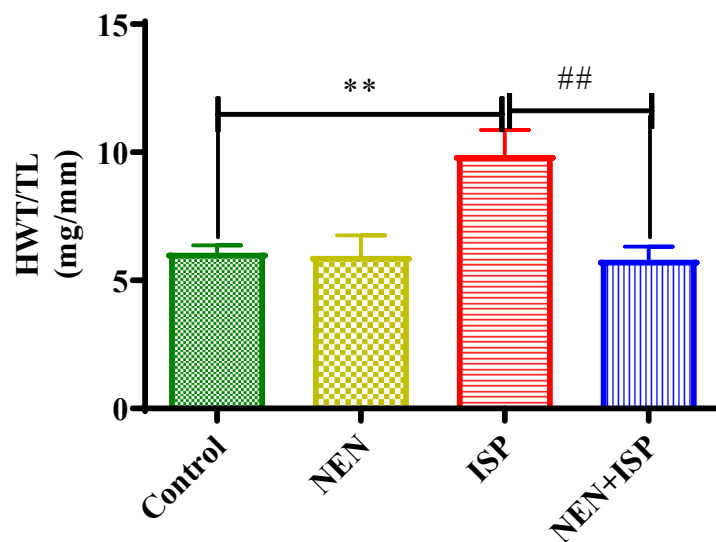


Fig. 4.53: Bar diagram showing heart weight to tibial length (HWT/TL) ratio of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (**, ##) on the bars present significant ($p<0.01$) difference between groups.

Table 4.43: Serum CK-MB level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	CK-MB (U/L)
Control (n=6)	54.44±4.69
Niclosamide ethanolamine-alone (n=4)	68.33±8.77
Isoprenaline-alone (n=6)	135.5±11.88*
Niclosamide ethanolamine+ISP (n=4)	178.3±38.43

Data are presented as mean±S.E.

* $p < 0.05$ vs control.

4.44 Effect of niclosamide ethanolamine (@ 1 mg/ kg body weight; I.P.) on serum LDH level in isoprenaline-induced cardiac injury in mice

The serum LDH level of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.44 and illustrated in Figure 4.55. A significantly ($p < 0.01$) increased serum LDH was observed in isoprenaline-alone administered cardiac injury group (1332 ± 67.40 U/L; n=6) compared to control group (1012 ± 18.58 U/L; n=6). However, niclosamide ethanolamine treatment did not improve serum LDH level (1249 ± 91.31 U/L; n=4) in comparison to ISP-alone administered group (1332 ± 67.40 U/L; n=6). LDH level of niclosamide ethanolamine-alone (1022 ± 33.27

Table 4.44: Serum LDH level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	LDH (U/L)
Control group (n=6)	1012±18.58
Niclosamide ethanolamine-alone (n=4)	1022±33.27
Isoprenaline-alone (n=6)	1332±67.40**
Niclosamide ethanolamine+ISP (n=4)	1249±91.31

Data are presented as mean±S.E.

** $p < 0.01$ vs control

U/L; n=4) and control (1012 ± 18.58 U/L; n=6) groups of mice were almost comparable to each other.

4.45 Effect of niclosamide ethanolamine (@ 1 mg/kg body weight) on the serum cholesterol level in ISP-induced cardiac injury in mice

Table 4.45 and Figure 4.56 depict the serum cholesterol level in control, niclosamide ethanolamine-alone, ISP-alone administered, and niclosamide ethanolamine-treated cardiac injury groups of mice. The cholesterol level was found significantly ($p < 0.05$) higher in ISP-alone administered group (93.03 ± 15.96 mg/dl; n=4) in comparison to control group (45.05 ± 6.45 mg/dl; n=3). No significant improvement in cholesterol level was observed in niclosamide ethanolamine-treated cardiac injury group (68.96 ± 10.08 mg/dl; n=4) compared to ISP-alone administered group (93.03 ± 15.96 mg/dl; n=4). The cholesterol level in niclosamide ethanolamine-alone (49.63 ± 2.74 mg/dl; n=4) and control (45.05 ± 6.45 mg/dl; n=3) groups of mice were almost comparable.

Table 4.45: Serum cholesterol level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Cholesterol (mg/dl)
Control group (n=3)	45.05 ± 6.45
Niclosamide ethanolamine-alone (n=4)	49.63 ± 2.74
Isoprenaline-alone (n=4)	$93.03 \pm 15.96^*$
Niclosamide ethanolamine+ISP (n=4)	68.96 ± 10.08

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control group.

4.46 Effect of niclosamide ethanolamine on serum triglyceride level in ISP-induced cardiac injury in mice

Data summarized in Table 4.46 and illustrated in Figure 4.57 show the serum triglyceride level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significantly ($p < 0.001$) increased level

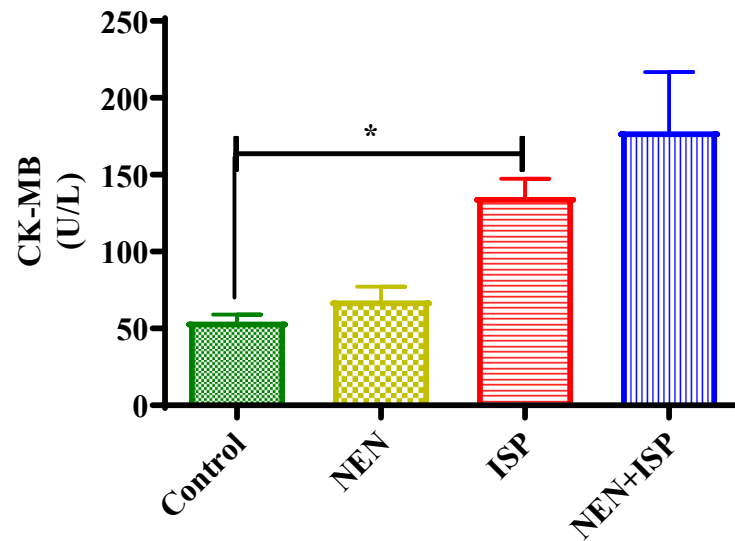


Fig. 4.54: Bar diagram showing serum CK-MB level of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between control and isoprenaline-alone administered groups.

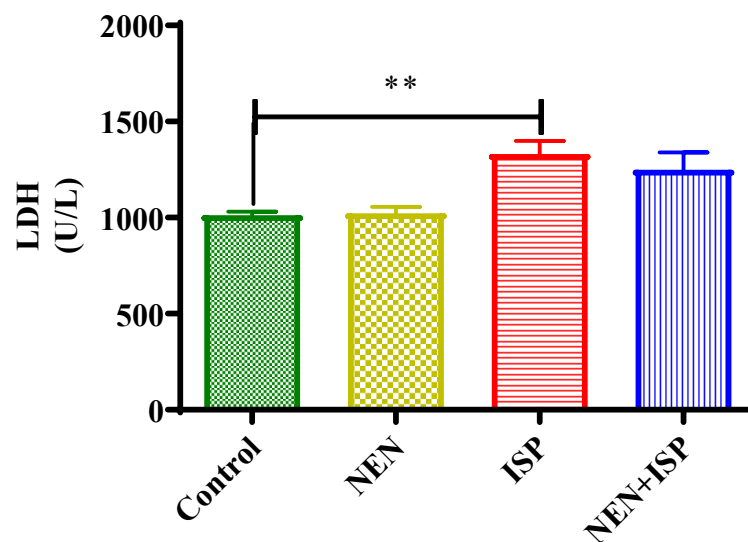


Fig. 4.55: Bar diagram showing serum LDH level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (**) on the bars present significant ($p<0.01$) difference between control and ISP-alone administered groups.

of serum triglyceride was observed in isoprenaline-alone administered group (190.2 ± 23.75 mg/dl; $n=6$) compared to control group (77.24 ± 7.69 mg/dl; $n=6$). No significant difference was observed in niclosamide ethanolamine-treated cardiac injury group (126.4 ± 15.01 mg/dl; $n=4$) in comparison to ISP-alone administered group (190.2 ± 23.75 mg/dl; $n=6$). Almost comparable triglyceride level was observed in niclosamide ethanolamine-alone (75.00 ± 11.88 mg/dl; $n=4$) and control (77.24 ± 7.69 mg/dl; $n=6$) groups of mice.

Table 4.46: Serum triglyceride level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Triglyceride (mg/dl)
Control (n=6)	77.24 ± 7.69
Niclosamide ethanolamine-alone (n=4)	75.00 ± 11.88
Isoprenaline-alone (n=6)	$190.2 \pm 23.75^{***}$
Niclosamide ethanolamine+ISP (n=4)	126.4 ± 15.01

Data are presented as mean $\pm$ S.E.

$^{***}p < 0.001$ vs control group.

4.47 Effect of niclosamide ethanolamine (@ 1 mg/kg body weight) on tissue catalase activity in ISP-induced cardiac fibrosis in mice

Data in Table 4.47 and Figure 4.58 depict catalase activity in heart tissue of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. The catalase activity was significantly ($p < 0.001$) reduced in ISP-alone administered group (0.009 ± 0.002 mmol H_2O_2 utilized/min/mg of proteins; $n=6$) in comparison to control group (0.057 ± 0.006 mmol H_2O_2 utilized/min/mg of proteins; $n=6$). Niclosamide ethanolamine treatment did not improve the tissue catalase activity (0.016 ± 0.004 mmol H_2O_2 utilized/min/mg of proteins; $n=4$) compared to the ISP-alone administered group (0.009 ± 0.002 mmol H_2O_2 utilized/min/mg of proteins; $n=6$). Catalase activities in niclosamide ethanolamine-alone (0.039 ± 0.004 mmol H_2O_2 utilized/min/mg of proteins; $n=4$) and control (0.057 ± 0.006 mmol H_2O_2 utilized/min/mg of proteins; $n=6$) groups of mice were almost comparable to each other.

Table 4.47: Catalase activity in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Catalase (mmol H ₂ O ₂ utilized/min/mg of proteins)
Control (n=6)	0.057±0.006
Niclosamide ethanolamine-alone (n=4)	0.039±0.004
Isoprenaline-alone (n=6)	0.009±0.002***
Niclosamide ethanolamine+ISP (n=4)	0.016±0.004

Data are presented as mean±S.E.

****p*<0.001 vs control.

4.48 Effect of niclosamide ethanolamine (@ 1 mg/kg body weight) on non-enzymatic antioxidant reduced glutathione (GSH) in ISP-induced cardiac injury in mice

The fold change in GSH in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.48 and illustrated in Figure 4.59. A significant (*p*<0.01) reduction was observed in GSH level in ISP-induced cardiac injury group (0.64±0.06 fold change; n=4) in comparison to the control group (1.00±0.07 fold change; n=3). No significant difference was observed in niclosamide ethanolamine-treated cardiac injury group (0.73±0.05 fold change; n=4) compared to isoprenaline-alone administered group (0.64±0.06 fold change; n=4). GSH

Table 4.48: Fold change of non-enzymatic antioxidant, GSH in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	GSH (fold change)
Control (n=3)	1.00±0.07
Niclosamide ethanolamine-alone (n=4)	0.90±0.03
Isoprenaline-alone (n=4)	0.64±0.06**
Niclosamide ethanolamine+ISP (n=4)	0.73±0.05

Data are presented as mean±S.E.

***p*<0.01 vs control group.

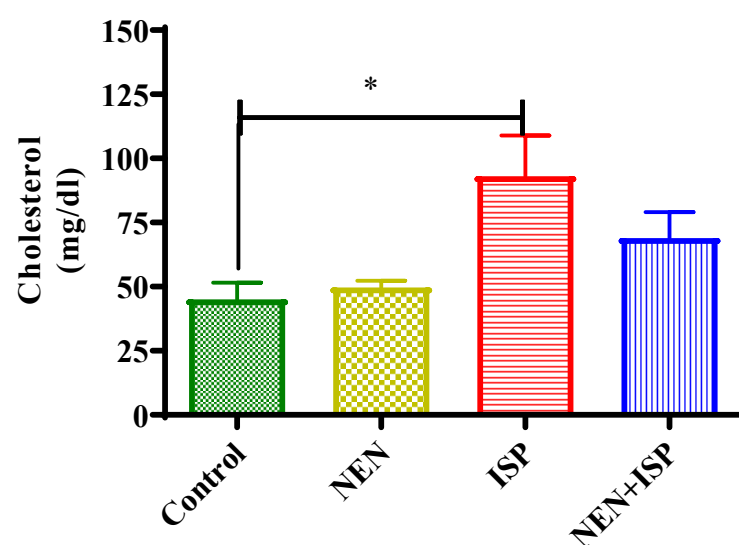


Fig. 4.56: Bar diagram showing the serum cholesterol level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between control and ISP-alone administered groups.

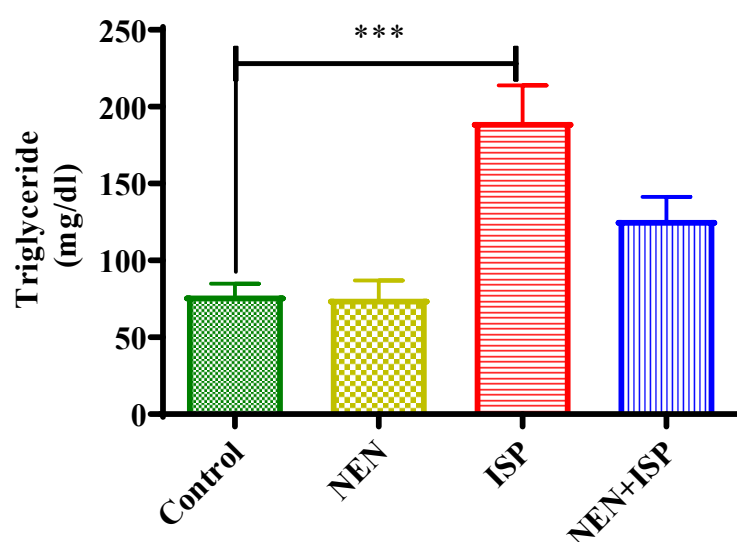


Fig. 4.57: Bar diagram showing the serum triglyceride level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (***) on the bars present significant ($p<0.001$) difference between control and ISP-alone group.

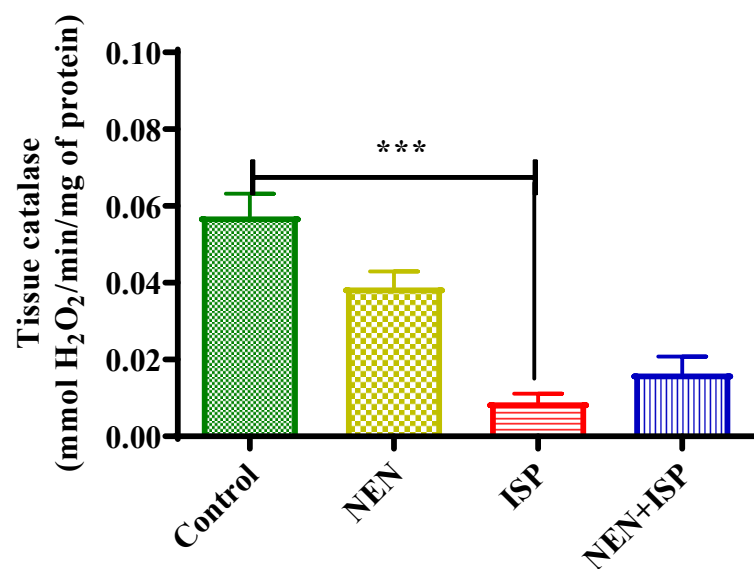


Fig. 4.58: Bar diagram showing the catalase activity in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***) on the bars present significant ($p < 0.001$) difference between groups.

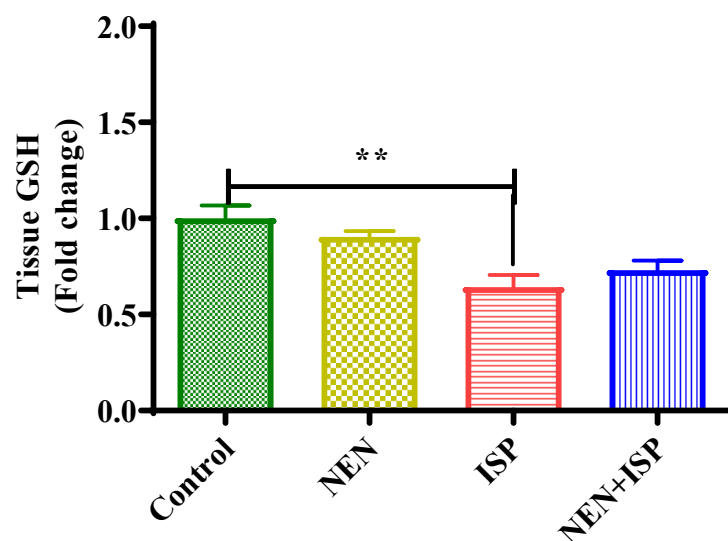


Fig. 4.59: Bar diagram showing the reduced glutathione (GSH) level in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (**) on the bars present significant ($p < 0.01$) difference between groups.

level was seen almost comparable between niclosamide ethanolamine-alone (0.90 ± 0.03 fold change; $n=4$) and control (1.00 ± 0.07 fold change; $n=4$) groups of mice.

4.49 Effect of niclosamide ethanolamine (@ 1 mg/kg body weight) on lipid peroxidation (LPO) in ISP-induced cardiac injury in mice

The MDA level in heart tissue of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.49 and illustrated in Figure 4.60. A significant ($p < 0.05$) increase in MDA level was observed in ISP-alone administered group (1.74 ± 0.18 nmol/mg of protein; $n=4$) in comparison to control group (1.09 ± 0.05 nmol/mg of protein; $n=3$). Niclosamide ethanolamine treatment did not improve the MDA level (1.87 ± 0.14 nmol/mg of protein; $n=4$) compared to the ISP-alone administered group (1.74 ± 0.18 nmol/mg of protein; $n=4$). The MDA level was found to be almost comparable in niclosamide ethanolamine-alone (1.24 ± 0.11 nmol/mg of protein; $n=4$) and control (1.09 ± 0.05 nmol/mg of protein; $n=3$) groups of mice.

Table 4.49: MDA level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	MDA (nmol/mg of protein)
Control ($n=3$)	1.09 ± 0.05
Niclosamide ethanolamine-alone ($n=4$)	1.24 ± 0.11
Isoprenaline-alone ($n=4$)	$1.74 \pm 0.18^*$
Niclosamide ethanolamine+ISP ($n=4$)	1.87 ± 0.14

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control group.

4.50 Effect of niclosamide ethanolamine (@ 1 mg/kg body weight) on tissue hydroxyproline level in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.50 and illustrated in Figure 4.61 depict the hydroxyproline level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac fibrosis group of mice. A significantly ($p < 0.05$) increased level of hydroxyproline

was observed in ISP-alone administered cardiac fibrosis group (7.97 ± 0.56 $\mu\text{g}/\text{mg}$ of tissue; $n=4$) in comparison to control group (4.89 ± 0.45 $\mu\text{g}/\text{mg}$ of tissue; $n=3$). Niclosamide ethanolamine treatment did not improve the hydroxyproline level in cardiac fibrosis (8.04 ± 1.08 $\mu\text{g}/\text{mg}$ of tissue; $n=4$) compared to the ISP-alone administered group (7.97 ± 0.56 $\mu\text{g}/\text{mg}$ of tissue; $n=4$). The hydroxyproline level was found to be almost comparable in niclosamide ethanolamine-alone (5.73 ± 0.53 $\mu\text{g}/\text{mg}$ of tissue; $n=4$) and control (4.89 ± 0.45 $\mu\text{g}/\text{mg}$ of tissue; $n=3$) groups of mice.

Table 4.50: Hydroxyproline level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Tissue hydroxyproline ($\mu\text{g}/\text{mg}$ of tissue)
Control ($n=3$)	4.89 ± 0.45
Niclosamide ethanolamine-alone ($n=4$)	5.73 ± 0.53
Isoprenaline-alone ($n=4$)	$7.97 \pm 0.56^*$
Niclosamide ethanolamine+ISP ($n=4$)	8.04 ± 1.08

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control group.

4.51 Effect of niclosamide ethanolamine (@ 1 mg/kg body weight) on tissue glucosamine level in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.51 and Figure 4.62 depict the fold change in glucosamine level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significantly ($p < 0.05$) increased level of glucosamine was observed in isoprenaline-alone administered group (3.93 ± 0.93 fold change; $n=6$) compared to control group (1.00 ± 0.35 fold change; $n=6$). No significant difference in glucosamine level was seen in niclosamide ethanolamine-treated cardiac injury group (4.19 ± 0.92 fold change; $n=4$) compared to ISP-alone administered group (3.93 ± 0.93 fold change; $n=6$). Glucosamine level was found to be almost comparable in niclosamide ethanolamine-alone (1.21 ± 0.37 fold change; $n=4$) and control (1.00 ± 0.35 fold change; $n=6$) groups of mice.

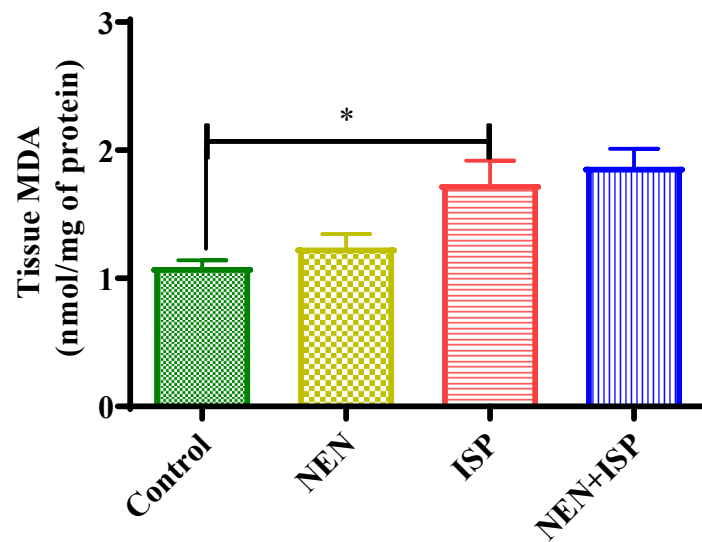


Fig. 4.60: Bar diagram showing the MDA level in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p < 0.05$) difference between control and ISP-alone administered groups.

Table 4.51: Glucosamine level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Tissue glucosamine (fold change)
Control (n=6)	1.00±0.35
Niclosamide ethanolamine-alone (n=4)	1.21±0.37
Isoprenaline-alone (n=6)	3.93±0.93*
Niclosamide ethanolamine+ISP (n=4)	4.19±0.92

Data are presented as mean±S.E.

* $p < 0.05$ vs control group.

C) Niclosamide ethanolamine (NEN @ 3 mg/kg body weight) study

4.52 Body weight at day zero and day 15th in different groups of mice

Body weights of mice at day zero and day 15th of the experiment in control, niclosamide ethanolamine-alone, isoprenaline-induced cardiac injury and niclosamide ethanolamine-treated cardiac injury groups are summarized in Table 4.52 and illustrated in Figure 4.63. A significant

Table 4.52: Body weight (g) at day zero and day 15th in mice of control, niclosamide ethanolamine (3 mg/kg body weight; I.P)-alone, isoprenaline-induced cardiac injury, and niclosamide ethanolamine (3 mg/kg body weight; I.P)-administered with isoprenaline (NEN+ISP) groups

Groups (mice)	Body weight (g) at zero day	Body weight (g) at 15 th day
	Mean±S.E.	Mean±S.E.
Control (n=10)	31.70±1.19	35.31±1.04****
Niclosamide ethanolamine (NEN; 3 mg/kg body weight)-alone (n=9)	31.82±1.29	37.90±1.42****
Isoprenaline-alone (n=12)	31.33±1.24	31.86±1.58
Niclosamide ethanolamine (NEN; 3 mg/kg body weight)+ISP (n=8)	28.06±1.17	31.18±0.74**

Data are presented as Mean±S.E.

**** $p < 0.0001$ & ** $p < 0.01$ vs day 15th

($p<0.0001$) increase in body weight on day 15th in comparison to day zero was observed in control (35.31 ± 1.04 g; $n=10$ vs 31.70 ± 1.19 g; $n=10$), and niclosamide ethanolamine-alone (37.90 ± 1.42 g; $n=9$ vs 31.82 ± 1.29 g; $n=9$) groups. In isoprenaline-alone administered group the body weight on day 15th and day zero were almost comparable (31.86 ± 1.58 g; $n=12$ vs 31.33 ± 1.24 g; $n=12$). Niclosamide ethanolamine-treated group showed significantly ($p<0.01$) higher body weight on day 15th compared to day zero (31.18 ± 0.74 g; $n=8$ vs 28.06 ± 1.17 g; $n=8$).

4.53 Percentage change in body weight from day zero to day 15th

Data summarized in Table 4.53 and illustrated in Figure 4.64 show the percentage change in body weight from day zero to day 15th in control, niclosamide ethanolamine-alone, ISP-alone administered and niclosamide ethanolamine-treated cardiac injury groups of mice. Least percentage increase in body weight was observed in ISP-alone group (1.62 ± 2.65 %; $n=12$), which was significantly ($p<0.05$) lower than percentage change in control group (11.86 ± 2.06 %; $n=10$). A significantly ($p<0.05$) higher percentage change was observed in niclosamide ethanolamine-treated cardiac injury group (11.78 ± 2.70 %; $n=8$) compared to isoprenaline-alone administered group (1.62 ± 2.65 %; $n=12$). However, percentage change in body weight between niclosamide ethanolamine-alone (19.35 ± 1.81 %; $n=9$) and control (11.86 ± 2.06 %; $n=10$) groups of mice were almost comparable.

Table 4.53: Percentage change in body weights during experimental period of the mice of control, niclosamide ethanolamine-alone, isoprenaline-induced cardiac injury, and niclosamide ethanolamine-administered with isoprenaline (NEN+ISP) groups of mice

Groups (mice)	Percentage change in body weight (%)
Control (n=10)	11.86 ± 2.06
Niclosamide ethanolamine-alone (n=9)	19.35 ± 1.81
Isoprenaline-alone (n=12)	$1.62\pm2.65^*$
Niclosamide ethanolamine (NEN)+ISP (n=8)	$11.78\pm2.70^\#$

Data are presented as mean $\pm$ SE.

* $p<0.05$ in comparison to control; $^\#p<0.05$ vs isoprenaline-alone administered group

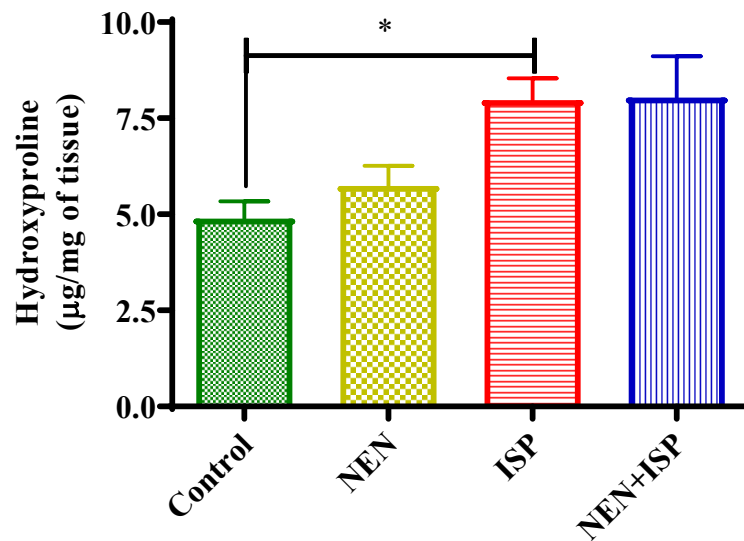


Fig.4.61: Bar diagram showing the hydroxyproline level in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between control and ISP-alone administered groups.

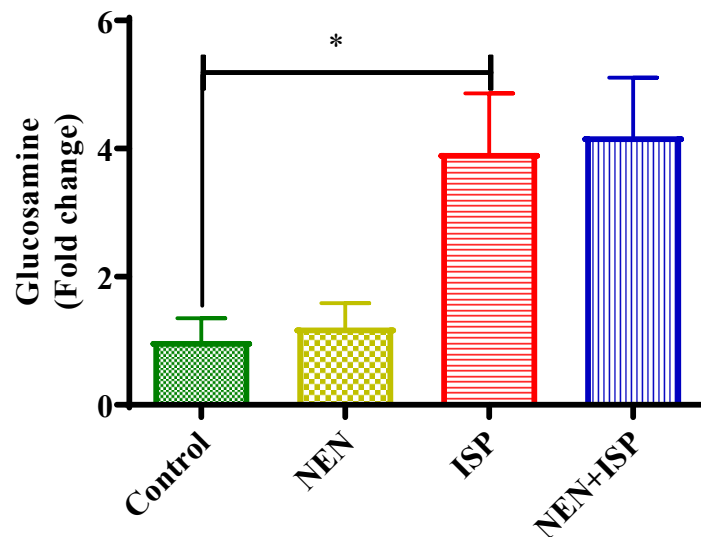


Fig. 4.62: Bar diagram showing the fold change in glucosamine level in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between control and ISP-alone administered groups.

