ANTI-BACTERIAL, ANTI-INFLAMMATORY AND SAFETY STUDIES OF CLOVE OIL IN RATS

BY

HUMBAL BRIJESH RAJESHBHAI
B.V.Sc. & A.H.
(Reg. No. 04-3195-2017)

DEPARTMENT OF VETERINARY PHARMACOLOGY & TOXICOLOGY
COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY
ANAND AGRICULTURAL UNIVERSITY
ANAND – 388 001 (GUJARAT)

2019

ANTI-BACTERIAL, ANTI-INFLAMMATORY AND SAFETY STUDIES OF CLOVE OIL IN RATS

A THESIS SUBMITTED TO THE
ANAND AGRICULTURAL UNIVERSITY
IN THE PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE

OF

MASTER OF VETERINARY SCIENCE

IN

VETERINARY PHARMACOLOGY AND TOXICOLOGY

BY

HUMBAL BRIJESH RAJESHBHAI
B.V.Sc. & A.H.
(Reg. No. 04-3195-2017)

COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY
ANAND AGRICULTURAL UNIVERSITY
ANAND – 388 001 (GUJARAT)

2019

ABSTRACT

"ANT-IBACTERIAL, ANTI-INFLAMMATORY AND SAFETY STUDIES OF CLOVE OIL IN RATS"

Name of Student	Name of Major Advisor
Humbal Brijesh Rajeshbhai	Dr. K. A. Sadariya

Department of Veterinary Pharmacology and Toxicology
College of Veterinary Science and Animal Husbandry
Anand Agricultural University, Anand 388001
Gujarat, INDIA

The present study was conducted to evaluate *in vitro* antibacterial, *in vivo* anti-inflammatory (100, 250 and 500 mg/kg) effects and safety of clove oil (50, 100 and 200 mg/kg) following repeated oral administration in wistar rats.

Screening of clove oil for antibacterial activity was done by the disc diffusion method. It was performed using an 18 h culture at 37°C in 10 ml of Muller Hinton agar (for *S. agalactiae* 5% defibrinated sheep blood was added). The test suspension was standardized to match 0.5 McFarland turbidity standard. The clove oil was suspended in 10% dimethylsulfoxide (DMSO) with tween 80 and sterilized by filtration through 0.45 µm membrane filter. Under aseptic condition, empty sterilized discs were impregnated with 50 µl of different concentrations (1:1, 1:2, 1:5, 1:10 and 1:20) of the respective clove oil and placed on the agar surface. Paper disc moistened with aqueous DMSO was placed on the seeded petriplate as a vehicle control. Standard discs containing antibacterial drug (cefotaxime, ampicillin, tetracycline and gentamicin) was used as reference control. The petri plates were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured.

The results revealed that the clove oil showed antibacterial activity with varying magnitudes. The zone of inhibition above 10 mm in diameter was taken as positive results. Both gram positive (*Staphylococcus aureus, Listeria monocytogenes* and *Streptococcus agalactiae*) and gram negative (*Salmonella typhimurium, Pseudomonas aeruginosa* and *Escherichia coli*) bacteria were sensitive to the clove oil. There was no inhibition in growth of bacteria with the vehicle control (10% DMSO with 0.5% v/v tween 80). Four antibacterial drugs (cefotaxime, ampicillin, tetracycline and gentamicin) were also tested against all six organisms and were found active against test bacteria.

The *in-vivo* anti-inflammatory assay of clove oil (*Syzygium aromaticum*) was carried out using rat paw edema method. Fifty rats were divided randomly into 10 groups each group of 5 males and 5 females. All rats were injected subcutaneously with 0.1 ml of a 10% w/v carrageenan suspension s/c as a local acute edema inducer after 30 minutes subsequent to oral administration of clove oil. Rats of control male and female groups were kept untreated. Rats of male and female standard control group were treated orally with indomethacin @ 10 mg/kg b.wt. as a reference drug, respectively. Clove oil was given orally to male and female rats at three different dose rate 100, 250 and 500 mg/kg b.wt., respectively. Edema was expressed as the increase in paw volume (ml). The paw volume was measured up to the tibiotarsal articulation. Volume of edematous paw was measured by using plethysmometer (PLM-01 plus, Orchid Scientific Instrument, India) at 0 hr (before treatment), 1, 2,3,4, 6 and 24 hours after treatments. Increase in paw thickness was measured by using digital plethysmometer and percent inhibition were calculated.

The results revealed that the clove oil showed anti-inflammatory effect with varying magnitudes at various doses in both male and female wistar rats. The anti-inflammatory effect of indomethacin was highest at 3h (41.75% in male, 42.99% in female) as compare to other doses of clove oil treated rats. The anti-inflammatory effect of clove oil was highest at 3h (35.77% in male, 35.46% in female) at the dose rate of 500mg/kg. At 3h all doses gave higher anti-inflammatory effect. Clove oil showed dose dependent anti-inflammatory activity in male and female rats.

The safety study of clove oil was conducted on forty (40) wistar rats divided into eight groups, each group contains 5 males and 5 females. Group I and V served as control. Clove oil administered orally at dose of 50, 100 and 200 mg/kg body weight once daily for 28 days in male rats of group II, III and IV as well as in female rats of group VI, VII and VIII, respectively.

The animals of all the groups were observed daily for clinical signs and mortality. Body weight and feed consumption of animals were monitored at weekly interval. At the end of experiment on 29^{th} day, blood samples were collected for the hematological and serum biochemical investigations. All the male and female rats were sacrificed at the end of experiment and subjected to post mortem examination. Organs (kidney, liver, spleen and heart) were collected for gross and histopathological examination.

In the present study, the test doses at 50, 100 and 200 mg/kg body weight were found to be safe. There was no significant difference observed in body weight of rats of control groups and no significant difference was observed in body weight of group II, III & IV of male rats and V, VII & VIII of female rats as compared to control group on day 7, 14, 21 and 28 days. No significant difference was observed in feed consumption of groups II, III and IV of male rats as compared to group I (male control) and also no significant difference was observed in female rats of group VI, VII and VIII as compared to group VI (female control) on 7^{th}, 14^{th}, 21^{st} and 28^{th} day of study.

No significant changes have been observed in Hb, RBCs, PCV, TLC, MCV, MCH and MCHC in clove oil treated male rats of group II, III and IV and female rats of group VI, VII and VIII at the end of experiment as compared to control male & female rats, respectively. No significant change have been observed in serum creatinine, BUN, bilirubin, AST, ALT, total cholesterol, total protein and albumin in clove oil treated male rats of group II, III and IV and in female rats of group VI, VII and VIII at the end of experiment on 28^{th} day as compared to male and female control rats, respectively.

Histopathology of organs like kidney, liver, spleen and heart from vehicle control rats (group I and V) did not showed any gross or microscopic changes. All organs collected showed normal microscopic structure. Whereas histopathology of kidney, liver, spleen and heart from clove oil treated male rats of group II, III and IV and in female rats of group VI, VII and VIII did not showed any marked gross or histopathological changes at the end of experiment on 28^{th} day as compared to male and female control groups, respectively. Clove oil was found safe following repeated oral administration @ 50, 100 and 200 mg/kg b.wt. for 28 days.

Dr. K. A. SADARIYA
Assistant Professor
Department of Veterinary Pharmacology & Toxicology
College of Veterinary Science and Animal Husbandry
Anand Agricultural University
Anand-388001, Gujarat (India)

CERTIFICATE

This is to certify that the thesis entitled **"ANTI-BACTERIAL, ANTI-INFLAMMATORY AND SAFETY STUDIES OF CLOVE OIL IN RATS"** submitted by **HUMBAL BRIJESH RAJESHBHAI** (**Reg. No. 04-3195-2017**) in partial fulfillment of the requirements for the award of the degree of **MASTER OF VETERINARY SCIENCE** in the subject of **VETERINARY PHARMACOLOGY AND TOXICOLOGY** of the Anand Agricultural University is a record of bonafide research work carried out by him under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Place: Anand
Date: /06/2019

Dr. K.A. Sadariya
Major Advisor

CERTIFICATE

This is to certify that I have no objection for supplying to any scientist only one copy of any part of this thesis at a time through reprographic process, if necessary, for rendering reference service in a library or documentation center.

Place: Anand
Date: / /2019

(Brijesh R. Humbal)

Dr. K.A. Sadariya
Major Advisor

DECLARATION

This is to certify that the whole of research work reported in the thesis in partial fulfillment of the requirements for the award of degree of MASTER OF VETERINARY SCIENCE in the subject of VETERINARY PHARMACOLOGY AND TOXICOLOGY is the result of investigation done by undersigned under the direct guidance and supervision of Dr. K. A. Sadariya, Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, AAU, Anand and no part of research work has been submitted to for any other degree so far.

Place: Anand
Date: / /2019

(Brijesh R. Humbal)

Dr. K.A. Sadariya
Major Advisor
Assistant Professor,
Depart of Veterinary Pharmacology and Toxicology,
College of Veterinary Science & Animal Husbandry
Anand Agricultural University, Anand

ACKNOWLEDGEMENT

It gives immense pleasure to express my deepest sense of gratitude and words of appreciation towards those, who helped me during the pursuit of my present study. It's my proud privilege and pleasure to acknowledge all those who are directly or indirectly involved.

Words are inadequate in the available lexicon to express my gratitude and sincere thanks to my major advisor **Dr. K. A. Sadariya,** Assistant Professor, Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University (AAU), Anand for his invaluable, judicious and constantly inspiring guidance with constructive criticism, persistent encouragement, active persuasion, diligent efforts and caring attitude through out the course of this study. It was indeed a valuable opportunity for me to pursue my M.V.Sc. study under a potent and brilliant man like him. The critical advices given have helped me to overcome many tough moments. He permitted complete freedom to me and I fervently hope that I have done something to do justice to the faith he had in me. If at all, an efflorescence element is witnessed in my work the entire credit goes to his blessings.

I take this opportunity in expressing my heartfelt thankfulness to my minor advisor **Dr. B. P. Joshi,** Professor, Department of Veterinary Pathology, College of Veterinary Science & Animal Husbandry, Anand for providing me continuous motivation, spirited guidance, indispensable suggestions during the study and for offering critical direction on the occasions required. I am also thankful to **Dr. D. J. Ghodasara (**Professor and Head) and **Dr. P. B. Jani** (Professor), Department of Veterinary Pathology College of Veterinary Science and Animal Husbandry, AAU, Anand.

I express my heart-felt gratitude to member of advisory committee **Dr. A. M. Thaker, Former Principal/Dean,** College of Veterinary Science & Animal Husbandry, Anand, for his consistent and invaluable inspirations, prolific and introspective guidance with constructive suggestions, deliberative discussions and active persuasion throughout the course of my study. I am also proudly thankful to him for his generous

attitude in providing necessary facilities to carry out the research work.

I wish to express my sincere gratitude and thanks to members of advisory committee **Dr. A. M. Pande**, Professor and Head, Department of Veterinary Physiology & Biochemistry, College of Veterinary Science & Animal Husbandry, Anand for their invaluable and critical suggestions and scholarly guidance, which served as a constant source of inspiration throughout the course of my study and research work. I am also thankful to him for providing the laboratory facilities and give permission to use automatic hematology analyzer and serum analyzer. I am also thankful to **Dr. M. M. Pathan**, Assistant Professor and departmental staff, Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, AAU, Anand for their cooperation and guidance during my research work.

I am gratefully obliged to **Dr. S. K. Bhavsar** Professor & Head, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science & Animal Husbandry, Anand, for his cooperation and ensuring the necessary laboratory facilities for timely completion of my research work. I am genuinely beholden by **Dr. R.J. Modi**, Assistant Professor, Department of Livestock production Management, College of Veterinary Science & Animal Husbandry, Anand for providing laboratory animal house facility for the research purpose.

I wish to express my sincere gratitude and thanks to members of advisory committee **Dr. B. B. Bhanderi,** Assistant Professor, **Dr. R. A. Mathkiya** Assistant Professor, Department of Veterinary Microbiology, Anand for their invaluable and critical suggestions and scholarly guidance, which served as a constant source of inspiration throughout the course of my study and research work. I am also thankful to him for providing the laboratory facilities and give permission to use laminar air flow and incubator.

I am profoundly thankful to **Dr. D.R. Kathiriya,** Director, Information Technology, AAU, for providing me the world class information technology services and necessary help whenever needed during the entire study period.

I am immensely thankful to my seniors and colleagues **Dr. Jayesh Maradiya, Dr. Bhavisha Jivani, Dr. Jaimin Prajapati, Dr. Jaydip Parmar and Dr. Bhavin Goswami** for their kind co-operation and unreserved help during the course of study. I am highly thankful to departmental staff Punjanibhai, Bhupatbhai, Vallabhbhai, Amitbhai (PGTC), Mrs. Himani and Library staff for their co-operation throughout the course of study.

I am really falling short of words to express my gratitude to my friends from different departments **Drs. Harpal, Jignesh, Ramdatt, Mukesh, Sohel, Digjay, Pratik, Aishwarya, Dhruvi, Prakash, Tapasvi, Priyank, Rajat, Jayvin, Jaymin** and others at the Department of Veterinary Pharmacology & Toxicology and Veterinary Pathology for their unreserved help, continuous motivation and well wishes as sources of constant inspiration for me.

It is like a drop in the ocean of words that can never reach its mark to acknowledge infinite love, blessings, sacrifices and constant encouragement of my mother and father, who have been the sole source of inspiration for me to proceed ahead in my life. I take this opportunity to dedicate this work to my **Parents** and Sister **Dixita Bena** and my **fiancee Jyoti**. I implore my profound thanks to my best friend **Nisarg Raviya** for his extraordinary support, patience and inspiration.

Finally, no words of admiration can justify the sacrifice of all those dumb rats who have contributed their blood and tissues for this research work. Last but not the least, I thank all the individuals who have in any way been associated with the completion of this work but have not been mentioned so far.

My essence thanks to Almighty God **Bhagwan Swaminarayan** and **Ravechi maa** who made everything possible. I know they are always there for me and always helps me to get out of all intriguing situations I get myself into.

Place : Anand (Humbal Brijesh R.)
Date : /07/2019

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
3.1	Composition of rat feed	25
3.2	Schedule for the preparation of Clove oil dilution	26
3.3	Schedule for the groups under treatments for anti-inflammatory activity in wistar rats	28
3.4	Schedule for the groups under treatments for safety study in wistar rats	29
4.1	Antibacterial activity of clove oil against *Salmonella typhemurium, P. aeruginosa, E.coli, S.aureus, L.monocytogenes* and *S.agalactiae* by using antibacterial sensitivity test	33
4.2	Effect of oral administration of clove oil on carrageenan-induced rat paw edema (ml) in male wistar rats (Mean ± SE, n=5)	41
4.3	Effect of oral administration of clove oil on carrageenan-induced rat paw edema (ml) in female wistar rats (Mean ± SE, n=5)	42
4.4	Percent inhibition of paw edema in wistar male rats treated with clove oil	42
4.5	Percent inhibition of paw edema in wistar female rats treated with clove oil	42
4.6	Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on body weight (g) of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)	48
4.7	Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on feed consumption (g) of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)	49
4.8	Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on Hb, RBCs, PCV and TLCs of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)	52
4.9	Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on MCV, MCH and MCHC of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)	54

4.10	Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on creatinine and BUN of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)	58
4.11	Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on AST, ALT, total cholesterol and bilirubin of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)	59
4.12	Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on total protein and albumin of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)	62

LIST OF FIGURES

FIGURE NO	TITLE	PAGE NO.
4.1	Antibiotic sensitivity test of standard controls against *S.typhemurium* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=gentamicin)	34
4.2	Antibiotic sensitivity test of clove oil concentrraions against *S.typhemurium* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)	34
4.3	Antibiotic sensitivity test of standard controls against *P.aeruginosa* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=gentamicin)	35
4.4	Antibiotic sensitivity test of clove oil concentrraions against *P.aeruginosa* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)	35
4.5	Antibiotic sensitivity test of standard controls against *E.coli* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=gentamicin)	36
4.6	Antibiotic sensitivity test of clove oil concentrraions against *E.coil* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)	36
4.7	Antibiotic sensitivity test of standard controls against *S.aureus* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=gentamicin)	37
4.8	Antibiotic sensitivity test of clove oil concentrraions against *S.aureus* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)	37
4.9	Antibiotic sensitivity test of standard controls against *L.monocytogenes* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=gentamicin)	38
4.10	Antibiotic sensitivity test of clove oil concentrraions against *L.monocytogenes* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)	38
4.11	Antibiotic sensitivity test of standard controls against *S.agalactiae* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=gentamicin)	39
4.12	Antibiotic sensitivity test of clove oil concentrraions against *S.agalactiae* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)	39
4.13	Effect of oral administration of clove oil on carrageenan-induced rat paw edema (ml) in male wistar rats	43
4.14	Effect of oral administration of clove oil on carrageenan-induced rat paw edema (ml) in female wistar rats	43

4.15	Percent inhibition of paw edema in wistar male rats treated with clove oil	44
4.16	Percent inhibition of paw edema in wistar female rats treated with clove oil	44
4.17	Effect of repeated oral administration of clove oil for 28 days on body weight of male (I-IV) and female (V-VIII) rats	48
4.18	Effect of repeated oral administration of clove oil for 28 days on feed consumption of male (I-IV) and female (V-VIII) rats	50
4.19	Effect of repeated oral administration of clove oil for 28 days on Hb of male (I-IV) and female (V-VIII) rats	52
4.20	Effect of repeated oral administration of clove oil for 28 days on RBC of male (I-IV) and female (V-VIII) rats	53
4.21	Effect of repeated oral administration of clove oil for 28 days on PCV of male (I-IV) and female (V-VIII) rats	53
4.22	Effect of repeated oral administration of clove oil for 28 days on TLC of male (I-IV) and female (V-VIII) rats	54
4.23	Effect of repeated oral administration of clove oil for 28 days on MCV of male (I-IV) and female (V-VIII) rats	55
4.24	Effect of repeated oral administration of clove oil for 28 days on MCH of male (I-IV) and female (V-VIII) rats	55
4.25	Effect of repeated oral administration of clove oil for 28 days on MCHC of male (I-IV) and female (V-VIII) rats	56
4.26	Effect of repeated oral administration of clove oil for 28 days on creatinine of male (I-IV) and female (V-VIII) rats	58
4.27	Effect of repeated oral administration of clove oil for 28 days on BUN of male (I-IV) and female (V-VIII) rats	59
4.28	Effect of repeated oral administration of clove oil for 28 days on AST of male (I-IV) and female (V-VIII) rats	60
4.29	Effect of repeated oral administration of clove oil for 28 days on ALT of male (I-IV) and female (V-VIII) rats	60

4.30	Effect of repeated oral administration of clove oil for 28 days on bilirubin of male (I-IV) and female (V-VIII) rats	61
4.31	Effect of repeated oral administration of clove oil for 28 days on total cholesterol of male (I-IV) and female (V-VIII) rats	61
4.32	Effect of repeated oral administration of clove oil for 28 days on total protein of male (I-IV) and female (V-VIII) rats	62
4.33	Effect of repeated oral administration of clove oil for 28 days on albumin of male (I-IV) and female (V-VIII) rats	63
4.34	Section of heart from male control rats (group I) showing normal architecture (H & E stain X 120).	66
4.35	Section of heart from clove oil treated male rats of group III (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).	66
4.36	Section of heart from female control rats (group V) showing normal architecture (H & E stain X 120).	67
4.37	Section of heart from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).	67
4.38	Section of liver from male control rats (group I) showing normal architecture (H & E stain X 120).	68
4.39	Section of liver from clove oil treated male rats of group III (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).	68
4.40	Section of liver from female control rats (group V) showing normal architecture (H & E stain X 120).	69
4.41	Section of liver from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).	69
4.42	Section of kidney from male control rats (group I) showing normal architecture (H & E stain X 120).	70
4.43	Section of kidney from clove oil treated male rats of group III (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).	70
4.44	Section of kidney from female control rats (group V) showing normal architecture (H & E stain X 120).	71

4.45	Section of kidney from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).	71
4.46	Section of spleen from male control rats (group I) showing normal architecture (H & E stain X 120).	72
4.47	Section of spleen from clove oil treated male rats of group III (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).	72
4.48	Section of spleen from female control rats (group V) showing normal architecture (H & E stain X 120).	73
4.49	Section of spleen from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).	73

ABBREVIATIONS AND SYMBOLS

$	Dollar
%	Per cent
<	Lesser than
>	Greater than
≤	Less than or equal to
µg/ml	Microgram per mililiter
µL	microliter
µl/g	Microliter per gram
µl/ml	Microliter per mililiter
^{0}C	Centigrade
ABST	Antibacterial sensitivity test
AD	Anno Domini
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATCC	American type culture collection
b.wt.	Body weight
BUN	Blood urea nitrogen
CEO	Clove essential oil
CFU	Colony forming unit
CPCSEA	Committee for the purpose of control and supervision of experiments on animals
DAM	Diacetyl-monoxime
DMSO	Dimethylsulfoxide
DNA	Deoxyribose nucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGF	Epidermal growth factor
EOs	Essential oils

et al.	et alibi
FG	Functional grade
FRAP	Ferric reducing antioxidant potential
g	gram
GC	Gas chromatography
h	Hour
H & E	Haematoxylin and eosin
Hb	Hemoglobin
HDL	High density lipoprotein
i.p.	Intraperitoneal
IC_{50}	Inhibitory concentration for 50 percent
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
K_3EDTA	Ethylenediaminetetraacetic acid
Kg	Kilogram
LD_{50}	Lethal dose for 50% of population
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
m	Meter
Max	Maximum
MBC	Minimum bactericidal concentrations
µg	Microgram
MCH	Mean corpuscular hemoglobin
MCV	Mean Corpuscular Volume
mg	Milligram
mg/dl	Milligram per deciliter
mg/kg	Milligram per kilogram

mg/ml	Milligram per miiliter
MHA	Mueller Hinton Agar
MIC	Minimum inhibitory concentrations
ml	Milliliter
ml/kg	Milliliter per kilogram
mm	Millimeter
MMP	Matrix metalloproteinase
MPE	Maximal percent effect
MS	Mass spectrometry
No.	Number
NOAEL	No observed adverse effect level
OECD	Organization for economic cooperation and development
ORAC	Oxygen radical absorbance capacity assay
p.o.	Per Os
PCV	Packed cell volume
ppm	Parts per million
RBCs	Red blood corpuscles
rpm	Revolution per minute
s/c	Sub cutaneous
SE	Standard error
SFE	Supercritical Fluid Extraction
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
SOD	Superoxide dismutase assay
SPSS	Statistical package for the social sciences
TC	Total cholesterol
TGF	Tumor growth factor
TLC	Total leukocyte counts

TNF	Tumor necrosis factor
UK	United Kingdom
US	United states
v/v	Volume in volume
VLDL	Very low density lipoprotein
w/v	Weight in volume
WBCs	White blood corpuscles
w/w	Weight in weight

CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
	ABSTRACT	iii
	ACKNOWLEDGEMENT	ix
	LIST OF TABLES	xi
	LIST OF FIGURES	xiii
	LIST OF ABBREVATION AND SYMBOLS	xvii
1	**INTRODUCTION**	**1-2**
2	**REVIEW OF LITERATURE**	**3-23**
	2.1 ESSENTIAL OILS	3
	2.2 CLOVE	4
	2.2.1 The Botanical Classification	4
	2.2.2 Components of Clove	5
	2.3 CLOVE OIL	5
	2.3.1 Source of Clove Oil	6
	2.3.2 Extraction of Clove Oil	6
	2.3.3 Composition of Clove Oil	7
	2.3.4 Use of Clove Oil in Food Industry	7
	2.3.5 Medicinal Use of Clove Oil	7
	2.4 ANTI-BACTERIAL ACTIVITY	8
	2.5 ANTI-INFLAMMATORY EFFECT	12
	2.6 ANTI-OXIDANT ACTIVITY	16
	2.7 ANTI-FUNGAL ACTIVITY	16
	2.8 ANALGESIC EFFECT	17
	2.9 ANTI-CANCER ACTIVITY	18
	2.10 SAFETY STUDIES	18
	2.10.1 Effect of Clove Oil on Body Weight	18
	2.10.2 Effect of Clove Oil on Feed Consumption	19
	2.10.3 Effect of Clove Oil on Haematology	19
	2.10.4 Effect of Clove Oil on Serum Biochemistry	20
	2.10.5 Effect of Clove Oil on Histopathology	22
3	**MATERIALS AND METHODS**	**24-32**
	3.1 LOCATION	24

	3.2 EXPERIMENTAL ANIMALS	24
	3.3 ACCLIMATIZATION	24
	3.4 HOUSING AND ENVIRONMENTAL CONDITIONS	24
	3.5 NUMBERING AND IDENTIFICATION	25
	3.6 DRUGS AND CHEMICALS	25
	3.7 PREPARATION OF CARRAGEENAN AND INDOMETHACIN SOLUTION	26
	3.8 *IN-VITRO* ANTIBACTERIAL SENSITIVITY ACTIVITY OF CLOVE OIL	26
	3.8.1 Sources of Test Organisms	26
	3.8.2 Preparation of Diffusion Solution	26
	3.8.3 Preparation of Different Concentration of Clove Oil	26
	3.8.4 Disc Diffusion Method	27
	3.9 *IN-VIVO* ANTI-INFLAMMATORY ACTIVITY OF CLOVE OIL	27
	3.10 SAFETY STUDY OF CLOVE OIL	28
	3.11 COLLECTION OF SAMPLES	29
	3.11.1 Collection of Blood	29
	3.11.2 Collection of Tissues	29
	3.12 OBSERVATIONS	30
	3.12.1 Clinical Signs and Mortality	30
	3.12.2 Body Weight	30
	3.12.3 Feed Consumption	30
	3.12.4 Hematological Estimation	30
	3.12.5 Serum Biochemical Parameters	30
	3.13 Histopathology	32
	3.14 Statistical Analysis	32
4	**RESULTS AND DISCUSSION**	**33-65**
	4.1 EVALUATION OF *IN VITRO* ANTIBACTERIAL ACTIVITY	33
	4.1.1 Antibacterial Sensitivity Testing / Disc diffusion Assay	33
	4.2 EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF CLOVE OIL IN WISTAR RATS	**40**
	4.3 SAFETY STUDY OF CLOVE OIL IN WISTAR RATS	45
	4.4 CLINICAL SIGNS AND MORTALITY	46
	4.5 EFFECT ON BODY WEIGHT	47
	4.6 EFFECT ON FEED CONSUMPTION	49
	4.7 HEMATOLOGICAL ESTIMATION	50

	4.7.1 Hemoglobin (Hb)	50
	4.7.2 Red Blood Cells (RBCs)	50
	4.7.3 Packed Cell Volume (PCV)	50
	4.7.4 Total Leucocytes Counts (TLCs)	51
	4.7.5 Mean Corpuscular Volume (MCV)	51
	4.7.6 Mean Corpuscular Hemoglobin (MCH)	51
	4.7.7 Mean Corpuscular Hemoglobin Concentration (MCHC)	51
	4.8 SERUM BIOCHEMICAL PARAMETERS	56
	4.8.1 Serum Creatinine	56
	4.8.2 Blood Urea Nitrogen (BUN)	56
	4.8.3 Aspartate Aminotransferase (AST)	56
	4.8.4 Bilirubin	57
	4.8.5 Alanine Aminotransferase (ALT)	57
	4.8.6 Total Cholesterol (TC)	57
	4.8.7 Total Protein	57
	4.8.8 Albumin	57
	4.9 HISTOPATHOLOGICAL FINDINGS	64
	4.9.1 Kidney, Liver, Spleen and Heart	64
5	**SUMMARY AND CONCLUSIONS**	**74-77**
	REFERENCES	i-xiii
	APPENDICES	i-iv

CHAPTER – I

INTRODUCTION

Essential oils (EOs) also called volatile or ethereal oils are aromatic oily liquid obtained from diffrent plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). The term 'essential oil' is thought to derive from the name coined by Paracelsus von Hohenheim in the 16th century. Essential oils are complex mixtures of low molecular weight (usually less than 500 daltons) compounds extracted by steam distillation, hydrodistillation or solvent extraction (Nakatsu *et al*., 2000). Most of the time bioactivities of a particular EO are decided by either one or two of its main components (Bakkali *et al*., 2008). Around 3000 essential oils have been produced by using atleast 2000 plant species, out of which 300 are important from the commercial point of view. The production of essential oils 40,000–60,000 tonnes per annum with estimated market value of 700 million US $, indicate that production and consumption of essential oils is increasing all over the World (Djilani and Dicko, 2012). Use of EOs in traditional systems of medicine is being practiced since ancient times in human history. Researchers from all over the world are trying to characterize a range of biological properties of Eos which includes antimicrobial, antiviral, antimutagenic, anticancer, antioxidant, anti-inflammatory, immunomodulatory and antiprotozoal activities (Bakkali *et al*., 2008).

Scientific name of clove is *Syzygium aromaticum* (*Eugenia caryophyllata*) belonging to Myrtaceae family. Clove is commonly called as "laving" in Gujarati and "laung" in Hindi. The name clove comes from the French name "clou" meaning nail (Charles, 2013). The clove is aromatic, dry, fully grown, but unopened flower bud of clove tree. Clove is a tree which is growing in islands of Indonesia, Tanzania, Sri Lanka, Madagascar, India and Malaysia (Arung *et al*., 2011). In India clove is mostly grown in the hilly tracts of Tamilnadu, Karnataka and Kerala. Clove oil is obtained by distillation of the leaves, flowers, buds and stem of the clove tree (Anderson *et al*., 1997). Clove contains 14-20% of volatile oil that includes eugenol, acetyleugenol, sesquiterpenes (α-and β-caryophyllenes) and small quantities of esters, ketones and alcohol. Clove also contains tannins, sitosterol and stigmosterol (Evans, 2001).

Clove is the oldest spice which has been in use for many years. In India, clove is used as a spice in foods. It was used for more than 2000 years for checking decay of the tooth and also for halitosis (bad breath). The oldest records of clove being used in china for medicinal property were as early as 240AD.

Clove relieves stomach pain, nausea and vomiting (Bhowmik *et al*., 2012). Clove has a deodorizing property and so used in perfumes and cosmetics (Daniel *et al*., 2009). It shows analgesic activity in people suffering from tooth pain (Tyler *et al*., 1988). The main component of the clove oil is the eugenol which possesses an anasthetic, analgesic and anti-inflammatory activity (Diaz *et al*., 1985). The essential oil of clove has biocidal activity against *Aedes albopictus* (tiger mosquitos), thereby helping in the control of malaria (Bhat and Kempraj, 2009). Stress which is very common in every individual can also be relieved with the help of hydro-alcoholic extracts of clove oil (Singh *et al.*, 2009).

Clove and its essential oil has been found effective in poultry to improve growth performance, control some intestinal pathogens and stimulate digestion and also showed strong antimicrobial, antifungal, anti-inflammatory, anesthetic, anti-carcinogenic, anti-parasitic and antioxidant activities (Mitsch *et al*., 2004; Najafi and Torki, 2010).

There are limited reports on the subacute toxicity study or safety study of clove oil in rats till date. The aim of this study was to evaluate the biological activity like antibacterial & anti-inflammatory activities of commercially available clove oil. So, the present study is undertaken with the following objectives.