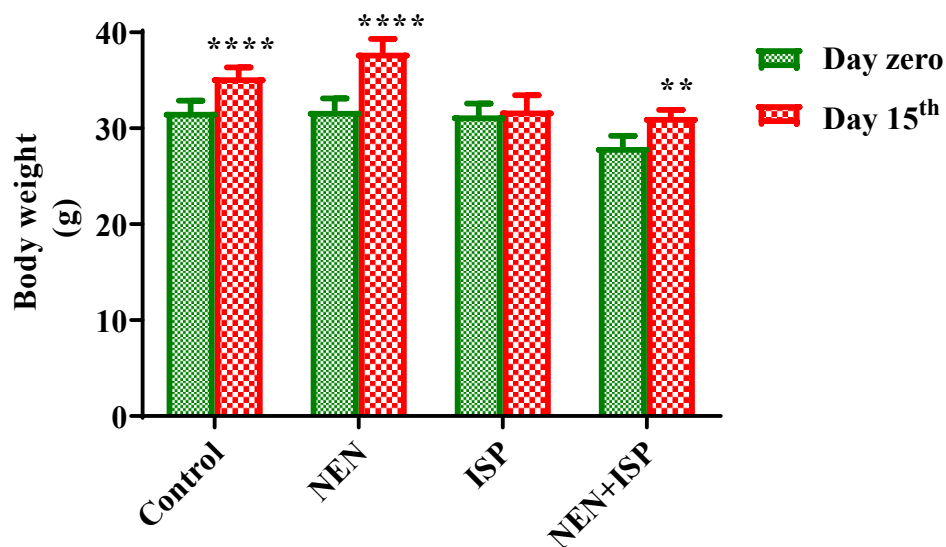


Fig. 4.63: Bar diagram showing the change in body weight on day zero and day 15th in mice of different groups. Data are analyzed by paired *t*-test. Different superscripts (****, **) on the bars present significant ($p < 0.0001$ -0.01) difference between day zero and day 15th.

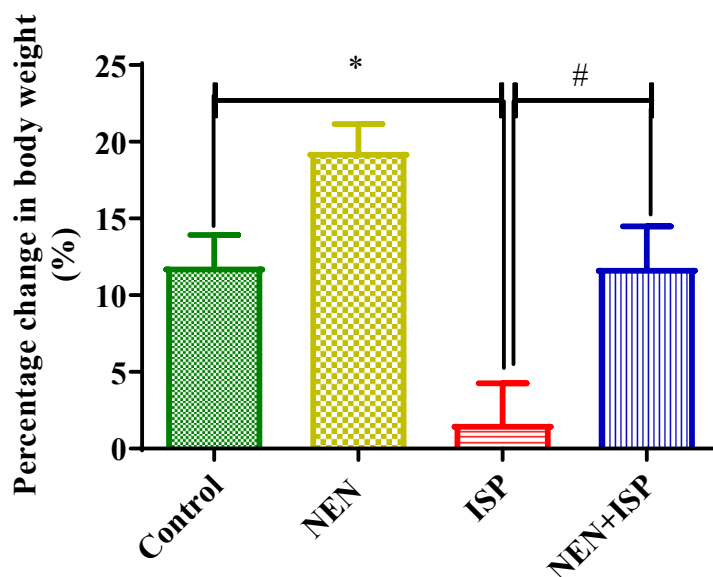


Fig. 4.64: Bar diagram showing the percentage change in body weight in different groups of mice during the experimental period. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

4.54 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) administration on heart weight in ISP-induced cardiac injury in mice

Table 4.54 and Figure 4.65 depict the absolute heart weight in different groups viz., control, niclosamide ethanolamine-alone, ISP-induced cardiac injury, and niclosamide ethanolamine administered with ISP (NEN+ISP) groups of mice. Data revealed that a significant ($p<0.001$) increase in absolute heart weight was observed in cardiac injury group (164.6 ± 4.35 mg; $n=12$) than the control group (135.1 ± 4.54 mg; $n=10$). Niclosamide ethanolamine treatment significantly ($p<0.01$) reduced the absolute heart weight (139.7 ± 3.01 mg; $n=8$) compared to the isoprenaline-alone administered group (164.6 ± 4.35 mg; $n=12$). No significant difference was noted in absolute heart weight in niclosamide ethanolamine-alone (146.2 ± 3.98 mg; $n=9$) and control (135.1 ± 4.54 mg; $n=10$) groups of mice.

Table 4.54: Absolute heart weight of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Absolute heart weight (mg)
Control (n=10)	135.1 $\pm$ 4.54
Niclosamide ethanolamine-alone (n=9)	146.2 $\pm$ 3.98
Isoprenaline-alone (n=12)	164.6 $\pm$ 4.35***
Niclosamide ethanolamine +ISP (n=8)	139.7 $\pm$ 3.01##

Data are presented as mean $\pm$ S.E.

*** $p<0.001$ vs control; ## $p<0.01$ vs isoprenaline alone group.

4.55 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on heart weight to body weight (HWT/BWT) ratio in ISP-induced cardiac fibrosis in mice

The HWT/BWT ratio of different groups of mice are summarized in Table 4.55 and illustrated in Figure 4.66. A significant ($p<0.001$) increase in HWT/BWT ratio was observed in ISP-induced cardiac injury group (5.26 ± 0.20 mg/g; $n=12$) compared to control group (3.84 ± 0.13 mg/g; $n=10$). Niclosamide ethanolamine treatment significantly ($p<0.05$) decreased the HWT/BWT ratio (4.51 ± 0.17 mg/g; $n=8$) in cardiac injury in comparison to ISP-alone

administered group of mice (5.26 ± 0.20 mg/g; $n=12$). HWT/BWT ratio was almost comparable between niclosamide ethanolamine-alone (3.89 ± 0.14 mg/g; $n=9$) and control (3.84 ± 0.13 mg/g; $n=10$) groups of mice.

Table 4.55: Heart weight to body weight ratio (HWT/BWT) of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	HWT/BWT ratio (mg/g)
Control ($n=10$)	3.84 ± 0.13
Niclosamide ethanolamine-alone ($n=9$)	3.89 ± 0.14
Isoprenaline-alone ($n=12$)	$5.26 \pm 0.20^{***}$
Niclosamide ethanolamine+ISP ($n=8$)	$4.51 \pm 0.17^{\#}$

Data are presented as mean $\pm$ S.E.

*** $p < 0.001$ control vs ISP; $^{\#}p < 0.05$ vs isoprenaline-alone group.

4.56 Effect of niclosamide ethanolamine (NEN @ 3 mg/kg body weight; I.P.) on heart weight to tibial length (HWT/TL) ratio in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.56 and illustrated in Figure 4.67 show the HWT/TL ratio in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significantly ($p < 0.001$) higher HWT/TL was observed in isoprenaline-alone administered group (7.56 ± 0.16 mg/mm; $n=12$) compared to control group (6.14 ± 0.19 mg/mm; $n=10$). Niclosamide ethanolamine-treated cardiac injury group showed a significantly ($p < 0.001$) lower HWT/TL (6.36 ± 0.14 mg/mm; $n=8$) than ISP-alone administered group (7.56 ± 0.16 mg/mm; $n=12$). No significant difference was noted in HWT/TL ratio between niclosamide ethanolamine-alone (6.55 ± 0.16 mg/mm; $n=9$) and control (6.14 ± 0.19 mg/mm; $n=10$) groups of mice.

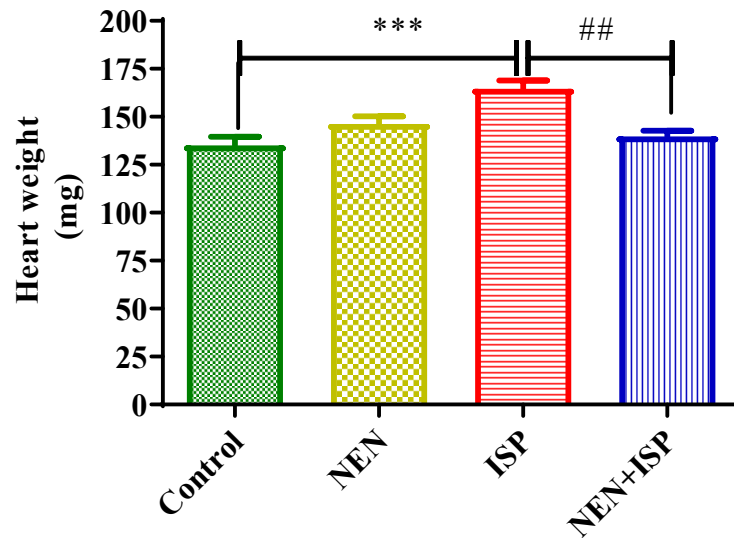


Fig. 4.65: Bar diagram showing absolute heart weight of control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, ##) on the bars present significant ($p<0.001-0.01$) difference between groups.

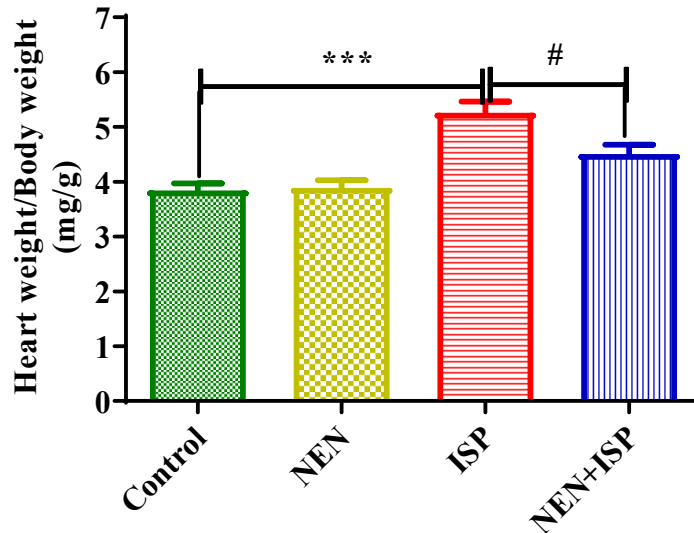


Fig. 4.66: Bar diagram showing the heart weight to body weight ratio (HWT/BWT) of control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, #) on the bars present significant ($p<0.001-0.05$) difference between groups.

Table 4.56: Heart weight to tibial length ratio (HWT/TL) of the control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	HWT/TL ratio (mg/mm)
Control (n=10)	6.14±0.19
Niclosamide ethanolamine-alone (n=9)	6.55±0.16
Isoprenaline-alone (n=12)	7.56±0.16 ^{***}
Niclosamide ethanolamine+ISP (n=8)	6.36±0.14 ^{###}

Data are presented as mean±S.E.

^{***}*p*<0.001 vs control; ^{###}*p*<0.001 vs isoprenaline-alone administered group.

4.57 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on serum CK-MB in isoprenaline-induced cardiac injury in mice

The CK-MB levels in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.57 and illustrated in Figure 4.68. Data revealed that CK-MB level was significantly (*p*<0.05) increased in ISP-alone administered group (187.8±25.61 U/L; n=6) than the control group (98.88±14.95 U/L; n=6). A significant (*p*<0.05) reduction in serum CK-MB level was observed in niclosamide ethanolamine-treated cardiac injury group (98.66±12.89 U/L; n=5) in comparison to the ISP-alone administered group (187.8±25.61 U/L; n=6). CK-MB level

Table 4.57: Serum CK-MB level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	CK-MB (U/L)
Control (n=6)	98.88±14.95
Niclosamide ethanolamine-alone (n=5)	105.3±17.18
Isoprenaline-alone (n=6)	187.8±25.61 [*]
Niclosamide ethanolamine+ISP (n=5)	98.66±12.89 [#]

Data are presented as mean±S.E.

^{*}*p*<0.05 vs control; [#]*p*<0.05 vs isoprenaline-alone administered group.

was found to be almost comparable in niclosamide ethanolamine-alone (105.3 ± 17.18 U/L; $n=5$) and control (98.88 ± 14.95 U/L; $n=6$) groups of mice.

4.58 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on serum LDH level in isoprenaline-induced cardiac injury in mice

Data summarized in Table 4.58 and illustrated in Figure 4.69 show the serum LDH level of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significant ($p < 0.05$) increased serum LDH was observed in ISP-induced cardiac injury group (2639 ± 215.3 U/L; $n=6$) compared to control group (1867 ± 129.3 U/L; $n=5$). However, niclosamide ethanolamine treatment moderately decreased (2321 ± 139.2 U/L; $n=7$) serum LDH level in comparison to ISP-alone administered group (2639 ± 215.3 U/L; $n=6$). LDH level of niclosamide ethanolamine-alone (2169 ± 201.0 U/L; $n=7$) and control (1867 ± 129.3 U/L; $n=5$) groups of mice were almost comparable to each other.

Table 4.58: Serum LDH level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	LDH (U/L)
Control group ($n=5$)	1867 ± 129.3
Niclosamide ethanolamine-alone ($n=7$)	2169 ± 201.0
Isoprenaline (ISP) ($n=6$)	$2639 \pm 215.3^*$
Niclosamide ethanolamine+ISP ($n=7$)	2321 ± 139.2

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control group.

4.59 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) treatment on the serum cholesterol level in ISP-induced cardiac injury in mice

Table 4.59 and Figure 4.70 depict the serum cholesterol level in control, niclosamide ethanolamine-alone, ISP-alone administered, and niclosamide ethanolamine-treated cardiac injury groups of mice. A significant ($p < 0.01$) elevation in cholesterol level was observed in

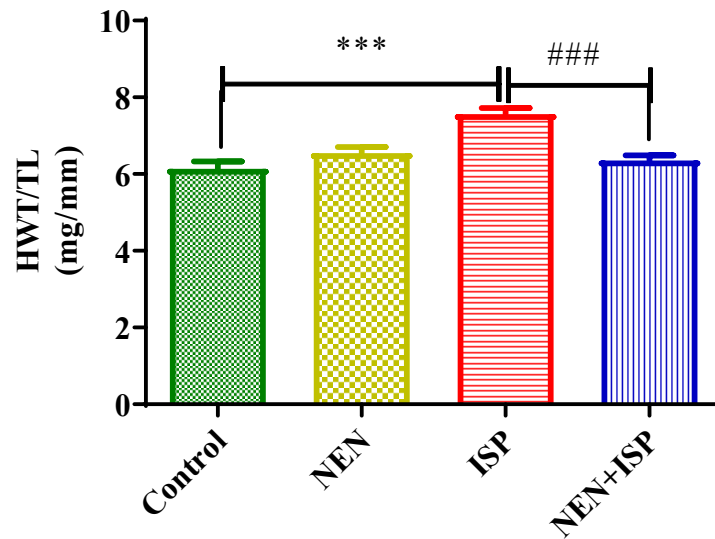


Fig. 4.67: Bar diagram showing heart weight to tibial length (HWT/TL) ratio of control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, ###) on the bars present significant ($p<0.001$) difference between groups.

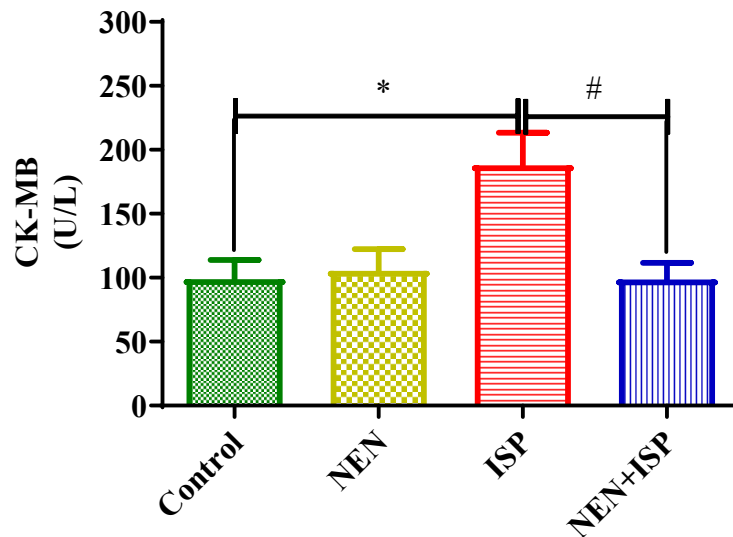


Fig. 4.68: Bar diagram showing serum CK-MB level of control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

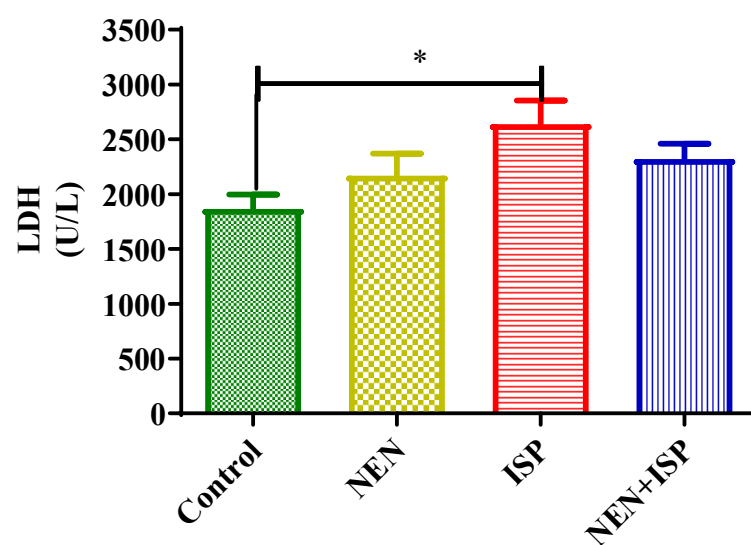


Fig. 4.69: Bar diagram showing serum LDH level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p < 0.05$) difference between control and ISP-alone administered groups.

ISP-alone administered group (113.0 ± 7.19 mg/dl; $n=10$) in comparison to control group (69.27 ± 10.83 mg/dl; $n=8$). However, niclosamide ethanolamine treatment moderately improved the cholesterol level (87.81 ± 6.24 mg/dl; $n=8$) in cardiac injury in comparison to ISP-alone administered group (113.0 ± 7.19 mg/dl; $n=10$). No significant alteration was observed in cholesterol level between niclosamide ethanolamine-alone (69.10 ± 5.99 mg/dl; $n=10$) and control (69.27 ± 10.83 mg/dl; $n=8$) groups of mice.

Table 4.59: Serum cholesterol level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Cholesterol (mg/dl)
Control group ($n=8$)	69.27 ± 10.83
Niclosamide ethanolamine-alone ($n=10$)	69.10 ± 5.99
Isoprenaline-alone ($n=10$)	$113.0 \pm 7.19^{**}$
Niclosamide ethanolamine+ISP ($n=8$)	87.81 ± 6.24

Data are presented as mean $\pm$ S.E.

$^{**}p < 0.01$ vs control.

4.60 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on serum triglyceride level in ISP-induced cardiac injury in mice

Data summarized in Table 4.60 and illustrated in Figure 4.71 show the serum triglyceride level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significantly ($p < 0.05$) higher level of serum triglyceride was found in ISP-alone administered group (94.32 ± 10.35 mg/dl; $n=8$) compared to control group (49.79 ± 11.49 mg/dl; $n=7$). Niclosamide ethanolamine treatment did not show a significant improvement in triglyceride level (60.27 ± 11.79 mg/dl; $n=7$) in cardiac injury group compared to ISP-alone group (94.32 ± 10.35 mg/dl; $n=8$). Almost comparable triglyceride level was observed in niclosamide ethanolamine-alone (57.39 ± 9.64 mg/dl; $n=8$) and control (49.79 ± 11.49 mg/dl; $n=7$) groups of mice.

Table 4.60: Serum triglyceride level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Triglyceride (mg/dl)
Control (n=7)	49.79±11.49
Niclosamide ethanolamine-alone (n=8)	57.39±9.64
Isoprenaline-alone (n=8)	94.32±10.35*
Niclosamide ethanolamine+ISP (n=7)	60.27±11.79

Data are presented as mean±S.E.

* $p < 0.05$ vs control.

4.61 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on high-density lipoprotein (HDL) cholesterol level in ISP-induced cardiac injury in mice

Data summarized in Table 4.61 and illustrated in Figure 4.72 depict HDL-c level in control, niclosamide ethanolamine-alone, isoprenaline-alone, and niclosamide ethanolamine-treated cardiac injury groups of mice. Data revealed that ISP-alone administration significantly ($p < 0.05$) decreased the HDL-c level (64.23 ± 4.14 mg/dl; $n=6$) in comparison to control group (77.83 ± 2.56 mg/dl; $n=6$). No significant improvement in HDL-c level was observed in niclosamide ethanolamine-treated cardiac injury group (72.09 ± 2.98 mg/dl; $n=6$) compared to the ISP-alone administered group (64.23 ± 4.14 mg/dl; $n=6$). The HDL-c level was found to

Table 4.61: Serum HDL-c level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	HDL-c (mg/dl)
Control (n=6)	77.83±2.56
Niclosamide ethanolamine-alone (n=6)	76.00±2.25
Isoprenaline-alone (n=6)	64.23±4.14*
Niclosamide ethanolamine+ISP (n=6)	72.09±2.98

Data are presented as mean±S.E.

* $p < 0.05$ vs control.

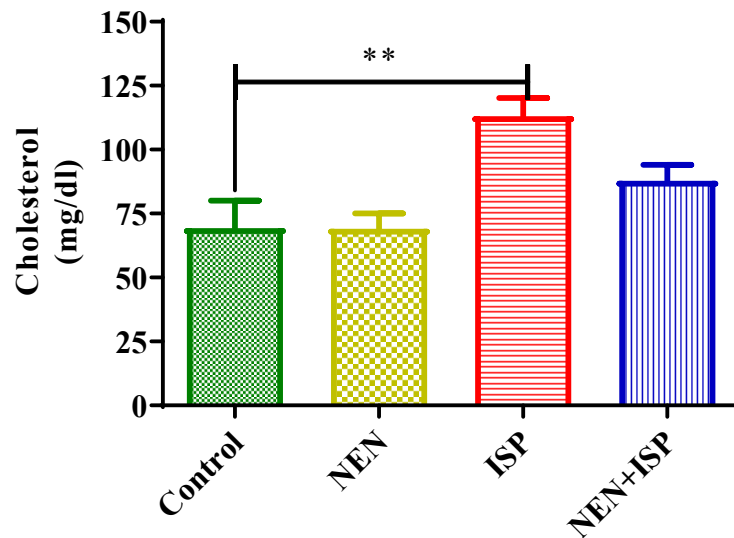


Fig. 4.70: Bar diagram showing the serum cholesterol level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (**) on the bars present significant ($p<0.01$) difference between ISP-alone and control groups.

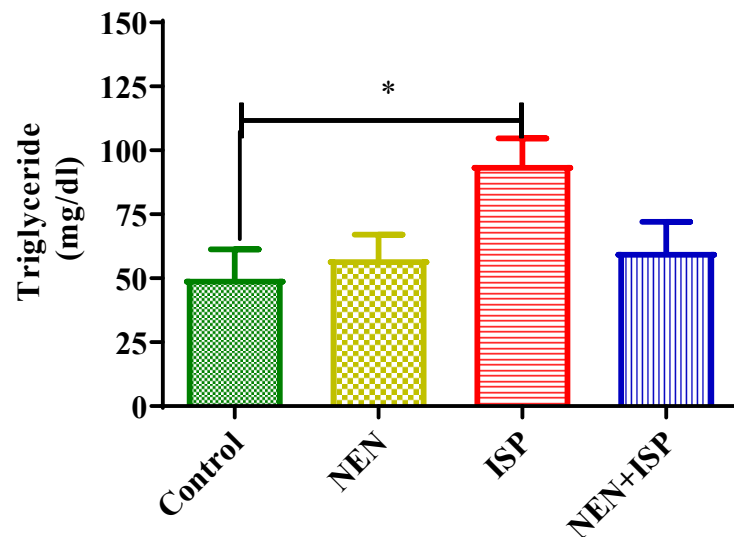


Fig. 4.71: Bar diagram showing the serum triglyceride level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between control and ISP-alone administered groups.

be almost comparable between niclosamide ethanolamine-alone (76.00 ± 2.25 mg/dl; $n=6$) and control (77.83 ± 2.56 mg/dl; $n=6$) groups of mice.

4.62 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on low-density lipoprotein cholesterol (LDL-c) level in ISP-induced cardiac injury in mice

Data in Table 4.62 and in Figure 4.73 show serum LDL- c level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significantly ($p < 0.05$) increased level of LDL-c was observed in ISP-alone administered group (62.91 ± 12.46 mg/dl; $n=6$) in comparison to control group (25.85 ± 6.62 mg/dl; $n=6$). However, no significant improvement was observed in LDL-c level in niclosamide ethanolamine-treated (34.32 ± 7.90 mg/dl; $n=6$) cardiac injury group in comparison to ISP-alone administered group (62.91 ± 12.46 mg/dl; $n=6$). LDL-c level was almost comparable between niclosamide ethanolamine-alone (27.17 ± 6.21 mg/dl; $n=6$) and control (25.85 ± 6.62 mg/dl; $n=6$) groups of mice.

Table 4.62: Serum LDL-c level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	LDL-c (mg/dl)
Control (n=6)	25.85 ± 6.62
Niclosamide ethanolamine-alone (n=6)	27.17 ± 6.21
Isoprenaline (ISP)-alone (n=6)	$62.91 \pm 12.46^*$
Niclosamide ethanolamine+ISP (n=6)	34.32 ± 7.90

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control.

4.63 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on HDL-c to LDL-c ratio (HDL-c/LDL-c) in ISP-induced cardiac injury in mice

As summarized in Table 4.63 and illustrated in Figure 4.74 depict the HDL-c/LDL-c ratio in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide

ethanolamine-treated cardiac injury groups of mice. Isoprenaline-administration significantly ($p<0.05$) decreased the HDL-c/LDL-c ratio (1.17 ± 0.17 ; $n=6$) compared to control group (4.42 ± 1.21 ; $n=6$). No significant improvement in HDL-c/LDL-c was observed in niclosamide ethanolamine-treated cardiac injury group (2.70 ± 0.62 ; $n=6$) than ISP-alone administered group (1.17 ± 0.17 ; $n=6$). HDL-c/LDL-c ratio was found to be almost comparable between niclosamide ethanolamine-alone (3.36 ± 0.55 ; $n=6$) and control (4.42 ± 1.21 ; $n=6$) groups of mice.

Table 4.63: Serum HDL-c/LDL-c ratio in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	HDL-c/LDL-c ratio
Control (n=6)	4.42±1.21
Niclosamide ethanolamine-alone (n=6)	3.36±0.55
Isoprenaline-alone (n=6)	1.17±0.17*
Niclosamide ethanolamine+ISP (n=6)	2.70±0.62

Data are presented as mean±S.E.

* $p<0.05$ vs control.

4.64 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on serum c-reactive protein (CRP) level in ISP-induced cardiac injury in mice

Data summarized in Table 4.64 and illustrated in Figure 4.75 show the CRP level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. Isoprenaline-alone administration significantly ($p<0.05$) increased CRP level (6.08 ± 1.01 mg/l; $n=7$) in comparison to the control group (3.33 ± 0.56 mg/l; $n=7$). A significant ($p<0.05$) reduction in CRP was observed in niclosamide ethanolamine-treated cardiac injury group (3.49 ± 0.48 mg/l; $n=7$) compared to ISP-alone administered group (6.08 ± 1.01 mg/l; $n=7$). Furthermore, almost comparable CRP level was noted in niclosamide ethanolamine-alone (4.01 ± 0.29 mg/l; $n=6$) and control (3.33 ± 0.56 mg/l; $n=7$) groups of mice.

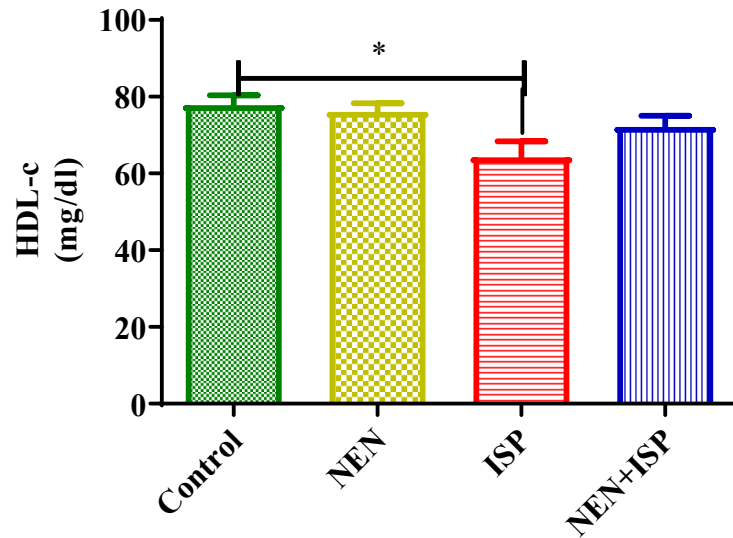


Fig. 4.72: Bar diagram showing the serum HDL-c in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscripts (*) on the bars present significant ($p < 0.05$) difference between control and isoprenaline-alone administered groups.

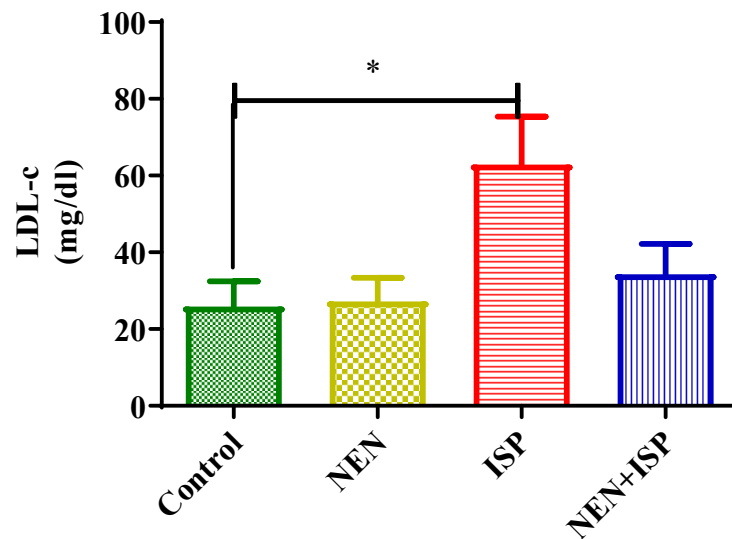


Fig. 4.73: Bar diagram showing the serum LDL-c in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscripts (*) on the bars present significant ($p < 0.05$) difference between control and ISP-alone administered groups.

Table 4.64: Serum CRP level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	CRP (mg/l)
Control (n=7)	3.33±0.56
Niclosamide ethanolamine-alone (n=6)	4.01±0.29
Isoprenaline-alone (n=7)	6.08±1.01*
Niclosamide ethanolamine+ISP (n=7)	3.49±0.48 [#]

Data are presented as mean±S.E.

* $p < 0.05$ vs control; [#] $p < 0.05$ vs isoprenaline-alone administered group.

4.65 Effect of niclosamide ethanolamine (NEN @ 3 mg/kg body weight; I.P.) on serum superoxide dismutase (SOD) activity in ISP-induced cardiac fibrosis in mice

Table 4.65 and Figure 4.76 depict the SOD activity in serum of control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significant ($p < 0.001$) decrease in SOD activity was observed in ISP-alone administered group (4.27 ± 0.11 units/mg of protein; $n=10$) in comparison to control group (5.44 ± 0.19 units/mg of proteins; $n=7$). Niclosamide ethanolamine treatment significantly ($p < 0.001$) increased the SOD activity (5.49 ± 0.16 units/mg of protein; $n=7$) in cardiac injury, compared to isoprenaline-alone administered group (4.27 ± 0.11 units/mg of protein; $n=10$). The SOD activity was found to be almost comparable in niclosamide ethanolamine-alone (4.99 ± 0.17 units/mg of proteins; $n=7$) and control (5.44 ± 0.19 units/mg of proteins; $n=7$) groups of mice.

Table 4.65: Serum SOD activity in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	SOD (units/mg of proteins)
Control (n=7)	5.44±0.19
Niclosamide ethanolamine-alone (n=7)	4.99±0.17
Isoprenaline-alone (n=10)	4.27±0.11 ^{***}
Niclosamide ethanolamine+ISP (n=7)	5.49±0.16 ^{###}

Data are presented as mean±S.E.

^{***}*p*<0.001 vs control; ^{###}*p*<0.001 vs ISP-alone administered group.

4.66 Effect of niclosamide ethanolamine (NEN @ 3 mg/kg body weight; I.P.) on serum catalase activity in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.66 and Figure 4.77 show the catalase activity in serum samples of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. ISP-administration significantly (*p*<0.05) reduced the serum catalase activity (3.58±0.89 mmol H₂O₂ utilized/min/mg of proteins; n=7) in comparison to the control group (9.71±1.25 mmol H₂O₂ utilized/min/mg of proteins; n=6). A significantly (*p*<0.05) increased catalase activity was observed in niclosamide ethanolamine-treated cardiac

Table 4.66: Serum catalase activity in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Catalase (mmol H ₂ O ₂ utilized/min/mg of proteins)
Control (n=6)	9.71±1.25
Niclosamide ethanolamine-alone (n=6)	10.51±1.62
Isoprenaline-alone (n=7)	3.58±0.89 [*]
Niclosamide ethanolamine+ISP (n=6)	9.48±1.49 [#]

Data are presented as mean±S.E.

^{*}*p*<0.05 vs control; [#]*p*<0.05 vs isoprenaline-alone group.

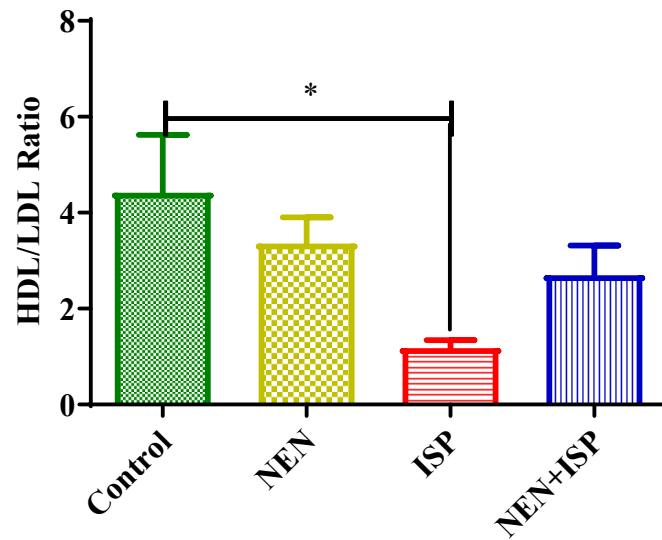


Fig. 4.74: Bar diagram showing the serum HDL-c/LDL-c ratio in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscripts (*) on the bars present significant ($p < 0.05$) difference between control and ISP-alone administered group.

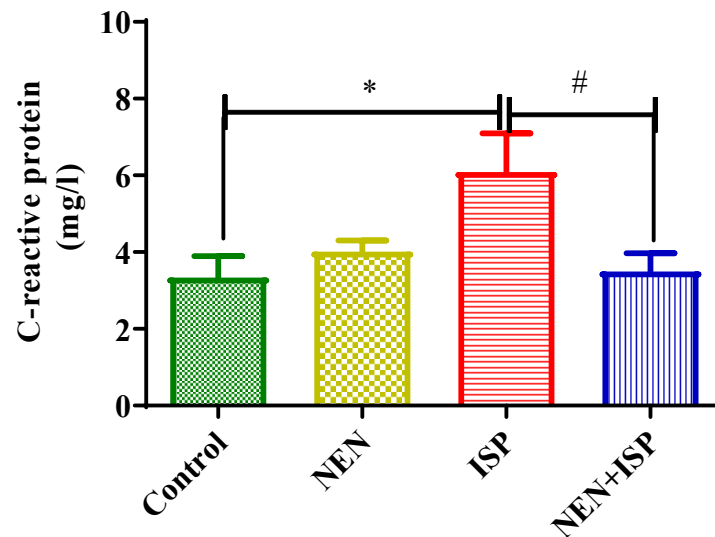


Fig. 4.75: Bar diagram showing the serum CRP level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

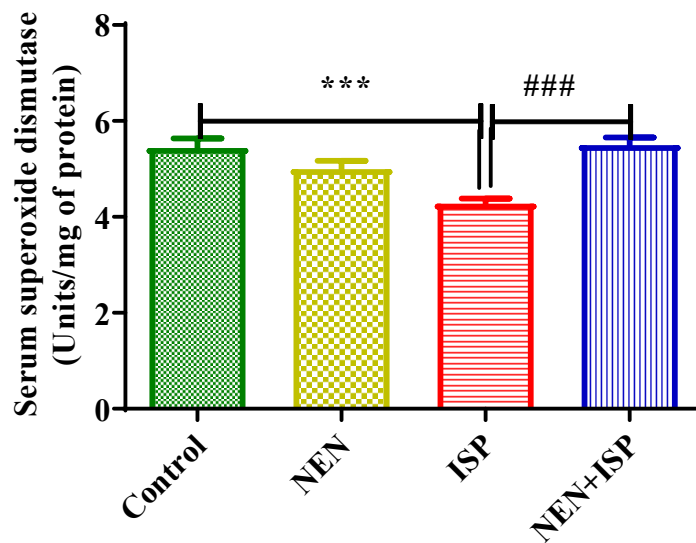


Fig. 4.76: Bar diagram showing the SOD activity in serum in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, ###) on the bars present significant ($p < 0.001$) difference between groups.

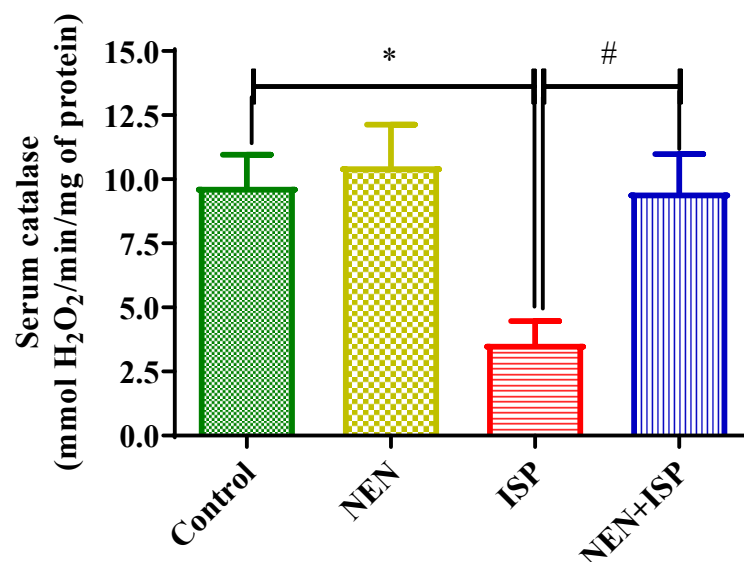


Fig. 4.77: Bar diagram showing the serum catalase activity in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

injury group (9.48 ± 1.49 mmol H_2O_2 utilized/min/mg of proteins; $n=6$) compared to the ISP-alone administered group (3.58 ± 0.89 mmol H_2O_2 utilized/min/mg of proteins; $n=7$). No significant difference in serum catalase activities was observed in niclosamide ethanolamine-alone (10.51 ± 1.62 mmol H_2O_2 utilized/min/mg of proteins; $n=6$) and control (9.71 ± 1.25 mmol H_2O_2 utilized/min/mg of proteins; $n=6$) groups of mice.

4.67 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on serum non-enzymatic antioxidant reduced glutathione (GSH) in ISP-induced cardiac injury in mice

The fold change in serum reduced glutathione in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.67 and illustrated Figure 4.78. Isoprenaline-alone administration significantly ($p < 0.05$) reduced the GSH level (0.72 ± 0.04 fold change; $n=10$) in comparison to the control group (1.00 ± 0.09 fold change; $n=7$). Serum GSH level was significantly ($p < 0.05$) increased in niclosamide ethanolamine-treated cardiac injury group (1.02 ± 0.06 fold change; $n=6$) compared to ISP-alone administered group (0.72 ± 0.04 fold change; $n=10$). Almost comparable level of serum GSH was observed between niclosamide ethanolamine-alone (1.05 ± 0.08 fold change; $n=8$) and control (1.00 ± 0.09 fold change; $n=7$) groups of mice.

Table 4.67: Fold change of serum GSH in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Serum GSH (Fold change)
Control ($n=7$)	1.00 ± 0.09
Niclosamide ethanolamine-alone ($n=8$)	1.05 ± 0.08
Isoprenaline-alone ($n=10$)	$0.72 \pm 0.04^*$
Niclosamide ethanolamine+ISP ($n=6$)	$1.02 \pm 0.06^\#$

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control; $^\#p < 0.05$ vs isoprenaline-alone group.

4.68 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on serum lipid peroxidation (LPO) in ISP-induced cardiac injury in mice

Data summarized in Table 4.68 and illustrated in Figure 4.79 depict the serum MDA of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury group of mice. A significant ($p<0.01$) increase in MDA level was observed in ISP-alone administered group (6.01 ± 0.32 nmol/ml of serum; $n=6$) in comparison to control group (3.64 ± 0.30 nmol/ml of serum; $n=7$). No significant difference was observed in MDA level in niclosamide ethanolamine-treated cardiac injury (4.28 ± 0.41 nmol/ml of serum; $n=6$) compared to ISP-alone administered (6.01 ± 0.32 nmol/ml of serum; $n=6$) groups. Almost comparable MDA level was seen between niclosamide ethanolamine-alone (3.41 ± 0.64 nmol/ml of serum; $n=6$) and control (3.64 ± 0.30 nmol/ml of serum; $n=7$) groups of mice.

Table 4.68: Serum MDA level in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	MDA (nmol/ml of serum)
Control (n=7)	3.64±0.30
Niclosamide ethanolamine-alone (n=6)	3.41±0.64
Isoprenaline-alone (n=6)	6.01±0.32**
Niclosamide ethanolamine+ISP (n=6)	4.28±0.41

Data are presented as mean±S.E.

** $p<0.01$ vs control group.

4.69 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on tissue superoxide dismutase (SOD) activity in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.69 and illustrated in Figure 4.80 depict SOD activity in heart tissue of control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significant ($p<0.05$) decrease in SOD activity was observed in ISP-alone administered group (10.11 ± 0.47 units/mg of protein; $n=6$) in comparison to control group (15.30 ± 1.52 units/mg of proteins; $n=7$). Niclosamide

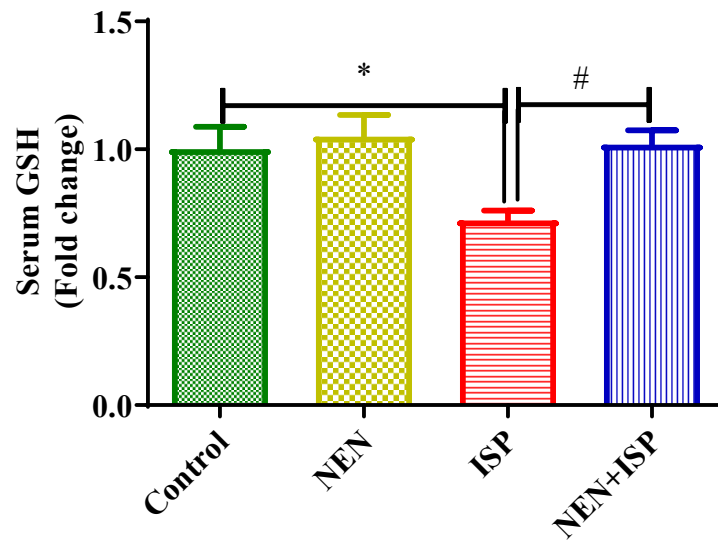


Fig. 4.78: Bar diagram showing the serum reduced glutathione (GSH) level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

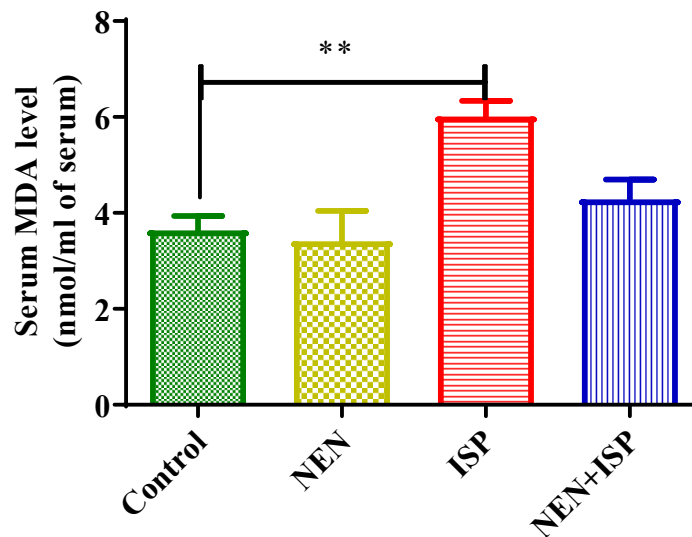


Fig. 4.79: Bar diagram showing the serum MDA level in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (**) on the bars present significant ($p<0.01$) difference between groups.

ethanolamine treatment significantly ($p<0.05$) increased the SOD activity in cardiac injury group (15.82 ± 1.07 units/mg of proteins; $n=7$) compared to ISP-alone administered group (10.11 ± 0.47 units/mg of protein; $n=6$). Furthermore, no significant difference was noted in SOD activity in niclosamide ethanolamine-alone (14.73 ± 1.31 units/mg of proteins; $n=6$) and control (15.30 ± 1.52 units/mg of proteins; $n=7$) groups of mice.

Table 4.69: Heart tissue SOD activity in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	SOD (units/mg of proteins)
Control ($n=7$)	15.30 ± 1.52
Niclosamide ethanolamine-alone ($n=6$)	14.73 ± 1.31
Isoprenaline-alone ($n=6$)	$10.11\pm0.47^*$
Niclosamide ethanolamine+ISP ($n=7$)	$15.82\pm1.07^\#$

Data are presented as mean $\pm$ S.E.

* $p<0.05$ vs control; $^\#p<0.05$ vs isoprenaline-alone administered group.

4.70 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on tissue catalase activity in ISP-induced cardiac fibrosis in mice

Data in Table 4.70 and Figure 4.81 depict catalase activity in heart tissue of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. A significant ($p<0.05$) reduction in catalase activity in heart was observed in ISP-alone administered group (0.33 ± 0.07 mmol H_2O_2 utilized/min/mg of proteins; $n=6$) in comparison to control group (1.28 ± 0.32 mmol H_2O_2 utilized/min/mg of proteins; $n=7$). Niclosamide ethanolamine treatment increased (non-significant) the tissue catalase activity (1.01 ± 0.13 mmol H_2O_2 utilized/min/mg of proteins; $n=7$) compared to the ISP-alone administered group (0.33 ± 0.07 mmol H_2O_2 utilized/min/mg of proteins; $n=6$). Catalase activities in niclosamide ethanolamine-alone (1.24 ± 0.29 mmol H_2O_2 utilized/min/mg of proteins; $n=6$) and control (1.28 ± 0.32 mmol H_2O_2 utilized/min/mg of proteins; $n=7$) groups of mice were almost comparable to each other.

Table 4.70: Catalase activity in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Catalase (mmol H ₂ O ₂ utilized/min/mg of proteins)
Control (n=7)	1.28±0.32
Niclosamide ethanolamine-alone (n=6)	1.24±0.29
Isoprenaline-alone (n=6)	0.33±0.07*
Niclosamide ethanolamine+ISP (n=7)	1.01±0.13

Data are presented as mean±S.E.

* $p < 0.05$ vs control.

4.71 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on tissue non-enzymatic antioxidant reduced glutathione (GSH) in ISP-induced cardiac fibrosis in mice

The fold change in GSH in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.71 and illustrated in Figure 4.82. A significant ($p < 0.05$) reduction was observed in GSH level in ISP-induced cardiac injury group (0.28 ± 0.04 fold change; $n=6$) in comparison to the control group (1.00 ± 0.24 fold change; $n=7$). Niclosamide ethanolamine treatment significantly ($p < 0.05$) increased GSH in cardiac injury group (1.03 ± 0.12 fold change; $n=6$) compared to ISP-alone administered group (0.28 ± 0.04 fold change; $n=6$). GSH level was observed almost comparable between niclosamide ethanolamine-alone (0.96 ± 0.23 fold change; $n=6$) and control (1.00 ± 0.24 fold change; $n=7$) groups of mice.

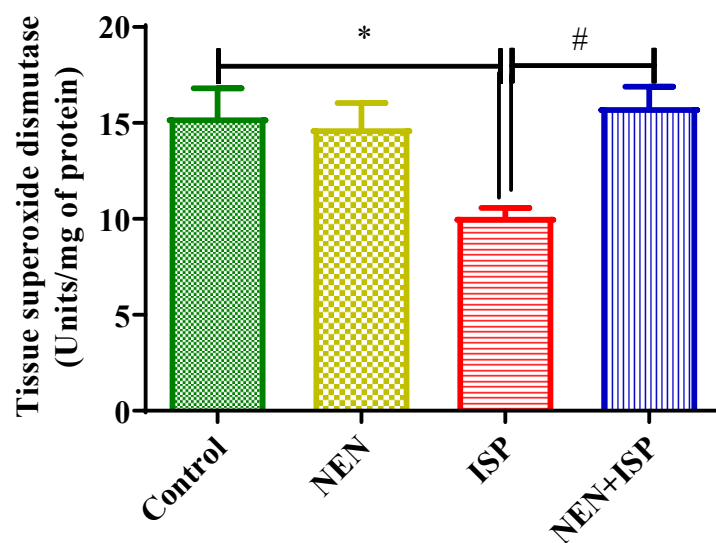


Fig. 4.80: Bar diagram showing the SOD activity in heart tissue in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

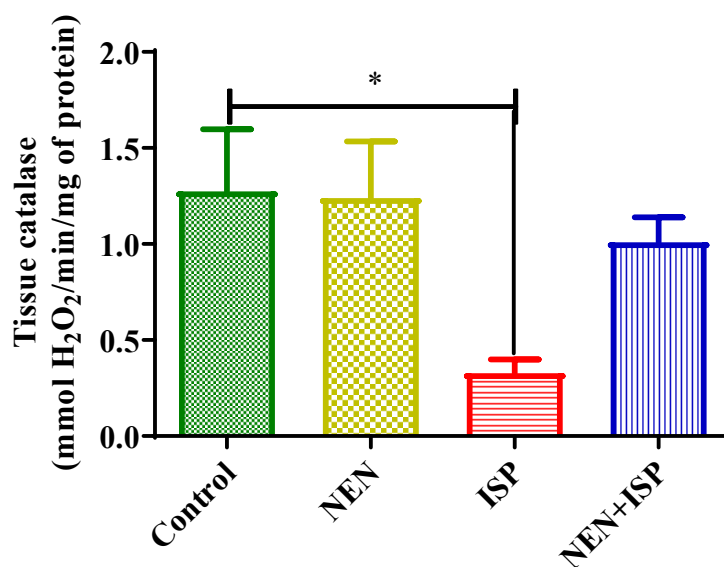


Fig. 4.81: Bar diagram showing the catalase activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between control and ISP-alone administered groups.

Table 4.71: Fold change of non-enzymatic antioxidant, GSH in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	GSH (Fold change)
Control (n=7)	1.00±0.24
Niclosamide ethanolamine-alone (n=6)	0.96±0.23
Isoprenaline-alone (n=6)	0.28±0.04*
Niclosamide ethanolamine+ISP (n=6)	1.03±0.12 [#]

Data are presented as mean±S.E.

* $p < 0.05$ vs control; [#] $p < 0.05$ vs isoprenaline-alone group.

4.72 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on tissue lipid peroxidation (LPO) in ISP-induced cardiac fibrosis in mice

The level of malondialdehyde (MDA) was estimated to assess the LPO in heart tissue. Table 4.72 and Figure 4.83 depict the MDA level in heart tissue of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury group of mice. A significant ($p < 0.01$) increase in MDA level was observed in ISP-alone administered group (2.82 ± 0.37 nmol/mg of protein; n=6) in comparison to control group (1.26 ± 0.24 nmol/mg of protein; n=7). Niclosamide ethanolamine treatment significantly ($p < 0.05$) reduced the MDA level (1.65 ± 0.19 nmol/mg of protein; n=6) compared to the ISP-alone administered

Table 4.72: MDA level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	MDA (nmol/mg of protein)
Control (n=7)	1.26±0.24
Niclosamide ethanolamine-alone (n=6)	1.51±0.16
Isoprenaline-alone (n=6)	2.82±0.37**
Niclosamide ethanolamine+ISP (n=6)	1.65±0.19 [#]

Data are presented as mean±S.E.

** $p < 0.01$ vs control group; [#] $p < 0.05$ vs ISP-alone administered group.

group (2.82 ± 0.37 nmol/mg of protein; $n=6$). The MDA level was found to be almost comparable in niclosamide ethanolamine-alone (1.51 ± 0.16 nmol/mg of protein; $n=6$) and control (1.26 ± 0.24 nmol/mg of protein; $n=7$) groups of mice.

4.73 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on tissue hydroxyproline level in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.73 and illustrated in Figure 4.84 show the hydroxyproline level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac fibrosis group of mice. A significantly ($p < 0.01$) increased level of hydroxyproline was observed in ISP-alone administered cardiac fibrosis group (14.40 ± 2.12 $\mu\text{g}/\text{mg}$ of tissue; $n=6$) in comparison to control group (5.16 ± 1.61 $\mu\text{g}/\text{mg}$ of tissue; $n=6$). Niclosamide ethanolamine treatment significantly ($p < 0.05$) reduced the hydroxyproline level in cardiac fibrosis (6.34 ± 1.26 $\mu\text{g}/\text{mg}$ of tissue; $n=6$) compared to the ISP-alone administered group (14.40 ± 2.12 $\mu\text{g}/\text{mg}$ of tissue; $n=6$). Almost comparable hydroxyproline level was observed in niclosamide ethanolamine-alone (5.48 ± 1.91 $\mu\text{g}/\text{mg}$ of tissue; $n=6$) and control (5.16 ± 1.61 $\mu\text{g}/\text{mg}$ of tissue; $n=6$) groups of mice.

Table 4.73: Hydroxyproline level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Tissue hydroxyproline ($\mu\text{g}/\text{mg}$ of tissue)
Control ($n=6$)	5.16 ± 1.61
Niclosamide ethanolamine-alone ($n=6$)	5.48 ± 1.91
Isoprenaline-alone ($n=6$)	$14.40 \pm 2.12^{**}$
Niclosamide ethanolamine+ISP ($n=6$)	$6.34 \pm 1.26^{\#}$

Data are presented as mean $\pm$ S.E.

$^{**}p < 0.01$ vs control group; $^{\#}p < 0.05$ vs ISP-alone administered group.

4.74 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on tissue glucosamine level in ISP-induced cardiac fibrosis in mice

The fold change in the glucosamine level in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized

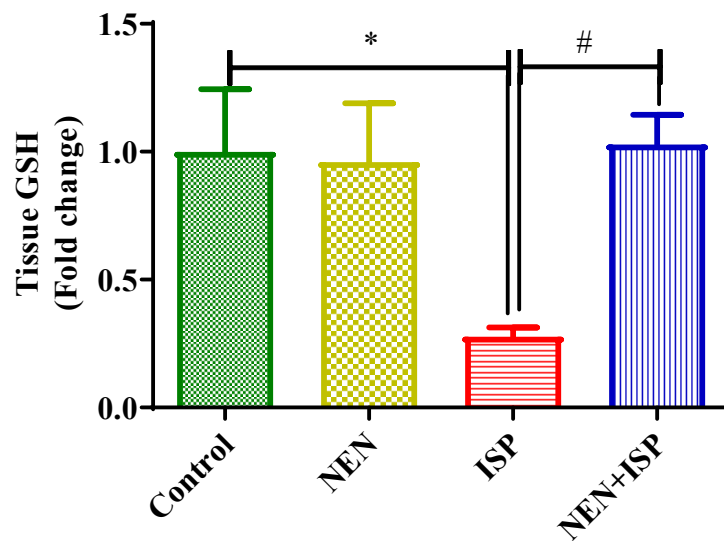


Fig. 4.82: Bar diagram showing the reduced glutathione (GSH) level in heart tissue in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups.

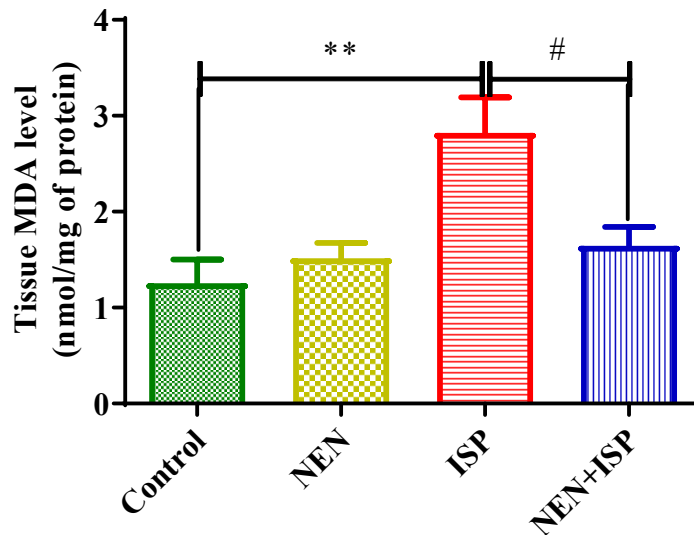


Fig. 4.83: Bar diagram showing the MDA level in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (**, #) on the bars present significant ($p<0.01-0.05$) difference between groups.

in Table 4.74 and Figure 4.85. The isoprenaline administration significantly ($p<0.001$) increased the glucosamine level (4.56 ± 0.53 fold change; $n=9$) compared to control group (1.00 ± 0.19 fold change; $n=6$). A significant ($p<0.05$) decrease in glucosamine level was observed in niclosamide ethanolamine-treated cardiac injury group (2.52 ± 0.34 fold change; $n=6$) compared to ISP-alone administrated group (4.56 ± 0.53 fold change; $n=9$). Glucosamine level was found to be almost comparable in niclosamide ethanolamine-alone (1.69 ± 0.36 fold change; $n=8$) and control (1.00 ± 0.19 fold change; $n=6$) groups of mice.

Table 4.74: Glucosamine level in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Tissue glucosamine (Fold change)
Control (n=6)	1.00 ± 0.19
Niclosamide ethanolamine-alone (n=8)	1.69 ± 0.36
Isoprenaline-alone (n=9)	$4.56\pm0.53^{***}$
Niclosamide ethanolamine+ISP (n=6)	$2.52\pm0.34^{\#}$

Data are presented as mean $\pm$ S.E.

*** $p<0.001$ vs control group; $^{\#}p<0.05$ vs ISP-alone administered group.

4.75 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on collagen type I in heart tissue in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.75 and illustrated in Figure 4.86 depict the fold change in collagen type I content of heart tissue in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac fibrosis groups of mice. Isoprenaline-alone administered cardiac injury group showed a significant ($p<0.05$) increase in collagen type I (1.66 ± 0.12 fold change; $n=6$) compared to control group (1.00 ± 0.14 fold change; $n=6$). A significant ($p<0.05$) reduction in collagen type I content of heart was observed in niclosamide ethanolamine-treated cardiac injury group (0.95 ± 0.18 fold change; $n=6$) compared to ISP-alone administered group (1.66 ± 0.12 fold change; $n=6$). Almost comparable collagen type I content was seen in niclosamide ethanolamine-alone (0.92 ± 0.19 fold change; $n=6$) and control (1.00 ± 0.14 fold change; $n=6$) groups of mice.