Objectives:

1) To study *in vitro* antibacterial activity of clove oil.
2) To study *in vivo* anti-inflammatory activity of clove oil in rats.
3) To study safety of repeated oral administration of clove oil at three different dosages for 28 days in rats.

CHAPTER – II

REVIEW OF LITERATURE

2.1 ESSENTIAL OILS

Essential oils (EO) are complex mixtures of low molecular weight (usually less than 500 daltons) compounds extracted by steam distillation, hydrodistillation or solvent extraction (Nakatsu *et al*., 2000). Essential oils are not strictly oils, but are often poorly soluble in water as are oils. Essential oils often have a pleasant odor and sometimes a distinctive taste and are therefore used in significant amounts in the flavoring and perfume industries (Burt, 2004). Around 3000 essential oils have been produced by using 2000 plant species, out of which 300 are important from the commercial point of view. 40,000–60,000 tonnes per annum production with estimated market value of 700 million US $, indicate that production and consumption of essential oils is increasing all over the World (Djilani and Dicko, 2012). Most of the time the bioactivities of a particular EO is decided by either one or two of its main components (Bakkali *et al*., 2008).

All of the EO producing plant families are rich in terpenoids. While, plant families like Apiaceae (Umbelliferae), Lamiaceae, Myrtaceae, Piperaceae and Rutaceae contain phenylpropanoids more frequently (Chami *et al*., 2004). Many commercially important plants belong to the family Myrtaceae. For example, *Melaleuca alternifolia*, *Eucalyptus globulus*, *Syzygium aromaticum* (*Eugenia caryophyllus*) and *Myrtus communis* produce EOs with well-known antibacterial, antifungal, antitumor, anticancer and antiviral properties (Burt, 2004; Hammer *et al*., 2006). Spices like clove, oregano, mint, thyme and cinnamon have been employed for centuries as food preservatives and as medicinal plants mainly due to its antioxidant and antimicrobial activities (Shaan *et al.*, 2005).

Plant essential oils possess various applications mainly in health, agriculture, cosmetic and food industries. Use of EOs in traditional systems of medicine is being practiced since ancient times in human history. These naturally occurring antimicrobials have extensive histories of their use in foods and can be identified from various components of the plants leaves, barks, stems, roots, flowers and fruits

(Rahman and Gray, 2002; Erasto *et al.,* 2004; Zhu *et al.,* 2004). EOs exhibit antimicrobial properties that may make them suitable alternatives to antibiotics (Chaves *et al*., 2008). Researchers from all over the world are trying to characterize a range of biological properties of EOs which includes antimicrobial, antiviral, antimutagenic, anticancer, antioxidant, anti-inflammatory, immunomodulatory and antiprotozoal activities (Bakkali *et al.,* 2008).

An important characteristic of EOs and their components is hydrophobicity, allowing the EOs to separate the lipids of the bacterial cell membrane and mitochondria and in the process cause the bacterial cell to become more permeable (Burt, 2004; Friedly *et al.,* 2009). The interaction of EOs with microbial cell membranes results in the growth inhibition of some Gram positive and Gram-negative bacteria (Calsamiglia *et al.,* 2007). Gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* are more susceptible to EOs than Gram-negative bacteria such as *Escherichia coli* and *Salmonella Enteritidis* (Chorianopoulos *et al*., 2004). Gram negative cells should be more resistant to plant EOs because they possess a hydrophilic cell wall (Kim *et al.,* 2011).

2.2 CLOVE

2.2.1 The Botanical Classification

- Kingdom: Plantae
- Clade: Angiosperms
- Clade: Eudicots
- Clade: Rosids
- Order: Myrtales
- Family: Myrtaceae
- Genus: Syzygium
- Species: *S. aromaticum*

Plants of the genus Eugenia (Syzygium), comprising of about 100 species, grow in tropical climate in which Clove (*Eugenia caryophyllata*) is a high (up to 15 m), evergreen tree of the family Myrtacae. It blooms twice a year, it yields for 60 years. Its fruits are edible and are used frequently to make spices. From the fifteen

year old plants about up to 4 kg of cloves can be obtained annually. Large spreading plants provide even 50 kg of dry cloves. Carnations, used as spices, are undeveloped buds. Cloves are harvested just before blooming, during the period from august to february, because at that time they contain the most considerable amount of oil. Buds reach the length of 10-18 mm, after drying they become dark brown. Whole cloves can be stored up to 24 months. Annual production of cloves in the world can reach several thousand tons (Gora, 2005).



The larger producer countries of clove are Indonesia, India, Malaysia, Sri Lanka, Madagascar and Tanzania specially the Zanzibar Island (Kamatou *et al.,* 2012). The clove tree is frequently cultivated in coastal areas at maximum altitudes of 200 m above the sea level. The production of flower buds, which is the commercialized part of this tree, starts after 4 years of plantation. Flower buds are collected in the maturation phase before flowering. The collection could be done manually or chemically-mediated using a natural phytohormone which liberates ethylene in the vegetal tissue, producing precocious maturation (Cortes-Rojas *et al.,* 2014).

2.2.2 Components of Clove

Clove represents one of the major vegetal sources of phenolic compounds as flavanoids, hydroxybenzoic acids, hydroxycinamic acids and hydroxyphenyl propens. Eugenol is the main bioactive compound of clove, which is found in concentration ranging from 9.38 to 14.65 g per 100 g of fresh plant material (Neveu *et al., 2010*).

2.3 CLOVE OIL

Clove oil was initially isolated by a German chemist Cordus Valerius, who lived in the first half of the sixteenth century. Since that time, it can be purchased at

pharmacies (Nowak *et al.,* 2012).

2.3.1 Source of Clove Oil

Clove oil can be obtained from distillation of buds, leaf or stem, each resulting in an oil having different characteristics of oil. Clove buds and stem are comminuted before distillation to break the oil cells and widen the surface so that the oil can be released more easily from the cells. Clove leaf does not need pre-treatment as it is already thin. The highest yield derived from high-quality clove bud is 20 %. In the UK, the finest oil containing 85–89 % eugenol, is obtained by water distillation.

Clove bud oil, a colourless or yellow liquid. Clove buds contain 15 to 20 % of oil by weight. The main oil constituents are eugenol (70–95 %), eugenol acetate (up to 20 %) and β-caryophyllene (12–17 %) **(**Guenther, 1950).

Clove stem oil, pale yellow, obtained after distillation is used in the mass market products. It is less expensive than the clove bud oil. Clove stem oil comprises 5–7 % of oil by weight, the major component being eugenol (90–95 %), while eugenyl acetate and β-caryophyllene and others are present in smaller amounts (Nurdjannah *et al*., 1990).

Clove leaf oil, dark brown liquid obtained after distillation of the dry leaves, is the main traded clove oil, because it is less expensive than the formers. It is used as a main source for the production of eugenol, which is used as an analgesic and as a raw material to produce synthetic vanillin. Clove leaf oil contains 3–4 % of oil by weight, the main component being eugenol (80–88 %) with low eugenyl acetate and high content of caryophyllene (Nurdjanah and Hardja, 1991; Hariono, 2009).

2.3.2 Extraction of Clove Oil

Clove oil is extracted from the *Syzygium aromaticum*. The raw material includes leaves and buds of plants. The quality of the oil depends on the origin and maturity of the buds, and the method of preparation. After fragmentation clove buds are carried out by steam distillation, which lasts from 8 to 24 hours. Cohobation and re-distillation of water increases the efficiency of the process, which is about 18% (Rutkowski *et al.,* 2003; Gora, 2005).

Clove oil is also obtained by the hydrodistillation using the *Clevenger* apparatus. Within 4 hours of distillation efficiency reaches 5%. The obtained oil is

dried with anhydrous sodium sulfate (Pourgholami *et al.*, 1999). Clove oil can also be obtained by extraction with ethanol and carbon dioxide in a supercritical fluid state. Supercritical fluid extraction leads to the receipt of high-quality essential oils, where the maximum content of the main ingredient, eugenol is 58.77%. The yield of this process is 19.56% (Guan *et al.*, 2007).

2.3.3 Composition of Clove Oil

Composition of clove oil altogether with its properties, depends on the origin of the plant, its growing season, the weather, time of day and air humidity. Another essential factor is the time which passes between the moment of picking up the raw material, and the production of oil. There are more than 100 components of clove oil which include heptan-2-one (0.05%), α-kopean + α-llangen (0.84%), caryophyllene (0.84%), α-humulen (1.06%), δ-kadinen (0.54%), eugenol (77.13%), eugenol acetate (5.04%) (Gora, 2005).

2.3.4 Use of Clove Oil in Food Industry

Clove and cinnamon oil acts as a natural preservative. It is a harmless component of food products. The mixture of these oils (1:1) inhibits the growth of mold, yeast and microbes. Probably their ingredients such as cinnamic aldehyde and eugenol, damage the cell walls of bacteria. Such properties can be used for storage of food products. Clove oil can also be used as an antioxidant and antimicrobial addition to oils, such as cotton oil. Addition of oil does not affect the color and appearance of cotton seed oil, in concentrations of 50 - 1200 ppm does not change its smell (Cimanga *et al.*, 2002).

2.3.5 Medicinal Use of Clove Oil

The clove oil, an important natural antibacterial drug, is used in many fields, including dentistry, pharmaceuticals and aromatherapy. It is used as an analgesic, antiseptic, warming, disinfectant and antibacterial because it inhibits the growth or kills most pathogens, such as: *E.coli*, *Mycobacterium phlei*, *Bacillus substilis*, *Streptococcus aureus*, *Aspergillus niger*, *Penicillum chrysogenum*. Clove oil is recommended for the treatment of sore throat, colds, catarrh and inflammation of the mucous membranes of the mouth. It is also helps to deal with any breathing problems, general weakness and neuralgia (Cimanga *et al.*, 2002; Gora, 2005; Nowak *et al.*, 2012).

Clove oil is an ingredient of many pharmaceutical preparations, ointments and pain killers. It is also a substrate for the production of dental analgesic preparations. In combination with zinc oxide is used to fill cavities in teeth. In addition, clove oil is included in mouthwash and gum liquids, toothpastes and preparations for disinfection of hands. Eugenol is also widely used in dentistry. Zinc-eugenol paste is a good for the filling of dental canals in treating periodontitis. Furthermore, the paste acts as antiseptic, local anesthetic and is resistant to moisture (Nowak *et al.*, 2012).

Clove oil is widely used in aromatherapy due to its activity. Massages and baths relieve various muscular and rheumatic pains, help with digestion problems, nausea and flatulence (Gora, 2005). However, clove oil can cause skin irritation and allergic reactions. It cannot be applied directly to the skin neither dissolved in vegetable oil nor in massage oil (Nowak *et al.*, 2012). It is also an important component of perfume products, especially those with an oriental flavor. It is also used for perfuming soaps (Gora, 2005).

2.4 ANTI-BACTERIAL ACTIVITY:

Dorman and Deans (2000) reported antibacterial activity of the volatile oil of clove (*Syzygium aromaticum*) against 25 different genera of bacteria. These included animal and plant pathogens, food poisoning and spoilage bacteria. The volatile oils exhibited considerable inhibitory effects against all the organisms under test while their major components demonstrated various degrees of growth inhibition.

Burt (2004) demonstrated antibacterial activity of essential oils (EOs) against *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Shigella dysenteria*, *Bacillus cereus* and *Staphylococcus aureus* at levels between 0.2 to 10 µl/ml. Gram-negative organisms are slightly less susceptible than gram-positive bacteria. A number of EO components have been identified as effective antibacterials, e.g. carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde and cinnamic acid having minimum inhibitory concentrations (MICs) of 0.05–5 µl/ml *in vitro*. A higher concentration is needed to achieve the same effect in foods. Studies with fresh meat, meat products, fish, milk, dairy products, vegetables, fruit and cooked rice have shown that the concentration needed to achieve a significant antibacterial effect is around 0.5–20 µl/g in foods and about 0.1–10 µl/ml in solutions for washing fruit and vegetables.

Perez-Conesa *et al.* (2006) investigated the antimicrobial efficacy of eugenol which was encapsulated in a micellar nonionic surfactant solution on four strains of *Listeria monocytogenes* (Scott A, 101, 108, and 310) and four strains of *Escherichia coli* O157:H7 (H1730, E0019, F4546, and 932). Colony biofilms of all *E. coli* O157:H7 strains were more sensitive to eugenol than *L. monocytogenes* strains. Eugenol was effective at 0.5% (w/w) concentration against *L. monocytogenes* 108. *L. monocytogenes* 310 was also sensitive to eugenol. Results suggest that eugenol recognized as safe essential oil compound which may offer a new means to control the growth of food pathogens such as *E. coli* O157:H7 and *L. monocytogenes* on food contact surfaces.

Sofia *et al.* (2007) evaluated the antimicrobial activity of clove. The activity was tested against three potent food borne pathogens, namely *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* using paper disc diffusion method, cup method and dilution method (qualitative). The results showed that the extracts of clove had good inhibitory action at 1% concentration. At 3% concentration, complete bactericidal effect was achieved.

Fu *et al.* (2007) evaluated the antimicrobial activity of the essential oils from clove (*Syzygium aromaticum* L.) and rosemary (*Rosmarinus officinalis* L.) as alone and in combination. The compositions of the oils were analysed by GC/MS. MIC against three Gram-positive bacteria, three Gram-negative bacteria and two fungi were determined for the essential oils and their mixtures. Also time-kill dynamic processes of clove and rosemary essential oils against *Staphylococcus epidermidis, Escherichia coli* and *Candida albicans* were tested. The MICs of clove oil ranged from 0.062% to 0.500% (v/v). The antimicrobial activity of combinations of the two essential oils indicated their additive, synergistic or antagonistic effects against individual microorganism tests. The time-kill curves of clove essential oil towards these three strains showed clear bactericidal and fungicidal properties.

Saeed *et al.* (2008) investigated the potential of using essential oil of clove (*Syzygium aromaticum*) as natural antibacterial agent against 100 isolates belonging to 10 different species of Gram negative bacilli viz., *Escherichia coli , Proteus mirabilis, Pseudomonas aeruginosa, Enterobacter aerogenes, Klebsiella ozaenae, Klebsiella pneumoniae, Serratia marcescens, Salmonella typhi, Shigella dysentriae* and *Vibrio cholera.* The screening was performed by standard disc diffusion method. Essential

oil of clove exhibited maximum activity against *V. cholerae* with 23.75± 3.03 mm mean diameter of zone of inhibition.

Ayoola *et al.* (2008) reported the antimicrobial activities of the clove oil against Gram-negative bacteria (*Escherichia coli* ATCC 35218, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella paratyphi*, *Citrobacter spp*. and *Enterobacter cloacae*), a Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923) and a fungus (*Candida albicans*) showed a broad spectrum of activity. The clove oil was active against all the microorganisms tested with a minimum zone diameter of 10 mm for *E. cloacae* and *E. coli* isolate and a maximum zone diameter of 35 mm for *C. albicans*.

Moon *et al.* (2011) found that the antibacterial activity of the clove oil was higher than β-caryophyllene but was similar to eugenol against all tested oral bacteria. Furthermore, the MIC and MBC were reduced to one half-one sixteenth as a result of the combination of clove oil or eugenol with antibiotics. The results suggest that the clove oil and eugenol could be employed as a natural antibacterial agent against cariogenic and periodontopathogenic bacteria.

Kumar *et al.* (2014) tested antimicrobial activity of Clove (*Syzygium aromaticum*) against two gram positive (*Bacillus cereus* and *Staphylococcus aureus*) and two gram negative (*Salmonella typhi* and *Escherichia coli*) pathogenic bacteria at different concentration (1000 ppm, 1500 ppm, 2000 ppm). Maximum effect of clove was shown on *Salmonella typhi* with zone of inhibition 23mm at 2000 ppm and minimum effect of clove was shown on *Escherichia coli* with zone of inhibition of 7mm at 1000 ppm.

Vanin *et al.* (2014) determined the antimicrobial and antioxidant activities of clove essential oil and eugenyl acetate produced by enzymatic esterification. Comparing the antibacterial activity of the essential oil of clove before and after esterification, they observed a significant decrease after esterification in the antimicrobial activity of eugenyl acetate, particularly with regard to minimum inhibitory concentration (MIC). Both eugenyl acetate and clove essential oil were most effective to the gram-negative than gram-positive bacteria group.

Abdullah *et al.* (2015) investigated the antimicrobial activity of clove bud oil by agar well diffusion method against four multidrug resistant strains namely

Acinetobacter baumannii, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus feacalis* as well as two standard strains, *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 27853). Clove bud oil exhibited inhibitory effects towards all the test organisms in which MICs ranged from 0.312% (v/v) to 1.25% (v/v). Based on this finding, it may be suggested that these clove bud oil may be used as natural antibacterial agent to treat infections caused by multidrug resistant bacteria.

Yadav *et al.* (2015) studied the effect of eugenol on *in vitro* biofilm and *in vivo* colonization by using microtiter plate assay and otitis media rat model, respectively. This study demonstrated that eugenol exhibits notable activity against *S.aureus*. Eugenol inhibited biofilm formation, disrupted the cell-to-cell connections, detached the existing biofilms, and killed the bacteria in biofilms of *S.aureus*. Therefore, eugenol may be used to control or eradicate *S. aureus* biofilm-related infections.

Shahavi *et al.* (2016) evaluated MIC and MBC of clove oil in aqueous solution by nanoemulsion. Result showed that this formulation of clove oil showed minimum inhibitory concentration against *Escherichia coli* and *Bacillus cereus* were 16 and 32 μg/ml, respectively, whereas minimal bactericidal concentrations were 16 and 64 μg/ml, respectively. Xu *et al.* (2016) exhibited the strong antibacterial activity of clove bud oil against *Staphylococcus aureus* (ATCC 25923) with a minimum inhibitory concentration (MIC) of 0.625 mg/ml and the antibacterial effects depended on its concentration and action time. Kill-time assays also confirmed that the clove oil had a significant effect on the growth rate of surviving *S. aureus*. Clove bud oil penetrates to the cytoplasmic membrane and then inhibits the normal synthesis of DNA and proteins that are required for bacterial growth. In this the results suggested that the effects of the clove bud oil on the growth inhibition of *S. aureus* may be at the molecular level rather than only physical damage.

Marchese *et al.* (2017) reported antimicrobial activity against a wide range of gram negative and gram-positive bacteria. They reported the effect of eugenol on multi-drug resistant microorganism. On the basis of collected data, eugenol represents a very interesting bioactive compound with broad spectrum antimicrobial activity against both planktonic and sessile cells belonging to food-decaying microorganism and human pathogens.

2.5 ANTI-INFLAMMATORY ACTIVITY:

Inflammation is a normal protective response induced by tissue injury or infection and functions to combat invaders in the body (microorganisms and non-self cells) and to remove dead or damaged host cells (Stevenson *et al.,* 2007).

In the inflammatory response there is an increase of permeability of endothelial lining cells and influxes of blood leukocytes into the interstitium, oxidative burst and release of cytokines (interleukins and tumor necrosis factor-α). At the same time, there is also an induction of the activity of several enzymes (oxygenases, nitric oxide synthases and peroxidases) as well as the arachidonic acid metabolism. In the inflammatory process there is also the expression of cellular adhesion molecules, such as intercellular adhesion molecule and vascular cell adhesion molecule (Darshan and Doreswamy, 2004).

In addition to the ability of some essential oils to scavenge free radicals, there is also evidence that some essential oils possess anti-inflammatory activity. For example, chamomile essential oil has been used for centuries as an anti-inflammatory and also for alleviating the symptoms associated with eczema, dermatitis and other pronounced irritation (Kamatou *et al.,* 2010). However, there are other examples of essential oils (eucalyptus, rosemary, lavender, millefolia) along with other plants (pine, clove and myrrh) that have been used as mixed formulations as anti-inflammatory agents (Hajhashemi *et al.,* 2004).

Carrageenan-induced mouse paw oedema is frequently used to determine the anti-inflammatory activity of diverse bioactive compounds such as plant extracts and essential oils (Lino *et al.,* 2005, Iscan *et al.,* 2006, Oyemitamn *et al.,* 2008, Juhás *et al.,* 2008, Apel *et al.,* 2010, Mendes *et al.,* 2010,). This method allows screening the anti-inflammatory of samples, very little information is given about its mechanism. The anti-inflammatory activity of essential oils may be attributed not only to their antioxidant activities but also to their interactions with signalling cascades involving cytokines and regulatory transcription factors and on the expression of pro-inflammatory genes.

Ozturk and Ozbek (2005) investigated the anti-inflammatory activity of clove (*Eugenia caryophyllata*) essential oil. The study involved eight groups; serum physiologic, ethyl alcohol, Indomethacin (3 mg/kg), etodolac (50 mg/kg), cardamom

(0.05 ml/kg), EC-I (0.025 ml/kg), EC-II (0.050 ml/kg), EC-III (0.100 ml/kg) and EC-IV (0.200ml/kg). Inflammation induced by lambda-carrageenan and measured by plethysmometer. Drugs were given intra-peritoneally. Three hours after the injections of the volume measurements of the right hind-paws were repeated and the difference between the volumes were compared. It was found that Indomethacin reduced the inflammation by 95.70%, EC-I by 46.55 %, EC-II by 90.15 %, EC-III by 66.94 % and EC-IV by 82.78%, respectively. The essential oil of *Eugenia caryophyllata* revealed an anti-inflammatory effect matching to that of Indomethacin at 0.05 and 0.2 ml/kg doses. As a result *Eugenia caryophyllata* essential oil extract was also have an anti-inflammatory effect.

Tanko *et al.* (2008) tested the ethanol extracts of *Syzygium aromaticum* flower bud for anti-inflammatory effects in formalin-induced hind paw edema in Wistar rats. Three doses of the ethanol extract (50, 100, and 200mg/kg body weight i.p.) were used for anti-inflammatory studies. The paw diameter was measured with the aid of a vernier caliper at 1, 2, 3, 4 and 5h after the injection of formalin. The ethanol extract of *S.aromatucum* produced 42, 45 and 52% inhibition of paw edema, respectively. The highest activity was obtained at the dose of 200mg/kg.

Daniel *et al.* (2009) evaluated the anti-inflammatory activity of eugenol in carrageenan-induced paw edema in rats. Eugenol was given orally 30 minutes prior to carrageenan injection at different dose (100, 200 and 400 mg/kg). The control group received an equivalent volume of water. Indomethacin (5 mg/kg, *p.o.*) and celecoxib (10 mg/kg, *p.o.*) were used as the reference drugs. At dose of 200 mg/kg, eugenol significantly inhibited carrageenan-induced edema.

Rodrigues *et al.* (2009) investigated the *in vivo* effect of hydroalcoholic extract of clove on pro-inflammatory cytokines (IL-1 and IL-6) production in mice. Treatment of mice with extract of clove was found to inhibit macrophages to produce both IL-1 and IL-6. The essential oil of clove also inhibited the production of these cytokines *in vitro*. Eugenol was found to be the major component of the clove extract and essential oil, and probably is the causative agent of cytokine inhibition.

Grespan *et al.* (2012) reported the efficacy of eugenol, a compound obtained from the essential oil of cloves (*Syzygium aromaticum*) in collagen-induced arthritis. Treatment with eugenol starting at the onset of arthritis (day 25) ameliorated these

clinical signs of collagen-induced arthritis. Results showed that eugenol lowered the levels of cytokines (tumor necrosis factor - α, interferon - γ and tumor growth factor - β) within the ankle joints.

Ahmad *et al.* (2012) reported the beneficial effect of aqueous extract of dried flower buds of *Syzygium aromaticum* (clove) in acute and chronic inflammation. Inflammation was induced in rats by injecting carrageenan in hind paw. Administration of the extract (1 g/kg body weight) inhibited the formation of oedema induced by carrageenan. When compared with the disease control, it is reported that it decrease the elevated levels of succinate dehydrogenase ($p<0.001$), xanthine oxidase ($p<0.05$), lipid peroxidation and increase the activity of catalase ($p<0.001$) and glutathione peroxidase ($p<0.01$) in the two animal models.

Bachiega *et al*. (2012) reported the anti-inflammatory effect of clove on cytokine production (interleukin -1b, IL-6 and IL-10) *in vitro*. Macrophages were incubated with clove (5, 10, 25, 50 or 100 mg/well) for 24 h. Concentrations that inhibited the production of cytokines were used before or after incubation with lipopolysaccharide (LPS), to verify a preventive or therapeutic effect. Clove (100 mg/well) inhibited IL-1b, IL-6 and IL-10 production and exerted an efficient action either before or after LPS challenge for all cytokines.

Taher *et al.* (2015) evaluated the analgesic and anti-inflammatory activity of clove oil in mice. The result revealed that clove oil significantly increased the reaction latency to pain after 60 min by 82.3% ($p < 0.05$) compared with morphine value of 91.7% ($p < 0.01$). Clove oil and indomethacin 50.6% ($p < 0.05$) and 70.4% ($p < 0.01$) inhibition of mouse paw edema induced by carrageenan, respectively.

Nikoui *et al.* (2017) studied the anti-inflammatory effect of clove oil in thirty adult male dogs which were divided into four group after a surgery. The first group received 25 mg/kg of clove oil while the second group was considered as a control. The third and fourth groups received betamethasone (20 mg/kg) and phenylbutazone (15 mg/kg) as anti-inflammatory and anti-pyretic agents, respectively. Results showed that in the clove oil treated animals significantly decreased amount of edema as compared to control ($P \leq 0.05$). Histopathology also revealed that the clove oil-treatment significantly reduced the inflammation.

Tsai *et al.* (2017) determined the effects of clove (*Syzygium aromaticum*)

extract against *Propionibacterium acnes* induced inflammatory responses. The results showed that clove extract significantly suppressed *P. acnes*-stimulated interleukin (IL)-1β, and IL-8 productions *in vitro*. In order to investigate their effects *in vivo,* histological assessment was examined and the observations demonstratded that clove extract inhibit *P. acnes* induced inflammatory responses.

Han and Parker (2017) investigated anti-inflammatory activity of a commercially available clove essential oil (CEO) in human dermal fibroblasts (skin disease model). They evaluated the effect of CEO on 17 protein biomarkers that play critical roles in inflammation and tissue remodeling. Four concentrations of CEO (0.011, 0.0037, 0.0012 and 0.00041% v/v) were studied. CEO at a concentration of 0.011% showed robust antiproliferative effects on human dermal fibroblasts. It significantly inhibited the increased production of several pro-inflammatory biomarkers and also inhibited tissue remodeling protein molecules. This study provides important evidence of CEO-induced anti-inflammatory and tissue remodeling activity in human dermal fibroblasts.

Saeed *et al.* (2017) evaluated the anti-inflammatory effect of the ethanolic extract of *Syzygium aromaticum* using 24 albino rats. The anti-inflammatory activity of the extract was tested in carrageenan induced paw edema in four equal groups of albino rats; groups 1 and 2 were treated with 250 and 500 mg/kg of the ethanolic extract, respectively, group 3 was treated with Indomethacin (as a reference standard compound) and group 4 was the untreated control. Oedema size was monitored at the 1, 2, 4, 6 and 24 hours after the treatment. The ethanolic extract of *S. aromaticum* showed significant ($P < 0.01$) decreased in the oedema size at efficacy rates of 79.41%, 82.39% and 63.92% for the dose, 500 mg/kg at the 2, 4 and 6 hour, respectively.

Singh *et al.* (2018) evaluated anti-inflammatory and anti-oxidant effect of the clove bud oil on some acnegenic pathogens. The essential oil showed an excellent dose dependent anti-inflammatory response with increasing concentration. However, the clove bud oil (1%, v/v) exhibited significant higher effect as compare to diclofenac standard.

Abdelrahman *et al.* (2018) evaluated clove for its potential to reduce inflammatory cytokines in carbon-tetrachloride induced liver injury rat model. At 10

weeks, liver histopathology was done and sera collected for liver and kidney function tests. Clove significantly reduced cytokines (TGF-β, TNF-α and EGF) and liver function enzymes (ALT, AST and GGT) activity as compared to carbon tetrachloride group.

2.6 ANTI-OXIDANT ACTIVITY

Chaieb *et al.* (2007) evaluated the antioxidant effect of the clove oil by measuring its 2,2- diphenyl-l-1-picrylhydrazil radical scavenging ability. Results showed that essential oil exhibited a very strong radical scavenging activity (IC_{50} = 0.2 µg/ml) when compared with the synthetic antioxidant (tert-butylated hydroxytoluene, IC_{50} = 11.5 µg/ml).

Dudonne *et al.* (2009) investigated for its antioxidant properties of aqueous extracts of clove using DPPH and ABTS radical scavenging capacity assay, oxygen radical absorbance capacity (ORAC) assay, superoxide dismutase (SOD) assay, and ferric reducing antioxidant potential (FRAP) assay. Clove aqueous extracts showed strong antioxidant properties and a high phenolic content (about 200 mg /g).

Gulcin (2011) estimated an antioxidant activity of eugenol by ferric thiocyanate method. Eugenol inhibited 96.7% (r2 = 0.9319) lipid peroxidation of a linoleic acid emulsion at a 15-µg/mL concentration. Butylated hydroxyanisole, butylated hydroxytoluene, a-tocopherol, and Trolox_ displayed 95.4% (r2 = 0.8482), 99.7% (r2 = 0.7798), 84.6% (r2 = 0.9272) and 95.6% (r2 = 0.8511) inhibition of peroxidation, respectively, at the 15-µg/mL concentration. According to the results of this study, eugenol showed powerful antioxidant activity and radical-scavenging activity.

2.7 ANTI-FUNGAL ACTIVITY

Abdel-Wahhab *et al.* (2005) investigated the ability of clove oil to scavenge free radicals generated during aflatoxicosis. The groups were treated for 30 days with *Syzygium aromaticum* oils with or without aflatoxin. The results indicated that *Syzygium aromaticum* oil given significant protection against aflatoxicosis to rats fed with aflatoxin-contaminated diet.

Omidbeygi *et al.* (2007) evaluated antifungal activity of essential oils of clove in culture medium and tomato paste. *Aspergillus xavus* were inoculated in Sabouraud

Dextrose Broth and tomato paste and then 0, 50, 200, 350 and 500 ppm of essential oils were added to each sample and then kept at 25±0.5 °C for 2 months. Results showed that clove essential oils could inhibit the growth of *A. xavus* at 500 ppm.

Park *et al.* (2007) investigated the potential of *Syzygium aromaticum* plant oil as natural antifungal agents by using the agar diffusion method. *Syzygium aromaticum* oil (clove oil) completely inhibited *Trichophyton mentagrophytes, Trichophyton rubrum* and *Microsporum gypseum* at a concentration of 0.2 mg/ml. Eugenol was the most effective antifungal constituent of clove oil against the dermatophytes.

Rana *et al.* (2011) evaluated antifungal potential of essential oil of *Syzygium aromaticum* against some common fungal pathogens of plants and animals *Fusarium moniliforme* NCIM-1100, *Fusarium oxysporum* MTCC-284, *Aspergillus spp*., *Mucor spp*., *Trichophyton rubrum* and *Microsporum gypseum*. All fungal species were found to be inhibited by the clove oil when tested through agar well diffusion method.

Hamini-Kadar *et al.* (2014) conducted study to find the efficiency of *Syzygium aromaticum* essential oil against three pathogenic fungus *viz Fusarium oxysporum*, *F. commune* and *F. redolens*. The results revealed that moderate to high level antifungal activities against tested microorganisms at all the tested concentration. The highest antifungal activity was recorded at 1μL/ml; on this concentration clove essential oil caused complete growth inhibition of *F. oxysporum* and *F. redolens* while the highest antifungal activity of oil against *F. commune* was observed at 0.5 μL/ml.