Table 4.75: Fold change of collagen type I content of heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Collagen type I (Fold change)
Control (n=6)	1.00±0.14
Niclosamide ethanolamine-alone (n=6)	0.92±0.19
Isoprenaline-alone (n=6)	1.66±0.12*
Niclosamide ethanolamine+ISP (n=6)	0.95±0.18 [#]

Data are presented as mean±S.E.

* $p < 0.05$ vs control group; [#] $p < 0.05$ vs isoprenaline-alone administered group.

4.76 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on connective tissue growth factor (CTGF) in heart tissue in ISP-induced cardiac fibrosis in mice

Table 4.76 and Figure 4.87 depict the fold change in CTGF level of heart tissue in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury group of mice. No significant difference was observed in CTGF level in control (1.00±0.07 fold change; n=6), niclosamide ethanolamine-alone (0.80±0.23 fold change; n=6), isoprenaline-alone (1.31±0.08 fold change; n=7) and niclosamide ethanolamine-treated cardiac injury (0.84±0.11 fold change; n=7) groups. However, an increased (non-significant) CTGF level was observed in ISP-alone administered group (1.31±0.08 fold change; n=7) in

Table 4.76: CTGF in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	CTGF (Fold change)
Control (n=6)	1.00±0.07
Niclosamide ethanolamine-alone (n=6)	0.80±0.23
Isoprenaline-alone (n=7)	1.31±0.08
Niclosamide ethanolamine+ISP (n=7)	0.84±0.11

Data are presented as mean±S.E.

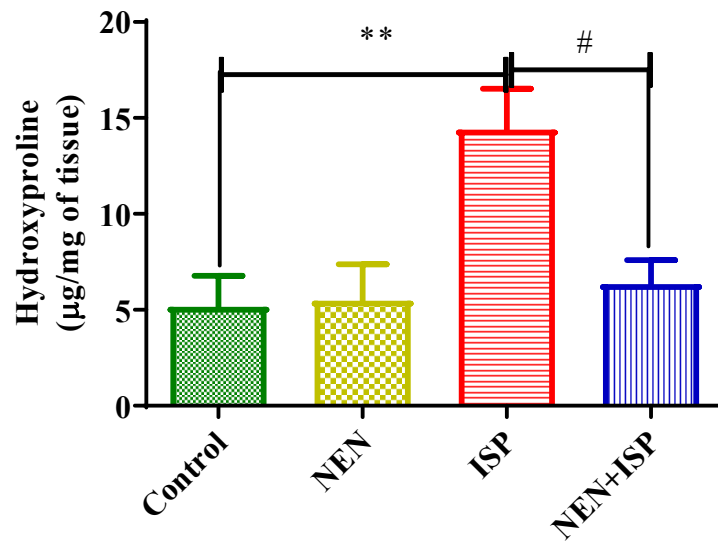


Fig. 4.84: Bar diagram showing the hydroxyproline level in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (**, #) on the bars present significant ($p<0.01-0.05$) difference between groups.

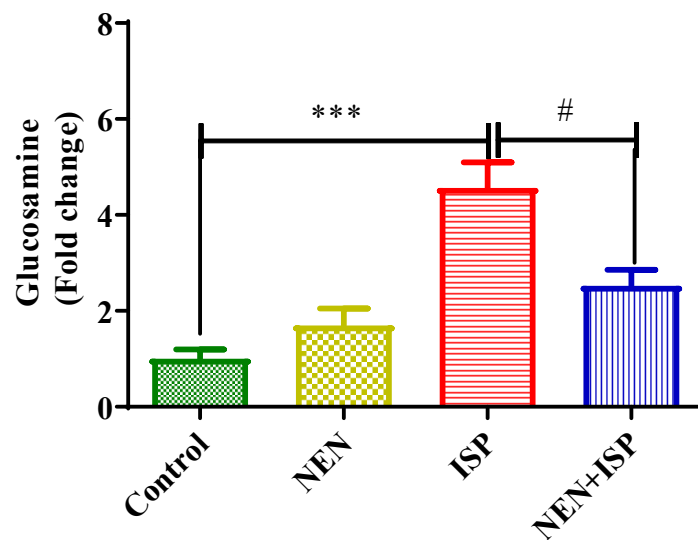


Fig. 4.85: Bar diagram showing the fold change in glucosamine level in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (***, #) on the bars present significant ($p<0.001-0.05$) difference between groups.

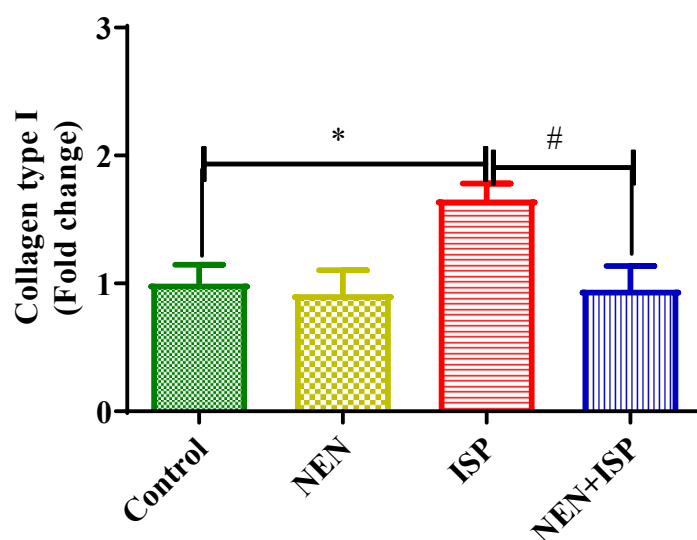


Fig. 4.86: Bar diagram showing collagen type I of heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

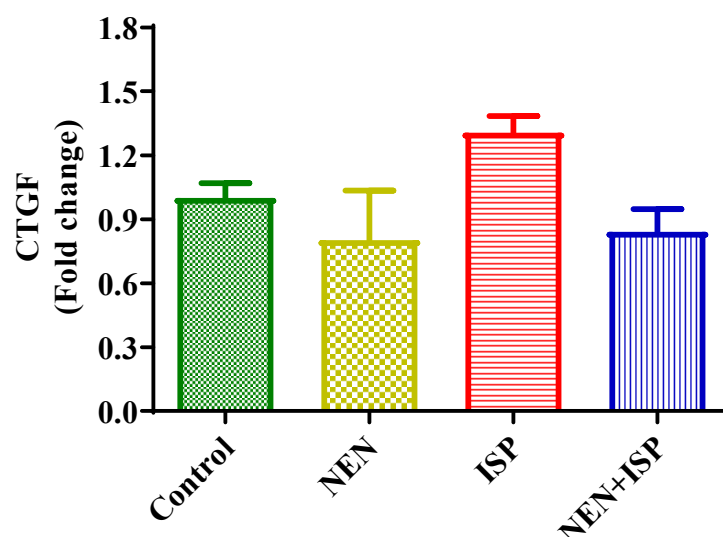


Fig. 4.87: Bar diagram showing the fold change in CTGF level in heart tissue in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test.

comparison to control group (1.00 ± 0.07 fold change; $n=6$). Furthermore, niclosamide ethanolamine treatment partially improved CTGF level (0.84 ± 0.11 fold change; $n=7$) in cardiac injury group, compared to ISP-alone administered (1.31 ± 0.08 fold change; $n=7$) groups of mice.

4.77 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on matrix metalloprotease-2 (MMP-2) activity in ISP-induced cardiac fibrosis in mice

The fold change in MMP-2 activity in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.77 and illustrated in Figure 4.88a. Figure 4.88b illustrate representative gel images showing MMP-2 activity. Data revealed that administration of ISP, significantly ($p < 0.05$) increased the MMP-2 activity in cardiac fibrosis (5.58 ± 1.96 fold change; $n=4$) compared to the control group (1.00 ± 0.00 fold change; $n=4$). A significant ($p < 0.05$) reduction was observed in MMP-2 activity in niclosamide ethanolamine-treated cardiac fibrotic group (1.04 ± 0.09 fold change; $n=4$) in comparison to ISP-alone administered group (5.58 ± 1.96 fold change; $n=4$). No significant difference was observed in MMP-2 activity in niclosamide ethanolamine-alone (1.61 ± 0.32 fold change; $n=4$) and control (1.00 ± 0.00 fold change; $n=4$) groups of mice.

Table 4.77: Fold change of MMP-2 activity in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	MMP-2 (Fold change)
Control (n=4)	1.00 ± 0.00
Niclosamide ethanolamine-alone (n=4)	1.61 ± 0.32
Isoprenaline-alone (n=4)	$5.58 \pm 1.96^*$
Niclosamide ethanolamine+ISP (n=4)	$1.04 \pm 0.09^\#$

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control group; $^\#p < 0.05$ vs ISP-alone administered group.

4.78 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on MMP-9 activity in ISP-induced cardiac fibrosis in mice

Table 4.78 and Figure 4.89a depict fold change in MMP-9 activity in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac fibrosis groups of mice. Figure 4.89b illustrates the representative gel image of MMP-9 activity in different groups. No significant change was observed in MMP-9 activity in control (1.00 ± 0.00 fold change; $n=2$), niclosamide ethanolamine-alone (0.86 ± 0.12 fold change; $n=2$), ISP-alone administered (1.19 ± 0.14 fold change; $n=2$) and niclosamide ethanolamine-treated cardiac fibrosis (1.06 ± 0.02 fold change; $n=2$) groups of mice.

Table 4.78: Fold change of MMP-9 activity in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	MMP-9 (Fold change)
Control (n=2)	1.00 ± 0.00
Niclosamide ethanolamine-alone (n=2)	0.86 ± 0.12
Isoprenaline-alone (n=2)	1.19 ± 0.14
Niclosamide ethanolamine+ISP (n=2)	1.06 ± 0.02

Data are presented as mean $\pm$ S.E.

4.79 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on Nrf-2 protein expression in heart tissue in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.79 and illustrated in Figure 4.90a depict the data of fold changes in Nrf-2 protein expression of heart tissue in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. The representative immune-blot images of Nrf-2 and GAPDH are illustrated in Figure 4.90b. A significant ($p < 0.05$) reduction in Nrf-2 protein expression was observed in ISP-alone administered group (0.51 ± 0.05 fold change; $n=3$) in comparison to control group (1.00 ± 0.00 fold change; $n=3$). Niclosamide ethanolamine treatment significantly ($p < 0.05$) increased the Nrf-2 protein expression (0.99 ± 0.18 fold change; $n=3$) in cardiac injury group compared to

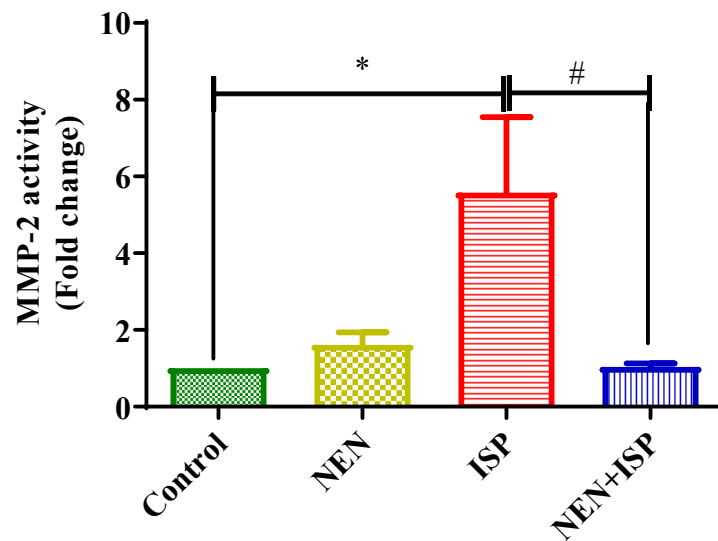


Fig. 4.88a: Bar diagram showing the fold change in MMP-2 activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

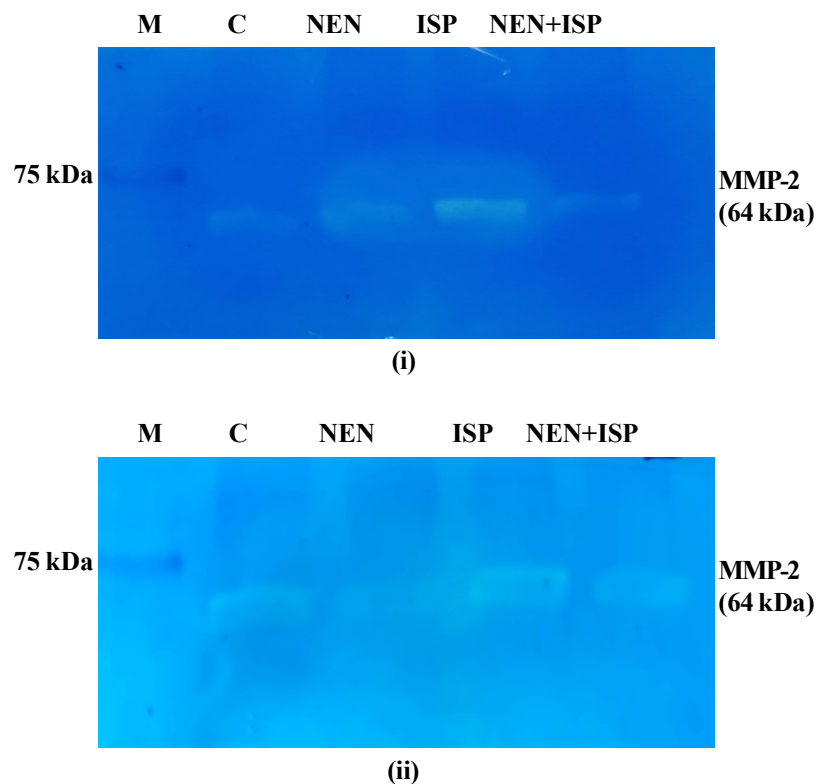


Fig. 4.88b: Representative images of gel (i & ii) showing MMP-2 activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. M-marker; C-control group; NEN-niclosamide ethanolamine-alone group; ISP-isoprenaline alone group; NEN+ISP- niclosamide ethanolamine +isoprenaline group.

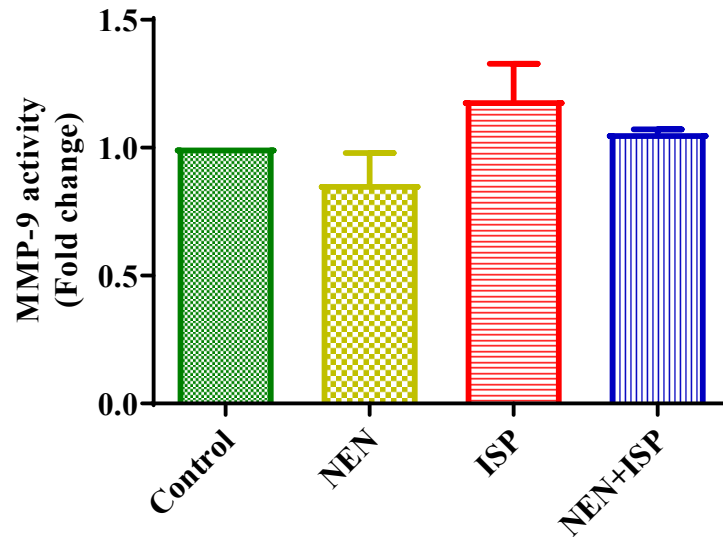


Fig. 4.89a: Bar diagram showing the fold change in MMP-9 activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test.

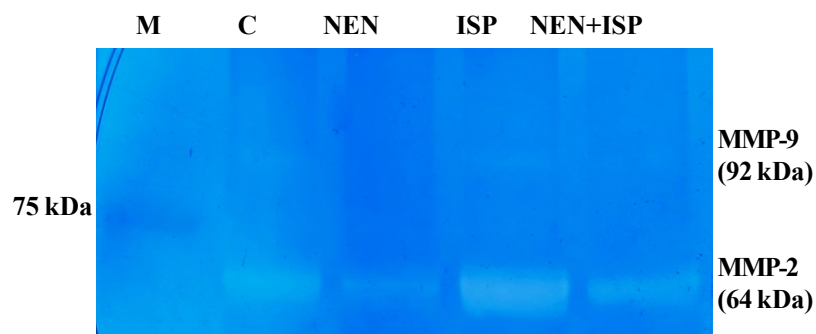


Fig. 4.89b: Representative image of gel showing MMP-9 activity in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. M-marker; C-control; NEN-niclosamide ethanolamine-alone; ISP- isoprenaline-alone; NEN+ISP- niclosamide ethanolamine + isoprenaline group.

ISP-alone administered group (0.51 ± 0.05 fold change; $n=3$). The Nrf-2 protein expression level was found to be almost comparable in control (1.00 ± 0.00 fold change; $n=3$) and niclosamide ethanolamine-alone (1.26 ± 0.06 fold change; $n=3$) groups of mice.

Table 4.79: Fold change of Nrf-2 protein expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Nrf-2 protein expression (Fold change)
Control ($n=3$)	1.00 ± 0.00
Niclosamide ethanolamine-alone ($n=3$)	1.26 ± 0.06
Isoprenaline-alone ($n=3$)	$0.51 \pm 0.05^*$
Niclosamide ethanolamine+ISP ($n=3$)	$0.99 \pm 0.18^\#$

Data are presented as mean $\pm$ S.E.

* $p < 0.05$ vs control group; $^\#p < 0.05$ vs ISP-alone administered group

4.80 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on NF- κ B protein expression in heart tissue in ISP-induced cardiac fibrosis in mice

Table 4.80 and Figure 4.91a show the fold change in NF- κ B protein expression in heart tissue of control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac injury groups of mice. Figure 4.91b illustrate the representative immunoblot images of NF- κ B protein expression. A significantly ($p < 0.05$) increased NF- κ B protein expression were observed in cardiac injury group (3.02 ± 0.74 fold change; $n=3$) compared to control (1.00 ± 0.00 fold change; $n=3$) group. Niclosamide ethanolamine treatment moderately improved the NF- κ B protein expression (1.13 ± 0.27 fold change; $n=3$) in comparison to ISP-alone administered (3.02 ± 0.74 fold change; $n=3$) group. No significant difference was observed in NF- κ B protein expression between niclosamide ethanolamine-alone (1.29 ± 0.31 fold change; $n=3$) and control (1.00 ± 0.00 fold change; $n=3$) groups of mice.

Table 4.80: Fold change of NF- κ B protein expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	NF- κ B (fold change)
Control (n=3)	1.00 $\pm$ 0.00
Niclosamide ethanolamine-alone (n=3)	1.29 $\pm$ 0.31
Isoprenaline-alone (n=3)	3.02 $\pm$ 0.74*
Niclosamide ethanolamine+ISP (n=3)	1.13 $\pm$ 0.27

Data are presented as mean $\pm$ S.E.

* p <0.05 vs control group.

4.81 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on TGF- β mRNA expression in heart in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.81 and illustrated in Figure 4.92 depict fold change in comparative mRNA expression of TGF- β in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac fibrosis groups of mice. The TGF- β and other mRNA expressions in different groups were normalized to corresponding GAPDH mRNA expression in heart tissue from different groups of mice. Figure 4.93 a & b illustrate real time PCR dissociation curve and amplification plot of GAPDH in heart tissue of different groups of mice. The mRNA expression of TGF- β was significantly (p <0.05) increased in isoprenaline-alone administered group (13.88 $\pm$ 5.71 fold change; n=5) in comparison to control group (1.00 $\pm$ 0.00 fold change; n=5). However, niclosamide ethanolamine treatment significantly (p <0.05) decreased the TGF- β mRNA expression in cardiac injury group (2.09 $\pm$ 0.76 fold change; n=5) compared to isoprenaline-alone group (13.88 $\pm$ 5.71 fold change; n=5). No significant difference in TGF- β mRNA expression was observed in niclosamide ethanolamine-alone (1.42 $\pm$ 0.62 fold change; n=5) and control (1.00 $\pm$ 0.00 fold change; n=5) groups of mice. Figure 4.94 a & b illustrate real time PCR dissociation curve and amplification plot of TGF- β in heart tissue of different groups of mice.

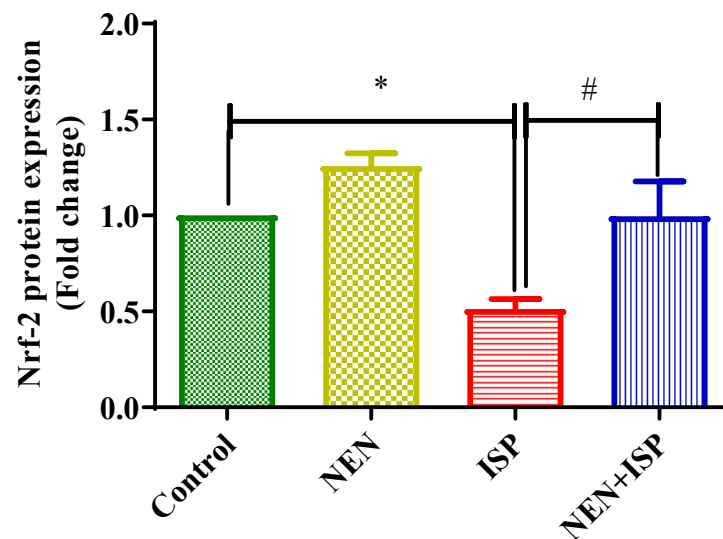


Fig. 4.90a: Bar diagram showing the fold change in Nrf-2 protein expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

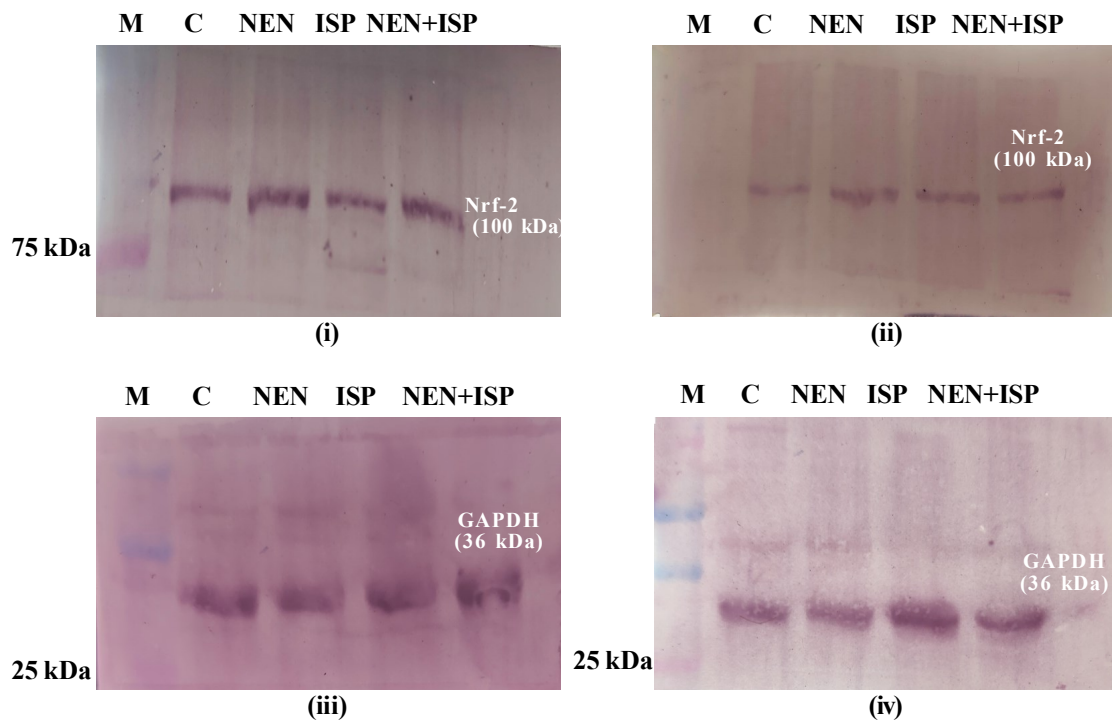


Fig. 4.90b: Representative immunoblot images showing Nrf-2 (i & ii) and GAPDH (iii & iv) protein expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. M-marker; C-control group; NEN-niclosamide ethanolamine-alone group; ISP-isoprenaline alone group; NEN+ISP- niclosamide ethanolamine+ISP group.

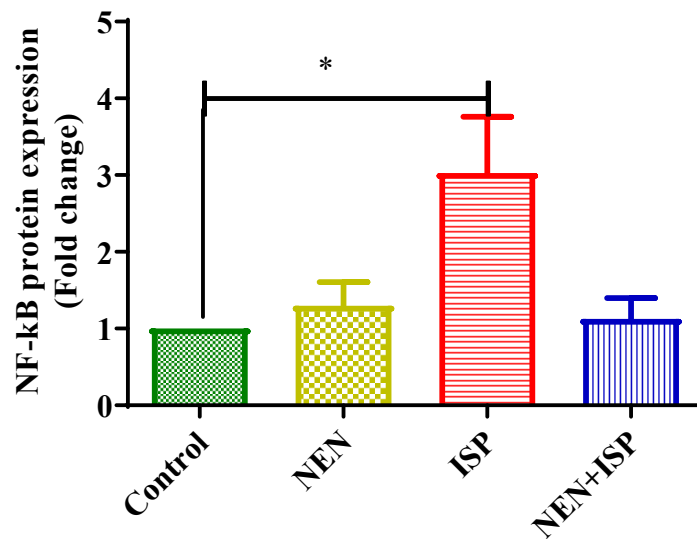


Fig. 4.91a: Bar diagram showing the fold change in NF- κ B protein expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p<0.05$) difference between control and ISP-alone administered groups.

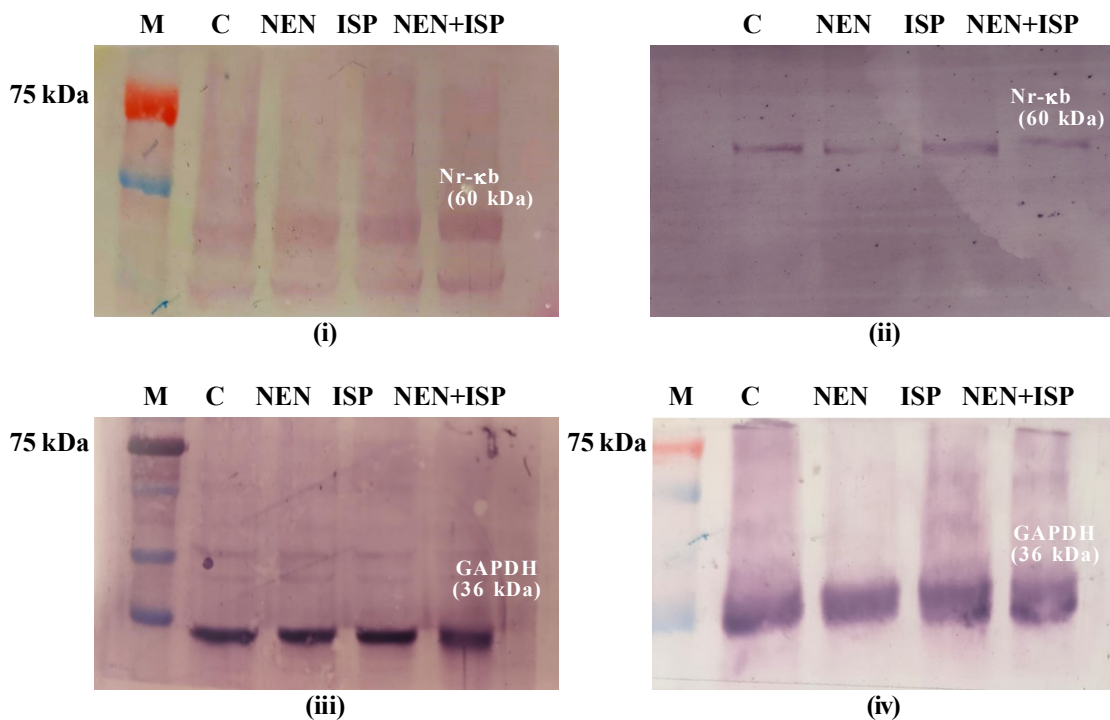


Fig. 4.91b: Representative image of immunoblot showing NF- κ B (i & ii) and GAPDH (iii & iv) protein expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. M-marker; C-control group; NEN-niclosamide ethanolamine-alone group; ISP-isoprenaline-alone group; NEN+ISP- niclosamide ethanolamine+ISP group.

Table 4.81: Comparative fold change in TGF- β mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	TGF- β mRNA expression (fold change)
Control (n=5)	1.00 $\pm$ 0.00
Niclosamide ethanolamine-alone (n=5)	1.42 $\pm$ 0.62
Isoprenaline-alone (n=5)	13.88 $\pm$ 5.71*
Niclosamide ethanolamine+ISP (n=5)	2.09 $\pm$ 0.76 [#]

Data are presented as mean $\pm$ S.E.

* p <0.05 vs control group; [#] p <0.05 vs ISP-alone administered group.