2.8 ANALGESIC EFFECT

Hosseini *et al.* (2011) investigated the analgesic effect of clove oil in mice. Fifty mice were divided into 5 groups: 1) saline control; 2) essential oil (EO) 2%, 3) EO 5%, 4) EO 10% and 5) EO 20%. The hot plate test (55±0.2 °C; Cut-off 60 sec) was performed as a base record 15 min before injection of drugs (Saline or 2, 5, 10 and 20% concentrations of Essential oil) and consequently repeated every 15 minutes after injection. Repeated measures ANOVA test showed that maximal percent effect (MPE) in animal groups treated by 5, 10 and 20% essential oil was significantly higher than saline group. Comparison between 4 treated groups showed that MPE in 10% essential group was higher than 2 and 5% groups however; there was no significant difference between 10% and 20% groups. The result of present study showed that clove essential oil has analgesic effect in mice using hot plate test.

2.9 ANTI-CANCER ACTIVITY

Ghosh *et al.* (2005) reported the inhibitory effect of eugenol on melanoma cells proliferation. Eugenol treatment produced a significant tumor growth delay (p 0.0057), an almost 40% decrease in tumor size, and a 19% increase in the median time to end point. More significantly, 50% of the animals in the control group died from metastatic growth, whereas none in the treatment group showed any signs of invasion or metastasis.

Nam *et al.* (2013) investigated the inhibitory effect of eugenol on matrix metalloproteinase (MMP) expression and activity. Eugenol was contained as a major ingredient in herbs such as clove. The inhibitory effects of eugenol on the activity and expression of MMP-9 activity related to metastasis were determined by using gelatin zymography and western-blot. The data showed that it inhibited MMP-9 activities in PMA-stimulated HT1080 cells. It was found that eugenol exerts inhibitory effects on MMP-9 via inactivation of extracellular-signal-regulated kinase. Results suggest that eugenol could be on melanoma cells proliferation available as an excellent agent for prevention of metastasis related to oxidative stress.

2.10 SAFETY STUDIES:

2.10.1 Effect of Clove Oil on Body Weight

Mishra and Singh (2008) reported that repeated clove bud extract oral administration @ 15, 30 and 60 mg/kg body weight in mice. Result showed no significant alteration in the body weight.

Adam *et al.* (2013) reported the effects of the aqueous extract of clove (50, 200, 400 and 800 mg/kg/day, PO) on body weight of wistar rats. After one week of dosing, the body weight gain @ 50, 200 and 400 mg/kg were lower ($p<0.05$) and @ 800 mg/kg treated rats showed no significant changes when compared to control rats. At the end of the experimental period (2 weeks), the body weight gain of rats in 200 mg/kg treated rats were significantly lower ($p<0.05$) than the controls, but no change was observed concerning to other test groups.

Issac *et al.* (2015) studied sub-acute toxicity of clove oil at doses of 0.5, 1.0 and 2.5 g/ kg body weight for 28 days in rats. Result showed that clove oil did not alter any body weight change compared to that of the control rats.

Vijayasteltar *et al.* (2016) investigated the safety of a standardized polyphenolic extract of clove buds (clovinol), as assessed by oral acute (5 g/kg b.wt. for 14 days) and subchronic (0.25, 0.5 and 1 g/kg b.wt. for 90days) toxicity studies in wistar rats. Clovinol treated (0.25, 0.5 and 1.0 g/kg b.wt.) animals showed no significant difference in the weight gain and growth rate among the male and female animals when compared to the untreated control group of animals.

Gashlan and Beladi (2016) also reported the effect of clove oil on b.wt. in diabetic rats. Initial body weights did not significantly differ among the groups. During the experimental period, the body weights were increase in all groups. At the end of the experiment, there was a very highly significant decrease ($P<0.001$) in the body weight of diabetic rats as compared to the control rats. There were highly significant increase ($P<0.01$) in the body weight of clove oil treated diabetic group 3 (300 mg/kg) and group 4 (600 mg/kg) as compared to untreated diabetic rats.

2.10.2 Effect of Clove Oil on Feed Consumption

Adam *et al.* (2013) reported that the repeated oral administration of aqueous extract of clove @ 50, 200, 400 and 800 mg/kg/day in wistar rats showed showed significant decrease in feed consumption as compare to control rats.

Issac *et al.* (2015) studied the effect of repeated oral administration of clove oil at doses of 0.5, 1.0 and 2.5 g/kg body weight for 28 days on feed consumption in rats. Result showed that clove oil has no significant effects on feed consumption in clove oil treated rats as compared to control rats.

Vijayasteltar *et al.* (2016) reported that administration of clovinol at 0.25 g/kg, 0.5 g/kg and 1 g/kg doses did not produce any significant difference ($p > 0.05$) in the food consumption of male and female rats when compared to untreated group of animals. Water consumption of the clovinol treated animals also remained unchanged when compared with untreated control animals.

2.10.3 Effect of Clove Oil on Haematology

Shalaby *et al.* (2011) studied toxicological effects of essential oil from Clove (500, 1000, 1500, 2000 and 2500 mg/kg b.wt.) on albino rats. Results revealed that the $1/10^{th}$ LD50 of clove oil showed no significant change in WBC count at 5^{th} dose as compared with normal animals, while at the 10^{th} dose of clove oil caused a significant increase in WBC count (+36.0%). Although the clove oil did not produce

any significant effect on RBC count at the 5th dose, there was a significant decrease at the 10th dose (9.4% below the normal level) whereas significant decrease ($P<0.01$) in haemoglobin concentration and platelet count at all the doses investigated.

Adam *et al.* (2013) studied effects of the aqueous extract of clove (50, 200, 400 and 800 mg/kg/day, PO) on haematology of Wistar rats. 50 mg/kg b.wt. treated rats showed the lower values ($p<0.05$) of MCH, MCHC and WBCs count than the control rats. Hb was significantly higher ($p<0.05$) in 200 & 400 mg/kg, RBCs was significantly higher in 50, 200 & 400 mg/kg and WBCs count was significantly higher ($p<0.05$) in 800 mg/kg b.wt. than the control rats. The values of PCV were high ($p<0.05$) 50, 200 & 400 mg/kg b.wt. as compared to control rats. The lymphocytes counts were higher ($p<0.05$) and neutrophils were lower ($p<0.05$) in the test groups than the control rats. The values of MCV remained unchanged.

Issac *et al.* (2015) reported the effect of oral administration of clove oil at doses of 0.5, 1.0 and 2.5 g/ kg body weight on haematology in rats. Clove oil did not produce any significant ($p >0.05$) changes in both haematological parameters. Haemoglobin, WBC, RBC, platelet counts and differential counts of treated animals remained in the normal range, when compared to the normal group rats.

Vijayasteltar *et al.* (2016) investigated the safety of a standardized polyphenolic extract of clove buds (Clovinol), as assessed by oral acute (5 g/kg b.wt. for 14 days) and subchronic (0.25, 0.5 and 1 g/kg b.wt. for 90 days) toxicity studies on wistar rats. Clovinol did not produce any significant ($p > 0.05$) changes in the hematological parameters. Hemoglobin, WBCs, RBCs, platelet counts and differential leucocyte counts (lymphocyte, eosinophil and neutrophils) in clovinol treated rats remained within normal range, when compared to the untreated control group of rats.

Saeed *et al.* (2017) studied safety assessment of ethanolic extract of *Syzygium aromaticum* in albino rats in which groups 1 and 2 were treated with 250 and 500 mg/kg of the extract, respectively, group 3 was treated with Indomethacin and group 4 was the untreated control. In all groups there were no significant ($P > 0.05$) changes in WBCs, RBCs, Hb and PCV as compared to control rats.

2.10.4 Effect of Clove Oil on Serum Biochemistry

Mishra and Singh (2008) reported the repeated clove bud extract administration @ 15, 30 and 60 mg/kg body weight on serum biochemical parameters

in mice. No significant differences were detected in ALT, AST and creatinine levels in serum of mice treated with clove bud extract as compare to control rats.

Shalaby *et al.* (2011) reported the effects of essential oil from clove @ 500, 1000, 1,500, 2,000 and 2,500 mg/kg b.wt. on serum biochemistry in albino rats. The result on liver function showed that clove essential oils produce a significant increase in the activity of GOT at all doses, but revealed significant ($P<0.05$) increase in GPT activity at the 5^{th} dose (11.9% above the normal level) and 10^{th} dose (27.2%). GOT and GPT activities were increased in liver of treated animals.

Adam *et al.* (2013) studied effects of oral administration of aqueous extract of clove @ 50, 200, 400 and 800 mg/kg/day on serum biochemical parameters in wistar rats. There was a significant decrease ($p<0.05$) in AST activity in 50 & 200 mg/kg b.wt. and increased activity ($p<0.05$) in 400 mg/kg b.wt. Increased activity ($p<0.05$) of ALT and cholesterol concentration and deceased activity of ALP and urea and cholesterol concentrations were observed in the test groups when compared to the control rats. Total protein was higher ($p<0.05$) in 400 & 800 mg/kg b.wt and that of bilirubin was higher in 400 mg/kg b.wt. as compared to control rats.

Issac *et al.* (2015) studied sub-acute toxicity of clove oil at doses of 0.5, 1.0 and 2.5 g/kg body weight in rats. Biochemical parameters related to hepatic and renal function showed normal values. Renal profile, liver function markers, creatinine, urea or the serum electrolyte levels in both male and female rats showed no significant ($p >0.05$) change as compare to the normal animals after 28 days of clove oil supplementation. Lipid profile remained unchanged with no significant variation in cholesterol, HDL, LDL and VLDL levels of both male and female rats and was comparable to that of normal group.

Gashlan and Beladi (2016) studied effects of clove oil on blood glucose level, lipid profile, lipid peroxidation and kidney function on diabetic rats. Diabetic groups treated with different doses (300 and 600 mg/kg) of clove oil showed an improvement in the levels of glucose, insulin, lipid profile, urea, creatinine and lipid peroxidation when compared with untreated diabetic group.

Vijayasteltar *et al.* (2016) investigated the safety of a standardized polyphenolic extract of clove buds (clovinol), as assessed by oral acute (5 g/kg b.wt. for 14 days) and subchronic (0.25, 0.5 and 1 g/kg b.wt. for 90 days) toxicity studies

on wistar rats. Clovinol administration did not produce any significant changes in biochemical parameters related to hepatic and renal function as compared to the untreated control group of animals. Lipid profile also remained unchanged with no significant ($p > 0.05$) variation in total cholesterol, HDL, LDL and VLDL cholesterol levels among both male and female rats, and were comparable to that of untreated control group.

Saeed *et al.* (2017) studied safety assessment of ethanolic extract of *Syzygium aromaticum* in albino rats in which groups 1 and 2 were treated with 250 and 500 mg/kg of the extract, respectively, group 3 was treated with Indomethacin and group 4 was the untreated control. The activities of AST and ALT showed no significant ($P > 0.05$) changes for all groups. Also all groups showed no significant ($P > 0.05$) changes in the concentration of total protein, albumin, billirubin urea and creatinine. No abnormal values were recorded in the control group.

2.10.5 Effect of Clove Oil on Histopathology

Shalaby *et al.* (2011) studied toxicological effects of essential oil from clove *(*500, 1000, 1,500, 2,000 and 2,500 mg/kg b.wt.) in albino rats. Liver sections of rats received an oral dose equal to $1/10^{th}$ LD50 of clove essential oil showed small vacuoles in hepatocytes along with structure of the hepatocytes appeared more or less like normal. The section of kidney also revealed congestion of the renal corpuscle, renal tubules, proximal convoluted tubules and distal convoluted tubules. Examination of kidney of treated rats by $1/10^{th}$ LD50 of clove essential oil showed lobulated renal corpuscles and the desquamation of the epithelial cells of the renal tubules.

Adam *et al.* (2013) studied effects of the aqueous extract of clove (50, 200, 400 and 800 mg/kg/day, PO) in wistar rats. After the end of the treatment period, no lesions were seen in the spleen, heart and liver of 200 mg/kg aqueous extract of clove treated rats and fatty cytoplasmic vacuolation of the centirlobular hepatocytes and hemorrhage seen in the liver and packing of the glomerular tubules, dilatation and necrosis of the renal tubules in the kidney of 400 mg/kg aqueous extract of clove treated rats, infilteration of lymphocytes and disquemination of the intestinal epithelium in the intestine of 800 mg/kg aqueous extract of clove treated rats.

Issac *et al.* (2015) studied sub-acute toxicity of clove oil at doses of 0.5, 1.0

and 2.5 g/kg body weight for 28 days in rats. Necropsy of the treated animals showed normal appearance of various organs and tissues. Clove oil treated rats showed normal cellular architecture similar to the normal group. The liver section of clove oil treated animals showed normal portal triads and central venous system; normal hepatocytes were arranged in cords with kupffer cells and showed normal sinusoidal spaces, which were identical with those from the normal animals. The tissue sections of spleen from clove oil treated animals showed normal lymphoid follicles with areas prominent in germinal centers. The kidney tissues of clove oil treated animals showed normal glomeruli with bowman's capsule and renal tubules. The interstitial tissues appeared normal with no apparent abnormalities when compared with the tissues of normal group of animals. The section of brain tissue of clove oil administered rats showed hyperplasia of the astrocytes with pleomorphism. There were occasional mitotic cells, normal glial cells and the interstitial tissues. The cerebellum also appeared normal. Overall cellular architecture and morphology of the clove oil treated animals were similar to the brain from normal animals.

Gashlan and Beladi (2016) studied effects on kidney tissues in diabetic groups treated with (300 and 600 mg/kg) of clove oil and normal groups treated with of clove oil (300 and 600 mg/kg) showed no alterations in morphology.

Vijayasteltar *et al.* (2016) investigated the safety of a standardized polyphenolic extract of clove buds (Clovinol), as assessed by oral acute (5 g/kg b.wt. for 14 days) and subchronic (0.25, 0.5 and 1 g/kg b.wt. for 90 days) toxicity studies in wistar rats. The histopathological examination of various organs of animals treated with 1.0 g/kg (b.wt.) of clovinol showed normal cellular architecture when compared with untreated groups of animals.

Saeed *et al.* (2017) studied safety assessment and potential anti-inflammatory effect of ethanolic extract of *Syzygium aromaticum* in albino rats. Groups 1 and 2 were treated with 250 and 500 mg/kg of the extract, respectively, group 3 was treated with ndomethacin and group 4 was the untreated control. *S. aromaticum* treated rats showed histopathological changes mainly, dilatation of the cortical tubules, congestion and dilatation of renal blood vessels. Liver showed congestion in the portal tract with slight necrosis of hepatocytes in liver.

CHAPTER – III

MATERIALS AND METHODS

3.1 LOCATION

The present study was carried out at the College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat. Rats were housed in cages at Laboratory Animal House Facility of Veterinary College. *In-vivo* anti-inflammatory assay of clove oil was carried out at Department of Veterinary Pharmacology and Toxicology, Veterinary College, AAU, Anand. Hematological and serum biochemical parameters were investigated at Department of Veterinary Physiology and Biochemistry, Veterinary College, AAU, Anand. Histopathological examination was carried out at Department of Veterinary Pathology, Veterinary College, AAU, Anand.

3.2 EXPERIMENTAL ANIMALS

The study was conducted on adult healthy male and female Wistar rats. 25 male rats (335 to 355 g) and 25 female (220 to 240 g) rats of 8-10 weeks of age were procured from Cadila Healthcare Ltd. (R & D Centre), Moraiya, Ahmedabad, Gujarat. All the protocols as per the CPCSEA guidelines on the care and use of laboratory animals were followed and approved by the Institutional Animal Ethics Committee (Project No. IAEC/279/VPT/2018) of Veterinary College, Anand. Rats were kept under constant observation during entire period of study.

3.3 ACCLIMATIZATION

All the rats were kept under acclimatization for 5 days prior to grouping and initiation of experiment.

3.4 HOUSING AND ENVIRONMENTAL CONDITIONS

All the rats were housed in polypropylene cages at Laboratory animal house facility in an environmentally controlled room with 22 ± 3 ºC temperature. Although the relative humidity was at least 30% and preferably not to exceed 70% other than during room cleaning, the aim was 50-60%. Light/dark cycles of 12/12 hours were provided throughout the study period. Rats were provided with standard pellet diet (SRK'S Scientist's Choice, Sangli, Maharashtra, India). The Composition of rat feed

is presented in the Table 3.1. Deionized water was provided *ad libitum* throughout the course of the experiment. All necessary managemental procedures were adopted to keep the rats free from stress.

Table 3.1: Composition of rat feed

Component	Unit
Crude protein (Min.)	20-21 %
Ether extract (Min.)	4-5 %
Crude fiber (Max.)	4 %
Ash (Max.)	8 %
Calcium (Min.)	1.2 %
Phosphorus (Min.)	0.6 %
Nitrogen free extract	54 %
Metabolizable energy	3600 K cal/kg
Pellet size	12 mm

(Composition as per SRK'S Scientist's Choice, Sangli, Maharashtra, India)

3.5 NUMBERING AND IDENTIFICATION

Identification of rats was done by body marking with picric acid solution prepared in water. Cage label and body marking were specific to identify the rats. Three rats were kept in one cage and marking on head, body and unmark indicated rat number 1, 2 and 3, respectively.

3.6 DRUGS AND CHEMICALS

Carrageenan from seaweed (Non-gelling, mixture of λ & κ carrageenan) was purchased from Sigma-Aldrich, India and Indomethacine 10 mg was purchased from local medical store of Anand district (Gujarat). Clove essential oil (Natural, Functional grade) was purchased from Sigma-Aldrich. All other reagents used of analytical grade like biochemical kits for estimation of serum biochemical parameters were purchased from Coral Clinical System (Goa, India). The standard antibacterial discs like ampicillin 10 mcg, gentamicin 10 mcg, tetracycline 3 0mcg and cefotaxime 30 mcg were purchased from HiMedia laboratories, India. The sterile blank discs were also purchased from HiMedia laboratories, India.

3.7 PREPARATION OF CARRAGEENAN AND INDOMETHACIN SOLUTION

For the preparation of 10% w/v carrageenan suspension, 0.5 gm carrageenan was weigh by digital analytical weighing balance which was dissolved in 5 ml of normal saline (10 %). For the preparation of Indomethacin suspension each 25 mg capsule was dissolved in 5 ml of distilled water so each ml contains 5 mg.

3.8 *IN-VITRO* ANTIBACTERIAL SENSITIVITY ACTIVITY OF CLOVE OIL

3.8.1 Sources of Test Organisms

The test bacterial organisms were procured from National Chemical Laboratory, Pune. Bacterial strains of six bacterial species, namely, *Streptococcus agalactiae* (ATCC 13813), *Listeria Monocytogenes* (ATCC 1911), *Staphylococcus aureus* (ATCC 6538P), *Pseudomonas aeruginosa* (ATCC19154), *Escherichia coli* (ATCC 10799), *Salmonella typhimurium* (ATCC 23564). Purity and viability of the organisms were checked by morphological, cultural and biochemical tests and maintained by periodical subculture.

3.8.2 Preparation of Diffusion Solution

For the preparation of 10% dimethylsulfoxide (DMSO), 10 ml of DMSO was dissolved in 90 ml of distilled water in measuring cylinder. For the preparation of diffusion solution, 0.5 ml of tween 80 was dissolved in 99.5 ml of 10% DMSO.

3.8.3 Preparation of Different Concentration of Clove Oil

Table 3.2: Schedule for the preparation of Clove oil dilution

Dilution	Clove oil (%)	Diffusion solution
1:1	Pure (100%)	-
1:2	0.5 ml (50%)	0.5 ml
1:5	0.2 ml (20%)	0.8 ml
1:10	0.1 ml (10%)	0.9 ml
1:20	0.05 ml (5%)	0.95 ml

3.8.4 Disc Diffusion Method

Screening of clove oil for antibacterial activity was done by the disc diffusion method. Three gram-positive and three gram-negative strains of bacteria were tested. It was performed using an 18 h culture at 37°C in 10 ml of Mueller Hinton Agar. The test suspension was standardized to match 0.5 McFarland turbidity standard which corresponds to approximately 10^5CFU/ml with sterile saline solution. Five hundred microliters of the suspensions were spread over the plates containing Mueller-Hinton agar (for *S. agalactiae* 5% defibrinated sheep blood was added) using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. The clove oil was suspended in 10% dimethylsulfoxide (DMSO) with tween 80 (0.5% v/v for easy diffusion) and sterilized by filtration through a 0.45 µm membrane filter. Under aseptic condition, empty sterilized discs (Whatman no. 5, 6 mm diameters) were impregnated with 50 µl of different concentrations (1:1, 1:2, 1:5, 1:10, 1:20) of the respective clove oil and placed on the agar surface (Wayne, 2002). Paper disc moistened with aqueous DMSO was placed on the seeded petriplate as a vehicle control. A standard disc containing antibacterial drug was used as reference control. All petridishes were sealed with sterile laboratory parafilm to avoid eventual evaporation of the test samples. The plates were left for 30 minutes at room temperature to allow the diffusion of oil and then they were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured with a vernier caliper. All the dilutions of clove oil were tested in triplicate against each bacteria.

3.9 *IN-VIVO* ANTI-INFLAMMATORY ACTIVITY OF CLOVE OIL

The *in-vivo* anti-inflammatory assay of clove oil (*Syzygium aromaticum)* was carried out using rat paw edema method as described by Winter *et al.*, (1962). Fifty rats were divided randomly to 10 groups each of 5 males and 5 females. All rats were injected subcutaneously with 0.1 ml of a 10% w/v carrageenan suspension (0.1 ml of a 1% suspension in 10% saline) s/c in the sub-planter region of the left hind limb as a local acute edema inducer after 30 minutes subsequent to oral administration of clove oil. Rats of male and female control groups were kept untreated. Rats of male and female standard control group were treated orally with indomethacin @ 10 mg/kg b.wt. as a reference drug, respectively. Clove oil was given orally to male and female rats @ three different dose rate 100, 250 and 500 mg/kg b.wt., respectively. Edema was expressed as the increase in paw volume (ml). The paw volume was measured up

to the tibiotarsal articulation. Volume of edematous paw was measured by using plethysmometer (PLM-01 plus, Orchid Scintific Instrument, India) at 0 hr (before treatment), 1, 2, 3, 4, 6 and 24 hours after treatments. Increase in paw thickness and percent inhibition were calculated. Treatment protocol to evaluate *in-vivo* activity of clove oil in rats is listed below.

Table 3.3: Schedule for the groups under treatments for anti-inflammatory assay in wistar rats

Group/ Treatment	Dose (mg/kg)	Route of administration	No. of animals/ Sex
Control- male	-	Oral	5(M)
Standard Control (Indomethacin)	10	Oral	5(M)
Clove oil	100	Oral	5(M)
Clove oil	250	Oral	5(M)
Clove oil	500	Oral	5(M)
Control- female	-	Oral	5(F)
Standard Control (Indomethacin)	10	Oral	5(F)
Clove oil	100	Oral	5(F)
Clove oil	250	Oral	5(F)
Clove oil	500	Oral	5(F)

3.10 SAFETY STUDY OF CLOVE OIL

The subacute oral toxicity studies of clove oil (*Syzygium aromaticum)* were carried out as per Organization for economic cooperation and development (OECD) guideline no. 407. Forty rats were divided into eight groups, each group contains 5 males and 5 females. Group I were served as the male control and group V served as female control group. Clove oil was administered orally at dose of 50, 100 and 200 mg/kg body weight once daily for 28 days to male rats of group II, III and IV and female rats of group VI, VII and VIII, respectively. The solutions were administered orally to rats directly in stomach by using rat oral feeding gavage for 28 days. Safety study protocol is mentioned below.

Table 3.4: Schedule for the groups under treatments for safety study in wistar rats

Group	Treatment	Dose (mg/kg)	Route of administration	No. of animals & sex
I	Control	-	Oral	5(M)
II	Clove oil	50	Oral	5(M)
III	Clove oil	100	Oral	5(M)
IV	Clove oil	200	Oral	5(M)
V	Control	-	Oral	5(F)
VI	Clove oil	50	Oral	5(F)
VII	Clove oil	100	Oral	5(F)
VIII	Clove oil	200	Oral	5(F)

3.11 COLLECTION OF SAMPLES

3.11.1 Collection of Blood

After 28 days of dosing period, on 29th day blood samples were collected from all the rats by retro-orbital plexuses puncture under light anesthesia with the help of capillary tube. Blood samples (0.5 ml) collected in K_3EDTA test tubes were utilized for hematological evaluation, whereas blood samples (1.5 ml) collected in centrifuge tubes without anticoagulant were allowed to clot at room temperature (26 ± 2°C) and then allow to clot for 2h. After that serum was harvested by centrifugation at 3000 rpm for 10 minutes and stored at -60^{o}C in ultra-low temperature freezer for biochemical analysis and analyzed within 24 hrs.

3.11.2 Collection of Tissues

After collection of blood samples on 29th day, all the rats were sacrificed by barbiturate over dose as per CPCSEA guideline. All sacrificed rats were subjected to post mortem examination to determine the presence/absence of gross and histopathological lesions. Detailed post mortem lesions from all the rats were recorded. Tissue samples *viz.*, kidney, liver, spleen, and heart were collected and preserved in 10% formalin solution for histopathological examination.

3.12 OBSERVATIONS

3.12.1 Clinical Signs and Mortality

All the rats belonging to group I to IV for male and group V to VIII for female were observed daily for any abnormal physical or behavioral changes and mortality throughout the dosing period.

3.12.2 Body Weight

Body weight of all rats of group I to IV for male and group V to VIII for female were taken initially on day 0 (before initiation of treatment) and then on 7^{th}, 14^{th}, 21^{st} and 28^{th} day of experimental period.

3.12.3 Feed Consumption

The quantity of feed was offered to all group of male and female rats housed in each cage based on the requirement and the same was recorded, left over of the feed was recorded every day to calculate feed consumed.

3.12.4 Hematological Estimation

Blood samples collected in test tubes with K_3EDTA were subjected to estimation of following hematological parameters on the day of blood collection by auto hematology analyzer (Mindray, BC-2800 Vet, Garnerville, New York).

1. Haemoglobin (Hb)
2. Total R.B.C. counts (RBCs)
3. Packed cell volume (PCV)
4. Total leukocyte counts (TLCs)
5. Mean Corpuscular Volume (MCV)
6. Mean Corpuscular Hemoglobin (MCH)
7. Mean Corpuscular Hemoglobin Concentration (MCHC)

3.12.5 Serum Biochemical Parameters

Serum biochemical parameters were estimated using standard assay kits (Coral Clinical System, Goa, India) with the help of auto serum chemistry analyser (Mindray BS-120, Mumbai, India).

1. Serum Creatinine
2. Blood Urea Nitrogen
3. Aspartate Aminotransferase (AST)
4. Alanine Aminotransferase (ALT)
5. Total Cholesterol
6. Total Bilirubin
7. Total Protein
8. Total Albumin

a) Serum Creatinine

Serum creatinine estimation was done by using creatinine kits (Modified Jaffe's Kinetic Method) using the manufacturer's protocol.

b) Blood Urea Nitrogen (BUN)

BUN estimation was carried out by Diacetyl-monoxime (DAM) method described by Wybenga *et al.* (1971).

c) AST (Aspartate aminotransferase)

AST (Aspartate aminotransferase) estimation was done by using AST kits (Modified IFCC method) using the manufacturer's protocol.

d) ALT (Alanine aminotransferase)

ALT (Alanine aminotransferase) estimation was done by using ALT kits (Modified IFCC method) using the manufacturer's protocol.

e) Total Cholesterol (TC)

Total cholesterol estimation was done by using kits (Colorimetric enzyme method) using the manufacturer's protocol.

f) Total Bilirubin

Total Bilirubin estimation was done by using kits (Modified Jaffe's Kinetic Method) using the manufacturer's protocol.

g) Total Protein

Total Protein estimation was done by using kits (Modified Jaffe's Kinetic Method) by following the manufacturer's protocol.

h) Total Albumin

Total Albumin estimation was done by using kits (Modified Jaffe's Kinetic Method) using the manufacturer's protocol.

3.13 HISTOPATHOLOGY

After recording gross lesions, the tissues were collected and fixed in 10 % formalin. The formalin fixed tissues were processed by paraffin wax embedding method of tissue sectioning. Sections from all the tissues were cut at 5-6 microns thickness with automatic section cutting machine (Leica, Automatic Microtome Machin, Germany) and were stained with Haematoxylin and Eosin (H & E) stains. The H & E stained slides were observed under microscope and lesions were recorded.

3.14 STATISTICAL ANALYSIS

One-way-analysis of variance (ANOVA) was used to compare the means of various parameters of *in vitro* anti-bacterial, *in vivo* anti-inflammatory and safety studies by using computer software SPSS (Version 25). Significant differences ($p<0.05$) between different experimental groups were analyzed by Duncan's test. All the data have been presented as mean ± SE.

CHAPTER – IV

RESULTS AND DISCUSSION

4.1 EVALUATION OF *IN VITRO* ANTIBACTERIAL ACTIVITY

4.1.1 Antibacterial Sensitivity Test / Disc Diffusion Assay

The present study was conducted by using 6 different bacterial species (3 Gram positive and 3 Gram negative) against 5 different ratio of clove oil (1:1, 1:2, 1:5, 1:10 and 1:20) compared with 4 different antibacterial drugs (cefotaxime, ampicillin, tetracycline and gentamicin). Mueller Hinton Agar (for *S. agalactiae* 5% defibrinated sheep blood was added) was used for the antibacterial sensitivity test. The test suspension was standardized to match 0.5 McFarland turbidity standard which corresponds to approximately 10^5CFU/ml with sterile saline solution. The clove oil was suspended in solution containing mixture of 10% dimethylsulfoxide (DMSO) and tween 80 (0.5% v/v) for easy diffusion. It was sterilized by filtration through a 0.45 μm membrane filter. After the incubation period, the zone of inhibition was measured in millimeter with a vernier caliper.

The results of the present study revealed that the clove oil showed antibacterial activity with varying magnitudes. The zone of inhibition above 10 mm in diameter was taken as positive results. Both gram positive and gram negative bacteria were sensitive to the clove oil. There was no inhibition in growth of bacteria with the vehicle control (10% DMSO with 0.5% v/v tween 80). Four antibacterial drugs (cefotaxime, ampicillin, tetracycline and gentamicin) were also tested against all six organisms and were found active against test bacteria. The anti-bacterial activity of 5 different concentrations of clove oil and four standard antibacterials are summarized in Table 4.1.

Clove oil (*Syzygium aromaticum*) at 1:1, 1:2, 1:5 and 1:10 concentartions were less active against *Salmonella typhimurium* than cefotaxime, ampicillin, tetracycline and gentamicin. Against *P.aeruginosa* 1:1, 1:2 and 1:5 concentrations of clove oil, tetracycline and gentamicin were equally active, while all concentrations of clove oil were less active than cefotaxime and ampicillin. Against *E. coli* 1:1, 1:2, 1:5 and 1:10 concentrations of clove oil and gentamicin were equally active, while all

concentrations of clove oil were less active than cefotaxime, ampicillin and tetracycline. Against *S. aureus* 1:1, 1:2 and 1:5 concentrations of clove oil and gentamicin were equally active, while all concentrations of clove oil were less active than cefotaxime, ampicillin and tetracycline. Against *L. monocytogenes* all concentrations of clove oil were less active than cefotaxime, ampicillin and tetracycline. Against *S.agalactiae* 1:1, 1:2, 1:5 and 1:10 concentrations of clove oil and tetracycline were equally active, while all concentrations of clove oil were less active than cefotaxime, ampicillin and gentamicin.