4.82 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on Smad-3 mRNA expression in heart in ISP-induced cardiac fibrosis in mice

The fold change in comparative Smad-3 mRNA expression in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-treated cardiac injury groups of mice are summarized in Table 4.82 and illustrated in Figure 4.95. Figure 4.96 a & b illustrate real time PCR dissociation curve and amplification plot of Smad-3 in heart tissue of different groups of mice. A significant (p <0.05) increase in the mRNA expression of Smad-3 was observed in isoprenaline-alone administered group (15.93 $\pm$ 6.38 fold change; n=4) in comparison to control group (1.00 $\pm$ 0.00 fold change; n=4). Niclosamide ethanolamine treatment significantly (p <0.05) reduced the Smad-3 mRNA expression in cardiac injury group (1.56 $\pm$ 0.52 fold change; n=4) compared to isoprenaline-alone administered group (15.93 $\pm$ 6.38 fold change; n=4). The Smad-3 mRNA expression in niclosamide ethanolamine-alone (2.07 $\pm$ 1.17 fold change; n=4) and control (1.00 $\pm$ 0.00 fold change; n=4) groups of mice were not different.

Table 4.82: Comparative fold change in Smad-3 mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Smad-3 mRNA expression (Fold change)
Control (n=4)	1.00±0.00
Niclosamide ethanolamine-alone (n=4)	2.07±1.17
Isoprenaline-alone (n=4)	15.93±6.38*
Niclosamide ethanolamine +ISP (n=4)	1.56±0.52 [#]

Data are presented as mean±S.E.

* $p < 0.05$ vs control group; [#] $p < 0.05$ vs ISP-alone administered group.

4.83 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on α -SMA mRNA expression in heart in ISP-induced cardiac fibrosis in mice

Table 4.83 and Figure 4.97 depict fold change in comparative mRNA expression of α -SMA in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac fibrosis groups of mice. Figure 4.98 a & b illustrate real time PCR dissociation curve and amplification plot of α -SMA in heart tissue of different groups of mice. The mRNA expression of α -SMA was significantly ($p < 0.05$) increased in isoprenaline-alone administered group (8.22±3.23 fold change; n=4) in comparison to control group (1.00±0.00 fold change; n=4). The α -SMA mRNA expression was moderately decreased in niclosamide ethanolamine-treated cardiac injury group (1.61±0.44 fold change; n=4) compared to isoprenaline-alone group (8.22±3.23 fold change; n=4). The α -SMA mRNA expression was found to be almost comparable in niclosamide ethanolamine-alone (1.68±0.32 fold change; n=4) and control (1.00±0.00 fold change; n=4) groups of mice.

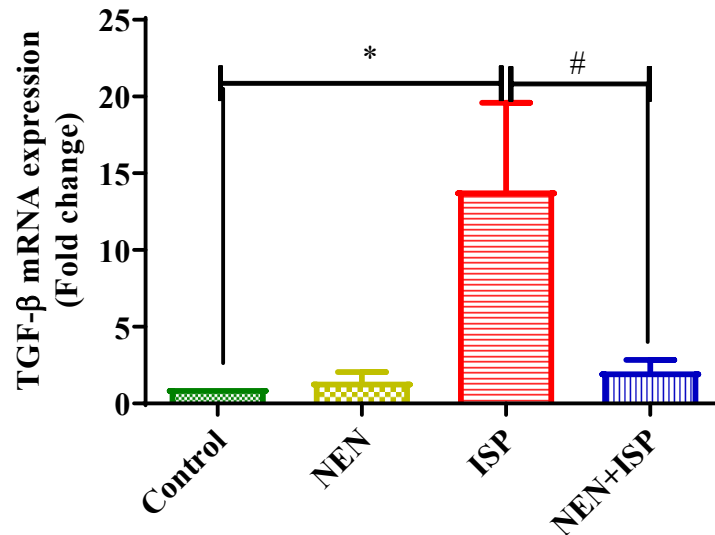


Fig. 4.92: Bar diagram showing the comparative fold change in TGF- β mRNA expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p<0.05$) difference between groups of mice.

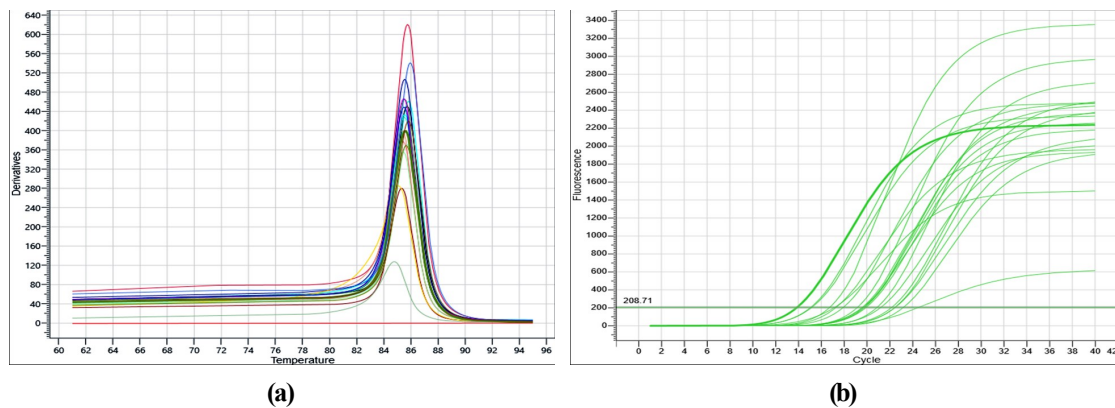
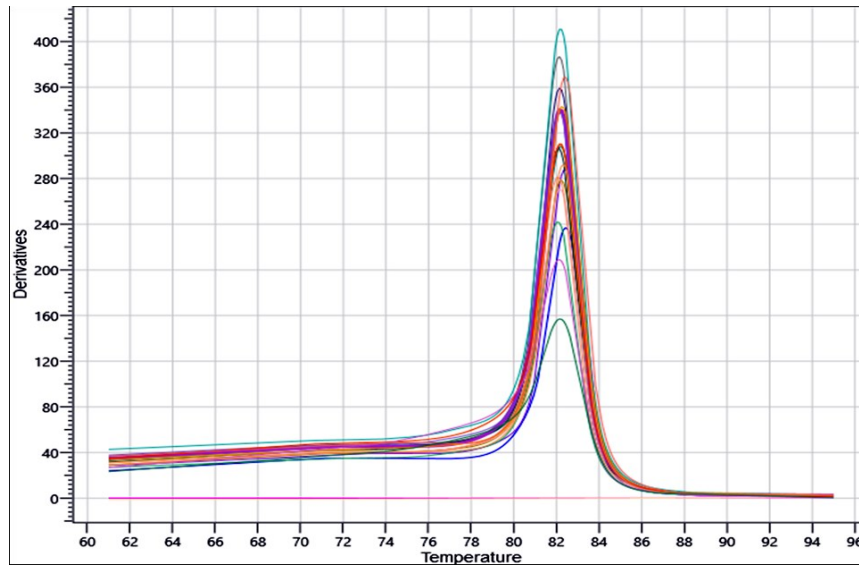
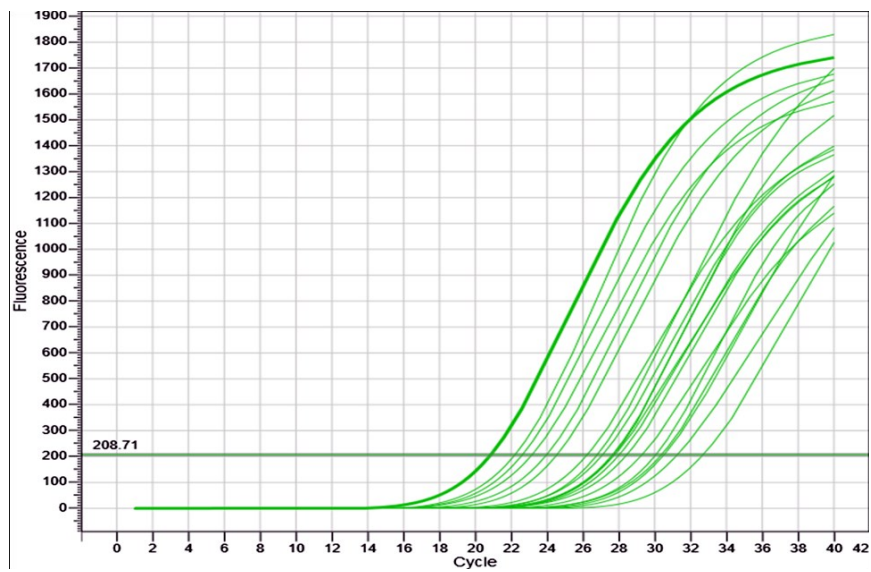


Fig. 4.93: qRT-PCR melt curve plot (a) and amplification plot (b) of GAPDH mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.



(a)



(b)

Fig. 4.94: qRT-PCR melt curve plot (a) and amplification plot (b) of TGF- β mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.

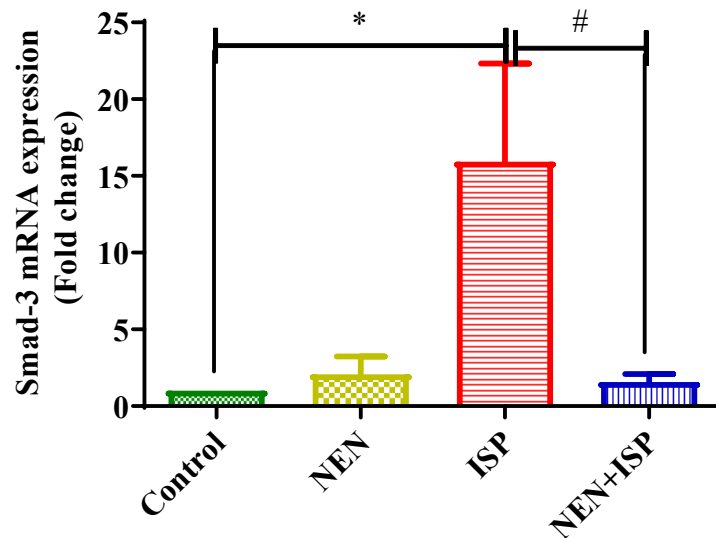


Fig. 4.95: Bar diagram showing the comparative fold change in Smad-3 mRNA expression in heart in control, NEN-alone, ISP-alone and NEN+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (*, #) on the bars present significant ($p < 0.05$) difference between groups.

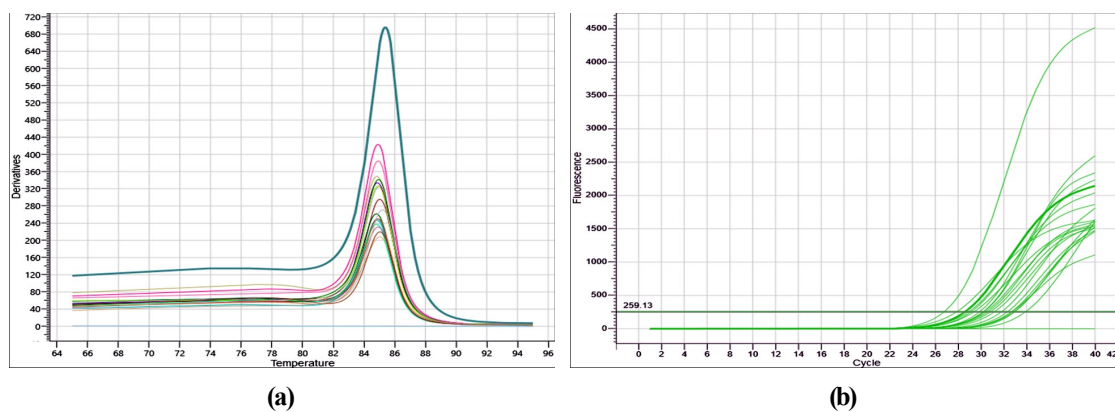


Fig. 4.96: qRT-PCR melt curve plot (a) and amplification plot (b) of Smad-3 mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.

Table 4.83: Comparative fold change in α -SMA mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	α -SMA mRNA expression (Fold change)
Control (n=4)	1.00 $\pm$ 0.00
Niclosamide ethanolamine-alone (n=4)	1.68 $\pm$ 0.32
Isoprenaline-alone (n=4)	8.22 $\pm$ 3.23*
Niclosamide ethanolamine+ISP (n=4)	1.61 $\pm$ 0.44

Data are presented as mean $\pm$ S.E.

* p <0.05 vs control group.

4.84 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on TNF- α mRNA expression in heart in ISP-induced cardiac fibrosis in mice

Data summarized in Table 4.84 and illustrated in Figure 4.99 depict fold change in comparative mRNA expression of TNF- α in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac fibrosis groups of mice. Figure 4.100 a & b illustrate real time PCR dissociation curve and amplification plot of TNF- α in heart tissue of different groups of mice. The mRNA expression of TNF- α was significantly (p <0.01) increased in isoprenaline-alone administered group (26.65 $\pm$ 6.12 fold change; n=4) in comparison to control group (1.00 $\pm$ 0.00 fold change; n=4). The TNF- α mRNA expression was significantly (p <0.01) decreased in niclosamide ethanolamine-treated cardiac injury group (6.51 $\pm$ 2.84 fold change; n=4) compared to isoprenaline-alone group (26.65 $\pm$ 6.12 fold change; n=4). TNF- α mRNA expression was not different in niclosamide ethanolamine-alone (3.03 $\pm$ 1.71 fold change; n=4) and control (1.00 $\pm$ 0.00 fold change; n=4) groups of mice.

Table 4.84: Comparative fold change in TNF- α mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	TNF- α mRNA expression (Fold change)
Control (n=4)	1.00 $\pm$ 0.00
Niclosamide ethanolamine-alone (n=4)	3.03 $\pm$ 1.71
Isoprenaline-alone (n=4)	26.65 $\pm$ 6.12**
Niclosamide ethanolamine+ISP (n=4)	6.51 $\pm$ 2.84 ^{##}

Data are presented as mean $\pm$ S.E.

** p <0.01 vs control group; ^{##} p <0.01 vs ISP-alone administered group.

4.85 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on IL-6 mRNA expression in heart in ISP-induced cardiac fibrosis in mice

Table 4.85 and Figure 4.101 depict fold change in comparative mRNA expression of IL-6 in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac fibrosis groups of mice. Figure 4.102 a & b illustrate real time PCR dissociation curve and amplification plot of IL-6 in heart tissue of different groups of mice. The mRNA expression of IL-6 did not significantly differ in isoprenaline-alone administered group (10.19 $\pm$ 5.62 fold change; n=4) in comparison to control group (1.00 $\pm$ 0.00 fold change; n=4). No significant difference was observed in the IL-6 mRNA expression in niclosamide ethanolamine-treated cardiac injury group (3.54 $\pm$ 0.93 fold change; n=4) compared to isoprenaline-alone group (10.19 $\pm$ 5.62 fold change; n=4). IL-6 mRNA expression was not different in niclosamide ethanolamine-alone (6.77 $\pm$ 3.81 fold change; n=4) and control (1.00 $\pm$ 0.00 fold change; n=4) groups of mice.

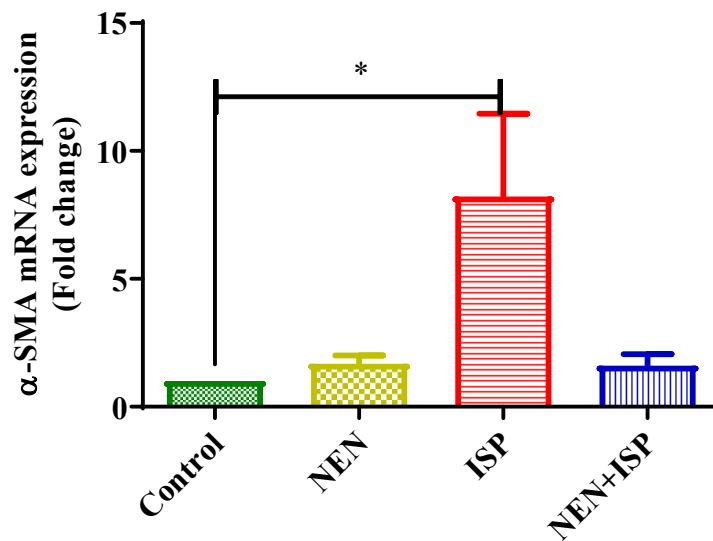


Fig. 4.97: Bar diagram showing the comparative fold change in α -SMA mRNA expression in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (*) on the bars present significant ($p < 0.05$) difference between control and isoprenaline-alone administered groups.

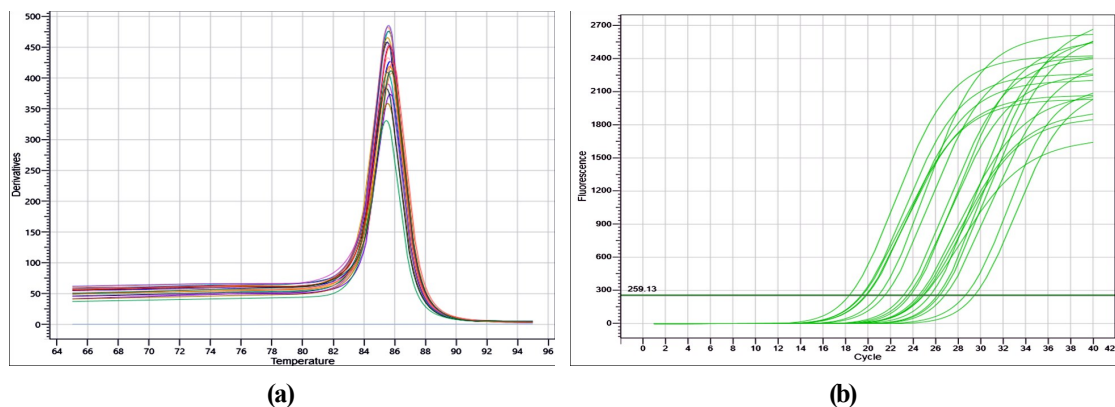


Fig. 4.98: qRT-PCR melt curve plot (a) and amplification plot (b) of α -SMA mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.

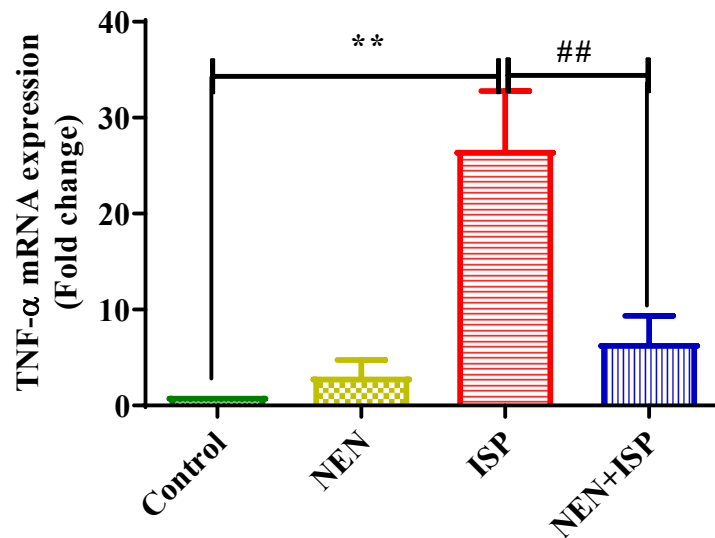


Fig. 4.99: Bar diagram showing the comparative fold change in TNF- α mRNA expression in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Different superscripts (**, ##) on the bars present significant ($p < 0.01$) difference between groups.

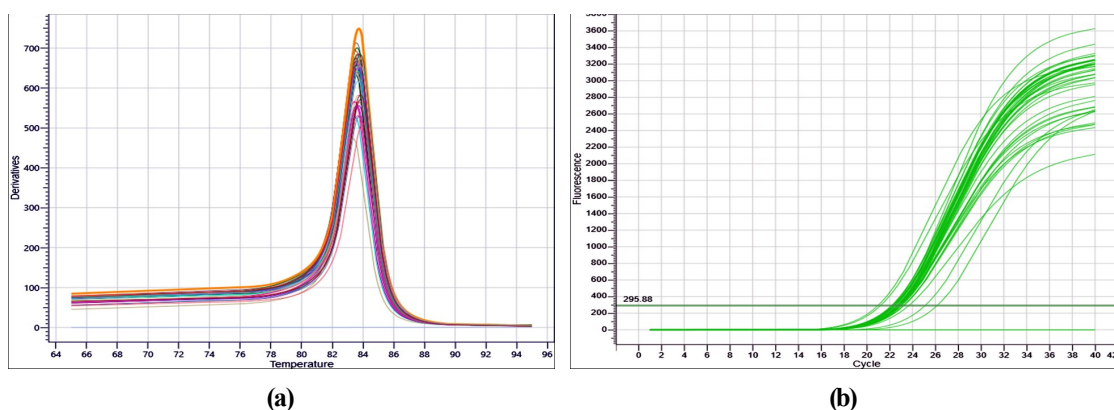


Fig. 4.100: qRT-PCR melt curve plot (a) and amplification plot (b) of TNF- α mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.

Table 4.85: Comparative fold change in IL-6 mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	IL-6 mRNA expression (fold change)
Control (n=4)	1.00±0.00
Niclosamide ethanolamine-alone (n=4)	6.77±3.81
Isoprenaline-alone (n=4)	10.19±5.62
Niclosamide ethanolamine+ISP (n=4)	3.54±0.93

Data are presented as mean±S.E.

4.86 Effect of niclosamide ethanolamine (3 mg/kg body weight; I.P.) on collagen type III mRNA expression in heart of mice

The comparative fold change of mRNA expression of collagen type III in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine-treated cardiac fibrosis groups of mice are summarized in Table 4.86 and illustrated in Figure 4.103. Figure 4.104 a & b show real time PCR dissociation curve and amplification plot of collagen type III in heart tissue of different groups of mice. No significant difference was observed in the mRNA expression of collagen type III in isoprenaline-alone administered (7.33 ± 3.40 fold change; n=4) and control groups (1.00 ± 0.00 fold change; n=4). Niclosamide ethanolamine treatment did not significantly improve the collagen type III mRNA expression (2.91 ± 0.96 fold change; n=4) compared to isoprenaline-alone group (7.33 ± 3.40 fold change; n=4). Collagen type III mRNA expression was not different in niclosamide ethanolamine-alone (3.18 ± 1.79 fold change; n=4) and control (1.00 ± 0.00 fold change; n=4) groups of mice.

Table 4.86: Comparative fold change in collagen type III mRNA expression in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Collagen type III mRNA expression (fold change)
Control (n=4)	1.00±0.00
Niclosamide ethanolamine-alone (n=4)	3.18±1.79
Isoprenaline-alone (n=4)	7.33±3.40
Niclosamide ethanolamine+ISP (n=4)	2.91±0.96

Data are presented as mean±S.E.

4.87 Histopathological examination of heart of niclosamide ethanolamine (3 mg/kg body weight; I.P.) study

Table 4.87 and Figure 4.105 depict the total scoring of inflammation and fibrosis of histopathological examination of heart of control, niclosamide ethanolamine-alone, ISP-alone administered and niclosamide ethanolamine-treated cardiac injury groups. The normal architecture of cardiomyocytes, normal striation of muscles and fibroblast cells were observed in control and niclosamide ethanolamine-alone administrated groups as evident by H & E staining. In cardiac injury group, extensive infiltration of mononuclear cells along with the proliferation of fibroblast cells was observed, which was improved in niclosamide ethanolamine-treated cardiac injury groups. MTS staining showed the normal striation of cardiac muscles with minimal collagen content (blue colored) in control and niclosamide ethanolamine-alone groups. Proliferative fibrotic cells with extensive collagen deposition were observed in ISP-induced cardiac injury group, which was improved in niclosamide ethanolamine-treated cardiac injury group. A significantly ($p<0.001$) higher total scoring of inflammatory infiltration and fibrosis was observed in cardiac injury group (5.00 ± 0.41 score; $n=4$) compared to control group (0.25 ± 0.25 score; $n=4$). Niclosamide ethanolamine treatment moderately improved the total scoring of inflammation and fibrosis (4.40 ± 0.75 score; $n=5$) in cardiac injury compared to ISP-alone administered (5.00 ± 0.41 score; $n=4$) group. No significant difference was observed in total scoring of control (0.25 ± 0.25 score; $n=4$) and niclosamide ethanolamine-

alone administered (0.50 ± 0.50 score; $n=4$) groups. Figure 4.106 (a, b, c, & d) and Fig. 4.107 (a, b, c, d) illustrate the microphotographs of H & E staining and MTS staining of different groups of mice.

Table 4.87: Total histopathological scoring of inflammation and fibrosis in heart tissue in control, niclosamide ethanolamine-alone, isoprenaline-alone and niclosamide ethanolamine-administered cardiac injury (NEN+ISP) groups of mice

Groups (mice)	Histopathological scoring
Control (n=4)	0.25 ± 0.25
Niclosamide ethanolamine-alone (n=4)	0.50 ± 0.50
Isoprenaline-alone (n=4)	$5.00 \pm 0.41^{***}$
Niclosamide ethanolamine+ISP (n=5)	4.40 ± 0.75

Data are presented as mean $\pm$ S.E.

$^{***}p < 0.001$ vs control group.



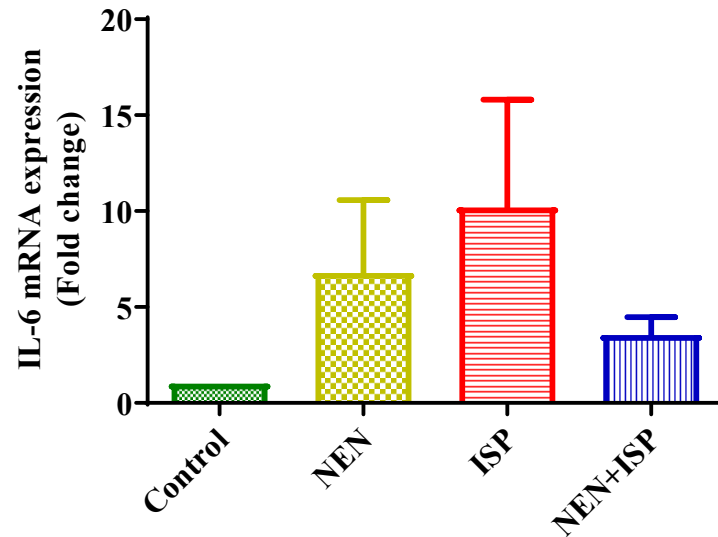


Fig. 4.101: Bar diagram showing the comparative fold change in IL-6 mRNA expression in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test.

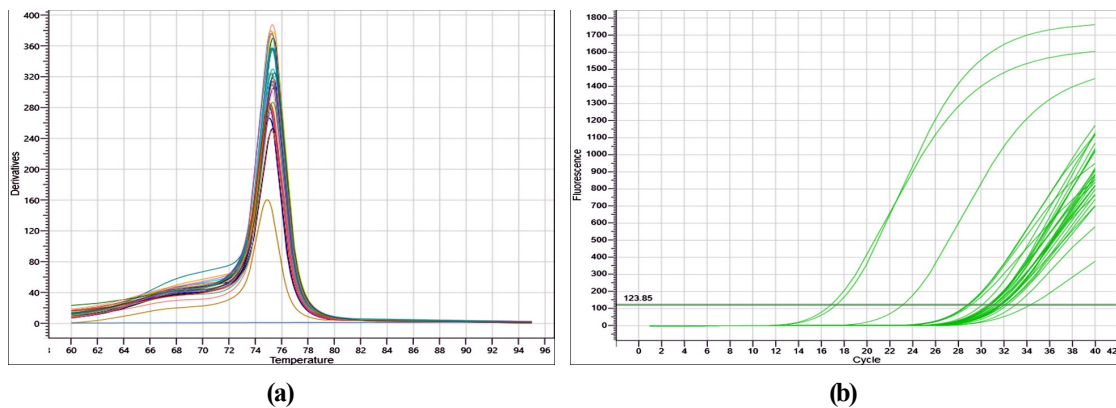


Fig. 4.102: qRT-PCR melt curve plot (a) and amplification plot (b) of IL-6 mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.

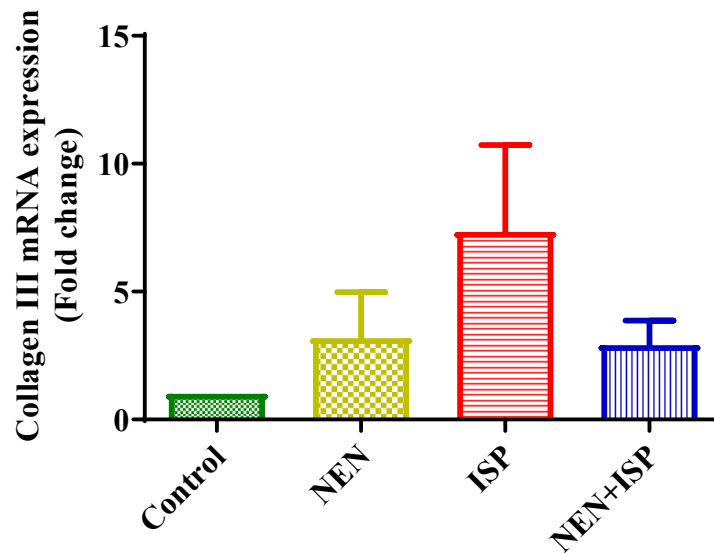


Fig. 4.103: Bar diagram showing the comparative fold change in collagen type III mRNA expression in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test.

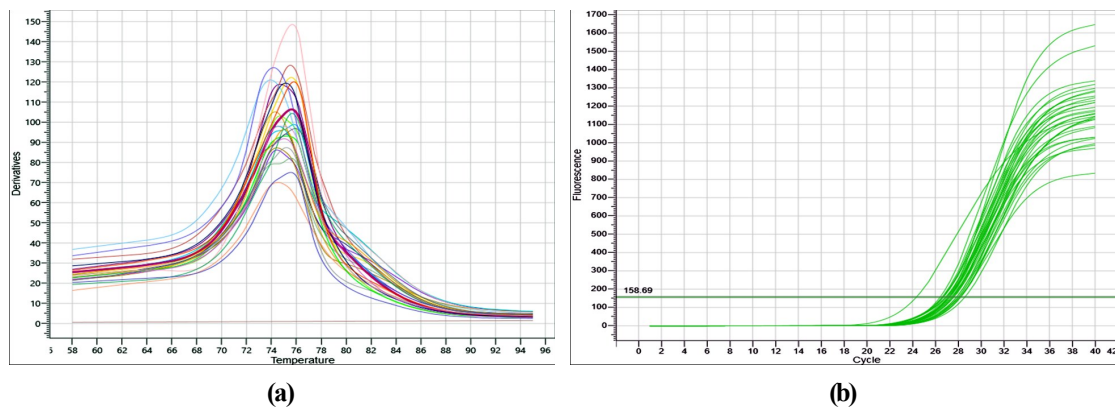


Fig. 4.104: qRT-PCR melt curve plot (a) and amplification plot (b) of collagen type III mRNA expression in the control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice.

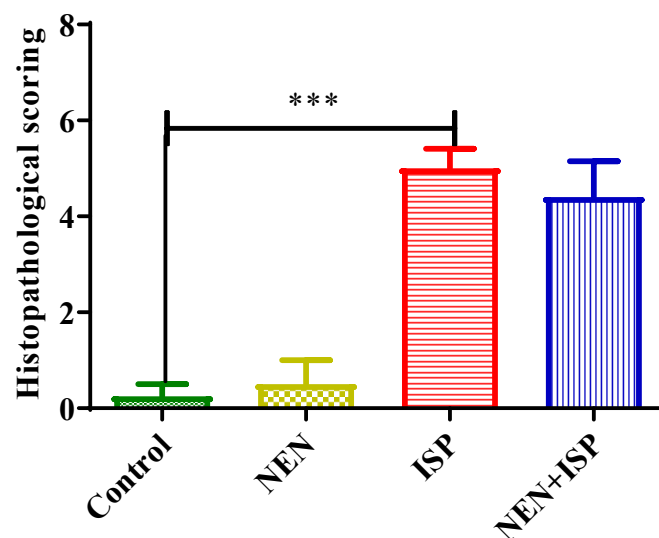


Fig. 4.105: Bar diagram showing the total histopathological scoring of inflammation and fibrosis in heart in control, niclosamide ethanolamine-alone, ISP-alone and niclosamide ethanolamine+ISP groups of mice. Data are analyzed by One-way ANOVA followed by *Tukey's post hoc* test. Superscript (***) on the bar present significant ($p < 0.001$) difference between control and ISP-alone administered groups.

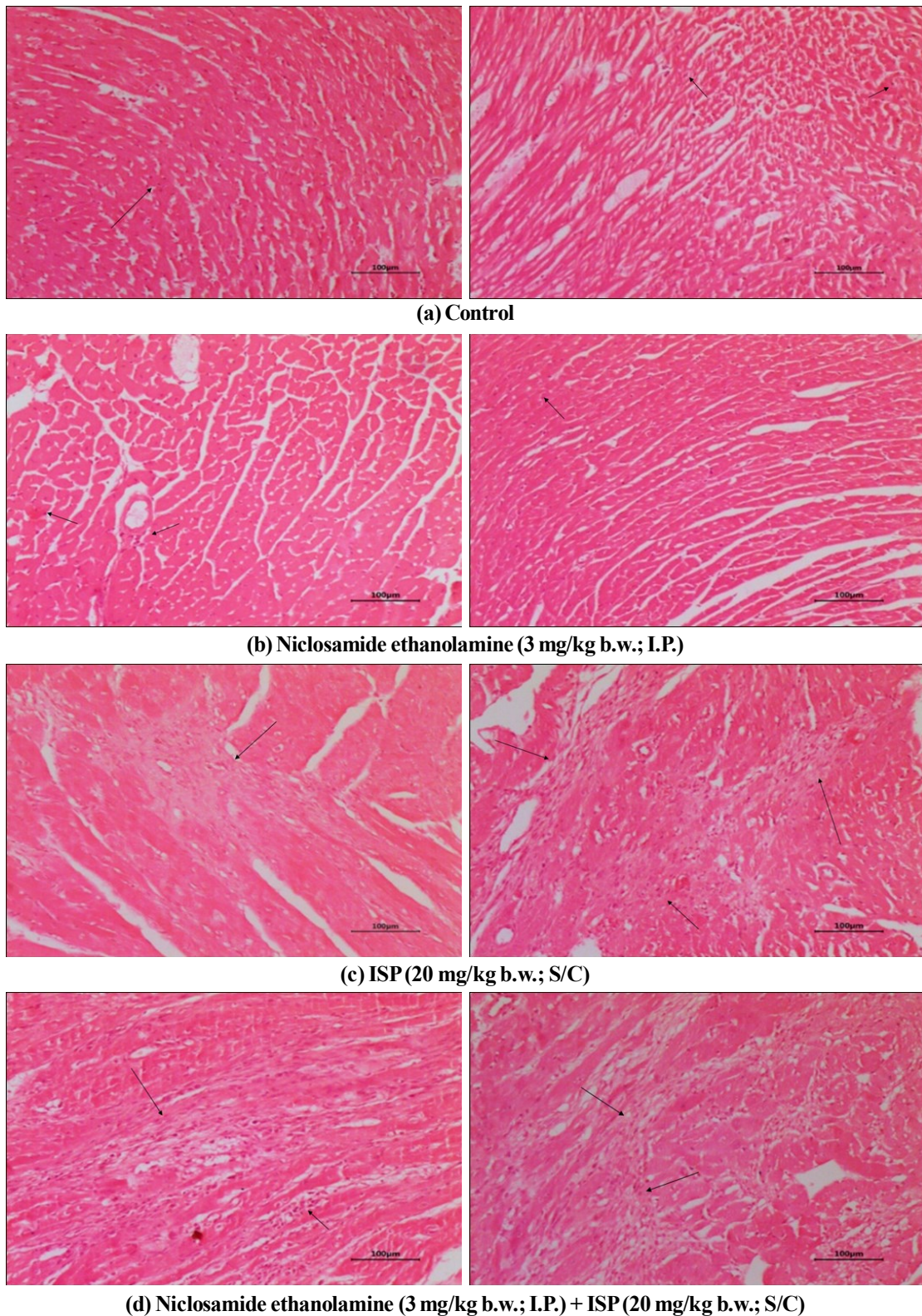


Fig. 4.106: Effect of niclosamide ethanolamine (NEN) on histopathology (H & E staining) of heart tissue in ISP-induced cardiac fibrosis in mice. a) Control mice showing the normal histoarchitecture of cardiomyocytes (arrow) (H & E, 100 X); b) Heart tissue of NEN-alone group showing normal cardiomyocytes with minimal inflammatory cell infiltration (arrow) (H & E, 100X); c) Heart tissue of ISP-induced cardiac injury showing extensive inflammatory cell infiltration and proliferative fibrotic cells (H & E, 100X); d) NEN-treated cardiac injury (NEN+ISP) mice heart showing moderate proliferation of fibroblast and inflammatory cell infiltration (arrow) (H & E, 100X).

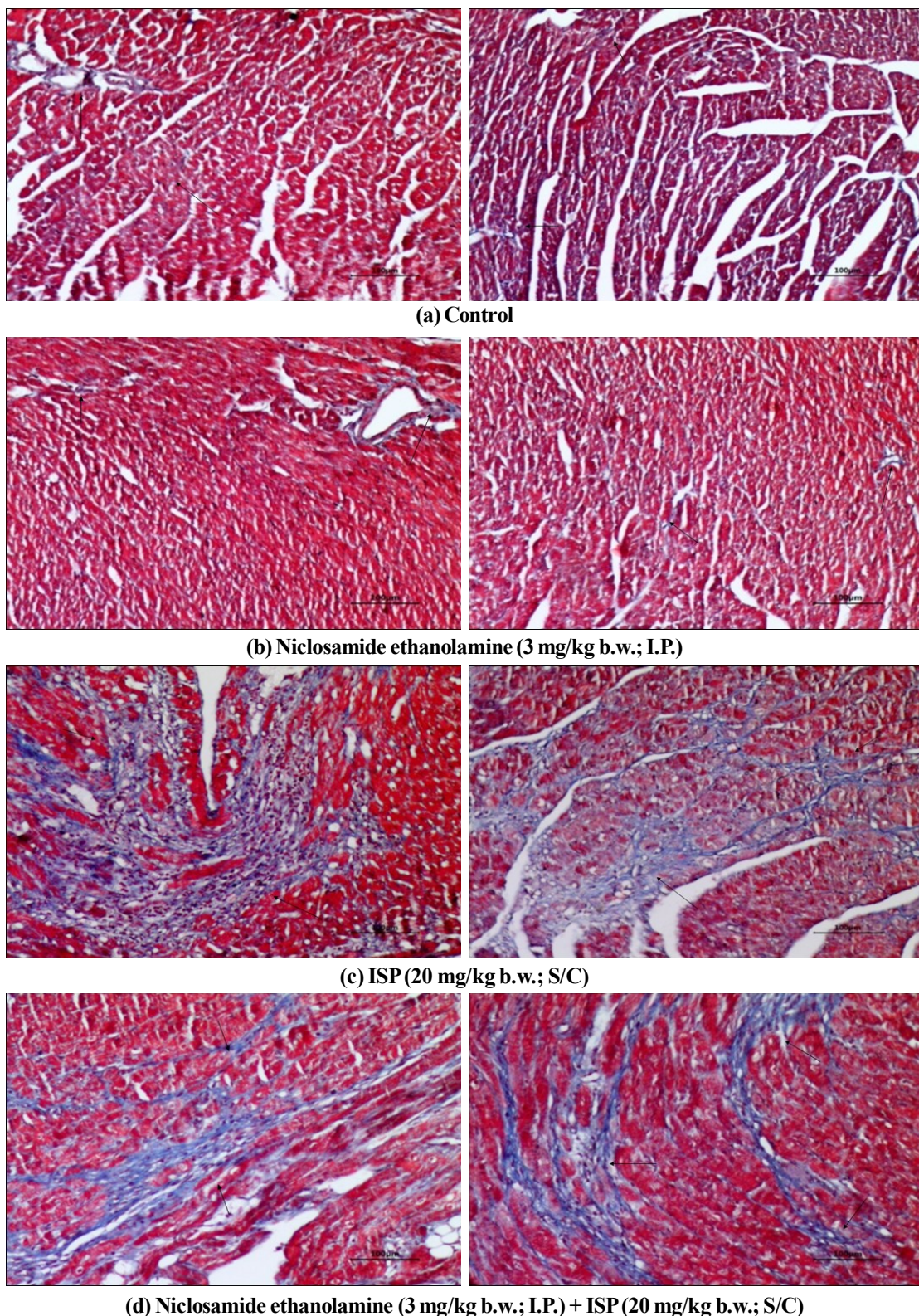
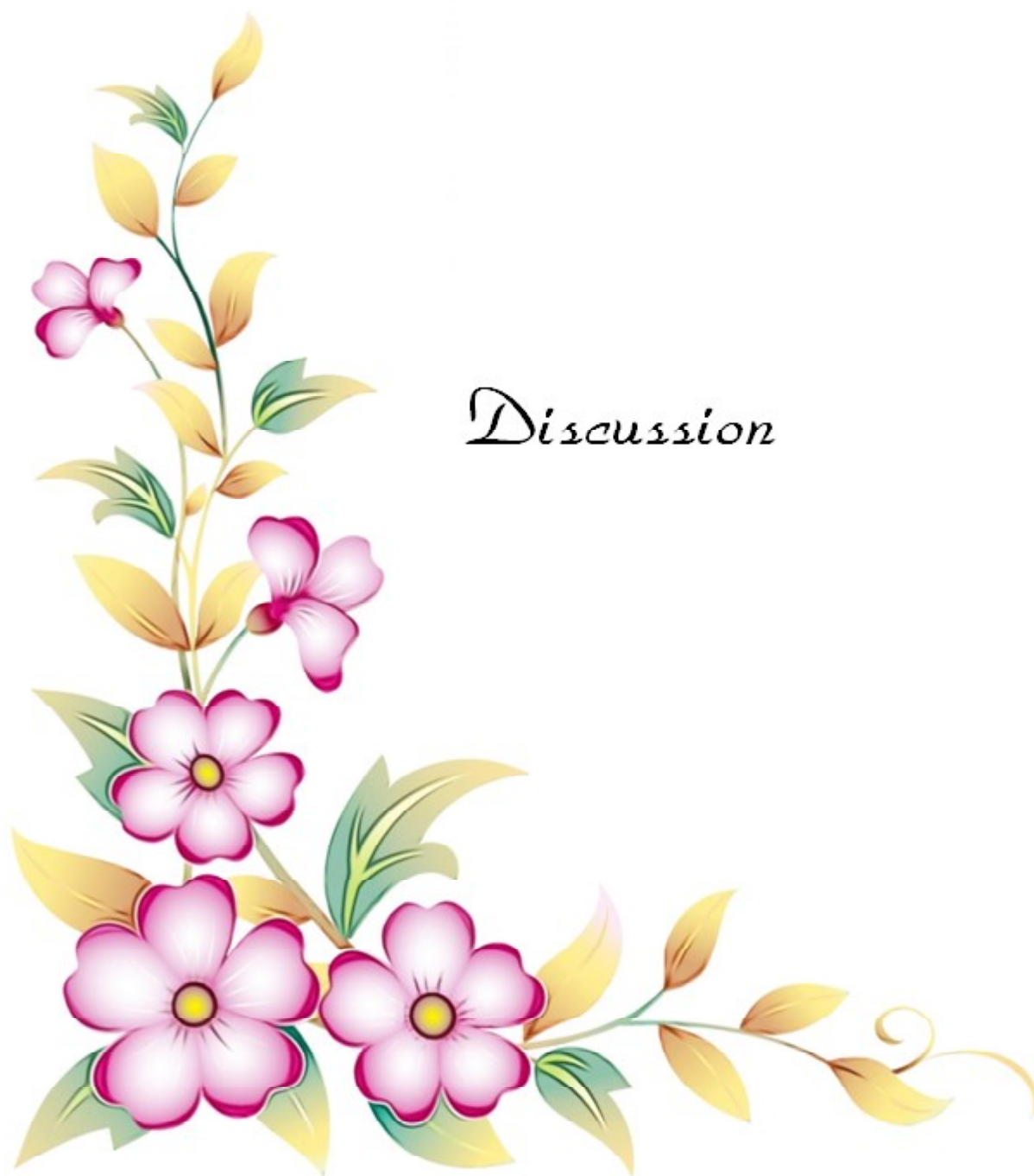


Fig. 4.107: Effect of niclosamide ethanolamine (NEN) on histopathology (Masson's Trichrome staining) of heart tissue in ISP-induced cardiac fibrosis in mice. a) Control mice showing mild proliferation of fibrotic cells and collagen deposition (arrow) (MTS; 100 X); b) Heart tissue of NEN-alone group showing mild proliferation of fibrotic cells with minimal collagen deposition (arrow) (MTS, 100X); c) Heart tissue of ISP-induced cardiac injury showing increased proliferation of fibroblasts in multifocal areas along with extensive collagen deposition (arrow) (MTS; 100X); d) NEN-treated cardiac injury (NEN+ISP) mice heart showing moderate proliferation of fibroblast and moderate collagen deposition (arrow) (MTS, 100X).



Discussion

Cardiovascular diseases (CVDs) remain a leading cause of death worldwide, posing significant challenges due to their widespread prevalence, morbidity, mortality, disability, and substantial economic burdens (Mensah *et al.*, 2019). Cardiovascular diseases (CVDs) affect both human and animals. Myocardial fibrosis, a consequence of cardiac remodeling post-myocardial infarction (MI), is associated with high morbidity and mortality. Cardiac fibroblasts (CFs) play a pivotal role in this process by proliferating and transforming into myofibroblasts, which deposit excessive extracellular matrix (ECM), particularly collagen. This accumulation stiffens the ventricle wall, leading to cardiac dysfunction and eventual heart failure (Yang *et al.*, 2021).

Experimental animal models are crucial for understanding cardiovascular disease mechanisms that facilitate the exploration of diagnostic and therapeutic avenues for cardiac diseases. Various animal models viz., spontaneously hypertensive, surgical, and drug-induced models are used for cardiac fibrosis research. Isoprenaline (ISP)-induced model is commonly used to induces cardiac hypertrophy, fibroblast activation, and fibrosis which mimic disease progression while being minimally invasive. ISP leads to cardiac energy depletion, causing biochemical and structural changes in cardiomyocytes. ISP-induced cardiac fibrosis involves oxidative stress, inflammation, MMP activation, and pro-fibrotic growth factor activation (Dai *et al.*, 2019).

Orlistat also known as tetrahydrolipstatin is a semisynthetic derivative of lipstatin which is an irreversible blocker of pancreatic lipase, and naturally obtained from *Streptomyces*

toxytricini (Barbier and Schneider, 1987). Orlistat is an over-the-counter anti-obesity drug approved by the Food and Drug Administration (FDA) in 1999 (Stephanie, 2007).

Niclosamide is an oral antihelminthic drug that used for the therapy of parasitic infection globally. Niclosamide acts as an oxidative phosphorylation uncoupler and interferes with helminth's energy metabolism, potentially by blocking the glucose uptake and production of adenosine triphosphate (ATP) (Frayha *et al.*, 1997; Swan, 1999; Chen *et al.*, 2018). Various activities viz., anti-oxidant, anti-inflammatory, anti-cancer activity and many more pharmacological activities of orlistat and niclosamide have been reported.

5.1 Orlistat study

Major findings of the orlistat study are as: isoprenaline @ 20 mg/ kg body weight via subcutaneous route for 14 consecutive days led to cardiac injury viz., cardiac hypertrophy, and cardiac fibrosis. Orlistat @ 30 mg/kg body weight, I.P. for 14 consecutive days significantly improved the cardiac hypertrophy parameters viz., absolute and relative heart weight, cardiac injury markers viz., CK-MB and LDH level and lipid profile. Orlistat had shown anti-oxidant effect in ISP-induced cardiac fibrosis by markedly enhancing the SOD activity, significantly increasing the catalase and reduced glutathione level and reduced MDA level in both serum and heart tissue. Furthermore, Nrf-2 protein expression was also significantly increased in orlistat-treated cardiac fibrosis group. Orlistat treatment reduced the collagen deposition and fibrotic markers as evident by hydroxyproline, collagen type I, glucosamine level and histopathology in heart tissue. Orlistat treatment also significantly improved the inflammatory mediatory viz., NF- κ B protein expression, inflammatory cell infiltration in histopathological examination of heart and mRNA expression for TNF- α , and IL-6. Fibrogenic modulators, including mRNA expression for TGF- β , SMAD-3, α -SMA, CTGF, and collagen I, collagen III, MMP-activation also improved in orlistat-treated cardiac injury.

Cardiac remodeling is an adaptive process however, chronic stimuli lead to the maladaptive remodeling causing pathological cardiac hypertrophy. Sustained activation of adrenoceptor is a key hallmark of pathological cardiac hypertrophy and serves as crucial factor for maladaptive cardiac remodeling, including myocardial hypertrophy, cardiac fibrosis

and apoptosis, which may lead to advancement of myocardial hypertrophy to the heart failure and mortality (Fu *et al.*, 2012; Li *et al.*, 2023). In the present study, body weight of the mice in all groups viz., control, isoprenaline-induced cardiac injury, orlistat-alone administered and orlistat-treated cardiac injury groups was significantly increased on day 15th compared to the zero day of experiment. However, the percentage change in body weight during experimental period was least and significantly lower in ISP-alone administered group compared to control group. A significantly higher percentage increase in body weight was observed in orlistat-treated cardiac injury group. Furthermore, the cardiac hypertrophy markers i.e. morphometric parameters viz., absolute heart weight, heart weight to body weight ratio (HWT/BWT) and heart weight to tibial length (HWT/TL) were significantly increased in ISP-induced cardiac fibrosis group which are in accordance with previous studies (Chen *et al.*, 2019; Rana *et al.*, 2023; Sharma *et al.*, 2023). Increased heart weight in isoprenaline-induced cardiac hypertrophy may be contributed by increased water content, edema of intramuscular space and inflammatory cells infiltration followed by necrosis of cardiomyocytes (Patel *et al.*, 2010; Ahmed *et al.*, 2023). Orlistat treatment significantly reduced these morphometric parameters like absolute and relative heart weight in ISP-induced cardiac fibrosis. Othman and co-worker (2022) demonstrated the lower diameter of cardiomyocyte in orlistat-treated obese rats, suggesting the cardiomyocyte hypertrophy ameliorating tendency of orlistat. Furthermore, another study has also reported that orlistat treatment significantly decreased the absolute left ventricular weight, left ventricular weight to body weight ratio, left ventricular wall thickness and cardiomyocyte diameter in pathological and physiological cardiac hypertrophy in obese rats (Singh and Krishan, 2010).

CK-MB is a predominant isoform of the CK enzyme in heart responsible for the energy metabolism in cardiomyocytes. LDH is an enzyme of the anaerobic metabolism. Both CK-MB and LDH are found in cardiac tissue and serve as cardiac injury markers. Isoprenaline causes the alteration in metabolism and insufficient oxygen supply to myocardial cell, resulting in cardiac damage. These degenerative changes in cardiac tissues lead to the leakage of these enzymes in bloodstream causing elevated level (Mert *et al.*, 2018; Song *et al.*, 2020; Ahmed *et al.*, 2023). Previous studies have reported the elevated level of serum cardiac injury markers

viz., CK-MB and LDH in ISP-induced cardiac injury (Song *et al.*, 2020; Shi *et al.*, 2022; Ahmed *et al.*, 2023; Rana *et al.*, 2023). In present study, we also found elevated level of serum CK-MB and LDH in ISP-induced cardiac injury group compared to control group. Orlistat-treatment significantly reduced these cardiac injury markers viz., CK-MB and LDH in ISP-induced cardiac injury. Present findings are in agreement with previous study which showed the lower level of serum CK-MB and LDH in orlistat-treated obese rats (Othman *et al.*, 2022).

Lipids are significant contributors to the development of cardiovascular diseases. Lipolysis is an important causal factor for isoprenaline-mediated myocardial injury. The activity of adenyl cyclase is enhanced by isoprenaline, leading to the increased cAMP formation which phosphorylate hormone-sensitive lipase followed by increased hydrolysis of triacylglycerol and myocardial lipid accumulation (Radhiga *et al.*, 2012). In the present study, a significant increase in total cholesterol, triglyceride, LDL-c along with decrease in HDL-c and HDL-c to LDL-c ratio were observed in ISP-induced cardiac injury group, which are in accordance with the previous reports (Peer *et al.*, 2008; Radhiga *et al.*, 2012; Panda *et al.*, 2017; Cinar *et al.*, 2020). Orlistat treatment significantly decreased the triglyceride level and increased HDL-c level in cardiac injury group. Furthermore, moderate improvement in cholesterol, LDL-c and HDL-c to LDL-c ratio was also observed in orlistat-treated groups. Singh and Krishan (2010) showed that orlistat treatment significantly reduced the cholesterol and triglyceride levels along with significant increase in HDL-c in experimental myocardial hypertrophy in obese rats. Several other studies have also reported the efficacy of the orlistat in lipid profile improvement viz., decreased cholesterol, triglyceride, LDL-c along with increased HDL-c level which are in agreement with present findings (Menezes-Rodrigues *et al.*, 2018; Zakaria *et al.*, 2021; Othman *et al.*, 2022; Javed *et al.*, 2023).

Catecholamines are crucial for regulating myocardial contractility and metabolism. Isoprenaline, a synthetic β -adrenoceptor agonist, triggers cardiotoxicity by generating free radicals through auto-oxidation. This process forms toxic compounds like amino-chrome, producing superoxide anions and hydrogen peroxide. Subsequently, highly reactive hydroxyl radicals form which leads damage in proteins, DNA, and lipids (Khalil *et al.*, 2015). Excessive

free radical production compromises the integrity and function of myocardial membranes. Over-production of oxidants and free radical exceed the endogenous antioxidant capacity of cells and lead to the oxidative stress. Additionally, these free radicals further propagate the damage by forming peroxy radicals through attacks on polyunsaturated fatty acids, resulting in lipid peroxidation and cardiac damage (Khalil *et al.*, 2015; Allawadhi *et al.*, 2018). The primary cellular free radical scavenging enzymes viz., SOD and catalase serve as cellular defence against oxidative injury. These enzymes prevent the highly reactive hydroxyl radical formation by scavenging the free radicals like superoxide anion and hydrogen peroxide (Khalil *et al.*, 2015). Malondialdehyde (MDA) is associated with lipid peroxidation and serves as indicator for the plasma membrane damage (Wu *et al.*, 2018).

In the present investigation, we found the increased level of MDA along with decreased enzymatic antioxidant viz., SOD, and catalase, and non-enzymatic anti-oxidant i.e. reduced glutathione (GSH) in both serum and heart tissue. Nrf2 is a redox-sensitive transcription factor activated in response to elevated levels of reactive oxygen species (ROS). Its activation leads to the transcription of genes encoding antioxidant enzymes (Althunibat *et al.*, 2022). A significantly decreased Nrf-2 protein expression was observed in heart tissue of ISP-induced cardiac injury group. Previous studies have also reported similar alteration in ISP-mediated oxidative stress causing cardiac injury (Wu *et al.*, 2018; Hasan *et al.*, 2020; Li *et al.*, 2023). In present investigation, orlistat treatment significantly increased the SOD, catalase and GSH level in both serum and heart in ISP-induced cardiac fibrosis group. MDA level in serum and heart were significantly reduced in orlistat treated group. Furthermore, orlistat treatment significantly increased the Nrf-2 protein expression in heart. These findings suggest the anti-oxidant activity of orlistat and may help to check the progression of cardiac fibrosis induced by ISP. The findings of current investigation are in agreement with the previous study which demonstrated increased SOD, and catalase, decreased MDA and upregulated Nrf-2 and other antioxidant gene expression in obese rats suggesting the anti-oxidant activity of orlistat in oxidative stress-mediated myocardial damage (Othman *et al.*, 2022). Another study by Zakaria and co-workers (2021) showed the protective and therapeutic effect of orlistat against high fed diet-induced metabolic dysfunction-associated fatty liver disease (MAFLD) rats by

activating Nrf-2 signaling, suggesting anti-oxidant activity of orlistat. Furthermore, Othman and co-workers (2022) also demonstrated the anti-oxidant activity of orlistat by improving the antioxidant enzymes and lipid peroxidation in aortic tissue in obese rats.

In animal models, exposure to the isoprenaline either acute or chronic leads to the necrosis, hypertrophy and fibrosis in heart. Excessive deposition of the collagen in extracellular matrix (ECM) is a characteristic feature of the pathological cardiac fibrosis (Borbély *et al.*, 2009). ECM serves as scaffolding for the various components of cell and helps to maintain homeostasis, coordinates contractile function and electrical coupling between cardiomyocytes (Martin and Blaxall, 2012). Under normal condition, cardiac fibroblasts produce ECM components, most commonly collagen type I and III and it is continuous process of newer collagen being synthesized and older being degraded. Adult human cardiac muscle is composed of approximately 85% of collagen type I and 11% of collagen type III (Hinderer and Schenke-Layland, 2019). Fibroblasts maintain this balance in collagen level by the production of cytokines, growth factors and MMPs (Baum and Duffy, 2011).

Myocardial injury leads to the abnormal regulation of the synthesis and degradation of ECM, resulting the accumulation of collagen and eventually myocardial fibrosis. Hydroxyproline is a post-translational product of proline hydroxylation which exclusively indicates the collagen content (Reddy and Enwemeka, 1996; Wang *et al.*, 2019). In the present study, the hydroxyproline in tissue was increased significantly in ISP-induced cardiac fibrosis group, which is in accordance to the previous reports (Liu *et al.*, 2015; Wang *et al.*, 2019; Jin *et al.*, 2020). Amino sugar glucosamine is an important precursor for glycosaminoglycan and glycoproteins viz., proteoglycan synthesis which are abundant component of extracellular matrix along with collagen (Jerosch *et al.*, 2011). Therefore, level of glucosamine indicates ECM deposition. A significantly increased level of glucosamine in heart tissue was observed in ISP-induced cardiac fibrosis in present investigation. During pathological remodelling, cardiac fibroblasts transdifferentiate into myofibroblasts which produce excessive collagen deposition, leading to progression of myocardial fibrosis (Yang *et al.*, 2021). Collagen content was significantly increased in ISP-induced cardiac fibrosis group as evident by increased collagen type I in heart tissue along with upregulated mRNA expression of collagen type I and collagen

type III in this group. Furthermore, collagen deposition in present study was also supported by histopathological examination of heart tissue by Masson's Trichrome staining which showed extensive proliferation of fibrous tissue and deposition of collagen fibers as blue coloured in ISP-induced cardiac fibrosis group compared to control group. Several earlier studies have also reported the increased hydroxyproline content, collagen type I, collagen type III in isoproterenol-mediated cardiac fibrosis models (Wang *et al.*, 2019; Yang *et al.*, 2021; Rana *et al.*, 2023). Orlistat treatment significantly reduced hydroxyproline and glucosamine level in heart tissue in ISP-induced cardiac fibrosis. Furthermore, moderate reduction in collagen type I and downregulation of mRNA expression in collagen type III along with significantly downregulated collagen type I mRNA in heart tissue were observed in orlistat-treated cardiac fibrosis. Present findings are supported by the previous study by Zakaria and co-workers (2021) in which they reported that orlistat as a reference drug decreased collagen deposition in Masson's Trichrome stain and fibrosis score liver in MAFLD in obese rats. Another earlier study also reported the decreased liver collagen content in orlistat administered group in obese and non-alcoholic fatty liver disease (Pai *et al.*, 2019). Furthermore, El-Baz and co-workers (2019) also reported that orlistat treatment as a reference drug reduced collagen type III in cardiac tissue in high fat diet fed obese rats.