Table 4.1: Antibacterial activity of clove oil against *Salmonella typhimurium, P. aeruginosa, E. coli, S. aureus, L. monocytogenes* and *S. agalactiae* by using antibacterial sensitivity test

	S.typhimurium	*P.aeruginosa*	*E.coli*	*S.aureus*	*L.Monocytogenes*	*S.agalactiae*
SA (1:1) (100%)	16.67 ± 0.33^{cd}	18.33 ± 0.33^{b}	20.00 ± 0.00^{b}	19.67 ± 1.20^{b}	25.00 ± 2.00^{bcd}	17.67 ± 1.76^{b}
SA (1:2) (50%)	16.33 ± 0.33^{cd}	17.33 ± 0.88^{b}	19.00 ± 0.00^{b}	18.67 ± 1.20^{b}	25.33± 1.33^{bcde}	17.00 ± 2.08^{b}
SA (1:5) (20%)	15.33 ± 0.67^{c}	15.33 ± 0.88^{b}	19.33 ± 0.67^{b}	18.33 ± 1.86^{b}	24.00± 0.00^{bc}	15.33 ± 2.40^{b}
SA (1:10) (10%)	12.00 ± 0.00^{b}	0.00	16.67 ± 0.88^{b}	15.33 ± 1.86^{ab}	20.67± 0.88^{b}	13.67 ± 2.1^{b}
SA (1:20) (5%)	0.00	0.00	8.67 ± 4.48^{a}	13.00 ± 1.1^{a}	11.33± 5.69^{a}	0.00
Cefotaxime	27.33 ± 0.33^{e}	27.00 ± 0.58^{c}	29.00 ± 1.00^{c}	28.33 ± 0.88^{c}	30.67± 0.67^{cde}	30.67 ± 0.88^{d}
Ampicillin	26.67 ± 0.33^{e}	30.33 ± 1.45^{c}	25.33 ± 1.20^{c}	31.67 ± 2.40^{c}	33.67± 2.85^{e}	23.33 ± 0.88^{c}
Tetracycline	21.33 ± 0.88^{d}	17.33 ± 5.3^{b}	25.00 ± 0.58^{c}	28.67 ± 1.86^{c}	34.33± 2.19^{de}	16.00 ± 1.00^{b}
Gentamicin	20.33 ± 2.40^{cd}	20.33 ± 1.86^{b}	18.00 ± 0.58^{b}	19.00 ± 0.58^{b}	31.67± 1.20^{cde}	29.67 ± 0.88^{d}

Mean value with dissimilar superscript in a column vary significantly at $p<0.05$
SA = *Syzygium aromaticum* oil (clove oil)

Similar results were reported by Dorman and Deans (1999). They reported significant inhibitory effect of *Syzigium aromaticum* oil against *E. coli* (13.6 mm), *S. aureous* (14.9 mm) and *P. aeruginosa* (14.0 mm). Similarly, Fu *et al.* (2007) reported antibacterial activity of clove oil against *E. coli* (16.3 mm), *S. aureous* (16.3 mm) and *P. aeruginosa* (9.5 mm) zone of inhibition. Result showed that essential oil

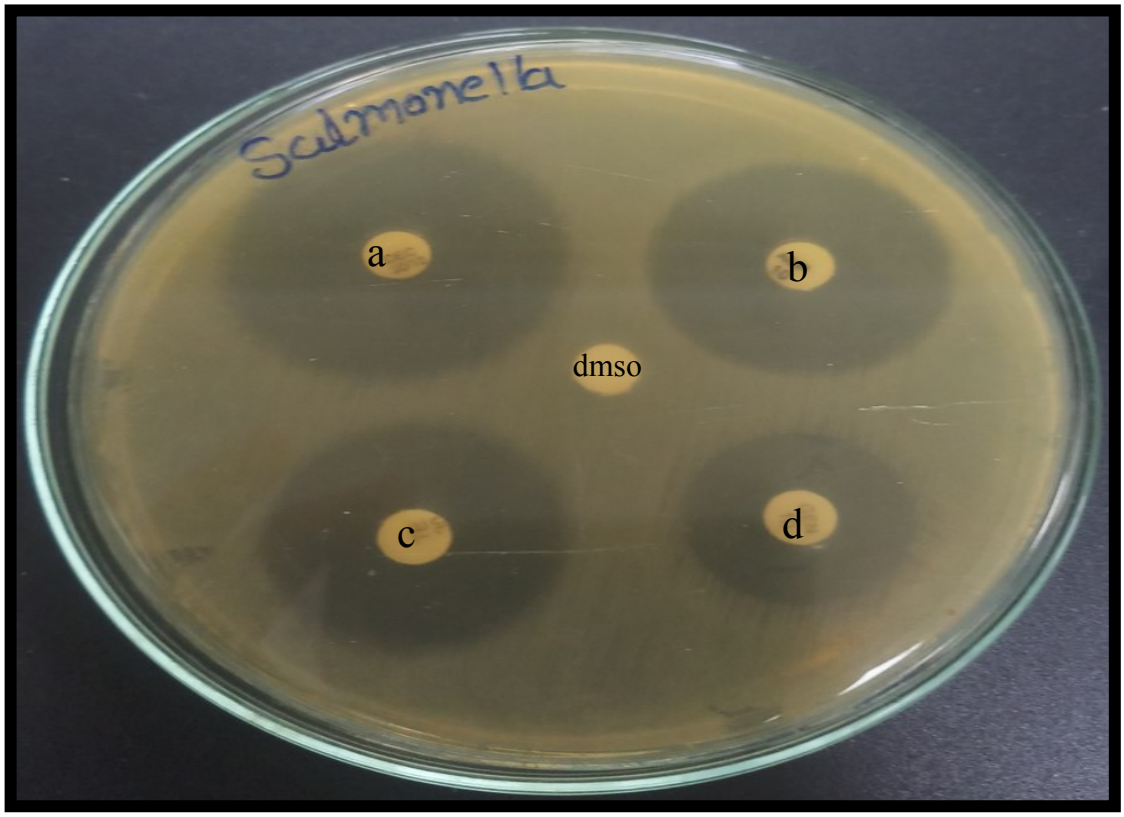

Figure 4.1: Antibacterial sensitivity test of standard controls against *S.typhimurium* (a= Cefotaxime, b = Ampicillin, c = Tetracycline, and d= Gentamicin)

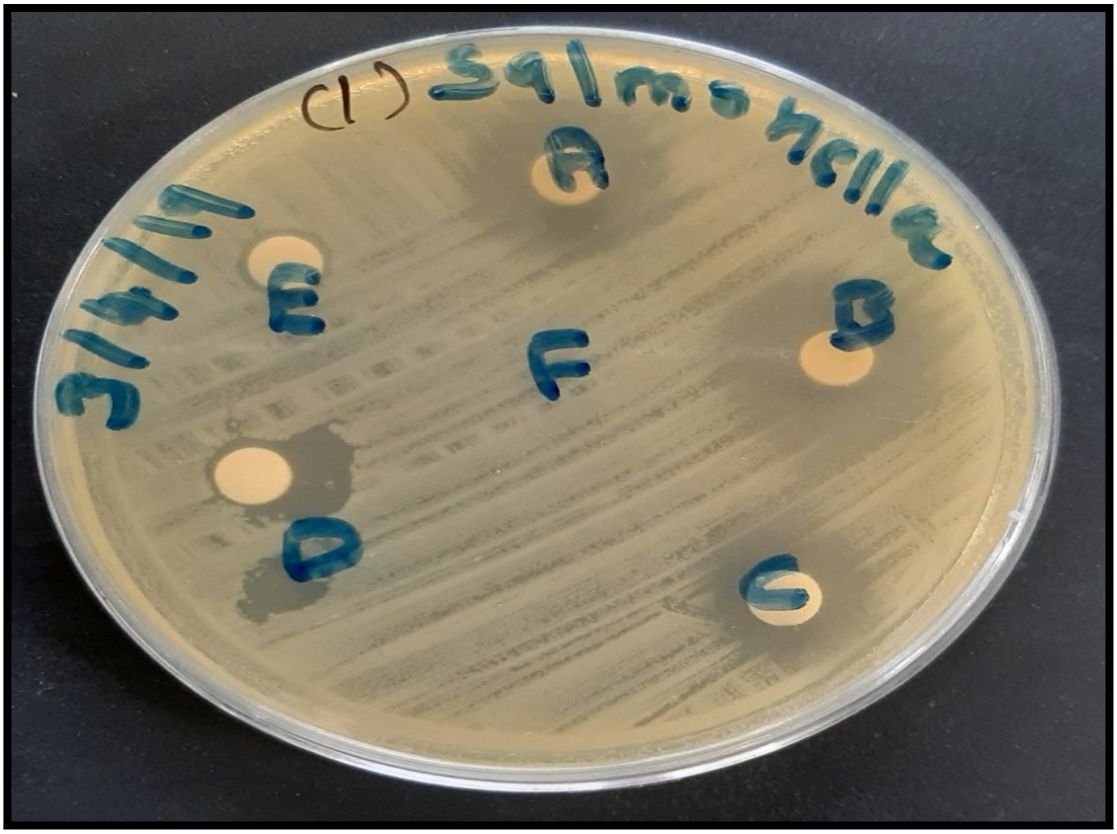

Figure 4.2: Antibacterial sensitivity test of clove oil concentrations against *S.typhimurium* (A= 1:1, B= 1:2, C= 1:5, D= 1:10, E= 1:20)

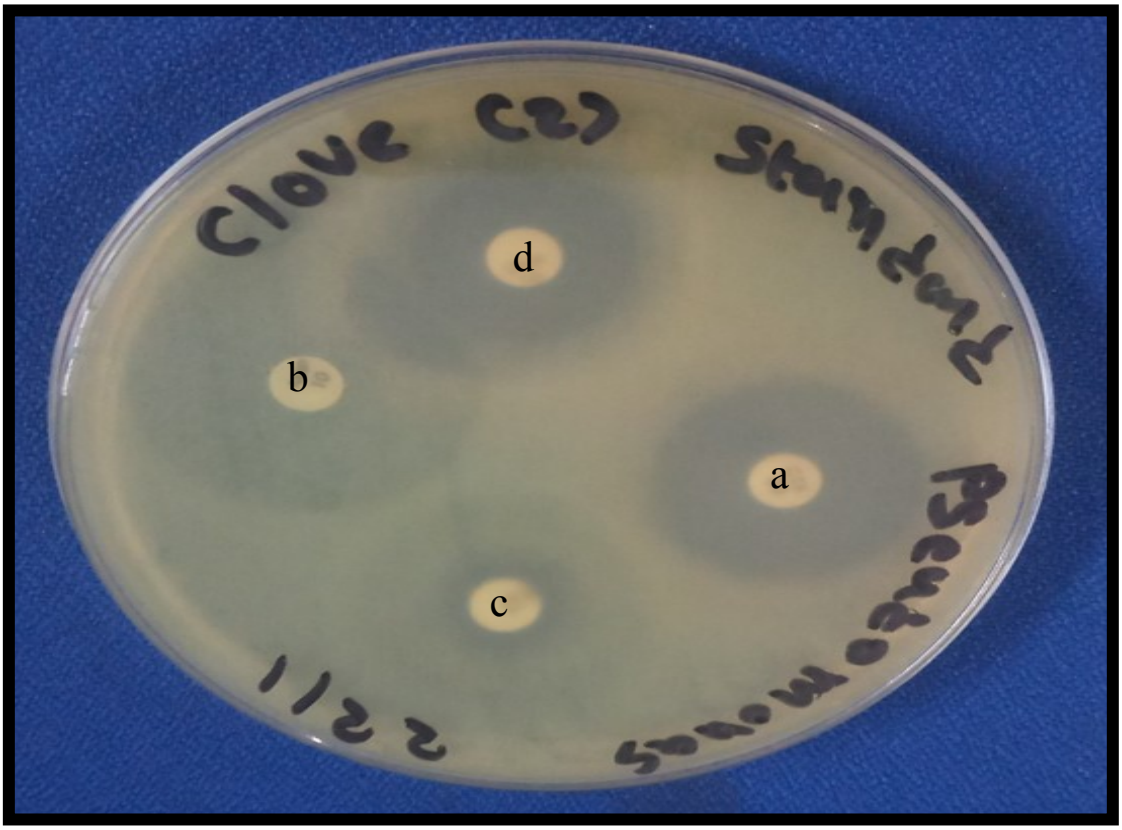

Figure 4.3: Antibacterial sensitivity test of standard controls against *P. aeruginosa* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=Gentamicin)

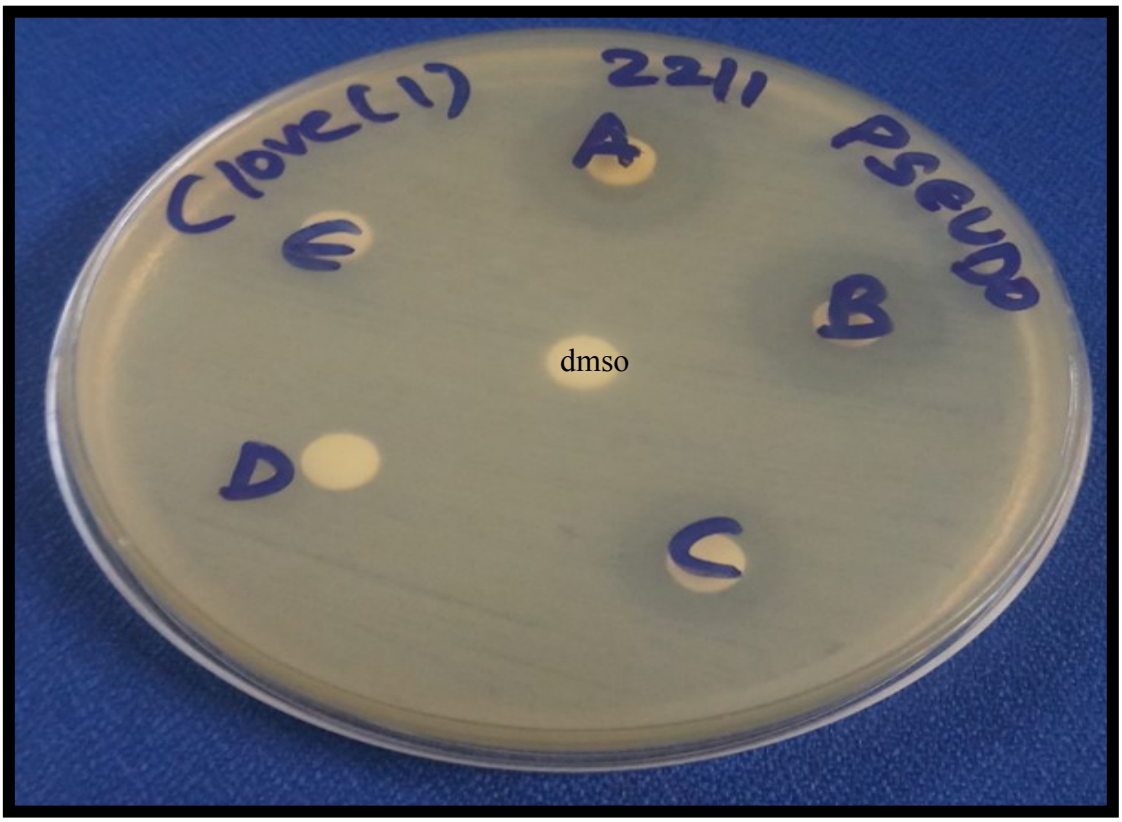

Figure 4.4: Antibacterial sensitivity test of clove oil concentrations against *P.aeruginosa* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)

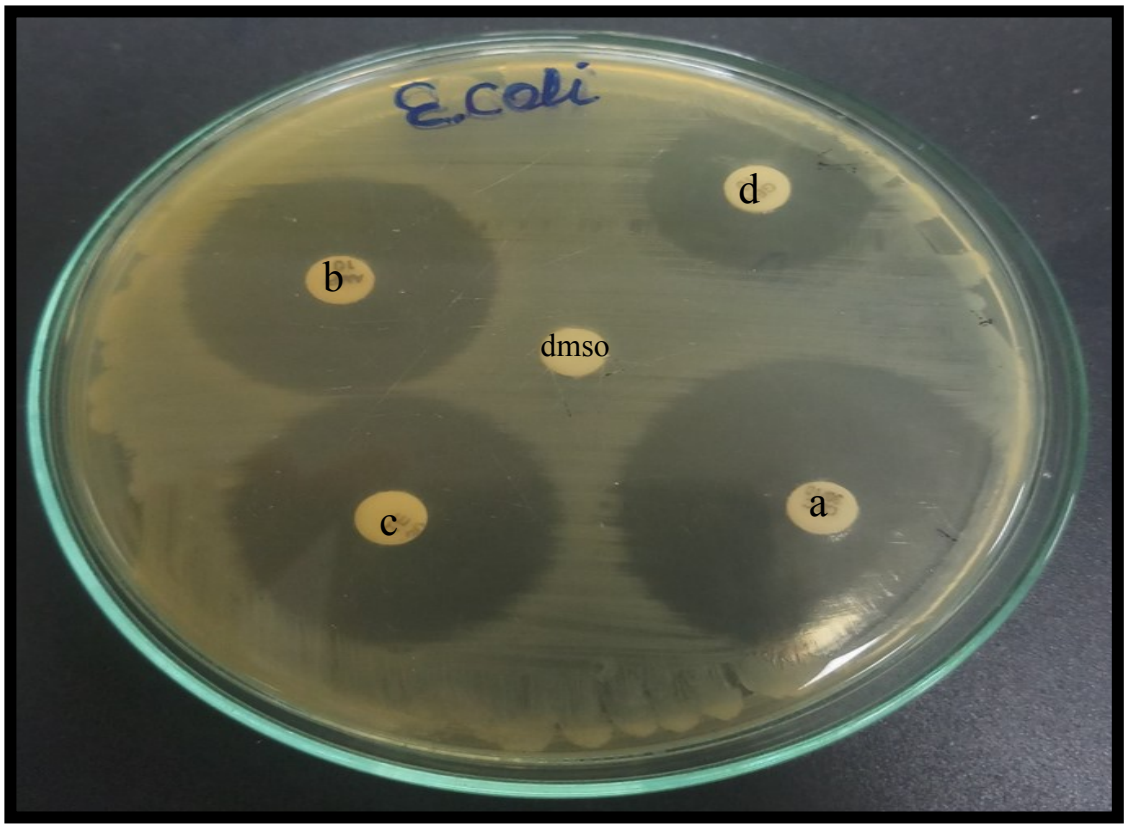

Figure 4.5 : Antibacterial sensitivity test of standard controls against *E. coli* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=Gentamicin)

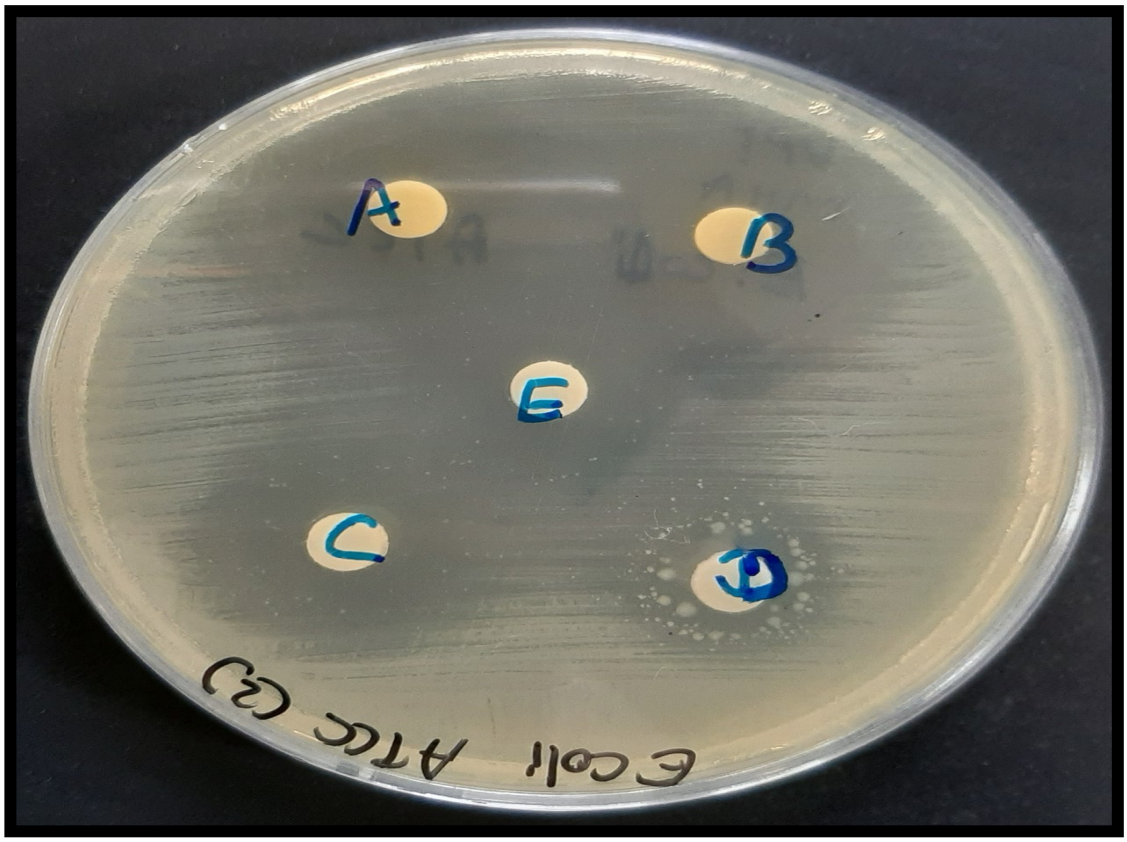

Figure 4.6: Antibacterial sensitivity test of clove oil concentrations against *E. coil* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)

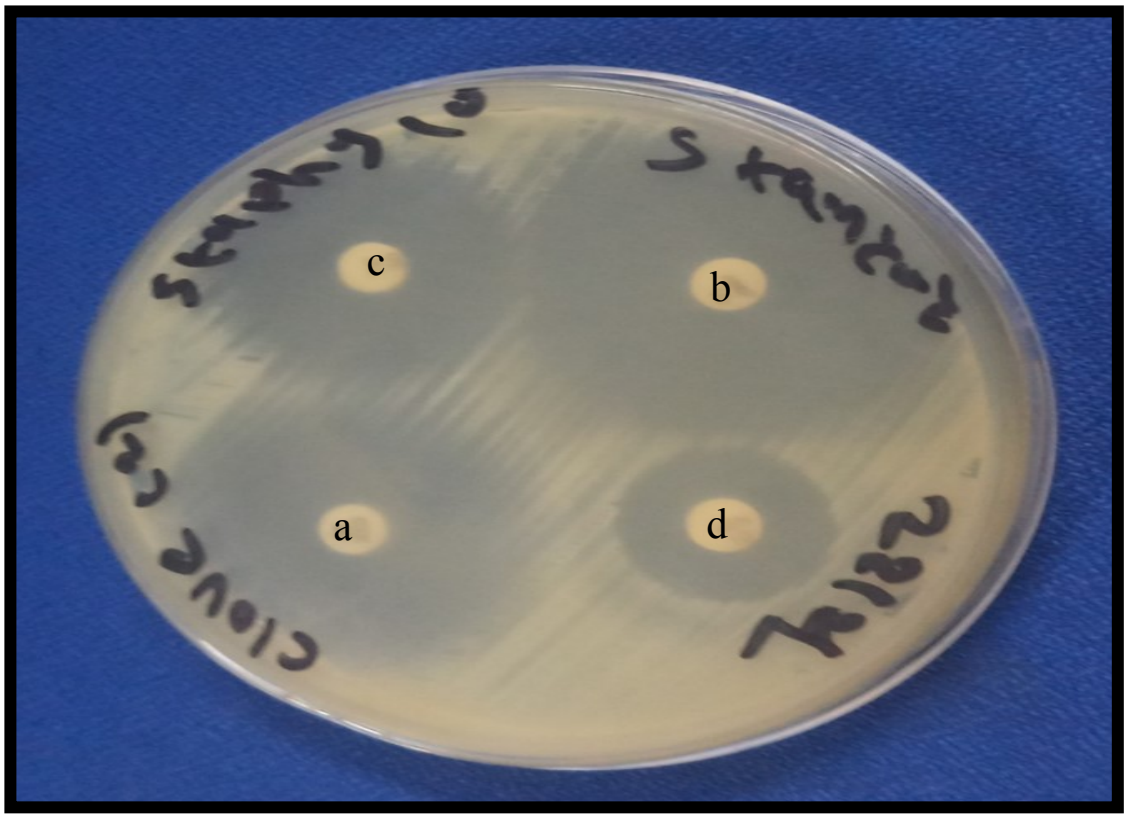

Figure 4.7: Antibacterial sensitivity test of standard controls against *S. aureus* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=Gentamicin)

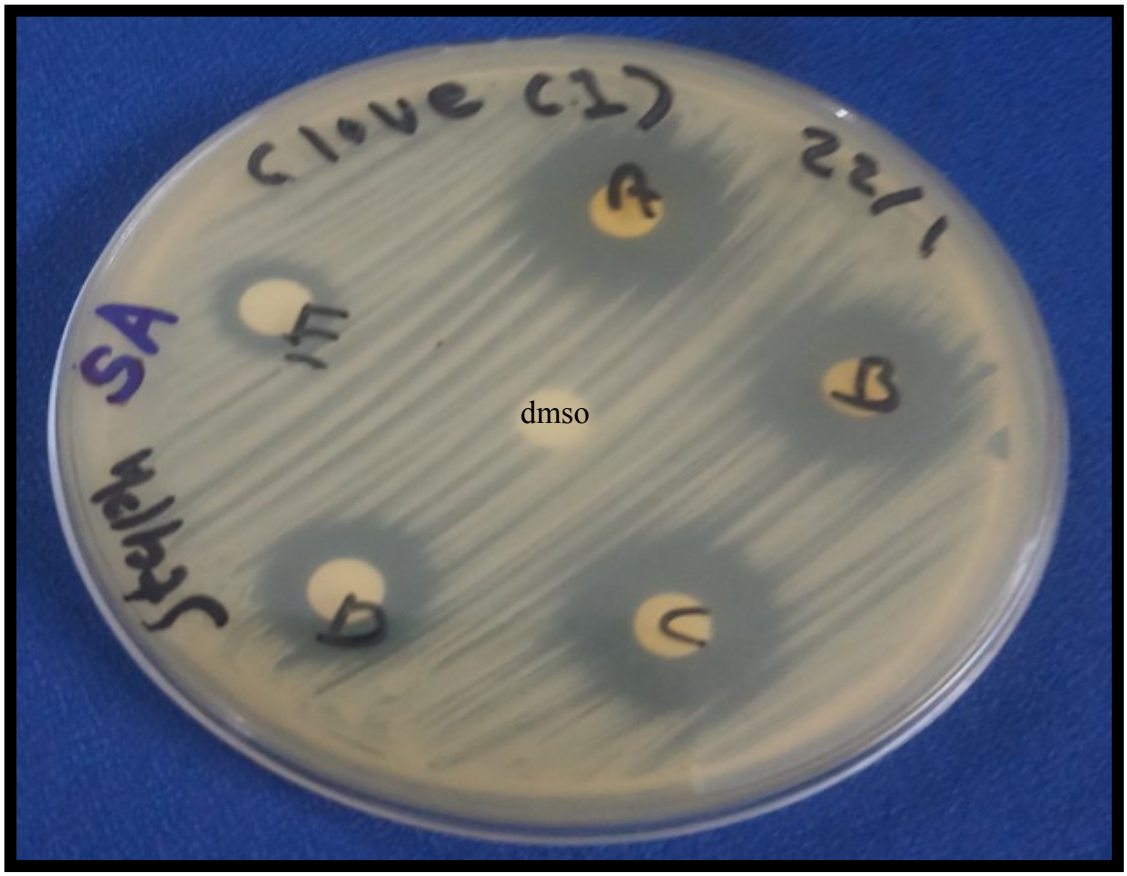

Figure 4.8: Antibacterial sensitivity test of clove oil concentrations against *S. aureus* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)

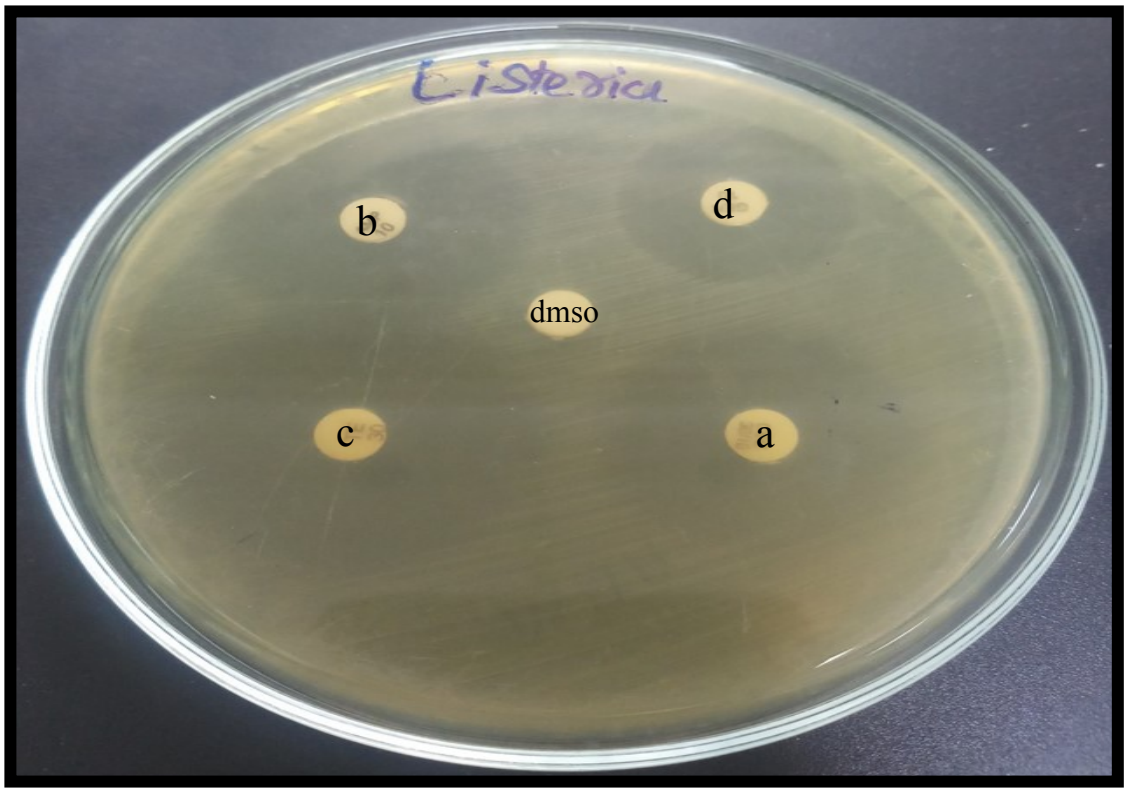

Figure 4.9: Antibacterial sensitivity test of standard controls against *L.monocytogenes* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=Gentamicin)