Cardiac insult leads to recruitment of immune and inflammatory cells viz macrophage, mast cells, granulocytes, dendritic cells, monocytes, B and T-lymphocytes to injury site (Murtha *et al.*, 2017). Nuclear factor-kappa β is one of the well-known transcription factors that regulates mainly inflammation-related genes. NF- κ B is activated by several pro-fibrotic stimuli, producing the ROS, growth factors, cytokines and hypoxia and directly regulates the fibrosis-related genes expression such as MMPs and fibronectin (Ma *et al.*, 2018). Myocardial injury triggers the inflammatory response and releases the pro-inflammatory cytokines viz., TNF- α and IL-6. These pro-inflammatory cytokines activate the cardiac fibroblast and transdifferentiate them in myofibroblasts that increase the ECM deposition, contributing to the cardiac fibrosis progression (Shi *et al.*, 2022). The activated NF- κ B further stimulates the production of TNF- α and IL-6 (Thangaiyan *et al.*, 2020). Several earlier studies reported the upregulation of inflammatory cytokines viz., TNF- α and IL-6 and NF- κ B in ISP-induced cardiac fibrosis

(Shaik *et al.*, 2020; Thangaiyan *et al.*, 2020; Shi *et al.*, 2022). We also observed a significantly upregulated NF- κ B protein expression and TNF- α mRNA expression in heart tissue in ISP-induced cardiac fibrosis. However, the IL-6 mRNA expression was moderately upregulated in ISP-induced cardiac fibrosis. This ISP-induced inflammatory condition was also supported by histopathological examination that revealed the more infiltration of inflammatory cells in cardiac tissue in ISP-alone group. C-reactive proteins (CRP) serves as crucial factor for the cardiovascular diseases and promotes the cardiac remodeling (Zhang *et al.*, 2010). The serum CRP level was significantly elevated in ISP-induced cardiac fibrosis group which is in accordance with the previous studies (Viswanadha *et al.*, 2020; Pan *et al.*, 2022). In the present investigation, orlistat treatment significantly reduced the NF- κ B protein expression, downregulated the TNF- α and IL-6 mRNA expression. In addition, we also observed reduction in inflammatory cell infiltration in cardiac tissue on histopathology and moderately reduced serum CRP in orlistat-treated cardiac fibrosis group. These findings are in consistent with earlier report which reported that orlistat either alone or in combination with metformin reduced the TNF- α and CRP in obese rats (Hamza and Alsolami, 2023). Furthermore, orlistat treatment also reduced NF- κ B in obesity-induced colorectal cancer (Jin *et al.*, 2021). Another study by Othman and co-workers (2022) also reported downregulation in NF- κ B and TNF- α mRNA expression in heart tissue in obese rats. In addition, it has also reported that orlistat as a reference drug reduced the IL-6 and CRP level in obese rats (Javed *et al.*, 2023).

Cardiac fibroblast cells play a crucial role in maintaining the balance of the extracellular matrix (ECM). In addition to synthesizing extracellular matrix proteins, they also produce metalloproteinases of the matrix (MMPs) and their inhibitors. Hence, proper regulation of MMPs and their inhibitors is essential for maintaining ECM homeostasis (Moore *et al.*, 2012). MMP-2 and MMP-9 are one of the key contributors of the myocardial remodeling and cardiac fibrosis. In the present study, mild changes were observed in MMP-9 activity. Whereas a significant increased activity of MMP-2 was observed in ISP-induced cardiac fibrosis group which is in accordance with the previous studies (Radhiga *et al.*, 2019; Thangaiyan *et al.*, 2020). Orlistat treatment significantly reduced the MMP-2 activity in ISP-induced cardiac fibrosis in present study.

Myocardial fibrosis is a complex condition of multiple mechanisms involving various growth factors viz., TGF- β and CTGF and other mediators. TGF- β 1 is a master profibrotic mediator that regulates the ECM production and tissue homeostasis and involved in fibrosis. TGF- β transmit the signals by phosphorylating and activating the downstream Smad proteins. These phosphorylated Smad proteins translocate into nucleus and act as transcription factor. Activation of the TGF- β 1/Smad-3 pathway enhances the collagen expression and promotes fibrosis (Wang *et al.*, 2019). In present study, ISP-alone administration upregulated the mRNA of TGF- β 1, Smad-3, collagen type I, and III, indicating the cardiac fibrosis. These findings are accordance to the multiple previous studies which showed the upregulated TGF- β 1, Smad-3 and collagen deposition (Che *et al.*, 2019; Wang *et al.*, 2019; Thangaiyan *et al.*, 2020). In present study, we observed a significant reduction in mRNA expression of TGF- β 1, Smad-3 and collagen type I in orlistat-treated cardiac fibrotic group. The previous study by Vasileva and co-workers (2021) showed that orlistat as a reference drug for anti-obesity downregulated the TGF- β 1 mRNA expression in obesity-related inflammation in human adipocytes.

CTGF is another important downstream modulator of TGF- β pathway. It is crucial pro-fibrotic marker that is activated by TGF- β transcriptionally and plays a pivotal role in facilitating tissue fibrosis and remodeling by stimulating cell adhesion, migration, angiogenesis, myofibroblast activation, and ECM deposition (Ning *et al.*, 2017; Li *et al.*, 2019). The results of present investigation showed an increased level of CTGF and its mRNA expression in heart in ISP-administered mice which are in accordance with the previous studies (Li *et al.*, 2013; Ning *et al.*, 2017; Li *et al.*, 2019). However, orlistat treatment moderately improved the CTGF level and mRNA expression in ISP-induced cardiac fibrosis. During pathological remodeling, increased cytokines, inflammatory cells, oxidative stress and upregulated growth factor viz., TGF- β 1 activates cardiac fibroblast and transforms them into myofibroblasts. α -SMA is a contractile protein that is expressed in myofibroblasts and promotes the excessive collagen production during fibrosis progression. Several previous studies have reported the upregulated α -SMA expression in ISP-induced cardiac fibrosis (Song *et al.*, 2020; Zhao *et al.*, 2020; Yang *et al.*, 2021). We also found significantly upregulated mRNA expression of α -SMA in ISP-alone administered mice which was significant downregulated in orlistat-treated mice.

5.2 Niclosamide ethanolamine (NEN @ 3 mg/kg body weight) study

Major findings of the niclosamide ethanolamine study are as: isoprenaline @ 20 mg/kg body weight via subcutaneous route for 14 consecutive days led to cardiac injury that progress to cardiac hypertrophy, and cardiac fibrosis. Niclosamide ethanolamine (NEN) @ 3 mg/kg body weight, I.P. on alternate days for 14 consecutive days significantly improved the cardiac hypertrophy parameters viz., absolute heart weight, and heart weight to tibial length. Furthermore, heart weight to body weight ratio, cardiac injury markers viz., CK-MB and LDH, lipid profile were moderately improved by niclosamide ethanolamine in ISP-induced cardiac injury. Niclosamide ethanolamine had also exhibited anti-oxidant effect in cardiac injury as evident by improved enzymatic anti-oxidant activities viz., SOD and catalase, non-enzymatic anti-oxidant i.e. GSH, and MDA level in both serum and heart. Furthermore, Nrf-2 protein expression was also significantly increased in niclosamide ethanolamine-treated cardiac fibrosis group. Collagen deposition and fibrotic markers viz., hydroxyproline and glucosamine in heart were also reduced in niclosamide ethanolamine-treated cardiac injury group which is also supported by histopathological examination. Niclosamide ethanolamine also showed anti-inflammatory activity as evident by reduced serum CRP, NF- κ B protein expression and downregulated mRNA expression for TNF- α and IL-6 and decreased inflammatory cell infiltration in histopathological examination. Moreover, niclosamide ethanolamine treatment also improved fibrogenic modulators, including TGF- β , SMAD-3, α -SMA, and collagen III, MMP-2 activation.

Cardiac hypertrophy is generally a compensatory response to increased workload during stress or injury. In chronic stimuli pathological cardiac hypertrophy progress to fibrosis marked by the buildup of extracellular matrix proteins like collagen type I and fibronectin (Ryu *et al.*, 2016). In present study, body weight of control, niclosamide-alone administered and niclosamide-treated cardiac injury groups were increased significantly on day 15th compared to the day zero. However, body weight was slightly increased in ISP-induced cardiac injury on day 15th compared to day zero. Furthermore, the percentage increase in body weight during experimental period was least and significantly lower in cardiac injury group. However, niclosamide ethanolamine treatment significantly increased the body weight during experimental

period in cardiac injury group. In current investigation, we also observed the significantly increased absolute heart weight, heart weight to body weight and heart weight to tibial length in ISP-induced cardiac injury group which is in accordance to previous study (Ryu *et al.*, 2016). Niclosamide ethanolamine treatment significantly decreased the absolute and relative heart weight in cardiac injury group. Fu and co-workers (2021) reported that orally administered niclosamide improved cardiac hypertrophy in pressure-overload mediated heart failure in mice. Another study has also reported that niclosamide ethanolamine treatment significantly decreased the kidney weight, kidney weight to body weight in db/db mice, suggesting it's renal hypertrophy ameliorating action (Han *et al.*, 2018).

Several previous studies reported that isoprenaline administration elevated the cardiac injury markers viz., CK-MB and LDH (Thangaiyan *et al.*, 2020; Rana *et al.*, 2023). In the present study we also found significantly increased level of CK-MB and LDH in ISP-induced cardiac injury group. Niclosamide ethanolamine treatment significantly decreased the CK-MB level along with moderately improved LDH in cardiac injury. The findings of current investigation are in agreement with previous study which showed moderately lower level of CK and LDH in niclosamide ethanolamine-treated diabetic kidney disease (Han *et al.*, 2018).

Lipid profile is one of the crucial factors for cardiovascular diseases and catecholamines serve as lipolytic hormones. In the present study, we found the significantly increased levels of total cholesterol, triglyceride, and LDL-c. We also observed decreased HDL-c and HDL-c/LDL-c level in cardiac injury group. These findings are in consistent with the earlier reports (Al-Rasheed *et al.*, 2015; Dhivya *et al.*, 2017). Niclosamide ethanolamine treatment moderately improved lipid profile viz., cholesterol, triglyceride, HDL-c, LDL-c and HDL-c/LDL-c in cardiac injury. Guo and co-workers (2017) reported that niclosamide ethanolamine improved the lipid profile by decreasing cholesterol, triglyceride and LDL-c along with increasing HDL-c in lysosomal acid lipase deficient mice. Another study has also reported that niclosamide administration significantly decreased cholesterol, triglyceride and LDL-c level along with increasing HDL-c in obese mice (Al-Gareeb *et al.*, 2017).

It is well established fact that isoprenaline stimulates the β -adrenoceptor to cause oxidative stress leading to inflammation and tissue damage (Hasan *et al.*, 2020). Furthermore,

ISP also triggers the free radical generation by amino-chrome formation. Imbalance between cellular antioxidants and free radical generation leads to oxidative stress and further propagate to lipid peroxidation and cardiac damage (Allawadhi *et al.*, 2018). Nrf-2 is a pivotal transcriptional factor that regulate the antioxidant genes such as SOD and catalase. In present study, we found decreased antioxidants viz., SOD, catalase and GSH both in serum and heart tissue homogenate. We also observed significantly higher level of MDA in serum and heart in cardiac injury group. Furthermore, Nrf-2 protein expression in heart was downregulated in cardiac injury group in current investigation. Earlier study by Chisty and co-workers (2024) reported the decreased antioxidants i.e. SOD, catalase and GSH along with elevated MDA level in isoprenaline-induced cardiac injury. The findings of current study are also in agreement with previous report which showed decreased antioxidants, increased MDA and downregulated Nrf-2 in ISP-induced cardiac injury. Niclosamide ethanolamine significantly increased the antioxidants, including SOD, catalase, and GSH with concurrent decreased MDA level in serum and heart tissue in cardiac injury. Furthermore, we also observed that niclosamide ethanolamine significantly upregulated Nrf-2 protein expression in heart in cardiac fibrosis. Previously Esmail and co-workers (2021) reported the increased antioxidants viz., SOD and GSH along with decreased MDA level in niclosamide-treated lipid fibrosis induced by bile duct ligation. The hepatoprotective activity of niclosamide ethanolamine in lipotoxic-stressed mice through Nrf-2 pathway activation has also been reported (Park *et al.*, 2019). Moreover, niclosamide ethanolamine exhibited nephroprotective activity via increasing catalase and promoting H₂O₂ scavenging in kidney disease (Han *et al.*, 2019).

Cardiac fibrosis involves the excessive buildup of extracellular matrix viz., collagen within the myocardium, representing a fundamental aspect of various cardiac pathological states (Kong *et al.*, 2014). Hydroxyproline is one of the key components of collagen that indicates the collagen content (Wang *et al.*, 2019). Being a precursor of glycosaminoglycan and glycoproteins, glucosamine indicates ECM deposition (Jerosch *et al.*, 2011). A significantly increased hydroxyproline, glucosamine and collagen type I were found in isoprenaline-induced cardiac fibrosis in the present investigation. Furthermore, moderately upregulated mRNA expression for collagen type III was observed in cardiac injury group. Furthermore, the

histopathological examination by Masson's Trichrome staining showed extensive proliferation of fibrous tissue along with excessive deposition of collagen in ISP-induced cardiac fibrosis. These findings are in accordance to the previous reports (Wang *et al.*, 2019; Rana *et al.*, 2023). Niclosamide ethanolamine significantly decreased the hydroxyproline, glucosamine and collagen type I in heart tissue in cardiac injury. Present findings were also supported by histopathological examination of heart by Masson's trichrome staining which showed reduction in proliferative tissue and collagen deposition. Gan and co-workers (2023) have also reported the similar alterations and showed that niclosamide nanoparticles decreased hydroxyproline, collagen type I deposition and improved the fibrosis score on Masson's trichrome staining of lung in bleomycin-induced pulmonary fibrosis. Another earlier study also reported the decreased hydroxyproline, and collagen in niclosamide ethanolamine treated pulmonary fibrosis in mice (Pei *et al.*, 2022). Present findings are also in agreement with another previous study which showed that niclosamide improved the cardiac fibrosis in pressure-overload induced heart failure by inhibiting the collagen secretion by cardiac fibroblasts (Fu *et al.*, 2021).

Inflammation also plays an important role in isoprenaline-induced cardiac fibrosis. Several previous studies have reported the upregulation of inflammatory mediators viz., C-reactive proteins, NF- κ B, TNF- α , IL-6 and more infiltration of inflammatory cells in cardiac fibrosis mediated by isoprenaline (Zhang *et al.*, 2010; Thangaiyan *et al.*, 2020; Shi *et al.*, 2022). In present study, we also found the significantly increased serum CRP, NF- κ B protein expression and TNF- α mRNA expression in heart in cardiac injury group. However, the mRNA expression for IL-6 in ISP-induced cardiac fibrosis was moderately upregulated. Furthermore, histopathological examination of heart in present study showed the increased infiltration of inflammatory cells and extensive proliferation of fibroblast cells in cardiac injury group. Niclosamide ethanolamine treatment significantly decreased the CRP, NF- κ B protein expression along with downregulated TNF- α mRNA expression in cardiac injury. IL-6 mRNA expression was moderately improved in niclosamide ethanolamine treated cardiac injury group. Furthermore, histopathological examination of heart tissue also supported these finding which showed reduction in inflammatory cell infiltration in niclosamide ethanolamine-treated cardiac injury group. The results are in agreement with previous study which showed significantly

reduced TNF- α along with moderately declined IL-6 in collagen-induced arthritis in rats (Al-Gareeb *et al.*, 2019). Another study by Esmail and co-workers (2021) reported that niclosamide treatment significantly reduced the inflammatory mediators viz., TNF- α , IL-6 and NF- κ B along with improved histopathological alterations i.e. inflammatory cell and fibrotic cell infiltration in bile duct ligation-induced liver fibrosis. Furthermore, Boyapally and co-workers (2019) also showed that TNF- α , IL-6 and NF- κ B decreased in lungs of niclosamide-treated pulmonary fibrosis.

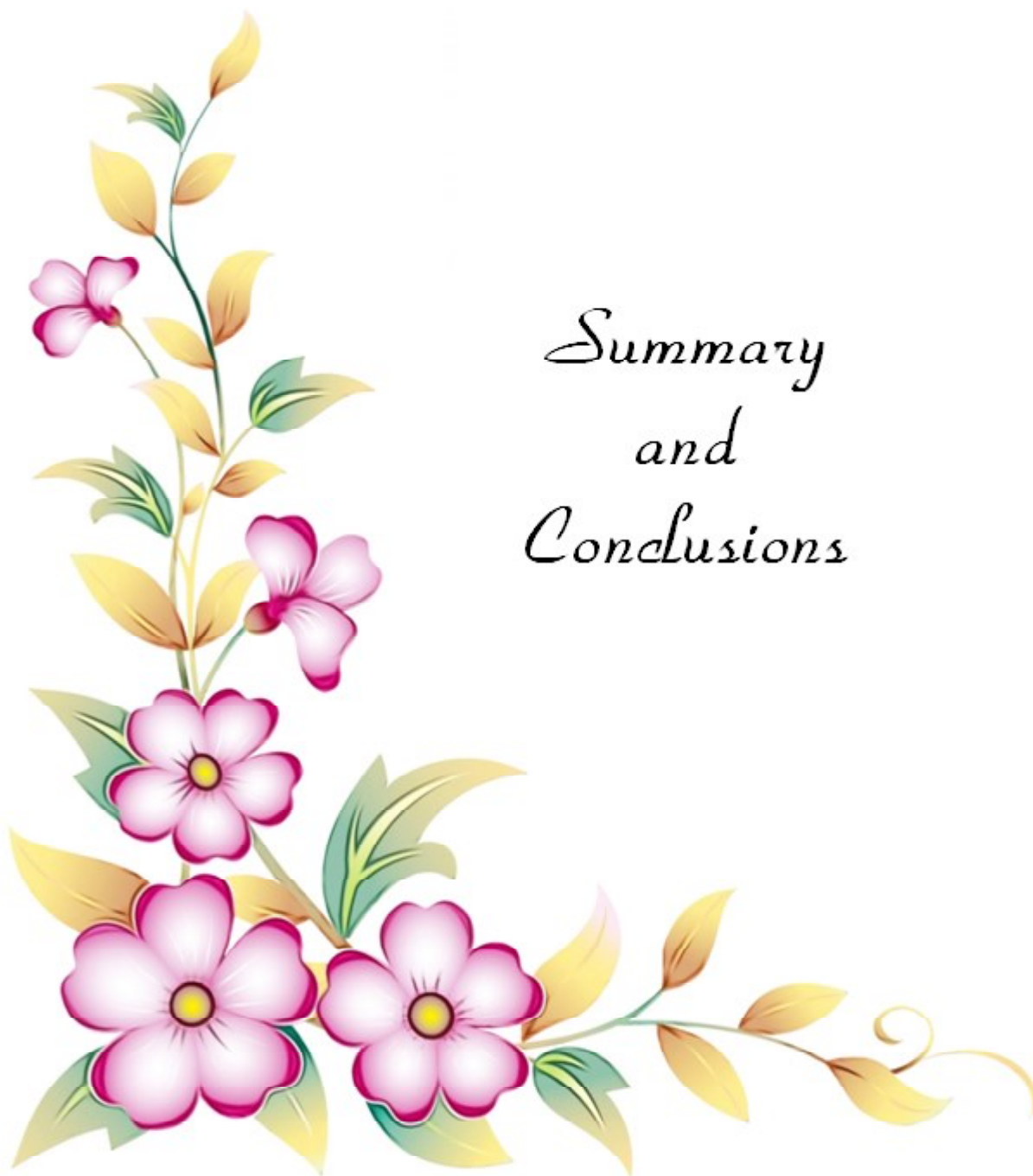
Matrix metalloproteinases (MMPs) and their inhibitors are the pivotal enzymes system that regulate myocardial matrix metabolism and ECM homeostasis. In present study, no significant alteration was observed in MMP-9 activity in among groups. Whereas, MMP-2 activity was significantly increased in ISP-induced cardiac fibrosis group which is in agreement with the earlier studies (Hori *et al.*, 2009; Radhiga *et al.*, 2019). In the current study, we found significantly reduced MMP-2 activity in niclosamide ethanolamine-treated cardiac injury. These findings are consistent to the previous study by Yu and co-workers (2017) reported that niclosamide downregulated the MMP-2 in human thyroid cancer in *in-vitro* study. Another study has also showed the downregulation of MMP-2 and MMP-9 in niclosamide-treated oral squamous cell carcinoma cells (Wang *et al.*, 2018).

Among various mechanistic pathways, smad-dependent TGF- β 1 signaling cascade plays key role in fibrosis. This pathway is initiated when ligands bind to specific receptors for TGF- β 1, triggering the activation of the TGF- β /Smad axis. Consequently, Smad2/3 proteins are phosphorylated and move into the nucleus, where they facilitate the transcription of genes associated with fibrosis (Yue *et al.*, 2010). Several previous studies have reported the upregulation in various fibrogenic mediators i.e. TGF- β 1, Smad-3, α -SMA and CTGF in isoprenaline-induced cardiac fibrosis (Ning *et al.*, 2017; Che *et al.*, 2019; Thangaiyan *et al.*, 2020). We also observed the significantly upregulated TGF- β 1, Smad-3 and α -SMA along with moderately increased CTGF level in heart tissue in cardiac injury. In current investigation a significantly downregulated mRNA expression of TGF- β 1 and Smad-3 was observed in niclosamide ethanolamine-treated cardiac injury group. Furthermore, moderately improved α -SMA mRNA expression and CTGF level was also found in niclosamide ethanolamine-

treated cardiac injury group. These findings are in agreement with the previous study by Boyapally and co-workers (2019) in which they reported that niclosamide downregulated the CTGF, TGF- β 1, Smad-3, and α -SMA expression in pulmonary fibrosis. Another previous study also showed the downregulation of TGF- β 1 in niclosamide ethanolamine-treated systemic lupus erythematosus (Han *et al.*, 2020). Several earlier studies have reported the potential of niclosamide in various conditions by targeting TGF- β 1/Smad signaling (Esmail *et al.*, 2021; Gan *et al.*, 2023; Spathakis *et al.*, 2023).



*Summary
and
Conclusions*



The present study was carried out to evaluate the effect of orlistat and niclosamide ethanolamine against isoprenaline (ISP)-induced cardiac fibrosis in mouse model. Cardiac fibrosis was induced by administering the ISP @ 20 mg/kg body weight via subcutaneous route for 14 consecutive days in cardiac fibrosis group. Orlistat was administered @ 30 mg/kg body weight through intraperitoneal route for 14 days in mice of isoprenaline (@ 20 mg/kg b.w.; S/C) co-administered group. Niclosamide ethanolamine was administered @ 3 mg/kg body weight alternatively through intraperitoneally for 14 days along with ISP (@ 20 mg/kg body weight; S/C). On day 15th mice were sacrificed under anaesthesia and blood and heart were collected for estimation of the various parameters viz., morphometric parameters, cardiac injury markers, lipid profile, fibrotic markers, protein expression and mRNA expression.

6.1 Major findings of orlistat study have been summarized below

1. Body weight during experimental period from day zero to day 15th was significantly ($p<0.0001-0.001$) increased in all groups. However, percentage increase in body weight was least and significantly ($p<0.01$) lower in ISP-induced cardiac fibrosis compared to control group. Orlistat treatment showed significantly ($p<0.05$) higher percentage change in body weight in ISP-induced cardiac fibrosis group.
2. Absolute heart weight and relative heart weight indices viz., heart weight to body weight (HWT/BWT) ratio and heart weight to tibial length ratio (HWT/TL) were significantly ($p<0.001$) increased in ISP-administered cardiac fibrosis group which were significantly ($p<0.001-0.01$) reduced in orlistat treated cardiac fibrosis (orlistat+ISP) group.

3. Cardiac injury markers viz., serum CK-MB and LDH levels were significantly ($p<0.01$ & $p<0.05$) increased in ISP-induced cardiac injury group. However, orlistat treatment significantly ($p<0.05$ & $p<0.001$) decreased the levels of these cardiac injury markers in cardiac injury (orlistat+ISP).
4. Serum total cholesterol level and triglyceride levels were significantly ($p<0.05$) elevated in ISP-induced cardiac injury group. Orlistat administration significantly ($p<0.01$) reduced the triglyceride levels in cardiac injury (orlistat+ISP). However, the serum total cholesterol level was improved non-significantly in orlistat-treated cardiac injury group.
5. ISP-administration significantly ($p<0.05$) decreased the HDL-c which was significantly ($p<0.01$) improved in orlistat-treated cardiac injury (orlistat+ISP) group.
6. LDL-c level was significantly ($p<0.01$) increased along with significant ($p<0.01$) reduction in HDL-c to LDL-c ratio in ISP-induced cardiac injury which were improved (non-significant) in orlistat-treated (orlistat+ISP) group.
7. In ISP-administered cardiac injury group, the level of serum CRP was increased significantly ($p<0.05$) which was improved (non-significant) in orlistat-treated cardiac fibrosis (orlistat+ISP) group.
8. ISP-alone administered group significantly ($p<0.05$) reduced the enzymatic antioxidant activities viz., SOD and catalase in serum which were significantly ($p<0.05$) enhanced in orlistat-treated cardiac injury (orlistat+ISP) group.
9. Serum non-enzymatic antioxidant activity viz., GSH level was significantly ($p<0.05$) decreased in ISP-induced cardiac injury which was significantly ($p<0.05$) improved in orlistat-treated cardiac injury (orlistat+ISP) group.
10. Orlistat treatment significantly ($p<0.05$) declined the serum MDA level in cardiac injury (orlistat+ISP) group which was significantly ($p<0.05$) enhanced in ISP-induced cardiac injury group.
11. Tissue SOD activity was decreased significantly ($p<0.05$) in ISP-induced injury group and the activity was increased markedly in orlistat-treated (orlistat+ISP) group.
12. ISP-administration significantly ($p<0.001$) reduced the tissue catalase activity which was significantly ($p<0.05$) improved in orlistat-treated cardiac injury (orlistat+ISP) group.

13. GSH level was significantly ($p<0.05$) decreased in heart tissue following ISP-alone administration which was significantly ($p<0.05$) elevated in orlistat-treated cardiac injury (orlistat+ISP) group.
14. Orlistat-treatment significantly ($p<0.05$) reduced the MDA level or LPO in cardiac fibrosis (orlistat+ISP) group which was significantly ($p<0.05$) increased in ISP-induced cardiac fibrosis.
15. Tissue hydroxyproline level was significantly ($p<0.05$) reduced in orlistat-treated cardiac fibrosis (orlistat+ISP) group, which was significantly ($p<0.001$) increased in ISP-alone administered group. Moreover, orlistat treatment also improved tissue glucosamine level significantly ($p<0.05$) which was increased significantly ($p<0.05$) following ISP-alone administration.
16. ISP-administration significantly ($p<0.05$) elevated the collagen type I in heart tissue homogenate which was moderately improved in orlistat-treated cardiac injury (orlistat+ISP) group.
17. CTGF level in heart tissue homogenate and its mRNA expression was increased in ISP-alone administered group which was moderately improved in orlistat-treated cardiac injury (orlistat+ISP) group.
18. MMP-2 activity in tissue was significantly ($p<0.001$) enhanced in ISP-induced cardiac injury group which was significantly ($p<0.01$) reduced in orlistat-treated injury (orlistat+ISP) group. However no significant alterations in MMP-9 were observed among groups.
19. Orlistat treatment significantly ($p<0.01$) increased the Nrf-2 protein expression in heart tissue in cardiac injury (orlistat+ISP) group which was significantly ($p<0.05$) decreased following ISP-administration.
20. Expression of NF- κ B protein in heart was significantly ($p<0.05$) enhanced in ISP-cardiac injury group which was significantly ($p<0.05$) downregulated in orlistat-treated cardiac injury (orlistat+ISP) group.
21. The mRNA expression of TGF- β was significantly ($p<0.01$) downregulated in orlistat-treated cardiac fibrosis (orlistat+ISP) group which was significantly ($p<0.05$) upregulated following ISP-administration.

22. ISP-induced cardiac fibrotic mice showed significantly ($p<0.05$) increased mRNA expression of Smad-3 in heart which was significantly ($p<0.05$) reduced in orlistat-treated (orlistat+ISP) mice.
23. Significantly ($p<0.05$) upregulated mRNA expressions of α -SMA and collagen type I were observed in ISP-induced cardiac fibrosis. However, orlistat treatment significantly ($p<0.05$) downregulated these mRNA expressions in cardiac fibrosis (orlistat+ISP).
24. The mRNA expression of TNF- α was increased significantly ($p<0.05$) in ISP-alone administered group which was significantly ($p<0.05$) decreased in orlistat-treated cardiac fibrosis (orlistat+ISP) group.
25. ISP-administration markedly increased mRNA expressions of collagen type III and IL-6 and this increased level was markedly improved in orlistat-treated (orlistat+ISP) group.
26. Histopathological examination showed that orlistat treatment improved the inflammatory cell infiltration and fibrotic score in cardiac fibrosis (orlistat+ISP) group which were observed higher in ISP-alone administered group.