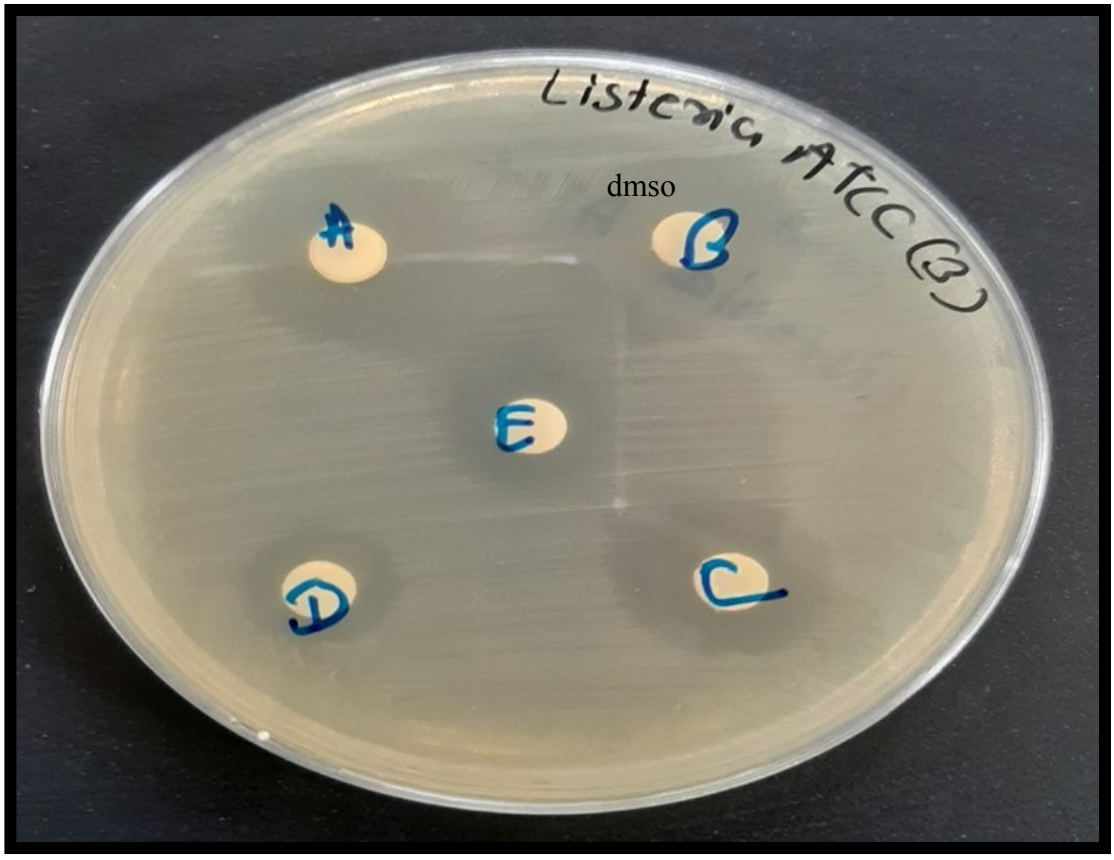

Figure 4.10: Antibacterial sensitivity test of clove oil concentrations against *L.monocytogenes* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)

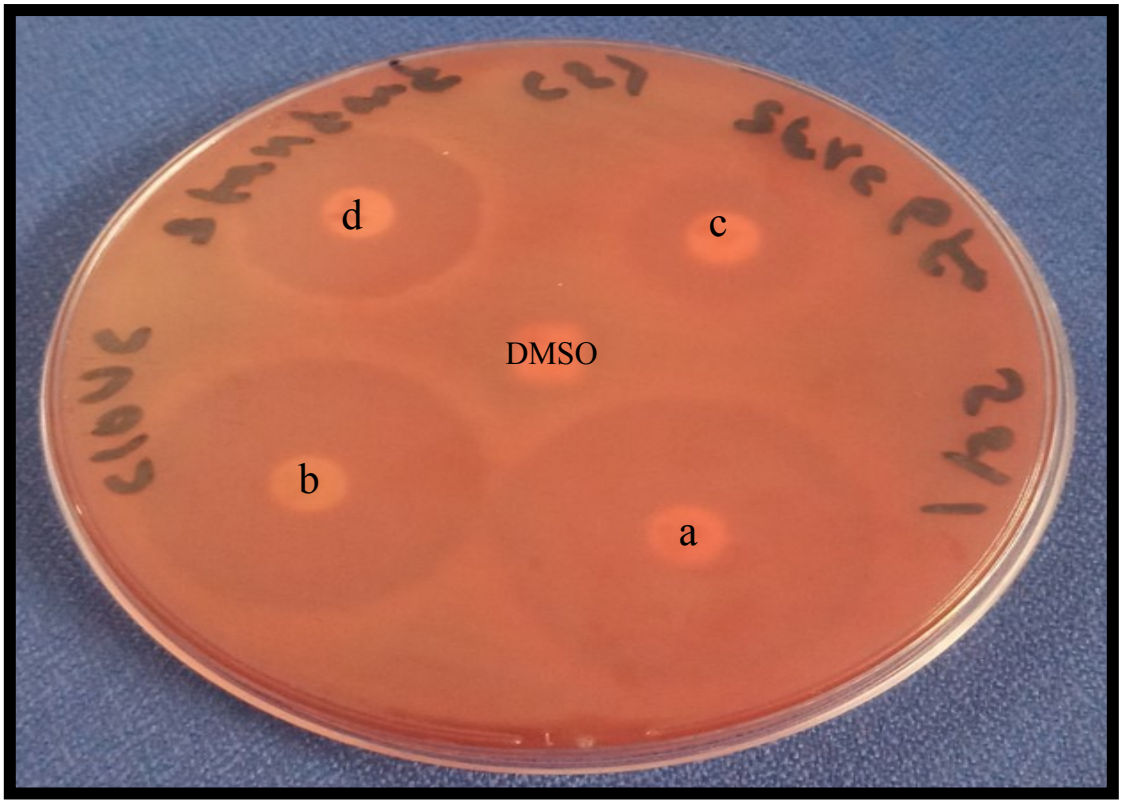

Figure 4.11: Antibacterial sensitivity test of standard controls against *S. agalactiae* (a=Cefotaxime, b=Ampicillin, c=Tetracycline, and d=Gentamicin)

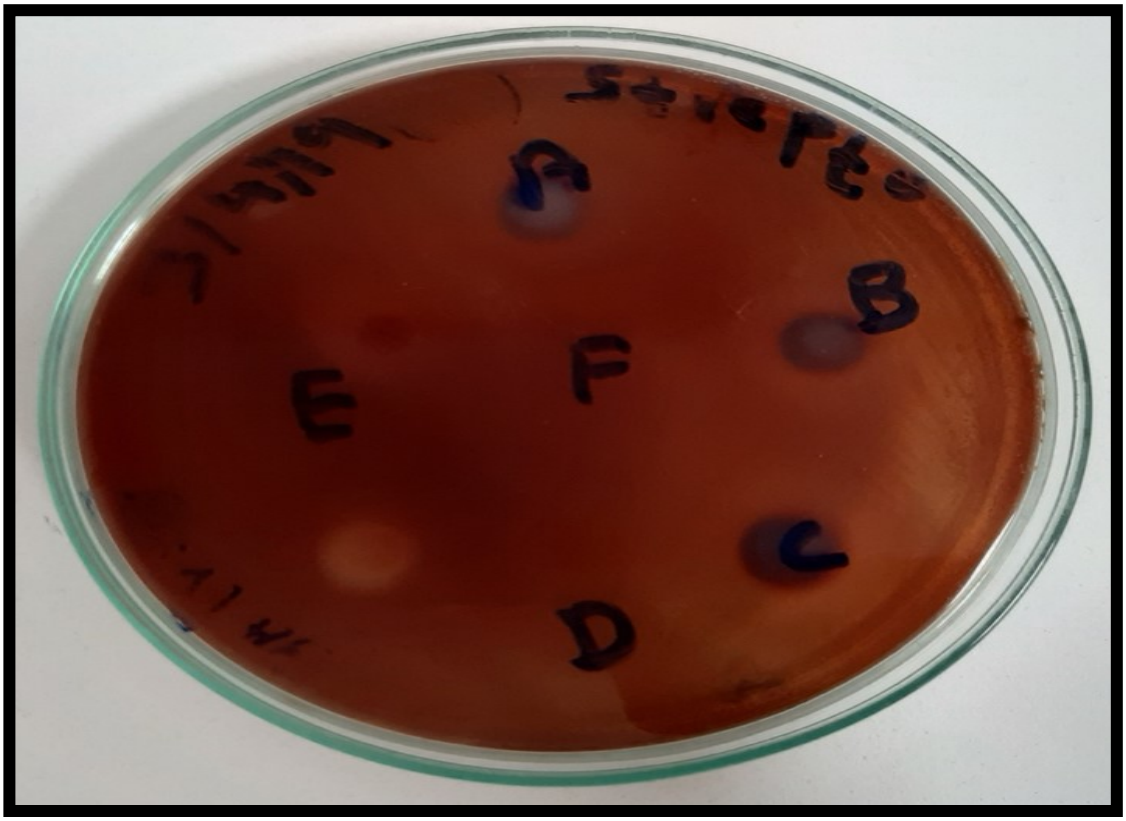

Figure 4.12: Antibacterial sensitivity test of clove oil concentrations against *S.agalactiae* (A=1:1, B=1:2, C=1:5, D=1:10, E=1:20)

may be useful as alternative to anti-infectious agents and as food preservatives. Sofia *et al.* (2007) evaluated antibacterial activity of clove extract against common foodborne pathogens and found effective against *E. coli* and *S .aureous* with 23.3 mm and 25.6 mm zone of inhibition, respectively at 3% concentration. Ayoola *et al.* (2008) also reported 23.00 mm zone of inhibition against *Escherichia coli* (ATCC35218) and 21.00 mm zone of inhibition against *Staphylococcus aureus* (ATCC25923) at 445 mg/ml concentration of *Syzigium aromaticum* oil. Result showed that *S. aromaticum oil* was active against all the microorganisms tested with a minimum zone diameter of 10 mm. Saeed and Tariq (2008) reported *in vitro* antibacterial activity of clove oil against gram negative bacteria and they reported 11.87 mm zone of inhibition against *E. coli*, 16.50 mm against *S. typhimurium* and 18.86 mm against *P. aeruginosa*. Result showed that the essential oil showed strong antibacterial activity against all bacterial isolates tested. Kumar *et al*. (2014) also reported antibacterial activity of Clove (*Syzygium aromaticum*) on different pathogenic bacteria and they reported 13 mm zone of inhibition against *E. coli*, 19.5 mm against *S. aureus* and 23 mm against *S .typhimurium* at 2000 ppm concentration. It was concluded that clove has antibacterial activity against the pathogens tested and it will be used as an alternative mode of anti-microbial treatment. Vanin *et al*. (2014) were reported 21.16 mm zone of inhibition against *E. coli*, 14.16 mm against *S. aureous*, 14.5 mm against *L. monocytogenes* and 15 mm against *P. aeruginosa* at 20 µL concentration of clove oil. Abdullah *et al*. (2015) studied antibacterial activity of clove oil on multidrug resistant bacteria and were reported zone of inhibition 25±1.4 against *P. aeruginosa* ATCC 27853, 22±1.6 against *S. aureous* ATCC 29213, 17±3.3 against *P. aeruginosa* and 20±2.8 against *S. aureous* at 10% concentration. The present study results demonstrated that clove oil possesses varying degree of antibacterial activity and it might act as the most obvious alternative.

4.2 EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF CLOVE OIL IN WISTAR RATS

The present study was conducted by using total 50 rats (5 males + 5 females in each group). All rats were injected subcutaneously with 0.1 ml of 10% w/v carrageenan suspension s/c in the sub-planter region of the left hind limb as a local acute edema inducer after 30 minutes subsequent to oral administration of clove oil. Rats of standard control group were treated orally with indomethacin @ 10 mg/kg

b.wt. as a reference drug in male and female rats, respectively. Clove oil was given orally to male and female rats of at three different dose rate 100, 250 and 500 mg/kg b.wt., respectively. Edema was expressed as the increase in paw volume (ml). The paw volume was measured up to the tibiotarsal articulation. Volume of edematous paw was measured by using plethysmometer at 0 hr (before treatment), 1, 2, 3, 4, 6 and 24 hours after treatments.

The result of anti-inflammatory effect was presented as change in paw volume (Table 4.2 and 4.3) and percentage inhibition (Table 4.4 and 4.5). The results revealed that the selected essential oil showed anti-inflammatory effect with various doses. The anti-inflammatory effect of indomethacin was highest at 3h (41.75% in male, 42.99% in female) as compare to other doses of clove oil. The anti-inflammatory effect of clove oil was highest at 3h (35.77% in male, 35.46% in female) at the dose rate of 500 mg/kg. In the present study clove oil showed dose dependent anti-inflammatory activity in both male and female at 3h all doses gave higher anti-inflammatory effect.

Table 4.2: Effect of oral administration of clove oil on carrageenan-induced rat paw edema (ml) in male wistar rats (Mean ± SE, n=5)

Group	0h	1h	2h	3h	4h	6h	24h
Control - M	0.72 ± 0.02	0.95 ± 0.01^{c}	1.14 ± 0.01^{d}	1.79 ± 0.03^{e}	1.72 ± 0.02^{d}	1.52 ± 0.02^{c}	0.91 ± 0.02^{b}
Indo-M	0.71 ± 0.02	0.82 ± 0.02^{a}	0.88 ± 0.03^{a}	1.04 ± 0.03^{a}	1.15 ± 0.02^{a}	1.16 ± 0.01^{a}	0.81 ± 0.04^{a}
SA-100-M	0.70 ± 0.01	0.88 ± 0.01^{b}	1.03 ± 0.02^{c}	1.38 ± 0.02^{d}	1.34 ± 0.01^{c}	1.29 ± 0.03^{b}	0.86 ± 0.04^{ab}
SA-250-M	0.71 ± 0.01	0.86 ± 0.01^{ab}	0.98 ± 0.01^{bc}	1.24 ± 0.01^{c}	1.22 ± 0.01^{b}	1.20 ± 0.01^{a}	0.83 ± 0.01^{ab}
SA-500-M	0.69 ± 0.02	0.84 ± 0.01^{ab}	0.94 ± 0.02^{b}	1.15 ± 0.02^{b}	1.16 ± 0.02^{a}	1.16 ± 0.04^{a}	0.81 ± 0.02^{a}

Mean value with dissimilar superscript in a column vary significantly at $p<0.05$
Indo-M= Indomethacin @ 10 mg/kg b.wt in male rats
SA-100-M = *Syzygium aromaticum* @ 100 mg/kg b.wt. in male rats
SA-250-M = *Syzygium aromaticum* @ 250 mg/kg b.wt. in male rats
SA-500-M = *Syzygium aromaticum* @ 500 mg/kg b.wt. in male rats

Table 4.3: Effect of oral administration of clove oil on carrageenan-induced rat paw edema (ml) in female wistar rats (Mean ± SE, n=5)

Group	0h	1h	2h	3h	4h	6h	24h
Control - F	0.53 ± 0.02	0.73 ± 0.02^{b}	1.01 ± 0.03^{b}	1.46 ± 0.05^{d}	1.4 ± 0.06^{c}	1.19 ± 0.04^{c}	0.74 ± 0.02^{b}
Indo-F	0.53 ± 0.02	0.63 ± 0.02^{a}	0.84 ± 0.03^{a}	0.83 ± 0.05^{a}	0.95 ± 0.06^{a}	0.90 ± 0.04^{a}	0.65 ± 0.02^{a}
SA-100-F	0.52 ± 0.01	0.67 ± 0.01^{a}	0.90 ± 0.03^{a}	1.14 ± 0.03^{c}	1.10 ± 0.02^{b}	1.01 ± 0.01^{b}	0.70 ± 0.02^{ab}
SA-250-F	0.53 ± 0.01	0.66 ± 0.03^{a}	0.87 ± 0.02^{a}	1.00 ± 0.02^{b}	0.98 ± 0.02^{a}	0.93 ± 0.01^{a}	0.69 ± 0.04^{ab}
SA-500-F	0.52 ± 0.01	0.64 ± 0.01^{a}	0.85 ± 0.05^{a}	0.94 ± 0.04^{b}	0.96 ± 0.02^{a}	0.92 ± 0.01^{a}	0.66 ± 0.01^{a}

Mean value with dissimilar superscript in a column vary significantly at $p<0.05$
Indo-F= Indomethacin @ 10 mg/kg b.wt in female rats
SA-100-F = *Syzygium aromaticum* @ 100 mg/kg b.wt. in female rats
SA-250-F = *Syzygium aromaticum* @ 250 mg/kg b.wt. in female rats
SA-500-F = *Syzygium aromaticum* @ 500 mg/kg b.wt. in female rats

Table 4.4: Percent inhibition of paw edema in wistar male rats treated with clove oil

Group	1h	2h	3h	4h	6h	24h
Indo-M	13.84	23.04	41.75	33.06	24.09	11.87
SA-100-M	7.64	10.06	22.87	21.86	15.49	5.55
SA-250-M	9.56	13.88	30.78	29.20	20.92	8.62
SA-500-M	11.64	17.17	35.77	32.56	23.71	10.78

Indo-M= Indomethacin @ 10 mg/kg b.wt in male rats
SA-100-M = *Syzygium aromaticum* @ 100 mg/kg b.wt. in male rats
SA-250-M = *Syzygium aromaticum* @ 250 mg/kg b.wt. in male rats
SA-500-M = *Syzygium aromaticum* @ 500 mg/kg b.wt. in male rats

Table 4.5: Percent inhibition of paw edema in wistar female rats treated with clove oil

Group	1h	2h	3h	4h	6h	24h
Indo-F	13.78	16.67	42.99	31.41	24.00	11.66
SA-100-F	7.85	11.16	21.16	20.52	15.10	5.35
SA-250-F	9.23	13.61	31.22	29.50	21.53	7.18
SA-500-F	12.20	15.52	35.46	30.76	22.81	10.95

Indo-F= Indomethacin @ 10 mg/kg b.wt in female rats
SA-100-F = *Syzygium aromaticum* @ 100 mg/kg b.wt. in female rats
SA-250-F = *Syzygium aromaticum* @ 250 mg/kg b.wt. in female rats
SA-500-F = *Syzygium aromaticum* @ 500 mg/kg b.wt. in female rats

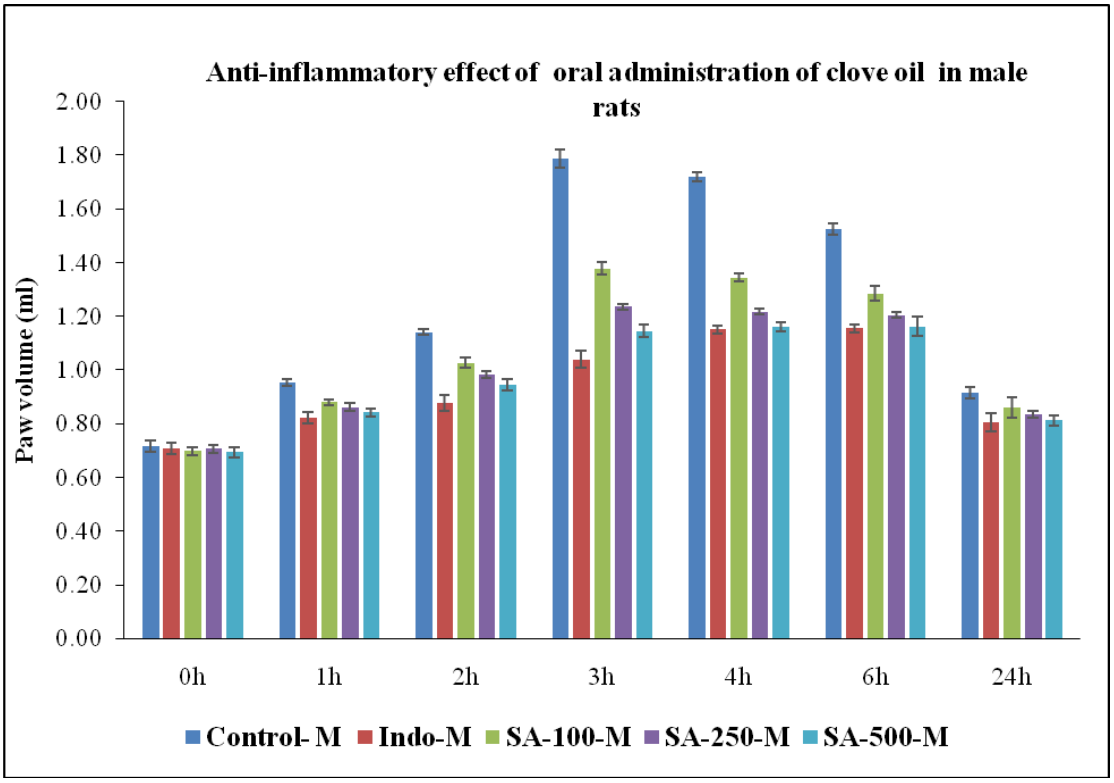

Figure 4.13: Effect of oral administration of clove oil on carrageenan-induced rat paw edema (ml) in male wistar rats

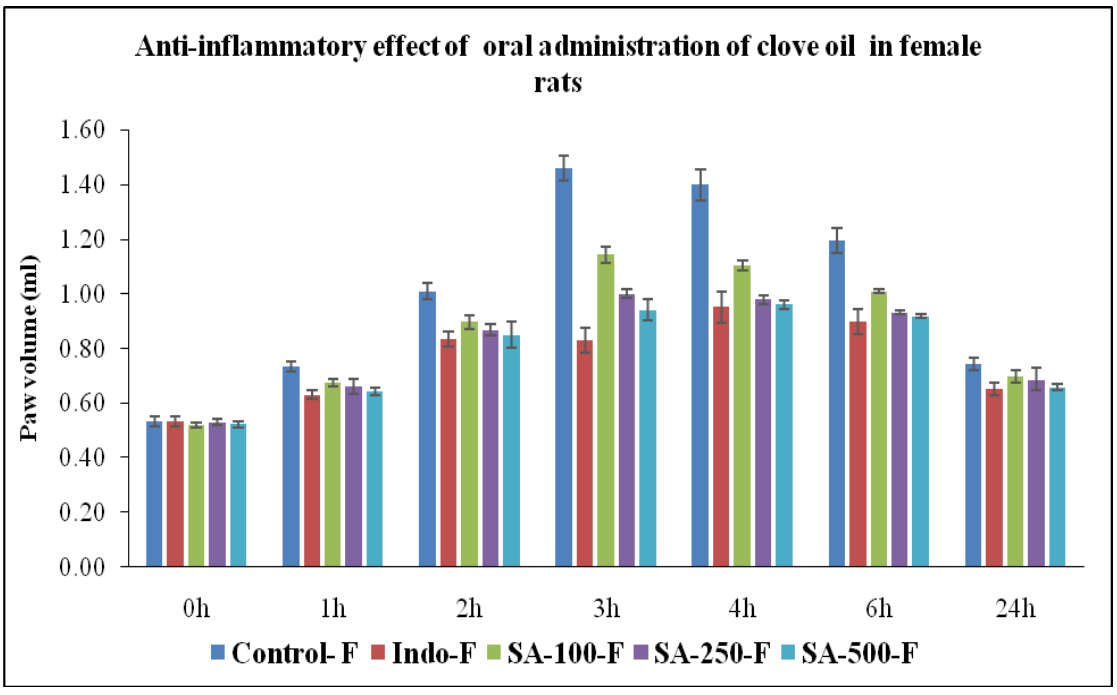

Figure 4.14: Effect of oral administration of clove oil on carrageenan-induced rat paw edema (ml) in female wistar rats

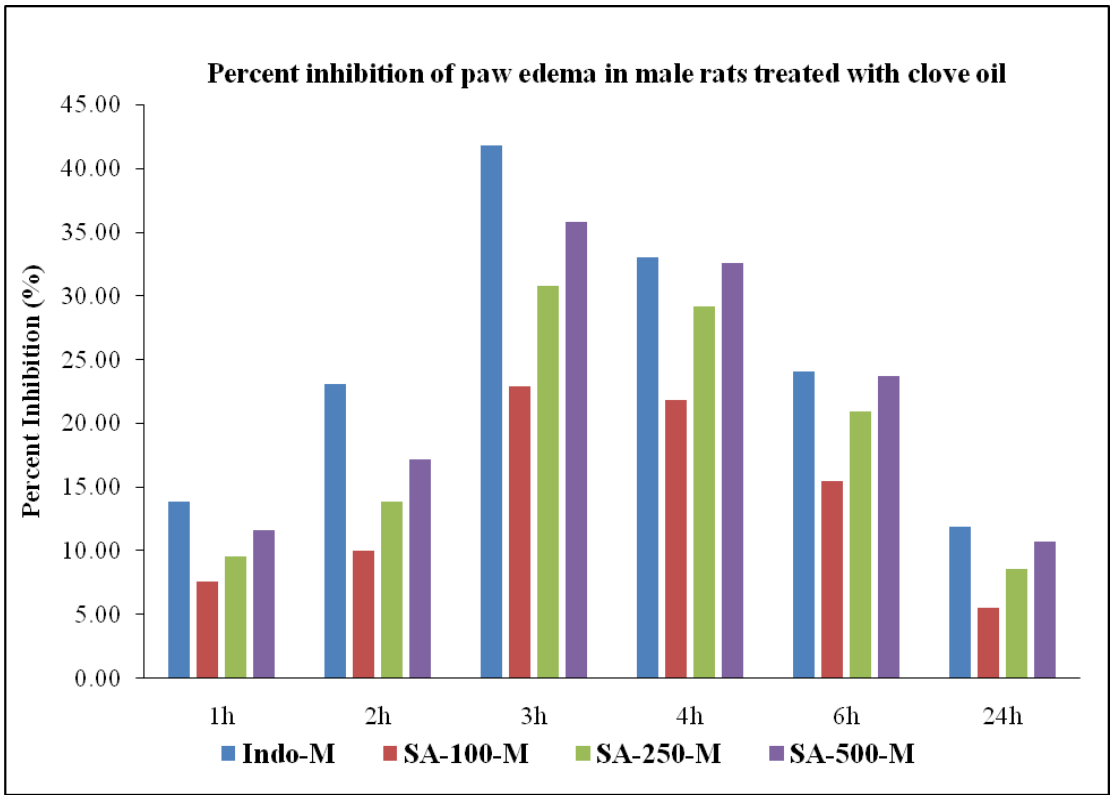

Figure 4.15: Percent inhibition of paw edema in wistar male rats treated with clove oil

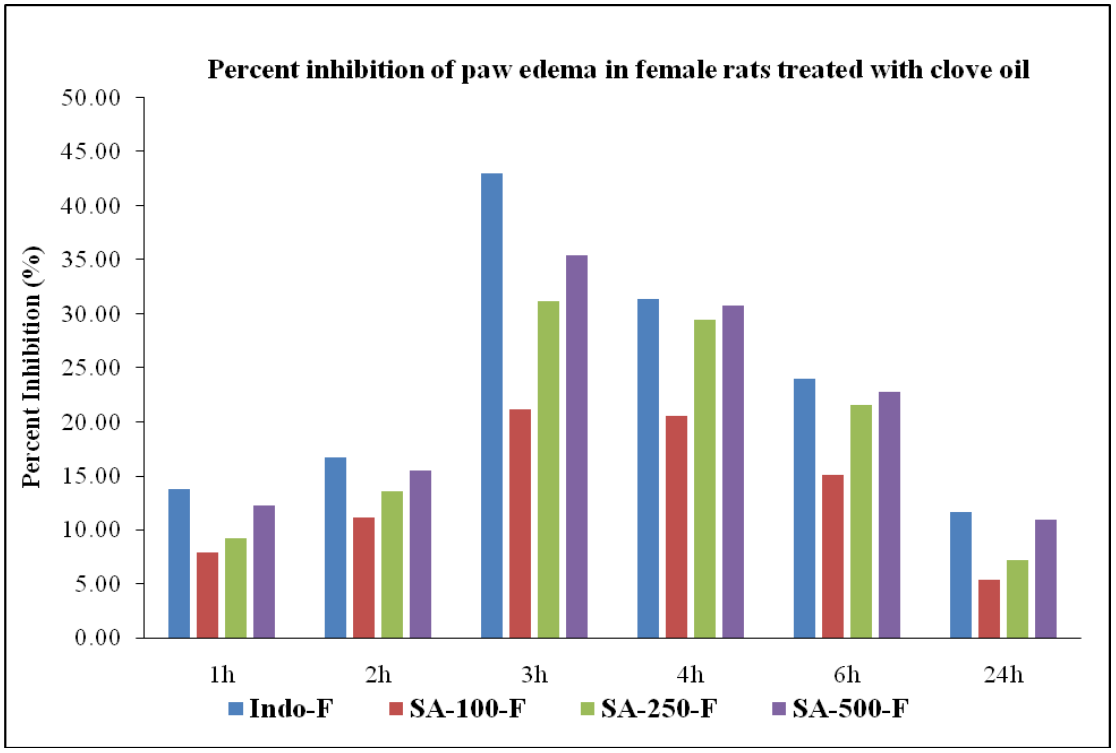

Figure 4.16: Percent inhibition of paw edema in wistar female rats treated with clove oil

Similarly, Ozturk and Ozbek (2005) reported the anti-inflammatory activity of *Eugenia caryophyllata* oil at 0.025, 0.050, 0.100 and 0.200 ml/kg b.wt. in carrageenan induced paw edema in rats. They reported 46.55, 90.15, 66.94 and 82.78 % inhibition of inflammation, respectively. Tanko *et al.* (2008) also reported anti-inflammatory activities of ethanolic extract of *syzygium aromaticum* flower bud in wistar rats paw edema model at 50, 100, and 200 mg/kg body weight. They reported 42, 45 and 52% inhibition of inflammation at 5h observation. The results obtained showed that the ethanolic extract significantly reduced the inflammation at all the dose given. Daniel *et al.* (2009) studied anti-inflammatory activities of eugenol oil by inflammatory exudates volume in carrageenan-induced paw edema in rats at 100, 200 and 400 mg/kg body weight. They reported that the oral administration of eugenol significantly inhibited paw edema at 22.2, 40 and 41.1% at 2-4 h after carrageenan injection and the inhibition rate was comparable to that of indomethacin group. Ahmad *et al.* (2012) reported anti-inflammatory activity of the aqueous extract of *syzygium aromaticum in vivo* in acute inflammation at 1 g/kg body weight in carrageenan induce paw edema model in rats. They reported 84% inhibition of paw edema as compare to control after 3h observation. Taher *et al.* (2015) studied anti-inflammatory activities of clove oil in mice at a dose of 33 mg/kg body weight (i.p.). Clove oil significantly suppressed the increased in paw thickness by 50.6% compared with control mice at 3h observation. Saeed *et al.* (2017) also reported potential anti-inflammatory effect of ethanolic extract of *syzygium aromaticum* at 250 and 500 mg/kg b.wt. in carrageenan induce paw edema in rats. The ethanolic extract of *S. aromaticum* showed significant decreased in the edema size at efficacy rates of 79.41, 82.39 and 63.92% for the dose 500 mg/kg body weight at the 2^{nd}, 4^{th} and 6^{th} h, respectively.

4.3 SAFETY STUDY OF CLOVE OIL IN WISTAR RATS

The present study was conducted on forty (40) male and female wistar rats dividing them in various groups having 10 (5 male + 5 female) rats in each group. Group I and group V served as vehicle control for male and female rats, respectively. Clove oil was administered at dose of 50, 100 and 200 mg/kg body weight (p.o.) once daily for 28 days to male rats group II, III & IV and female rats group VI, VII and VIII, respectively.

The animals of all the groups were observed daily for clinical signs and mortality. Body weight and feed consumption of animals were monitored at weekly interval. The blood sample were subjected to the hematological and serum biochemical investigations. All the rats were sacrificed at the end of experiment and subjected to post mortem examination for gross observation and collection of organs (kidney, liver, spleen and heart) for histopathological examination. The results obtained from the present study are as under.

Similarly, Mishra and Singh (2008) studied safety assessment of *Syzygium aromaticum* flower bud (clove) extract with respect to testicular function in mice at the dose of 15, 30 and 60 mg/kg and reported that *Syzygium aromaticum* flower bud (clove) extract did not induce any systemic toxicity at the doses tested. Vijayasteltar *et al.* (2016) reported safety assessment of a standardized polyphenolic extract of clove buds (clovinol) and reported that clovinol was safe in rats with no observed adverse effect level (NOAEL) of 1 g/kg body weight per day. Saeed *et al.* (2017) studied safety assessment of ethanolic extract of *syzygium aromaticum* in albino rats at 250 mg/kg, 500 mg/kg, and 1000 mg/kg and they reported that *S. aromaticum* ethanolic extract showed no mortality or signs of toxicity to rats at the doses used.

4.4 CLINICAL SIGNS AND MORTALITY

There were no clinical signs observed in all clove oil treated male and female rats as well as control group rats also did not show any noticeable clinical signs. There were no mortality in rats of any treatment and control group.

Similarly, Issac *et al.* (2015) reported that sub-acute toxicity studies of clovinol (polyphenol-rich extract of clove buds) at doses of 0.5, 1.0 and 2.5 g/kg b.wt. and confirmed that it did not induce any mortality, abnormal clinical or behavior signs. Vijayasteltar *et al.* (2016) studied safety assessment of a standardized polyphenolic extract of clove buds and reported that oral administration of clovinol at 0.25, 0.5 and 1.0 g/kg body weight did not produce any mortality, adverse effects or clinical sign during the 14 days acute toxicity study. Saeed *et al.* (2017) studied safety assessment of ethanolic extract of *syzygium aromaticum* in albino rats at 250 mg/kg, 500 mg/kg and 1000 mg/kg and they reported that *S. aromaticum* ethanolic extract showed no mortality or clinical signs in rats.

4.5 EFFECT ON BODY WEIGHT

Effect of 28 days oral administration of clove oil (*Syzygium aromaticum*) on body weight of Wistar rats are shown in Table-4.6 and Figure-4.17.

No significant difference were observed in body weight of male and female rats of different treatment groups and control group rats on day 0.

No significant difference were observed in body weight of male rats of different treatment groups (II, III and IV) on day 7, 14, 21 and 28 at dose of 50, 100 and 200 mg/kg as compared to rats of male control group (I). Similarly, no significant difference were observed in body weight of female rats of different treatment groups (VI, VII and VIII) on day 7, 14, 21 and 28 at dose of 50, 100 and 200 mg/kg as compared to rats of female control group (V).

Similarly, Issac *et al.* (2015) reported that sub-acute toxicity studies of clovinol at doses of 0.5, 1.0 and 2.5 g/kg b.wt., and they showed that clove oil treated rats did not induce any significant changes in body weight as compared to that of control group. Mishra and Singh (2008) studied safety assessment of *Syzygium aromaticum* flower bud (clove) extract with respect to testicular function in mice at the dose of 15, 30 and 60 mg/kg and reported that treatment had no significant effect on the body weight of animals, and all the animals maintained a healthy appearance throughout the investigation. Vijayasteltar *et al.* (2016) studied safety assessment of a standardized polyphenolic extract of clove buds at 0.25, 0.5 and 1.0 g/kg body weight which showed no significant difference in the weight gain and growth rate among the male and female animals when compared to the untreated control group of animals.

In contrast, Adam *et al.* (2013) studied effects of the aqueous extract of clove (50, 200, 400 and 800 mg/kg/day, *p.o.*) on wistar rats reported that the body weight gain of rats in group 3 was significantly lower ($p<0.05$) than the control rats, but no change was observed concerning to other treatment groups. Gashlan and beladi (2016) reported high significant increase ($P<0.01$) in the body weight of clove oil treated diabetic rats at 300 and 600 mg/kg as compared to untreated diabetic control group.

Table-4.6: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on body weight (g) of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	0 day	7 day	14 day	21 day	28 day
I	Control-M	336 ± 8.12	350 ± 10.95	354 ± 8.72	369 ± 7.65	375 ± 7.58
II	SA-50-M	344 ± 15.68	352 ± 19.85	364 ± 18.87	369 ± 20.27	380 ± 17.03
III	SA-100-M	348 ± 5.39	374 ± 5.10	381 ± 6.96	387 ± 1020	401 ± 3.32
IV	SA-200-M	353 ± 4.90	367 ± 6.63	375 ± 15.49	389 ± 7.14	398 ± 9.17
V	Control-F	222 ± 3.74	228 ± 3.74	226 ± 4.00	234 ± 8.12	249 ± 5.57
VI	SA-50-F	232 ± 7.35	246 ± 2.45	248 ± 4.90	244 ± 5.10	250 ± 7.07
VII	SA-100-F	228.4 ± 2.66	236 ± 2.45	236 ± 8.12	238 ± 2.00	246 ± 2.92
VIII	SA-200-F	238 ± 4.90	244 ± 13.27	238 ± 11.58	244 ± 7.48	244 ± 5.10

SA-50-M= *Syzygium aromaticum* oil @ 50 mg/kg b.wt. in male rats
SA-100-M= *Syzygium aromaticum* oil @ 100 mg/kg b.wt. in male rats
SA-200-M= *Syzygium aromaticum* oil @ 200 mg/kg b.wt. in male rats
SA-50-F= *Syzygium aromaticum* oil @ 50 mg/kg b.wt. in female rats
SA-100-F= *Syzygium aromaticum* oil @ 100 mg/kg b.wt. in female rats
SA-200-F= *Syzygium aromaticum* oil @ 200 mg/kg b.wt. in female rats

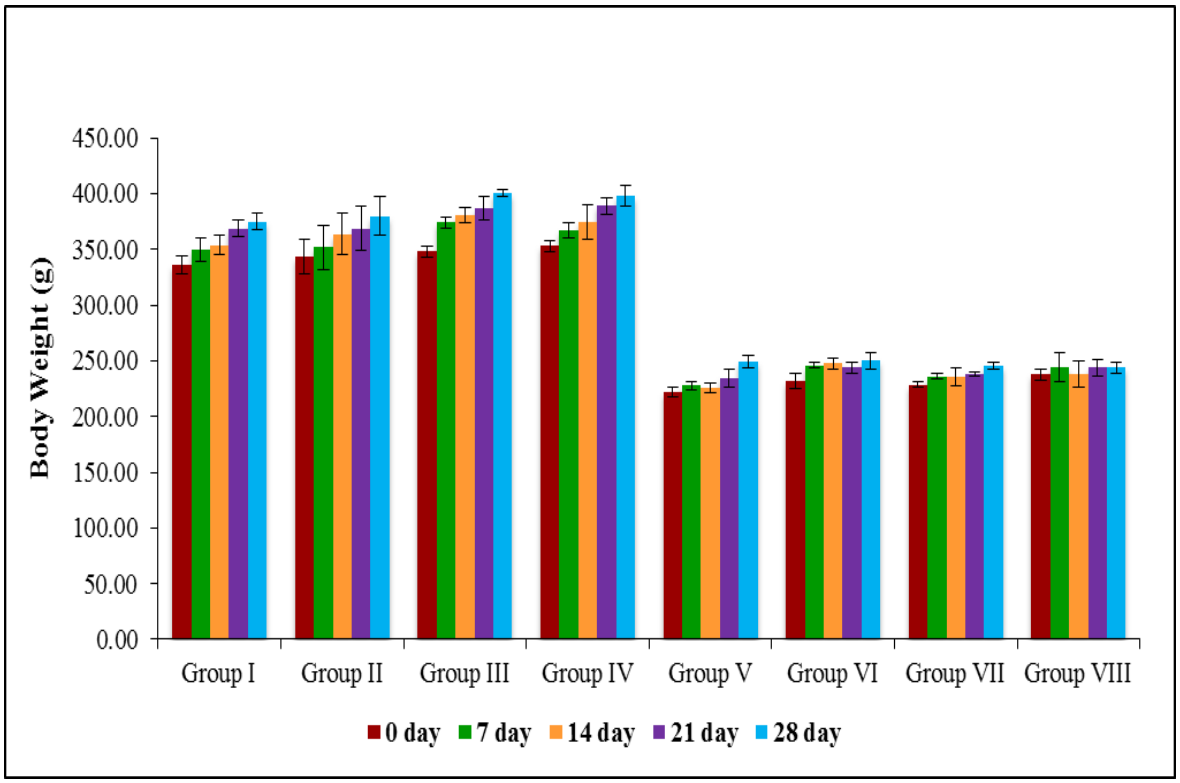

Figure 4.17: Effect of repeated oral administration of clove oil for 28 days on body weight of male (I-IV) and female (V-VIII) rats

4.6 EFFECT ON FEED CONSUMPTION

Effect of 28 days oral administration of clove oil (*Syzygium aromaticum*) on feed consumption of wistar rats have been shown in Table-4.7 and Figure 4.18.

There were no significant difference observed in feed consumption of different treatment groups of male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to male and female control rats (I & V, respectively) on 1st, 2nd, 3rd & 4th week.

Similarly, Issac *et al.* (2015) reported no significant change in feed consumption in clovinol treated rats at doses of 0.5, 1.0 and 2.5 g/kg b.wt. as compare to control rats. Vijayasteltar *et al.* (2016) also reported that repeated oral administration of extract of clove buds at 0.25, 0.5 and 1.0 g/kg did not produce any change in feed consumption.

In contrast, Adam *et al.* (2013) reported that repeated oral administration of aqueous extract of clove (50, 200, 400 and 800 mg/kg/day) showed significant decrease in feed consumption in rats as compare to control rats.

Table-4.7: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on feed consumption (g) of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	Week 1	Week 2	Week 3	Week 4
I	Control-M	19.76 ± 0.24	20.69 ± 1.69	21.07 ± 0.36	21.86 ± 0.43
II	SA-50-M	19.19 ± 2.05	17.98 ± 0.12	19.29 ± 0.71	20.95 ± 0.48
III	SA-100-M	20.60 ± 1.55	20.12 ± 2.02	20.95 ± 1.90	21.90 ± 0.95
IV	SA-200-M	18.02 ± 0.83	18.31 ± 1.12	18.67 ± 0.67	20.36 ± 1.21
V	Control-F	21.05 ± 1.49	21.93 ± 0.79	19.62 ± 0.40	21.64 ± 0.21
VI	SA-50-F	19.05 ± 0.57	20.57 ± 0.86	19.79 ± 1.07	19.00 ± 0.43
VII	SA-100-F	18.83 ± 0.98	19.29 ± 0.43	19.59 ± 0.84	18.93 ± 0.79
VIII	SA-200-F	18.57 ± 0.71	20.71 ± 0.57	18.44 ± 0.94	19.02 ± 1.69

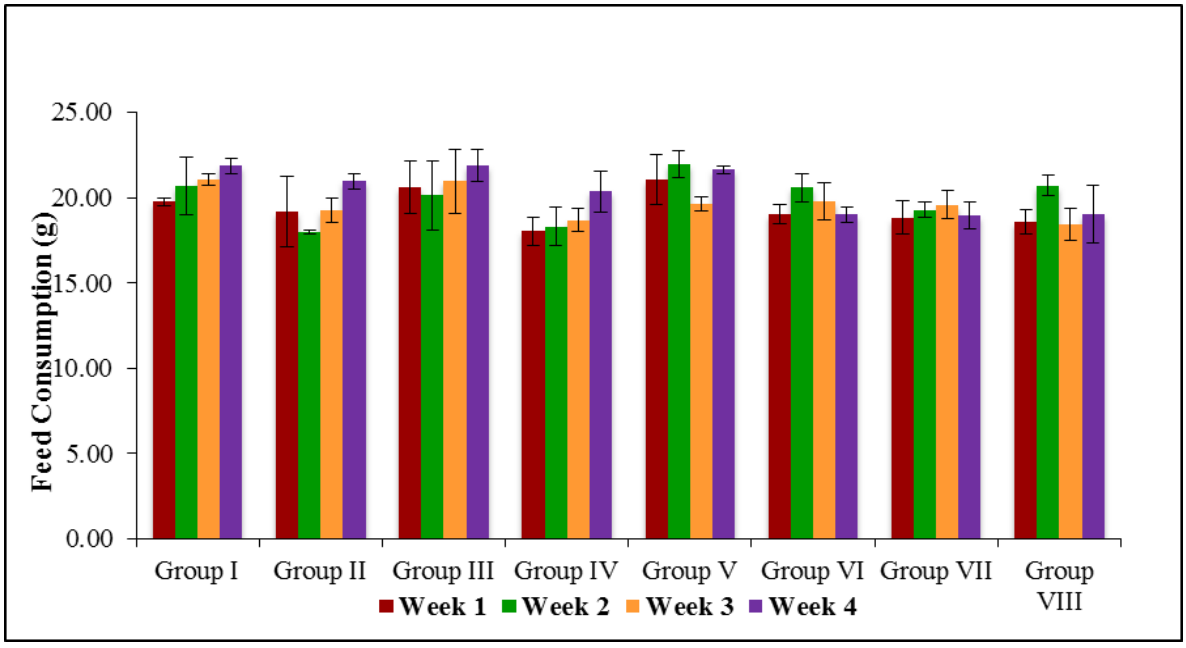

Figure 4.18: Effect of repeated oral administration of clove oil for 28 days on feed consumption of male (I-IV) and female (V-VIII) rats

4.7 HAEMATOLOGICAL ESTIMATION

The results obtained after 28 days oral administration of clove oil (*Syzygium aromaticum*) to male and female rats on Hb, RBCs, PCV and TLCs counts are presented in Table 4.8 and Figure 4.19 to 4.22. Whereas the results obtained after 28 days oral administration of clove oil (*Syzygium aromaticum*) to male and female rats on MCV, MCH and MCHC are presented in Table 4.9 and Figure 4.23 to 4.25.

4.7.1 Hemoglobin (Hb)

No significant changes have been observed in Hb level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.7.2 Red Blood Cells (RBCs)

No significant changes have been observed in RBCs in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.7.3 Packed Cell Volume (PCV)

No significant changes have been observed in PCV level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.7.4 Total Leucocytes Counts (TLCs)

No significant changes have been observed in TLCs in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.7.5 Mean Corpuscular Volume (MCV)

No significant changes have been observed in MCV level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.7.6 Mean Corpuscular Hemoglobin (MCH)

No significant changes have been observed in MCH level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.7.7 Mean Corpuscular Hemoglobin Concentration (MCHC)

No significant changes have been observed in MCHC level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

Similarly, Issac *et al.* (2015) studied sub-acute toxicity testing of clovinol (polyphenol-rich extract of clove buds) at doses of 0.5, 1.0 and 2.5 g/kg b.wt. for 28 days in wistar rats. Report of result showed that clovinol did not produce any significant ($p > 0.05$) changes in haemoglobin, WBC and RBC counts of treated animals. Vijayasteltar *et al.* (2016) studied safety assessment of a standardized polyphenolic extract of clove buds at 0.25, 0.5 and 1.0 g/kg body weight for 90 days in wistar rats & the results showed that clovinol did not produce any significant ($p > 0.05$) changes in the hematological parameters like hemoglobin, WBC, RBC and platelet counts. Saeed *et al.* (2017) also reported that repeated oral administration of *syzygium aromaticum* in albino rats at 250, 500 and 1000 mg/kg showed no significant ($P > 0.05$) changes in Hb, PCV, WBCs and RBCs as compared to control rats.

In contrast, Shalaby *et al.* (2011) reported toxicological effects of clove oil @ 500, 1000, 1500, 2000 and 2500 mg/kg b.wt. for 30 days (total 10 doses) in albino rats. They reported that on 10th dose (on 30 day) showed significant increase in WBC

and significant decrease in Hb and RBCs. Adam *et al.* (2013) reported the effects of repeated oral administration of aqueous extract of clove @ 50, 200, 400 and 800 mg/kg/day in wistar rats. Result of 50 mg/kg treated rats showed significat low values ($p<0.05$) of MCH, MCHC and WBCs than the control rats. Significantly higher values of Hb, PCV, RBCs and WBCs were recorded @ 400 mg/kg treated rats as compared to control rats whereas the values of MCV remained unchanged at all doses as compare to the control rats.

Table-4.8: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on Hb, RBCs, PCV and TLCs of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	HB (g/dl)	RBCs (10^6/µL)	PCV (%)	TLCs (10^3/µl)
I	Control-M	15.46 ± 0.58	9.06 ± 0.49	44.76 ± 2.03	6.63 ± 0.31
II	SA-50-M	16.76 ± 0.87	10.03 ± 0.66	50.36 ± 2.22	6.42 ± 0.16
III	SA-100-M	15.80 ± 0.37	9.80 ± 0.35	50.34 ± 1.45	6.60 ± 0.18
IV	SA-200-M	16.46 ± 0.45	9.93 ± 0.40	49.45 ± 2.89	6.40 ± 0.39
V	Control-F	15.18 ± 0.58	8.98 ± 0.76	46.95 ± 1.45	6.73 ± 0.42
VI	SA-50-F	15.88 ± 0.15	8.87 ± 0.12	44.62 ± 0.72	5.98 ± 0.41
VII	SA-100-F	15.30 ± 0.16	8.78 ± 0.03	44.62 ± 2.07	6.52 ± 0.18
VIII	SA-200-F	16.76 ± 0.35	10.63 ± 1.03	50.56 ± 3.03	6.36 ± 0.13

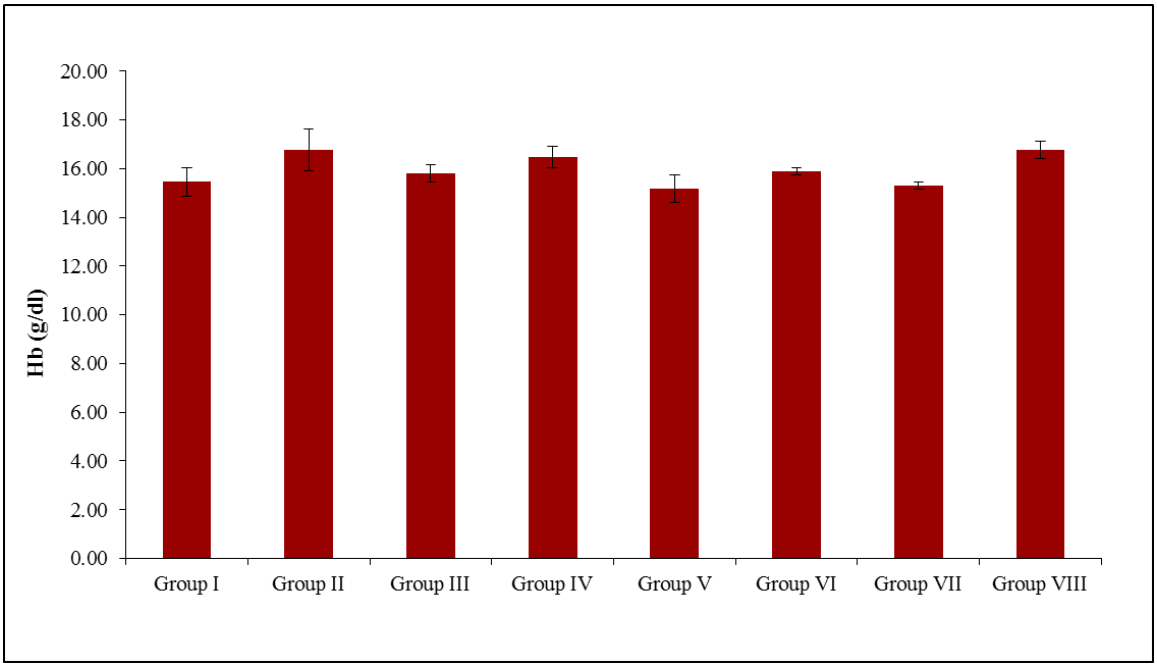

Figure 4.19: Effect of repeated oral administration of clove oil for 28 days on Hb of male (I-IV) and female (V-VIII) rats

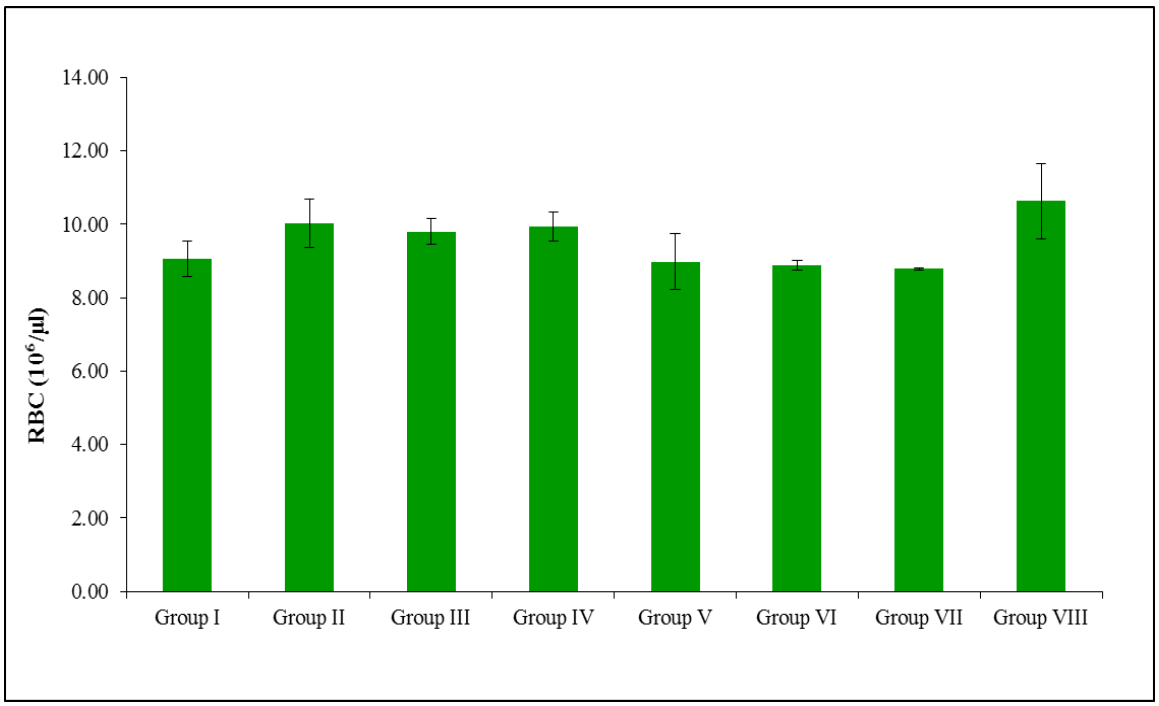

Figure 4.20: Effect of repeated oral administration of clove oil for 28 days on RBCs of male (I-IV) and female (V-VIII) rats

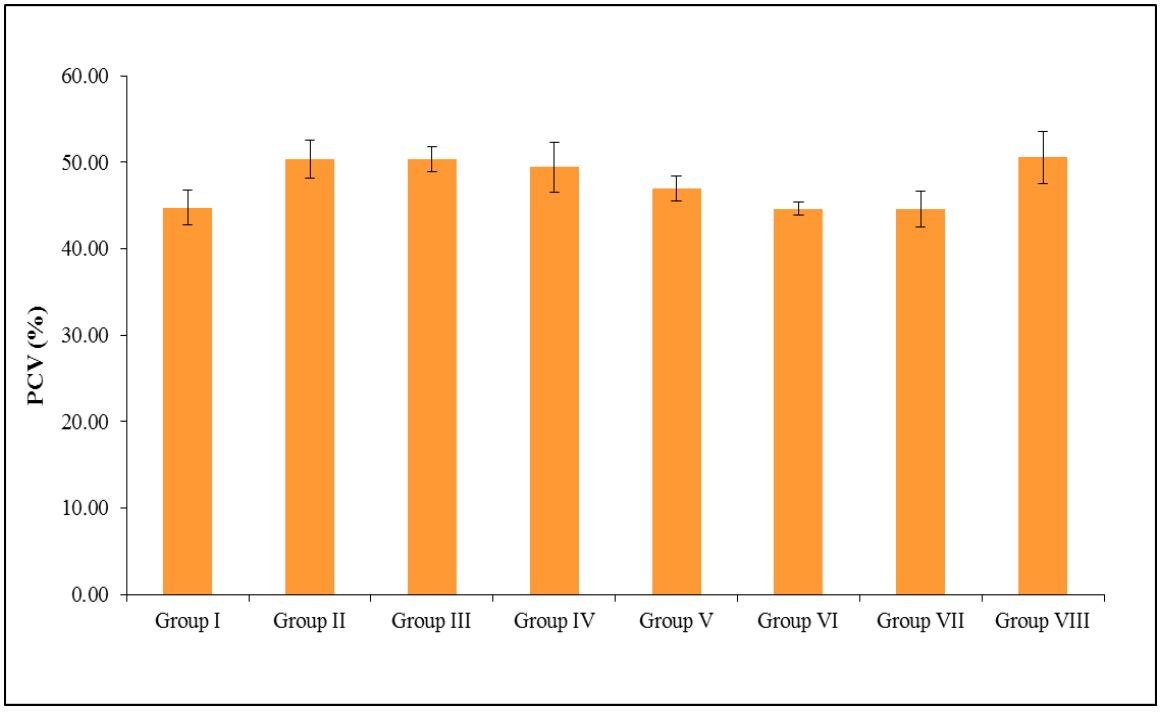

Figure 4.21: Effect of repeated oral administration of clove oil for 28 days on PCV of male (I-IV) and female (V-VIII) rats

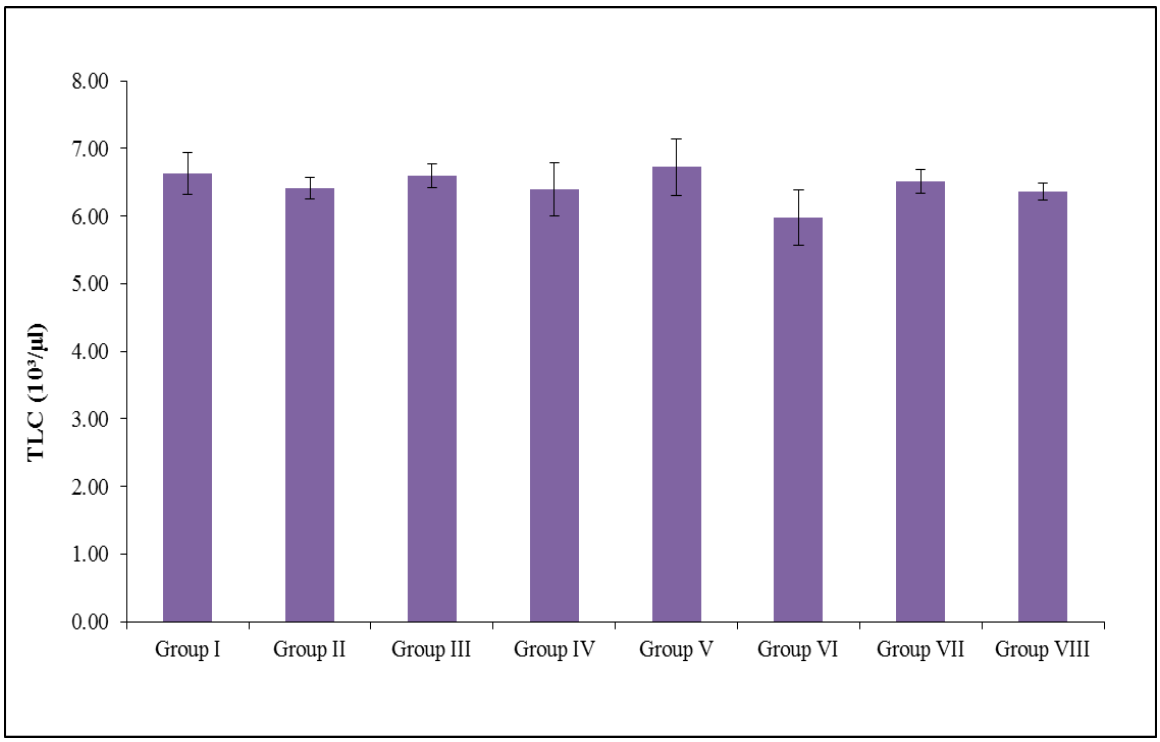

Figure 4.22: Effect of repeated oral administration of clove oil for 28 days on TLC of male (I-IV) and female (V-VIII) rats

Table-4.9: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on MCV, MCH and MCHC of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	MCV (fl)	MCH (pg)	MCHC (g/dl)
I	Control-M	50.09 ± 3.67	17.28 ± 1.11	34.74 ± 1.72
II	SA-50-M	51.31 ± 4.93	16.80 ± 0.54	33.70 ± 2.62
III	SA-100-M	51.71 ± 2.70	16.17 ± 0.34	31.54 ± 1.50
IV	SA-200-M	49.80 ± 2.05	16.62 ± 0.27	33.62 ± 1.63
V	Control-F	53.33 ± 3.48	17.32 ± 1.39	32.55 ± 2.08
VI	SA-50-F	50.28 ± 0.52	17.91 ± 0.24	35.61 ± 0.45
VII	SA-100-F	51.87 ± 2.28	17.79 ± 0.13	34.55 ± 1.44
VIII	SA-200-F	48.64 ± 3.60	16.19 ± 1.12	33.47 ± 1.35