In conclusion, orlistat treatment improved the morphometric parameters, cardiac injury markers, oxidative stress, lipid profile, inflammatory markers and MMPs in ISP-induced cardiac fibrosis. Furthermore, orlistat treatment also showed improvement in collagen content, mRNA expression for the fibrotic mediators viz., TGF- β 1, Smad-3, α -SMA, and inflammatory mediators i.e. TNF- α and IL-6 in ISP-induced cardiac fibrosis. Therefore, it can be postulated that orlistat attenuated the ISP-induced cardiac fibrosis through anti-oxidant, anti-inflammatory and TGF- β 1/Smad-3 signaling modulation.

6.2 Major findings of niclosamide ethanolamine (NEN @ 1 mg/kg body weight) are summarized as follows

1. Body weight significantly ($p<0.05$) decreased after day 15th following ISP-induced cardiac injury. Niclosamide ethanolamine (NEN) @ 1 mg/kg body weight did not show any impact on body weight in cardiac injury (NEN+ISP) group.

2. Niclosamide ethanolamine (@ 1 mg/kg body weight) treatment significantly ($p<0.01$) reduced absolute heart weight and heart weight to tibial length (HWT/TL) ratio in cardiac injury (NEN+ISP) which were significantly ($p<0.05$; $p<0.01$) increased in ISP-induced cardiac injury.
3. Heart weight to body weight (HWT/BWT) ratio was significantly ($p<0.05$) increased following ISP-administration which was not significantly improved in NEN (@ 1 mg/kg body weight)-treated cardiac injury (NEN+ISP).
4. NEN @ 1 mg/kg body weight did not improve the cardiac injury markers viz., CK-MB and LDH in cardiac injury (NEN+ISP) which was significantly ($p<0.05$; $p<0.01$) increased in ISP-induced cardiac injury.
5. Serum cholesterol and triglyceride levels were not improved in NEN (@ 1 mg/kg body weight) treatment in cardiac injury (NEN+ISP) which were increased following ISP-administration.
6. NEN (@ 1 mg/kg body weight) treatment did not restore the anti-oxidant activities viz., catalase and GSH in heart tissue in cardiac injury (NEN+ISP) which were significantly reduced in ISP-induced cardiac injury.
7. Tissue MDA level was significantly ($p<0.05$) increased in ISP-induced cardiac injury which was not improved in NEN (@ 1 mg/kg body weight)-treated cardiac injury (NEN+ISP).
8. NEN (@ 1 mg/kg body weight) treatment did not improve the fibrotic markers viz., hydroxyproline and glucosamine in cardiac injury (NEN+ISP) which were increased significantly ($p<0.05$) in ISP-induced cardiac injury.

In conclusion, niclosamide ethanolamine at 1 mg/kg body weight via intraperitoneally on alternate days for initial eight days in ISP-induced cardiac injury did not improve the cardiac injury, lipid profile, oxidative stress and fibrotic markers. Therefore, we decided to increase the dose of NEN to 3 mg/kg body weight, alternatively for 14 days.

6.3 Major findings of niclosamide ethanolamine (NEN @ 3 mg/kg body weight; I.P) are summarized as follows:

1. Body weight during experimental period from day zero to day 15th was not changed in ISP-induced cardiac injury. Niclosamide ethanolamine (NEN @ 3 mg/kg body weight) treatment significantly ($p<0.01$) increased the body weight during experimental period in cardiac injury (NEN+ISP).
2. The percentage increase in body weight was least and significantly ($p<0.05$) lower in ISP-induced cardiac fibrosis. Niclosamide ethanolamine (@ 3 mg/kg body weight) treatment showed significantly ($p<0.05$) higher percentage change in body weight in cardiac fibrosis (NEN+ISP) group.
3. Niclosamide ethanolamine (@ 3 mg/kg body weight) significantly ($p<0.01$) decreased the absolute heart weight in cardiac injury (NEN+ISP) which was significantly ($p<0.001$) increased in ISP-alone group.
4. The relative heart weight indices viz., heart weight to body weight (HWT/BWT) ratio and heart weight to tibial length ratio (HWT/TL) were significantly ($p<0.001$) increased in ISP-administered cardiac fibrosis group which were significantly ($p<0.05-0.001$) reduced in niclosamide ethanolamine-treated cardiac fibrosis (NEN+ISP) group.
5. ISP-administration significantly ($p<0.05$) increased serum CK-MB and LDH level. Niclosamide ethanolamine (@ 3 mg/kg body weight) treatment significantly ($p<0.05$) reduced serum CK-MB in cardiac injury group. LDH level was moderately improved in NEN-treated cardiac injury (NEN+ISP) group.
6. Serum total cholesterol level and triglyceride levels were significantly ($p<0.01$; $p<0.05$) elevated in ISP-induced cardiac injury group which were moderately improved in niclosamide ethanolamine (@ 3 mg/kg body weight) treatment in cardiac injury (NEN+ISP) group.
7. Niclosamide ethanolamine (@ 3 mg/kg body weight) treatment moderately increased serum HDL-c and HDL-c/LDL-c ratio in cardiac injury (NEN+ISP) which were significantly ($p<0.05$) decreased in ISP-induced cardiac injury (NEN+ISP) group.

8. LDL-c level was significantly ($p<0.05$) increased in ISP-induced cardiac injury which was improved (non-significant) in niclosamide ethanolamine-treated cardiac injury (NEN+ISP) group.
9. Niclosamide ethanolamine (@ 3 mg/kg body weight) significantly ($p<0.05$) reduced serum CRP in cardiac injury (NEN+ISP) which was significantly ($p<0.05$) elevated following ISP-administration.
10. ISP-administration significantly ($p<0.001$) reduced serum SOD which was significantly ($p<0.001$) enhanced in niclosamide ethanolamine-treated cardiac injury (NEN+ISP) group.
11. Niclosamide ethanolamine (@ 3 mg/kg body weight) treatment significantly ($p<0.05$) increased the serum catalase and GSH level in ISP-induced cardiac injury.
12. Serum MDA level was significantly ($p<0.01$) increased in ISP-induced cardiac injury which was moderately improved in niclosamide ethanolamine treatment cardiac injury (NEN+ISP) group
13. Niclosamide ethanolamine @ 3 mg/kg body weight significantly ($p<0.05$) increased tissue SOD activity along with moderate improvement in tissue catalase activity in cardiac injury (NEN+ISP) which were decreased significantly ($p<0.05$) in ISP-induced injury group.
14. ISP-administration significantly ($p<0.05$) reduced the tissue GSH level which was significantly ($p<0.05$) improved in niclosamide ethanolamine-treated cardiac injury (NEN+ISP) group.
15. MDA level was significantly ($p<0.01$) increased in heart tissue following ISP-alone administration which was significantly ($p<0.05$) decreased in niclosamide ethanolamine-treated cardiac injury (NEN+ISP) group.
16. Niclosamide ethanolamine (@ 3 mg/kg body weight)-treatment significantly ($p<0.05$) improved the hydroxyproline level in cardiac fibrosis (NEN+ISP) group which was significantly ($p<0.01$) increased in ISP-induced cardiac fibrosis.
17. Tissue glucosamine level was significantly ($p<0.05$) reduced in niclosamide ethanolamine-treated cardiac fibrosis (NEN+ISP) which was significantly ($p<0.001$) increased in ISP-alone administered group.

18. ISP-administration significantly ($p<0.05$) elevated the collagen type I in heart tissue homogenate which was significantly ($p<0.05$) reduced in niclosamide ethanolamine-treated cardiac injury (NEN+ISP) group.
19. CTGF level in heart tissue homogenate was increased (non-significant) in ISP-alone administered group which was moderately improved in niclosamide ethanolamine-treated cardiac injury (NEN+ISP) group.
20. Niclosamide ethanolamine treatment significantly ($p<0.05$) reduced the MMP-2 activity in tissue in cardiac injury group (NEN+ISP) which was significantly ($p<0.05$) increased in cardiac injury group. However no significant alterations in MMP-9 were observed among groups.
21. Nrf-2 protein expression in heart was significantly ($p<0.05$) reduced in ISP-induced cardiac injury group which was significantly ($p<0.05$) increased in niclosamide ethanolamine-treated cardiac injury (NEN+ISP) group.
22. Expression of NF- κ B protein in heart was significantly ($p<0.05$) enhanced in ISP-cardiac injury group which was moderately reduced in niclosamide ethanolamine-treated cardiac injury (NEN+ISP) group.
23. The mRNA expression of TGF- β was significantly ($p<0.05$) upregulated in ISP-induced cardiac fibrosis which was significantly ($p<0.05$) downregulated in niclosamide ethanolamine-treated cardiac injury (NEN+ISP) group.
24. ISP-administration showed significantly ($p<0.05$) increased mRNA expression of Smad-3 in heart which was significantly ($p<0.05$) reduced in niclosamide ethanolamine-treated (NEN+ISP) mice.
25. Significantly ($p<0.05$) upregulated mRNA expression of α -SMA was observed in ISP-induced cardiac fibrosis. However, niclosamide ethanolamine (@ 3 mg/kg body weight) treatment moderately downregulated α -SMA mRNA expressions in cardiac fibrosis (NEN+ISP).
26. Niclosamide ethanolamine (@ 3 mg/kg body weight) treatment significantly ($p<0.01$) decreased mRNA expression of TNF- α in cardiac injury (NEN+ISP) group which was significantly ($p<0.01$) increased in ISP-induced cardiac fibrosis.

27. ISP-administration markedly increased mRNA expressions of collagen type III and IL-6 and this increased level was markedly improved in niclosamide ethanolamine-treated (NEN+ISP) group.
28. Histopathological examination showed extensive infiltration of inflammatory cells and collagen deposition in isoprenaline-induced cardiac fibrosis, which were improved in niclosamide ethanolamine-treated cardiac injury (NEN+ISP).

In conclusion, niclosamide ethanolamine (3 mg/kg body weight) treatment improved the morphometric parameters, cardiac injury markers, lipid profile, oxidative stress, and MMPs in ISP-induced cardiac fibrosis. Furthermore, collagen content, mRNA expression for the fibrotic mediators viz., TGF- β 1, Smad-3, α -SMA, and inflammatory mediators i.e. TNF- α and IL-6 were improved in niclosamide ethanolamine-treated cardiac fibrosis group. Therefore, it can be postulated that niclosamide ethanolamine @ 3 mg/kg body weight attenuated the ISP-induced cardiac fibrosis through anti-oxidant, anti-inflammatory and TGF- β 1/Smad-3 signalling modulation.



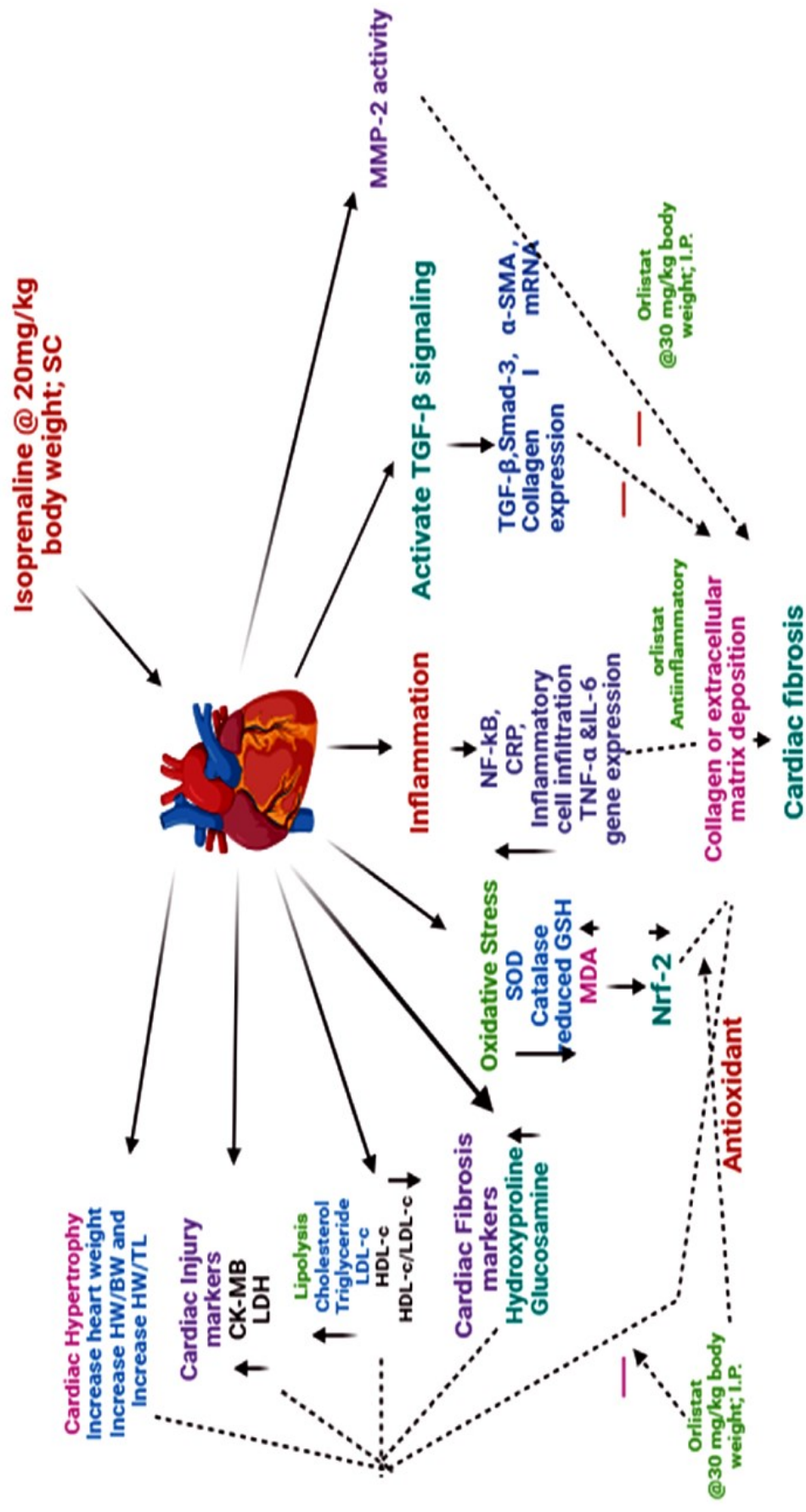


Fig. 5: Mechanistic signaling of orlistat-treated cardiac fibrosis

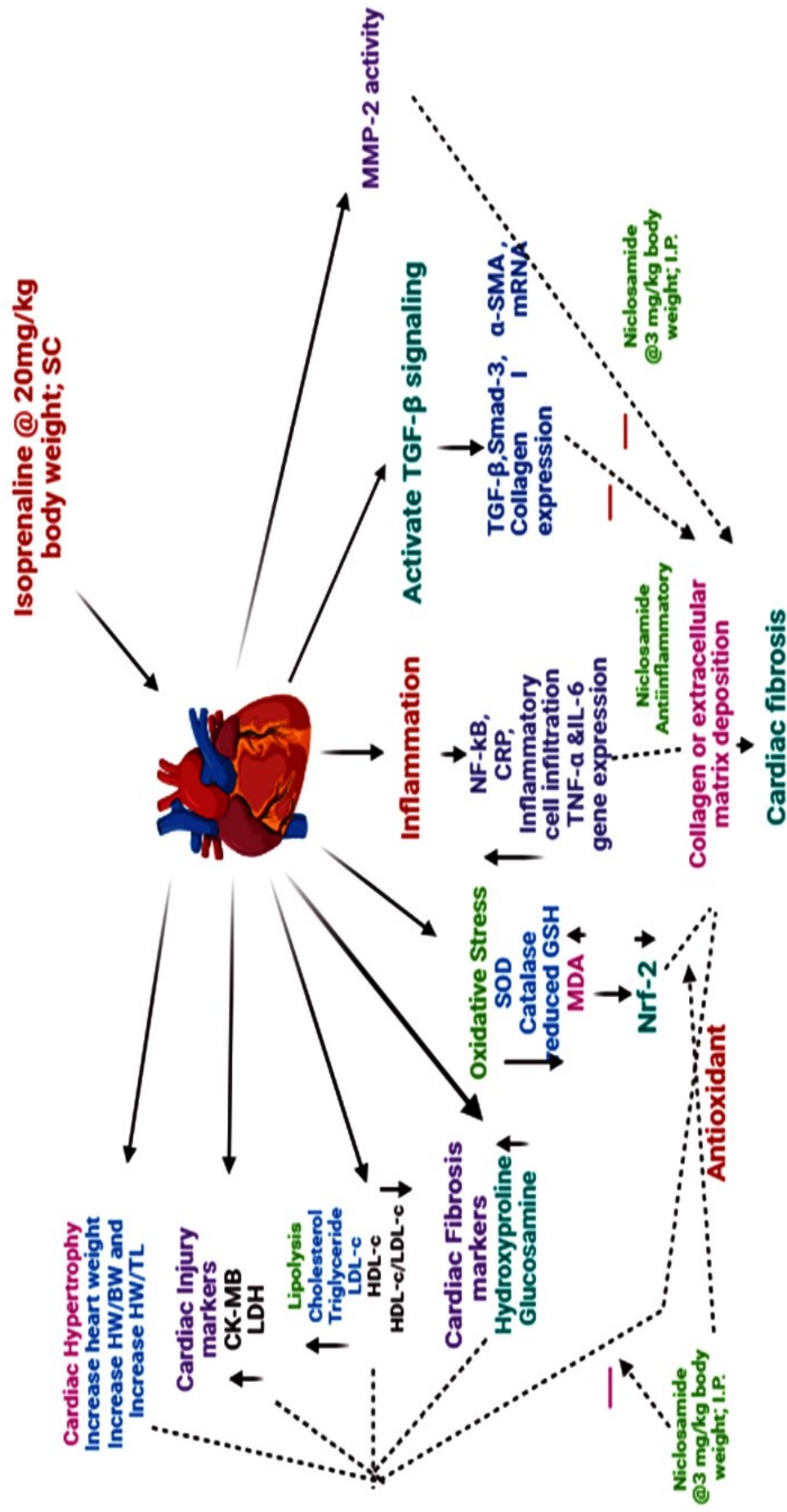


Fig. 6: Mechanistic signaling of nicosamide ethanolamine (NEN)-treated cardiac fibrosis



The present study was undertaken to assess the effect of orlistat and niclosamide ethanolamine against isoprenaline (ISP)-induced cardiac fibrosis in mouse model. Isoprenaline was administered @ 20 mg/kg body weight through a subcutaneous route for 14 days in mice to induce cardiac fibrosis. Administration of the orlistat was done @ 30 mg/kg body weight through the intraperitoneal route for 14 days in the cardiac fibrosis affected mice. Niclosamide ethanolamine was administered @ 3 mg/kg body weight (intraperitoneally) on alternate day for 14 days. After 24 hours of the last dose of isoprenaline, mice of all groups were sacrificed under anaesthesia and heart and blood samples were collected to estimate various parameters. Orlistat treatment significantly improved the morphometric parameters, cardiac injury markers viz., CK-MB and LDH, lipid profile and fibrotic markers i.e. hydroxyproline and glucosamine in ISP-induced cardiac injury group. Orlistat exhibited the anti-oxidant activity as evident by increased antioxidants, decreased lipid peroxidation and upregulated Nrf-2 protein expression in cardiac injury. Furthermore, ISP-induced cardiac injury increased inflammatory mediators viz., CRP, NF- κ B protein expression and upregulated TNF- α and IL-6 mRNA expression. Orlistat treatment significantly downregulated the NF- κ B protein expression and TNF- α mRNA expression in cardiac injury. However, CRP and IL-6 were moderately improved in orlistat-treated cardiac injury. Moreover, orlistat treatment also moderately resolved the histopathological alterations of ISP-induced cardiac fibrosis by reducing fibrogenesis and inflammatory cell infiltrations. Isoprenaline administration significantly upregulated the mRNA expression of fibrogenic mediators TGF- β 1, Smad-3, α -SMA and collagen type I and MMP-2 activity. However, CTGF and collagen type III mRNA expression were moderately enhanced in ISP-induced cardiac injury. Histopathological examination by Masson's Trichrome staining (MTS) also supported the increased collagen deposition in cardiac injury group. Orlistat treatment significantly downregulated the mRNA expression of fibrogenic mediators viz., TGF- β 1, Smad-3, α -SMA and collagen type I and MMP-2 activity along with moderate improvement in CTGF and collagen type III mRNA expression in cardiac injury. Moreover, the collagen deposition in heart tissue was also improved in orlistat-treated cardiac injury as evident by MTS staining. Niclosamide ethanolamine (NEN) @ 3 mg/kg body weight significantly improved the morphometric parameters and CK-MB in cardiac injury. Serum LDH and lipid profile were moderately improved in niclosamide ethanolamine-treated cardiac injury. Niclosamide ethanolamine increased antioxidants and Nrf-2 protein expression, and decreased lipid peroxidation by exerting the antioxidant activity against ISP-induced cardiac injury. Tissue hydroxyproline, glucosamine and collagen type I were significantly increased in ISP-induced cardiac fibrosis, which were significantly reduced by niclosamide ethanolamine-treated cardiac fibrosis. NEN treatment significantly decreased CRP level, and TNF- α mRNA expression in cardiac injury. Furthermore, NEN also reduced NF- κ B protein expression, IL-6 and resolved the infiltration of inflammatory cell and fibrotic cell in cardiac fibrosis. The mRNA expression of TGF- β and Smad-3 and MMP-2 activity were significantly decreased in NEN-treated cardiac fibrosis. Moreover, CTGF level and α -SMA mRNA expression were moderately improved by NEN-treatment in cardiac injury. NEN-treatment also reduced the collagen deposition and proliferation of fibrotic tissue in cardiac injury as evident on MTS staining.



लघु सारांश

वर्तमान अध्ययन, मूषक मॉडल में आईसोप्रिनालिन-प्रेरित हृदय तंतुमयता (कार्डियक फाइब्रोसिस) के खिलाफ ऑर्लिस्टैट और निकलोसामाइड इथेनॉलमाइन के प्रभाव का आकलन करने के लिए किया गया था। हृदयतंतुमयता प्रेरित करने के लिए आईसोप्रिनालिन (आई.एस.पी.) @ 20 मिलीग्राम/किलोग्राम शारीरिक वजन की दर से 14 दिनों के लिए उप चर्म मार्ग के माध्यम से दिया गया था। ऑर्लिस्टैट को 30 मि.ग्रा./कि.ग्रा. शारीरिक वजन की दर से आई.पी. माध्यम से 14 दिनों के लिए उपचार समूह में आईसोप्रिनेलीन (उपचर्म) के साथ दिया गया। निकलोसामाइड इथेनॉलमाइन (इंट्रापेरिटोनियल) को 14 दिनों के लिए वैकल्पिक दिन पर 3 मिलीग्राम/किलोग्राम शरीर के वजन की दर से उपचार समूह में दिया गया था। आइसोप्रिनालाईन की अंतिम खुराक के 24 घंटों के बाद, सभी समूहों के चूहों को एनेस्थीसिया के तहत सेक्रिफाइस किया गया और विभिन्न मापदण्डों का अनुमान लगाने के लिए हृदय और रक्त के नमूने एकत्र किए गए। ऑर्लिस्टैट उपचार ने आईएसपी-प्रेरित हृदयतंतुमयता (कार्डियक फाइब्रोसिस) में मॉर्फोमेट्रिक मापदण्ड, हृदय क्षति अंक गणक जैसे सीके-एमबी और एलडीएच, लिपिड प्रोफाइल और फाइब्रोटिक मार्कर यानी हाइड्रोक्सीप्रोलाइन और ग्लूकोसामाइन में काफी सुधार किया। ऑर्लिस्टैट ने एंटी-ऑक्सीडेंट क्रिया का प्रदर्शन किया जो कि बढ़े हुए एंटीऑक्सीडेंट, कम लिपिड पेरॉक्सीडेशन और हृदय की चोट में एनआरएफ-2 प्रोटीन की अभिव्यक्ति में वृद्धि से स्पष्ट है। इसके अलावा, आईएसपी-प्रेरित हृदय की चोट ने सूजन मध्यस्थों जैसे सीआरपी, एनएफ-कप्पा बी प्रोटीन अभिव्यक्ति और टीएनएफ-अल्फा और आईएल-6 एमआरएनए अभिव्यक्ति में वृद्धि की। ऑर्लिस्टैट उपचार ने हृदय की चोट में एनएफ-कप्पा-बी प्रोटीन अभिव्यक्ति और टीएनएफ-अल्फा एमआरएनए अभिव्यक्ति को काफी कम कर दिया। हालाँकि, ऑर्लिस्टैट-उपचारित हृदय संबंधी चोट में सीआरपी और आईएल-6 में मामूली सुधार हुआ था। इसके अलावा, ऑर्लिस्टैट उपचार ने फाइब्रोजेनेसिस और सूजन करने वाली कोशिकाओं के घुसपैठ को कम करके आईएसपी-प्रेरित कार्डियक फाइब्रोसिस के हिस्टोपैथोलॉजिकल परिवर्तनों को भी काफी कम किया। आइसोप्रिनालाईन ने फाइब्रोजेनिक मध्यस्थों टीजीएफ-बीटा 1, स्मैड-3, अल्फा-एसएमए और कोलेजन प्रकार 1 के एमआरएनए अभिव्यक्ति और एमएमपी-2 की गतिविधि को महत्वपूर्ण रूप से बढ़ा दिया। हालाँकि, आईएसपी-प्रेरित हृदय की चोट में सीटीजीएफ और कोलेजन प्रकार 3 एमआरएनए अभिव्यक्ति को मामूली रूप से बढ़ाया गया था। एमटीएस स्टेनिंग ने भी कोलेजन के जमाव का समर्थन किया। ऑर्लिस्टैट उपचार ने फाइब्रोजेनिक मध्यस्थों जैसे टीजीएफ-बीटा 1, स्मैड-3, अल्फा-एसएमए और कोलेजन प्रकार 1 एमआरएनए अभिव्यक्ति और एमएमपी-2 गतिविधि को काफी हद तक कम कर दिया, साथ ही हृदय की चोट में सीटीजीएफ और कोलेजन प्रकार 3 एमआरएनए अभिव्यक्ति में मध्यम सुधार हुआ। इसके अलावा, एमटीएस स्टेनिंग से स्पष्ट है कि ओर्लिस्टैट-उपचारित हृदय की चोट में हृदय के ऊतकों में कोलेजन जमाव में भी सुधार हुआ था। निकलोसामाइड इथेनॉलमाइन (एनईएन) @ 3 मिलीग्राम/किग्रा शरीर के वजन ने हृदय संबंधी चोट में सीरम एलडीएच और लिपिड प्रोफाइल में मामूली सुधार हुआ। निकलोसामाइड इथेनॉलमाइन ने एंटीऑक्सीडेंट और एनआरएफ-2 प्रोटीन अभिव्यक्ति को बढ़ाया, और आईएसपी-प्रेरित हृदय की चोट के खिलाफ एंटीऑक्सीडेंट गतिविधि को बढ़ाकर लिपिड पेरॉक्सीडेशन को कम किया। आईएसपी-प्रेरित कार्डियक फाइब्रोसिस में ऊतक हाइड्रोक्सीप्रोलाइन, ग्लूकोसामाइन और कोलेजन प्रकार 1 में काफी वृद्धि हुई थी, जो निकलोसामाइड इथेनॉलमाइन-उपचारित कार्डियक फाइब्रोसिस द्वारा काफी कम हो गई थी। एनईएन उपचार से सीआरपी स्तर और हृदय की चोट में टीएनएफ-अल्फा एमआरएनए अभिव्यक्ति में काफी कमी आई। इसके अलावा, एनईएन उपचार ने एनएफ-कप्पाबी प्रोटीन अभिव्यक्ति, आईएल-6 को भी कम कर दिया और कार्डियक फाइब्रोसिस में सूजन करने वाली कोशिका और फाइब्रोटिक कोशिका की घुसपैठ को कम किया। एनईएन-उपचारित कार्डियक फाइब्रोसिस में टीजीएफ-बीटा और स्मैड-3 एमआरएनए अभिव्यक्ति और एमएमपी-2 गतिविधि में काफी कम हो गई थी। इसके अलावा, हृदय की चोट में एनईएन-उपचार द्वारा सीटीजीएफ स्तर और अल्फा-एसएमए एमआरएनए अभिव्यक्ति में काफी हद तक सुधार हुआ था। जैसा कि एमटीएस स्टेनिंग पर स्पष्ट है, एनईएन-उपचार ने हृदय की चोट में कोलेजन जमाव और फाइब्रोटिक ऊतक के प्रसार को भी कम कर दिया। ओर्लिस्टैट 30 मिलीग्रा./कि.ग्रा. शरीर वजन की दर से आइसोप्रिनेलीन से होने वाले कार्डियक फाइब्रोसिस से बचाव किया। निकलोसामाइड इथेनोलामीन 3 मिलीग्राम/कि.ग्रा. शरीर वजन की दर से देने पर आइसोप्रोलीन प्रेरित कार्डियक फाइब्रोसिस को रोकने में लाभकारी सिद्ध हुआ।

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