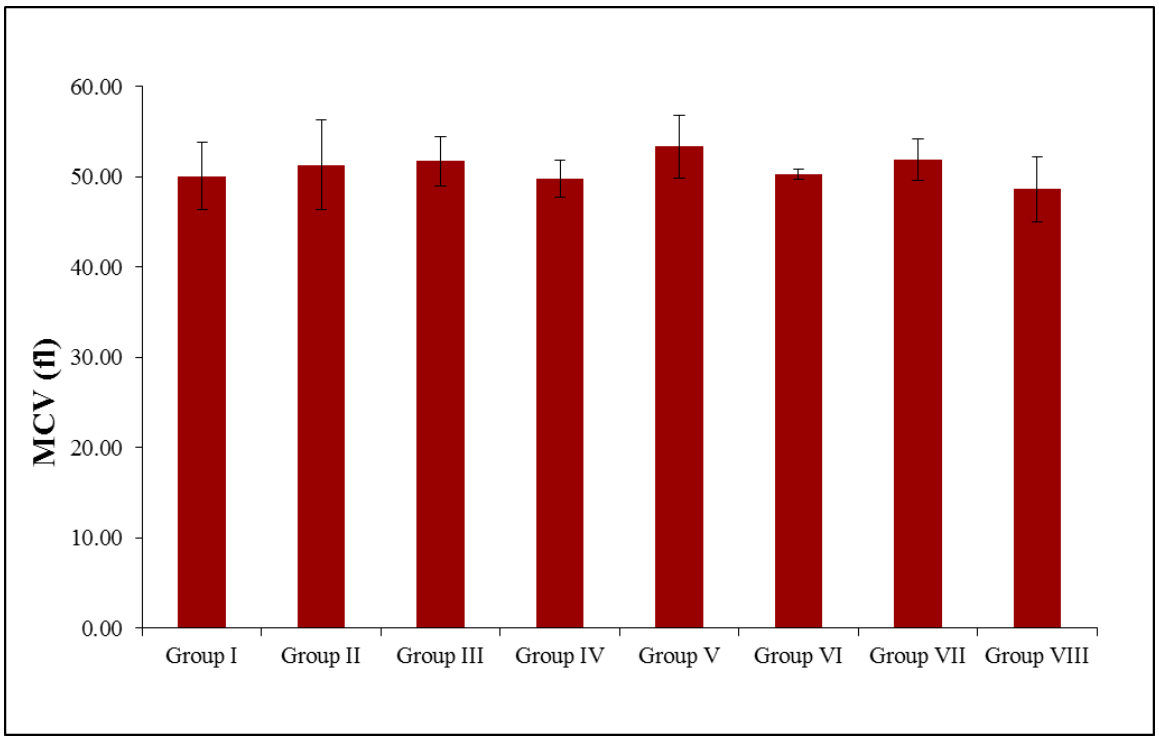

Figure 4.23: Effect of repeated oral administration of clove oil for 28 days on MCV of male (I-IV) and female (V-VIII) rats

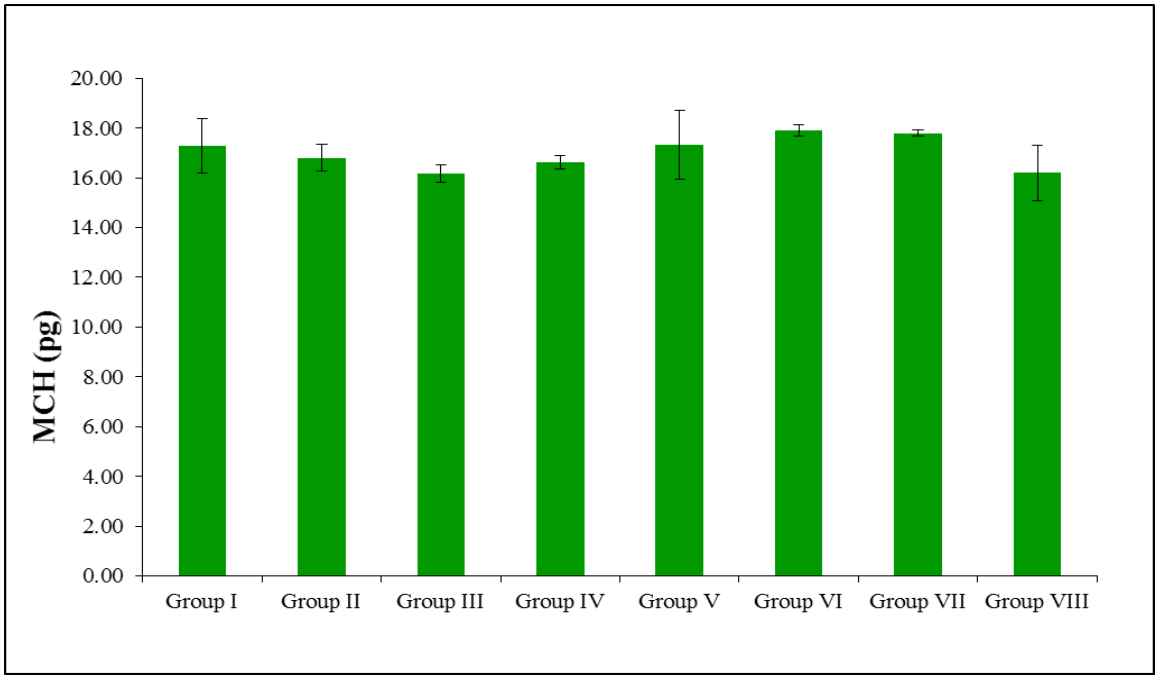

Figure 4.24: Effect of repeated oral administration of clove oil for 28 days on MCH of male (I-IV) and female (V-VIII) rats

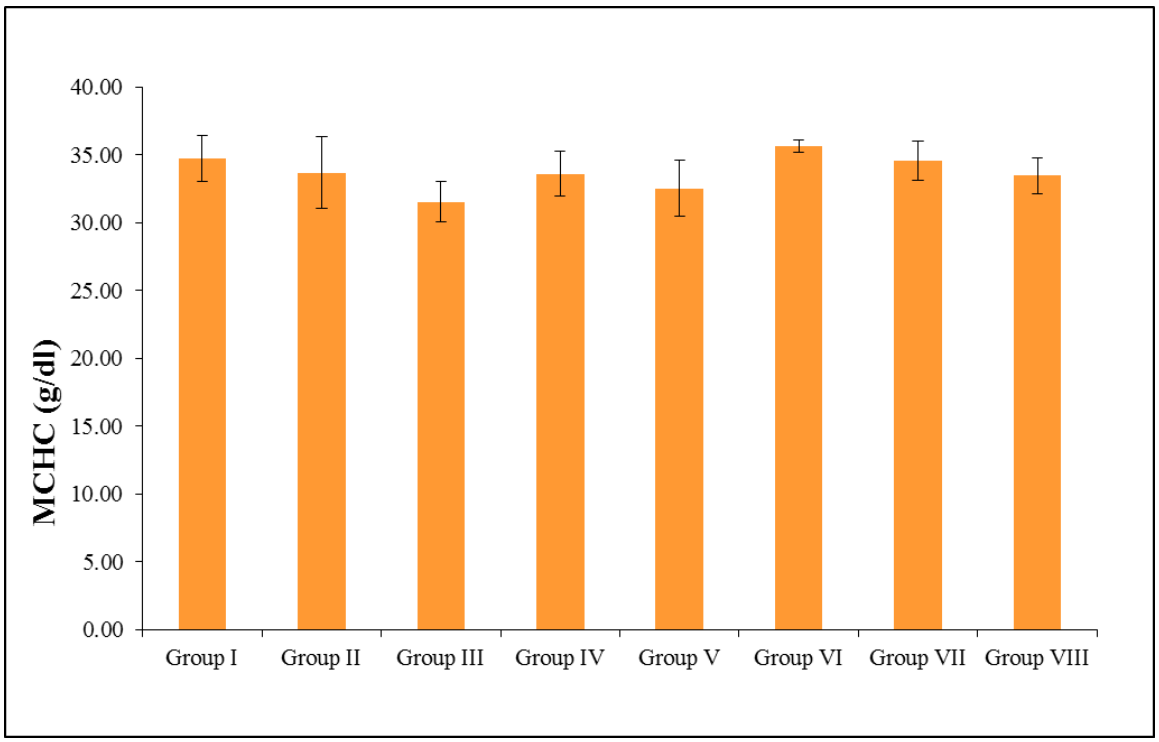

Figure 4.25: Effect of repeated oral administration of clove oil for 28 days on MCHC of male (I-IV) and female (V-VIII) rats

4.8 SERUM BIOCHEMICAL PARAMETERS

The results obtained after 28 days oral administration of clove oil (*Syzygium aromaticum*) to male and female rats on serum creatinine and BUN are presented in Table 4.10 and Figure 4.26 to 4.27.

4.8.1. Serum Creatinine

No significant changes have been observed in serum creatinine level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.8.2 Blood Urea Nitrogen (BUN)

No significant changes have been observed in BUN level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.8.3 Aspartate Aminotransferase (AST)

The results obtained after 28 days oral administration of clove oil (*Syzygium aromaticum*) to rats on AST, ALT, bilirubin and total cholesterol are presented in

Table 4.11 and Figure 4.28 to 4.31.

No significant changes have been observed in AST level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.8.4 Alanine Aminotransferase (ALT)

No significant changes have been observed in ALT level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.8.5 Total Bilirubin

No significant changes have been observed in bilirubin level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.8.6 Total Cholesterol (TC)

No significant changes have been observed in TC level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.8.7. Total Protein

The results obtained after 28 days oral administration of clove oil (*Syzygium aromaticum*) to rats on Total protein and Albumin are presented in Table 4.12 and Figure 4.32 to 4.33.

No significant changes have been observed in total protein level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

4.8.8. Total Albumin

No significant change haves been observed in albumin level in clove oil treated male rats (II, III and IV) and female rats (VI, VII and VIII) as compared to control rats (I & V, respectively) at the end of experiment on 28th day.

Table-4.10: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on creatinine and BUN of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	Creatinine (mg/dl)	BUN (mg/dl)
I	Control-M	0.38 ± 0.02	17.07 ± 0.83
II	SA-50-M	0.50 ± 0.07	16.61 ± 1.75
III	SA-100-M	0.55 ± 0.07	18.92 ± 0.81
IV	SA-200-M	0.58 ± 0.06	19.12 ± 0.49
V	Control-F	0.50 ± 0.03	18.42 ± 0.65
VI	SA-50-F	0.43 ± 0.09	19.83 ± 0.81
VII	SA-100-F	0.54 ± 0.05	16.91 ± 0.62
VIII	SA-200-F	0.47 ± 0.07	17.81 ± 1.69

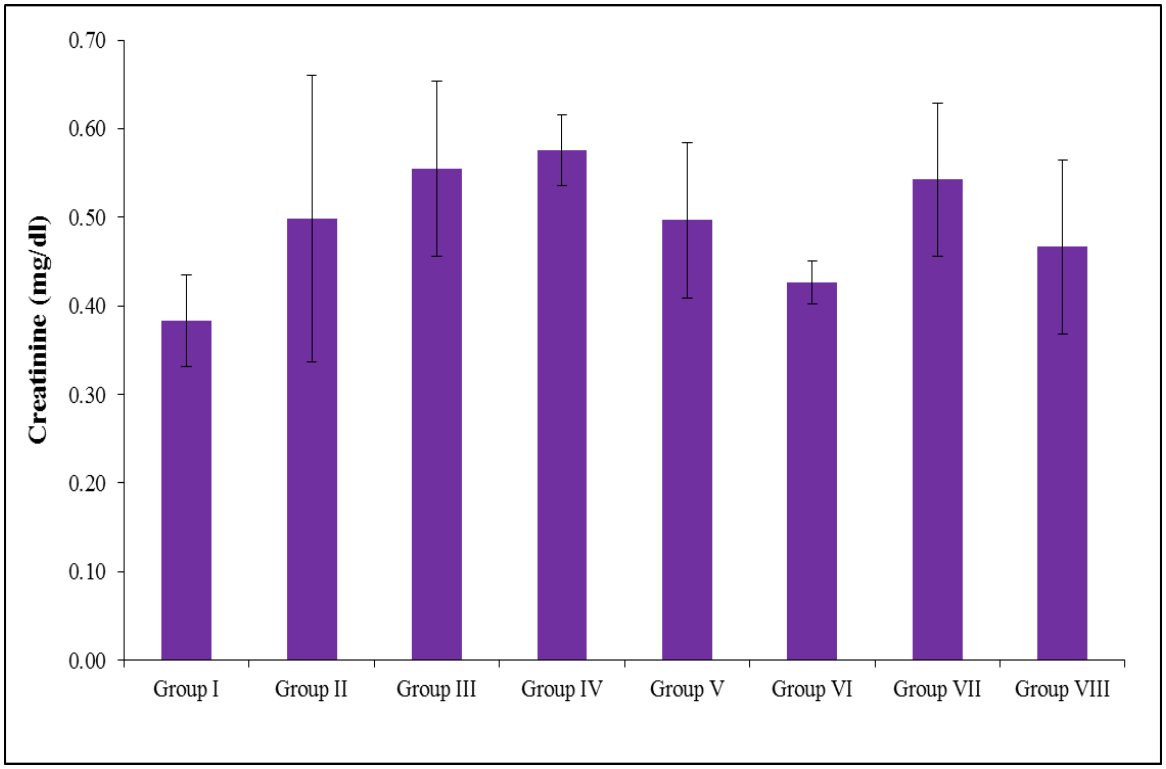

Figure 4.26: Effect of repeated oral administration of clove oil for 28 days on creatinine of male (I-IV) and female (V-VIII) rats

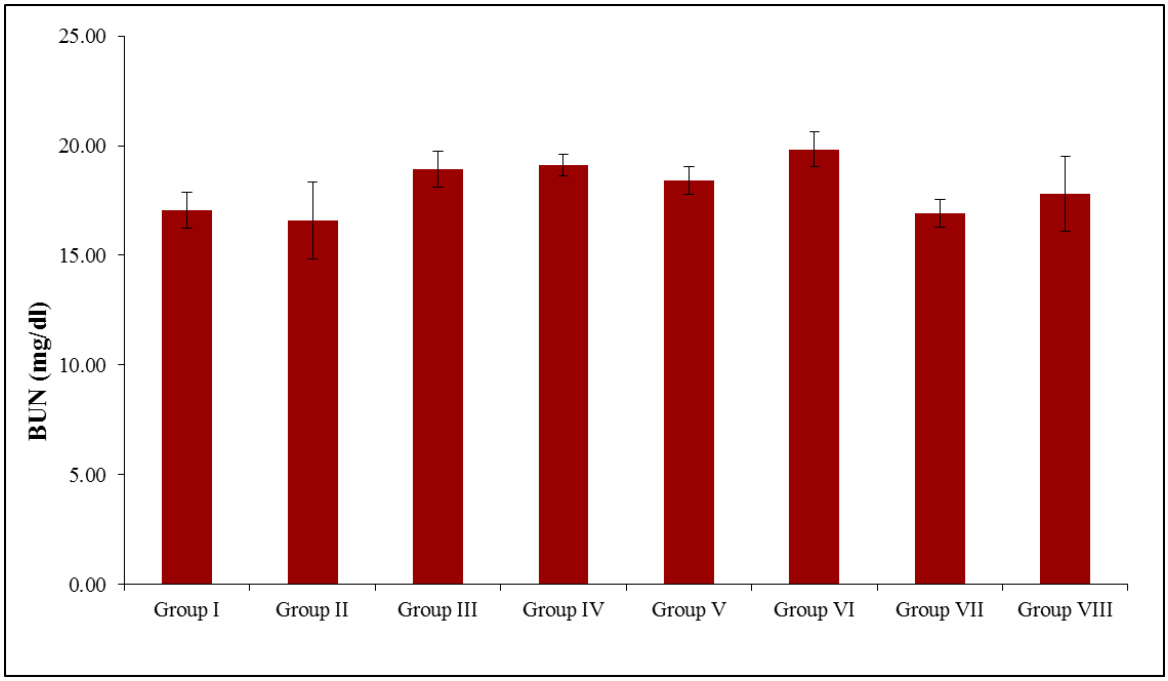

Figure 4.27: Effect of repeated oral administration of clove oil for 28 days on BUN of male (I-IV) and female (V-VIII) rats

Table-4.11: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on AST, ALT, total cholesterol and bilirubin of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	AST (U/l)	ALT (U/l)	Bilirubin (mg/dl)	TC (mg/dl)
I	Control-M	66.28 ± 2.99	137.95 ± 7.24	0.67 ± 0.05	102.45 ± 4.09
II	SA-50-M	60.63 ± 5.49	136.45 ± 5.57	0.55 ± 0.16	95.35 ± 6.06
III	SA-100-M	80.55 ± 9.98	147.75 ± 6.97	0.57 ± 0.10	89.44 ± 4.17
IV	SA-200-M	69.08 ± 3.32	144.63 ± 4.94	0.75 ± 0.04	103.80 ± 6.10
V	Control-F	61.69 ± 6.67	137.87 ± 9.09	0.77 ± 0.09	83.32 ± 5.92
VI	SA-50-F	68.24 ± 9.36	141.01 ± 5.62	0.51 ± 0.02	88.17 ± 9.54
VII	SA-100-F	65.23 ± 3.83	137.25 ± 2.31	0.57 ± 0.09	90.85 ± 10.41
VIII	SA-200-F	63.10 ± 3.48	134.24 ± 4.56	0.70 ± 0.10	87.46 ± 3.60

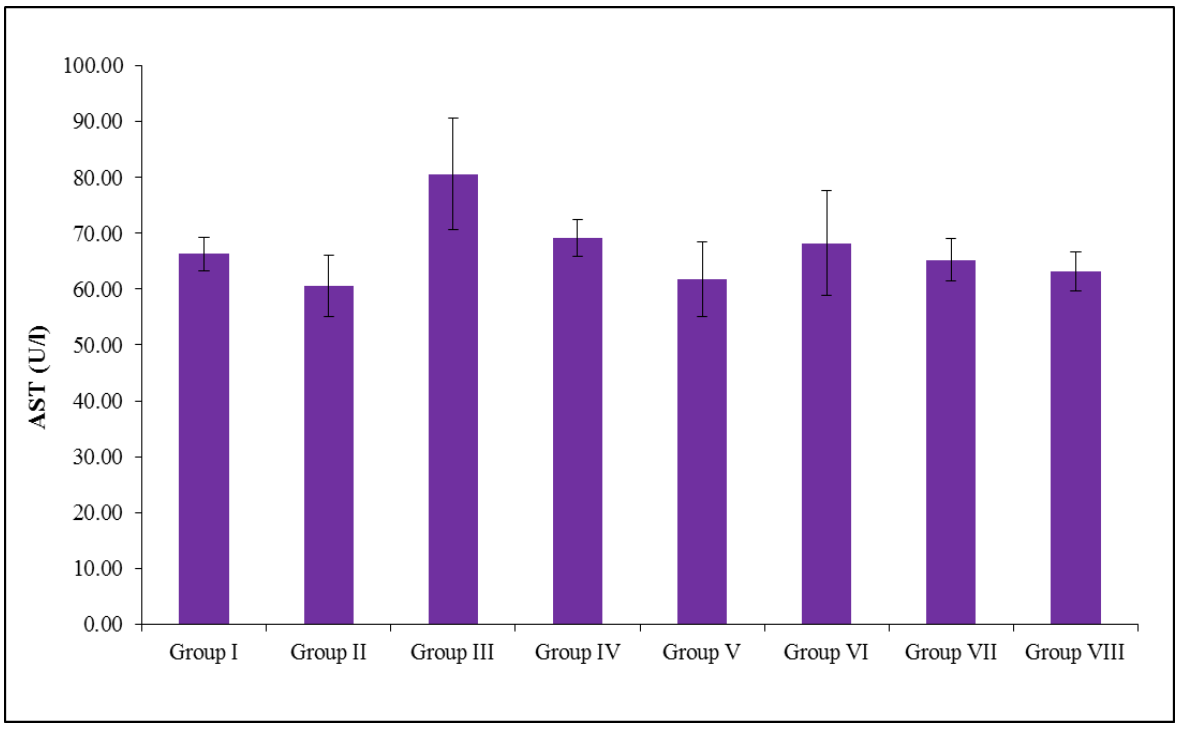

Figure 4.28: Effect of repeated oral administration of clove oil for 28 days on AST of male (I-IV) and female (V-VIII) rats

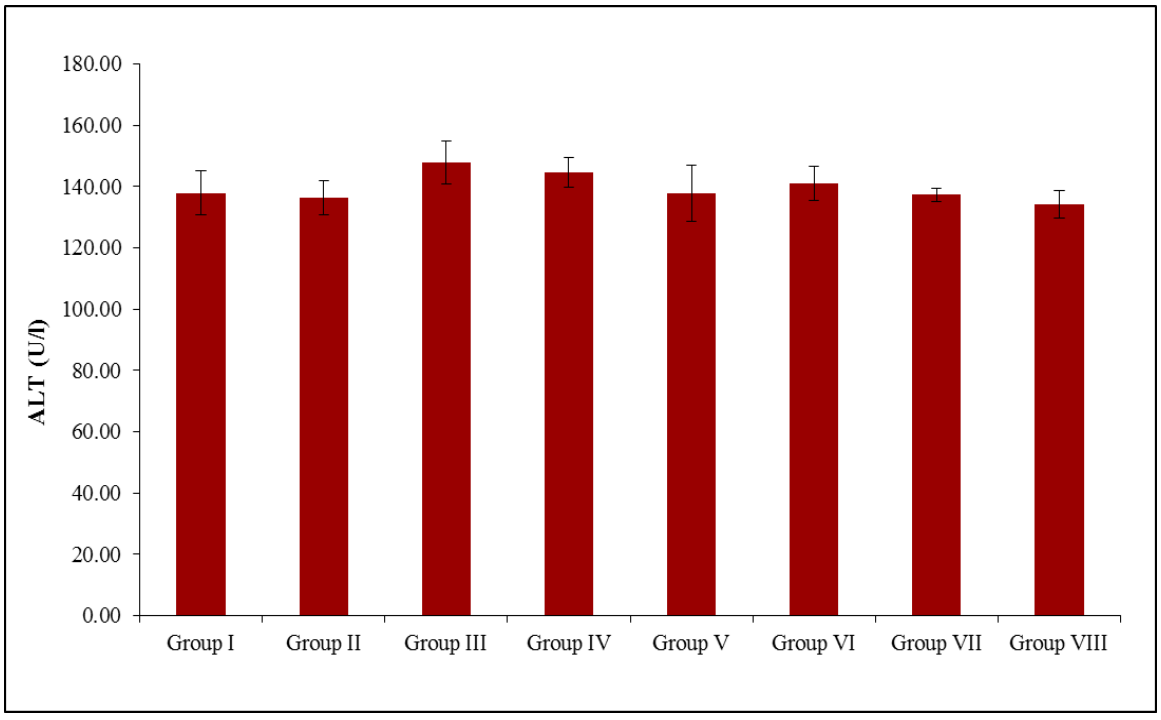

Figure 4.29: Effect of repeated oral administration of clove oil for 28 days on ALT of male (I-IV) and female (V-VIII) rats

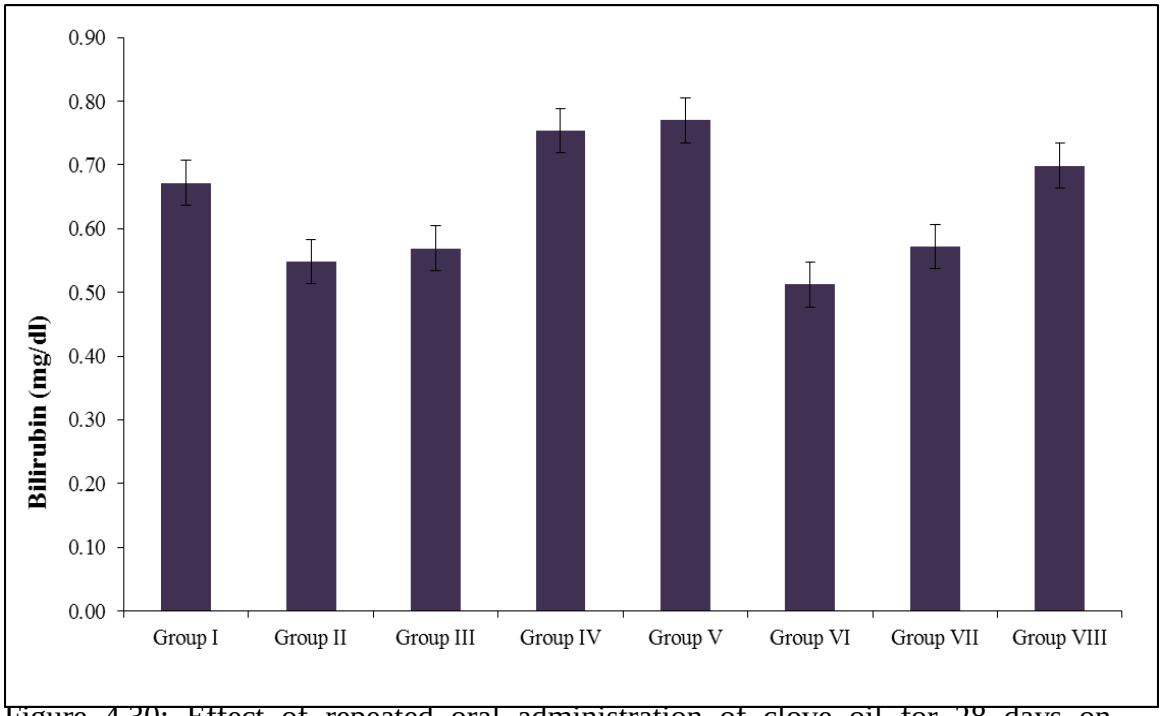

Figure 4.30: Effect of repeated oral administration of clove oil for 28 days on bilirubin of male (I-IV) and female (V-VIII) rats

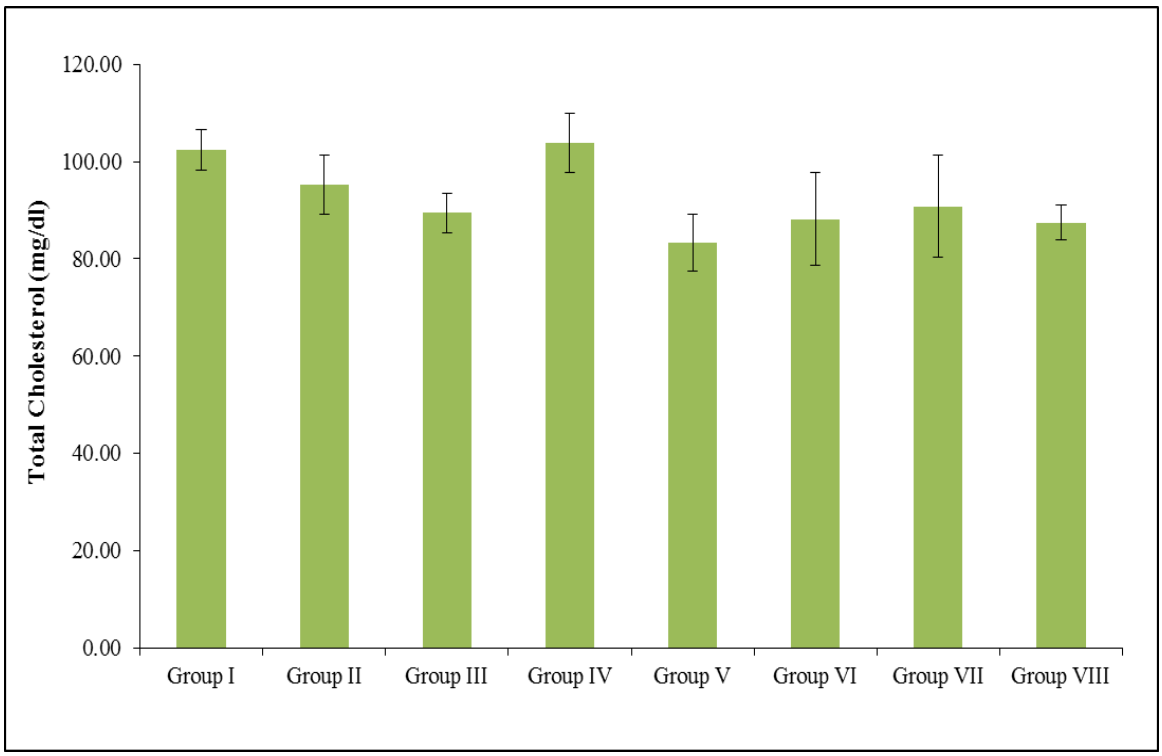

Figure 4.31: Effect of repeated oral administration of clove oil for 28 days on total cholesterol of male (I-IV) and female (V-VIII) rats

Table-4.12: Effect of repeated oral administration of clove oil (*Syzygium aromaticum*) for 28 days on total protein and albumin of male (I-IV) and female (VI-VIII) rats (Mean ± S.E.; n=5)

Group	Treatment	Total Protein (g/dl)	Albumin (g/dl)
I	Control-M	6.00 ± 0.35	4.38 ± 0.26
II	SA-50-M	5.87 ± 0.41	4.13 ± 0.19
III	SA-100-M	6.07 ±0.16	4.08 ± 0.06
IV	SA-200-M	5.73 ± 0.26	4.12 ± 0.16
V	Control-F	5.91 ± 0.16	4.44 ± 0.12
VI	SA-50-F	6.35 ± 0.14	4.45 ± 0.10
VII	SA-100-F	6.48 ± 0.35	4.28 ± 0.20
VIII	SA-200-F	6.38 ± 0.32	4.48 ± 0.06

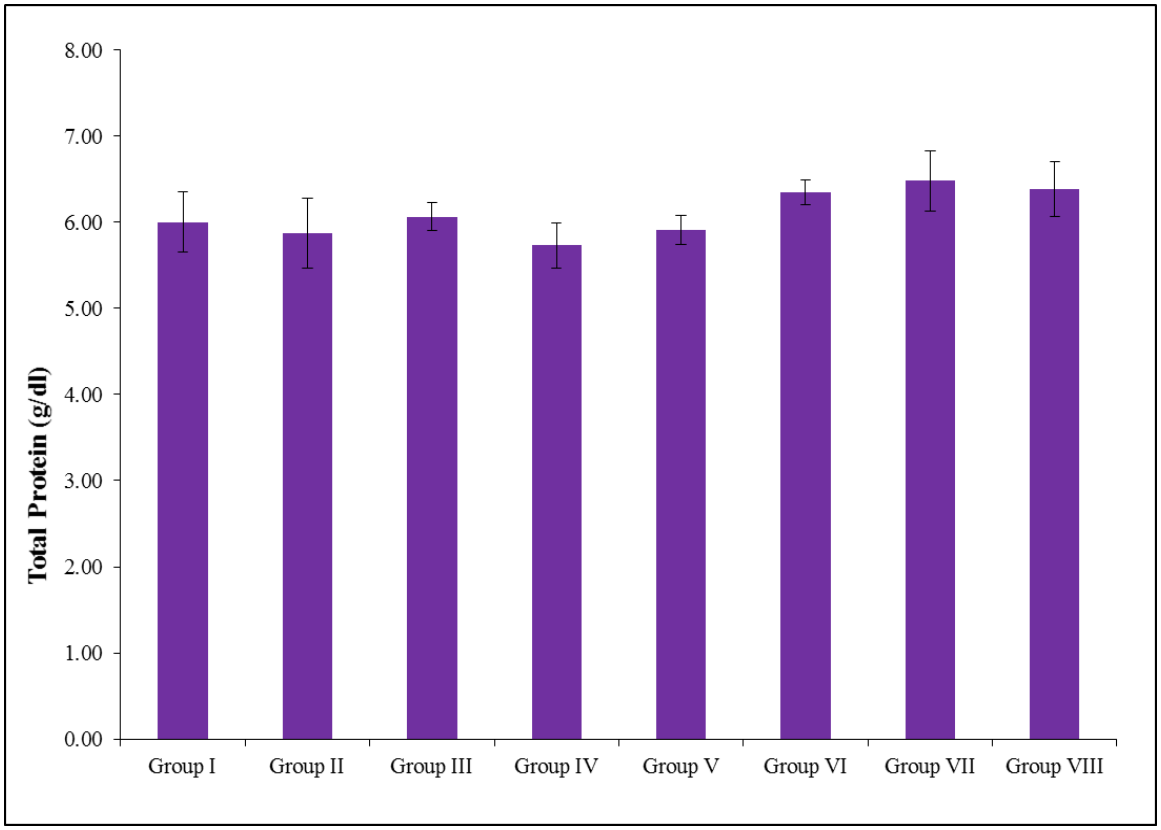

Figure 4.32: Effect of repeated oral administration of clove oil for 28 days on total protein of male (I-IV) and female (V-VIII) rats

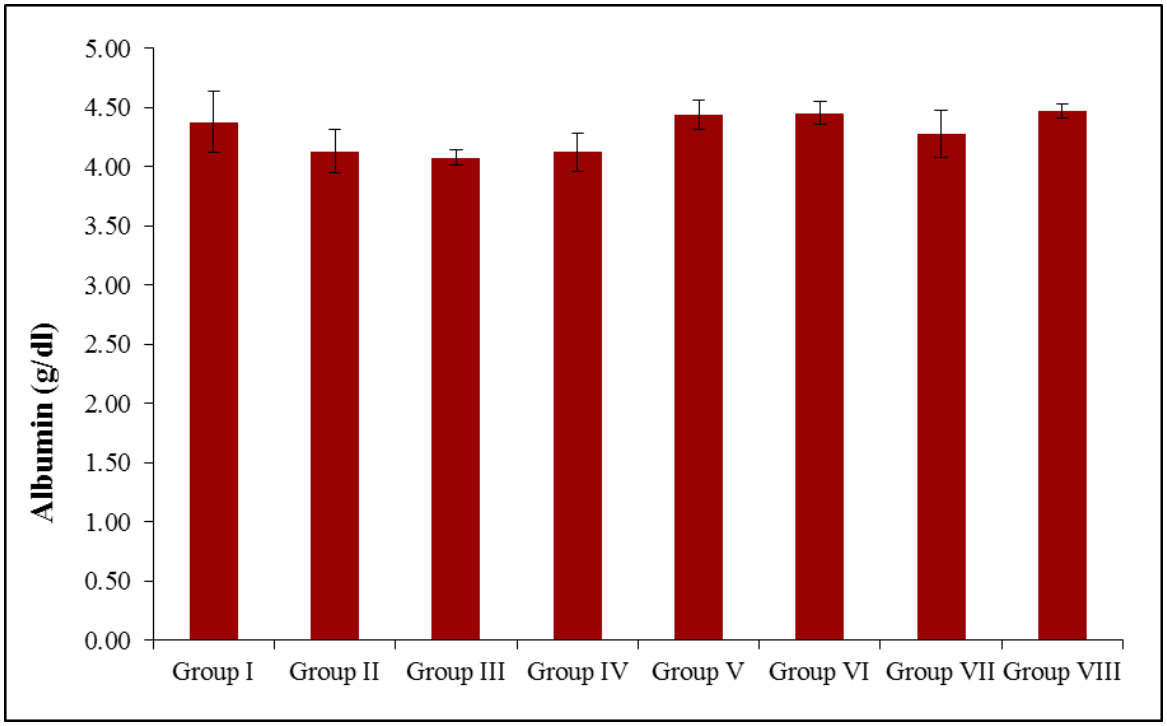

Figure 4.33: Effect of repeated oral administration of clove oil for 28 days on albumin of male (I-IV) and female (V-VIII) rats

Similarly, Mishra and Singh (2008) reported that repeated oral administration of *Syzygium aromaticum* flower bud (clove) hexane extract at the dose of 15, 30 and 60 mg/kg for 35 days in mice showed no significant difference in creatinine, AST and ALT levels as compared to control mice. Issac *et al.* (2015) studied sub-acute toxicity of clovinol (polyphenol-rich extract of clove buds) at doses of 0.5, 1.0 and 2.5 g/kg b.wt in both male and female rats showed no significant ($p > 0.05$) change in biochemical parameters like creatinine, urea, hepatic function and cholesterol levels as compare to control group. Vijayasteltar *et al.* (2016) studied safety assessment of a standardized polyphenolic extract of clove buds at 0.25, 0.5 and 1.0 g/kg body weight for 90 days in wistar rats. The result showed that treatment group did not produce any significant changes on biochemical parameters related to renal function, hepatic function and cholesterol level in compared to untreated control male and female rats. Saeed *et al.* (2017) studied safety assessment of ethanolic extract of *syzygium aromaticum* in albino rats at 250, 500 and 1000 mg/kg for 21 days in rats and result showed no significant ($P > 0.05$) changes in the levels of bilirubin, urea, creatinine, AST, ALT, TP and albumin as compare to control rats.

In contrast, Shalaby *et al.* (2011) studied toxicological effects of clove oil @ 500, 1000, 1500, 2000 and 2500 mg/kg b.wt. in rats. Result showed that clove oil produce a significant increase in the activity of AST and ALT as compred to control rats. Adam *et al.* (2013) reported the effects of repeated oral administration of aqueous extract of clove @ 50, 200, 400 and 800 mg/kg/day on serum biochemical parameters in wistar rats. Result showed that significat increase in AST, ALT and cholesterol level @ 200 mg/kg treated rats wherease significant increased in total protein and bilirubin level @ 400 mg/kg treated rats compared to control rats.

4.9 HISTOPATHOLOGICAL FINDINGS

All the rats were sacrificed on 29th day of experiment and detailed post-mortem examination was performed. Tissues like kidney, liver, spleen and heart were collected for histopathological examination from all the rats during necropsy. No appreciable gross changes were observed in kidney, liver, spleen and heart of any experimental rats and are depicted in figure 4.34 to 4.49.

4.9.1 Kidney, Liver, Spleen and Heart

Histopathology of organs like kidney, liver, spleen and heart from vehicle control rats (group I) did not showed any gross or microscopic changes. All organs collected showed normal microscopic structure. Histopathology of kidney, liver, spleen and heart from clove oil treated male rats of II, III, IV groups and female rats of VI, VII and VIII groups did not showed any marked gross or histopathological changes as compared to vehicle control rats.

Similarly, Shalaby *et al.* (2011) studied toxicological effects of essential oil from clove (500, 1000, 1500, 2000, and 2500 mg/b.w.) on Albino rats showed that the structure of the hepatocytes appeared more or less like normal but few specimens also revealed slight congestion of the blood vessels in the portal area and associated with inflammatory infiltration. Issac *et al.* (2015) studied sub-acute toxicity studies of clovinol (polyphenol-rich extract of clove buds) at doses of 0.5, 1.0 and 2.5 g/kg b.wt. reported that the histopathological examination of various organs of animals treated with 2.5 g/kg b.wt. clovinol showed normal cellular architecture similar to control rats. The liver section of clovinol treated rats showed normal portal triads and central venous system as compare to normal rats. The tissue sections of spleen from clovinol treated rats showed normal lymphoid follicles with area of prominent in germinal

center. The kidney tissues of clovinol treated rats showed normal glomeruli with bowman's capsule and renal tubules. Gashlan and Beladi (2016) reported that repeated oral administration of clove oil @ 300 and 600 mg/kg of clove oil showed no alterations in morphology as compare to normal rats treated. Vijayasteltar *et al.* (2016) studied safety assessment of a standardized polyphenolic extract of clove buds at 0.25, 0.5 and 1.0 g/kg body weight reported that the histopathological examination of various organs of animals treated with 2.5 g/kg b.wt. polyphenolic extract of clove has showed normal cellular architecture similar to the control group. The liver section of polyphenolic extract of clove treated rats showed normal portal triads and central venous system, the tissue sections of spleen also showed normal lymphoid follicles and the kidney tissues showed normal glomeruli with bowman's capsule and renal tubules as compare to control rats.

In contrast, Shalaby *et al.* (2011) studied toxicological effects of clove oil from (500, 1000, 1500, 2000 and 2500 mg/kg b.wt.) in albino rats showed lobulated renal corpuscles and the desquamation of the epithelial cells of the renal tubules. Adam *et al.* (2013) studied effects of the oral administration of aqueous extract of clove (50, 200, 400 and 800 mg/kg/day) on wistar rats reported fatty cytoplasmic vacuolation of the centrilobular hepatocytes in the liver of 200 mg/kg aqueous extract treated rats and fatty cytoplasmic vacuolation of the centirlobular hepatocytes and hemorrhage in the liver of 400 mg/kg aqueous extract treated rats and packing of the glomerular tubules, dilatation and necrosis of the renal tubules in the kidney of 400 mg/kg aqueous extract. Saeed *et al.* (2017) studied safety assessment of ethanolic extract of *syzygium aromaticum* in albino rats at 250, 500 and 1000 mg/kg showed histopathological changes mainly like dilatation of the cortical tubules, congestion and dilatation in renal blood vessels and liver showed congestion in the portal tract with slight necrosis of hepatocytes in treated rats.

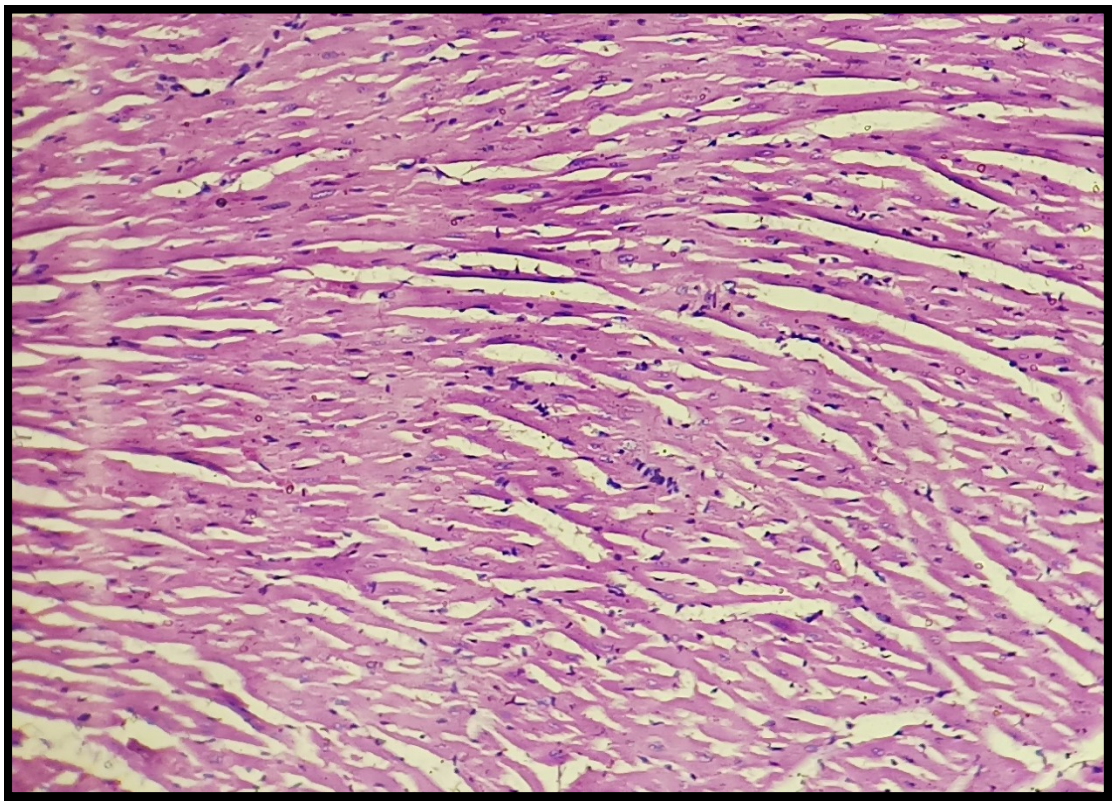

Figure 4.34 : Section of heart from male control rats (group I) showing normal architecture (H & E stain X 120).

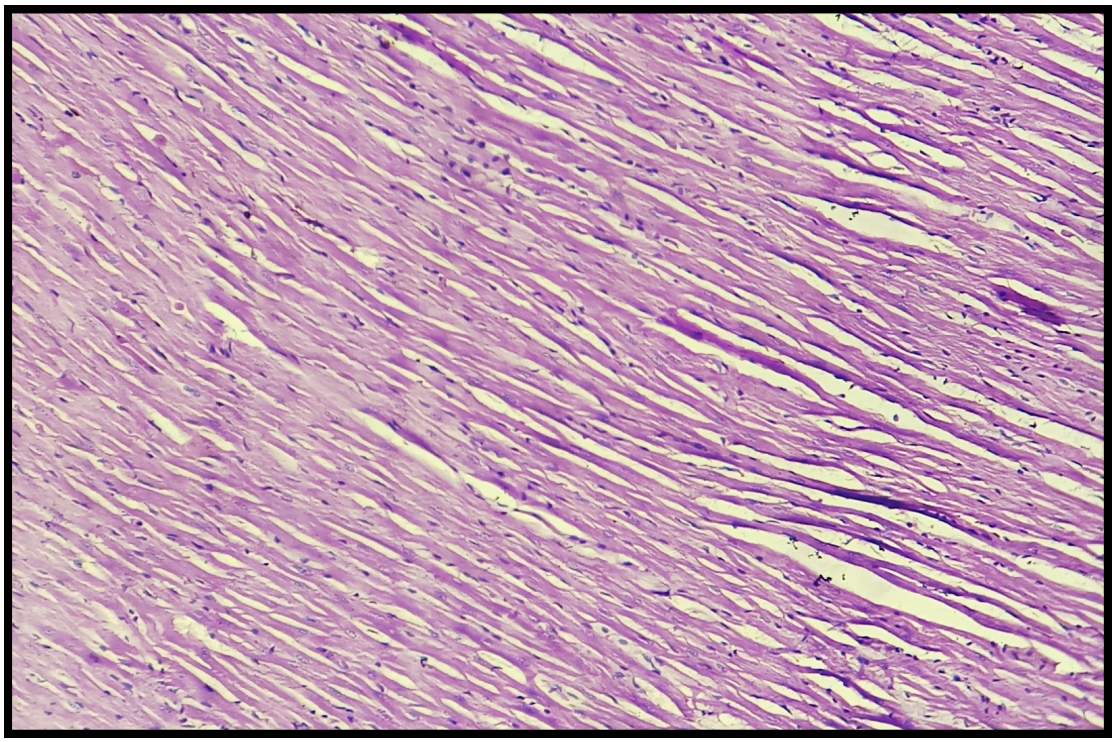

Figure 4.35 : Section of heart from clove oil treated male rats of group III (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).

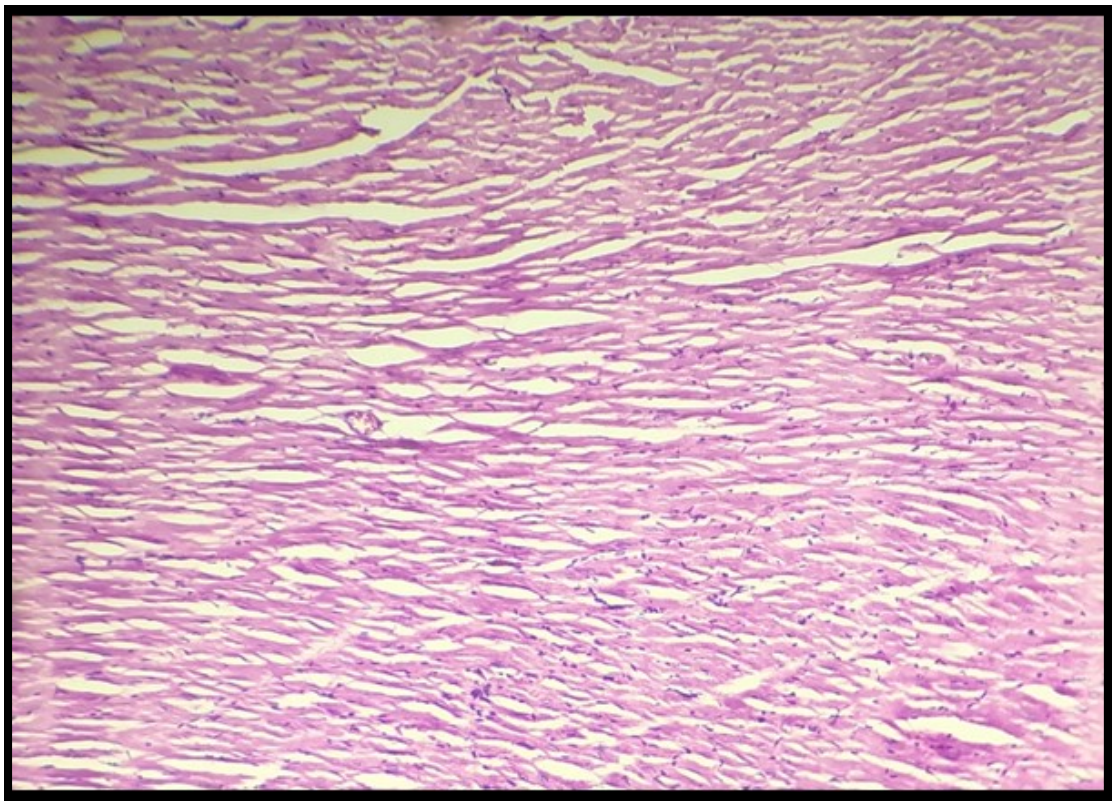

Figure 4.36 : Section of heart from female control rats (group V) showing normal architecture (H & E stain X 120).

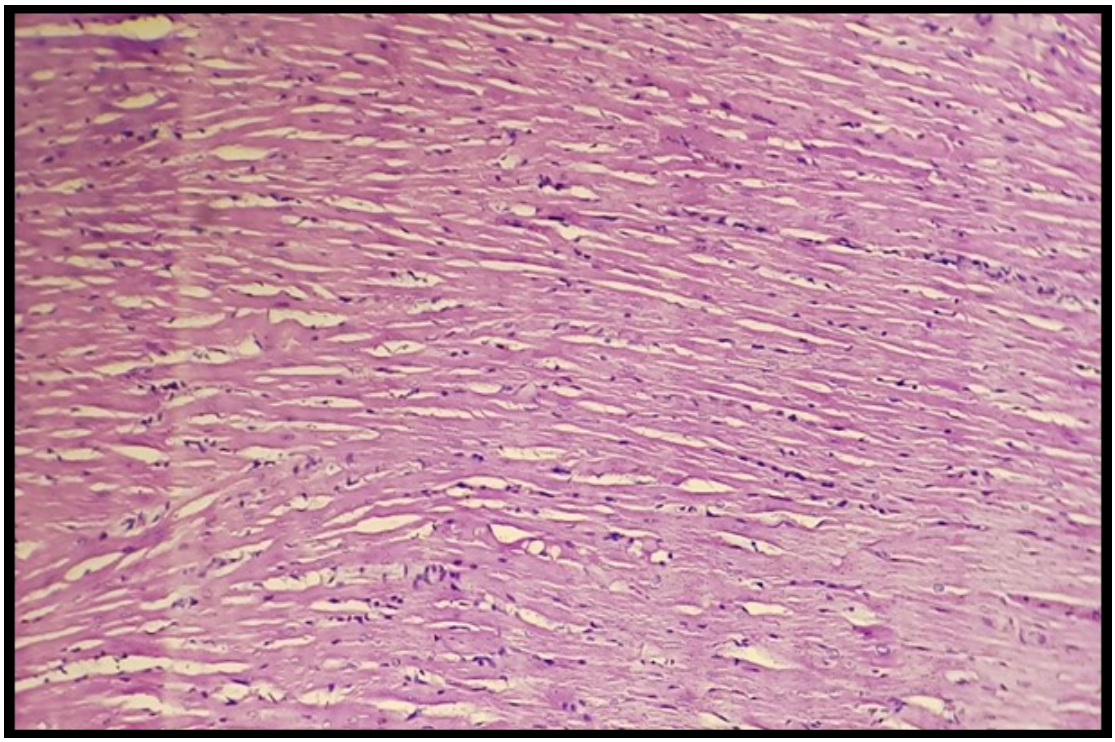

Figure 4.37 : Section of heart from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120)..

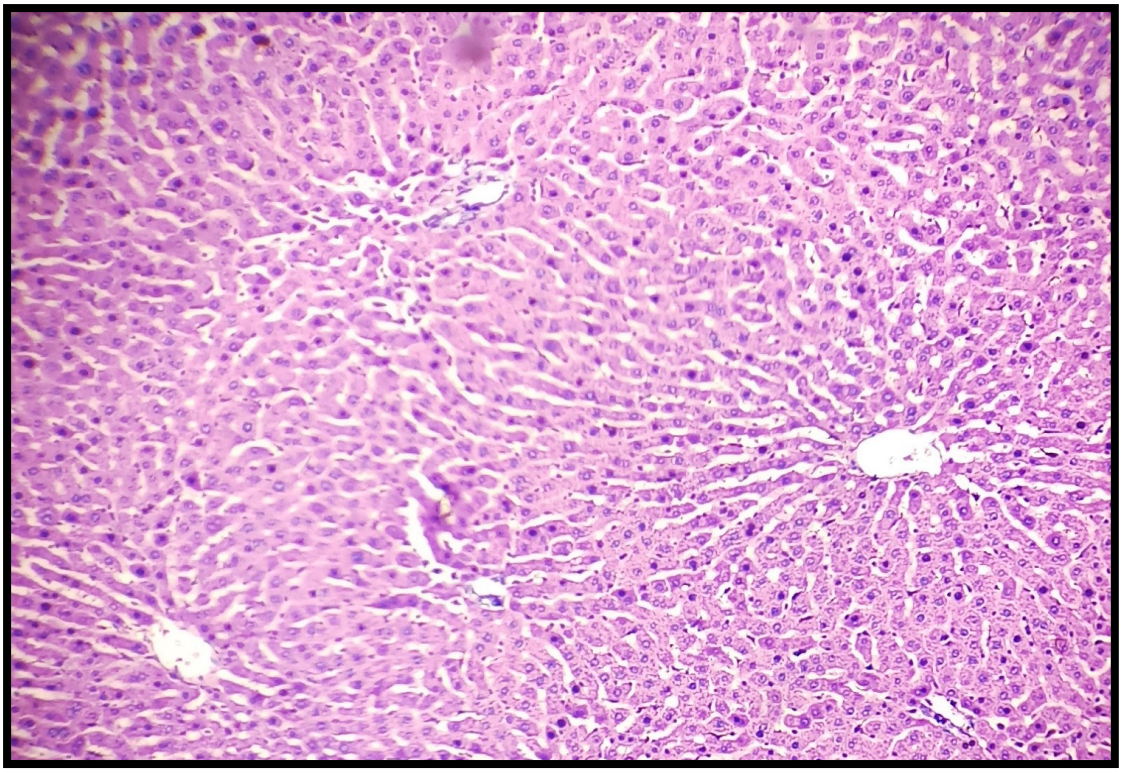

Figure 4.38 : Section of liver from male control rats (group I) showing normal architecture (H & E stain X 120).

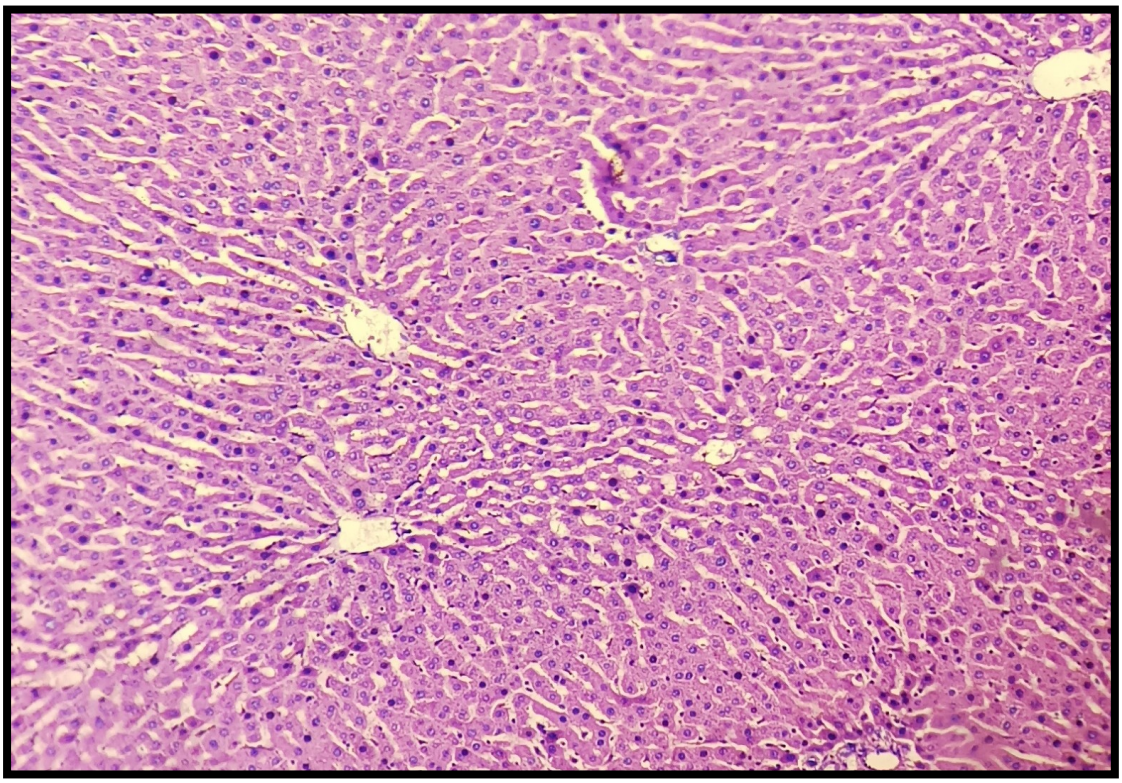

Figure 4.39: Section of liver from clove oil treated male rats of group III (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).

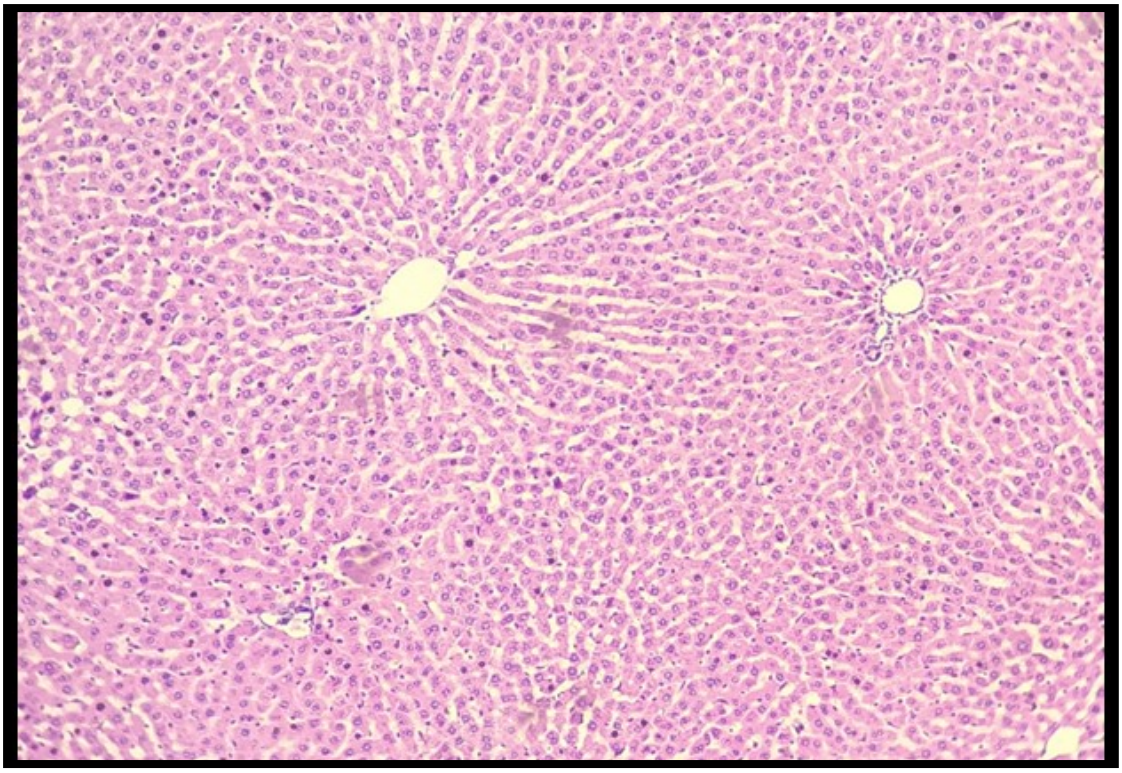

Figure 4.40 : Section of liver from female control rats (group V) showing normal architecture (H & E stain X 120).

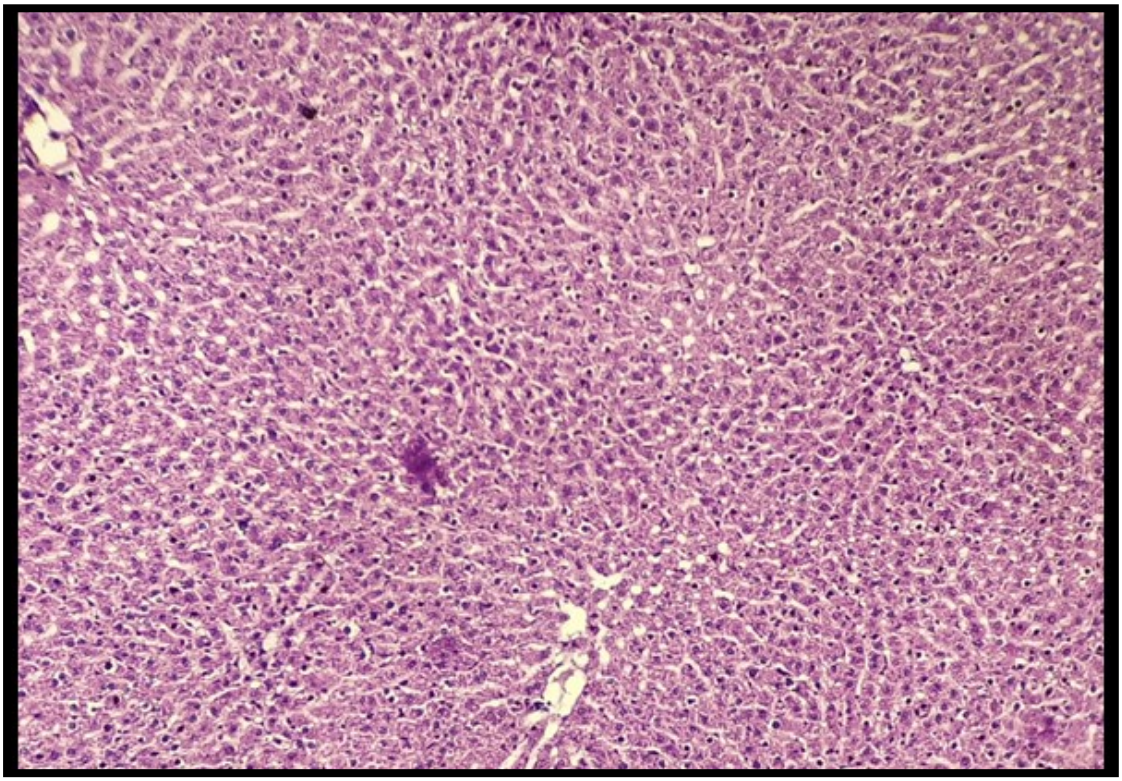

Figure 4.41 : Section of liver from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120)..

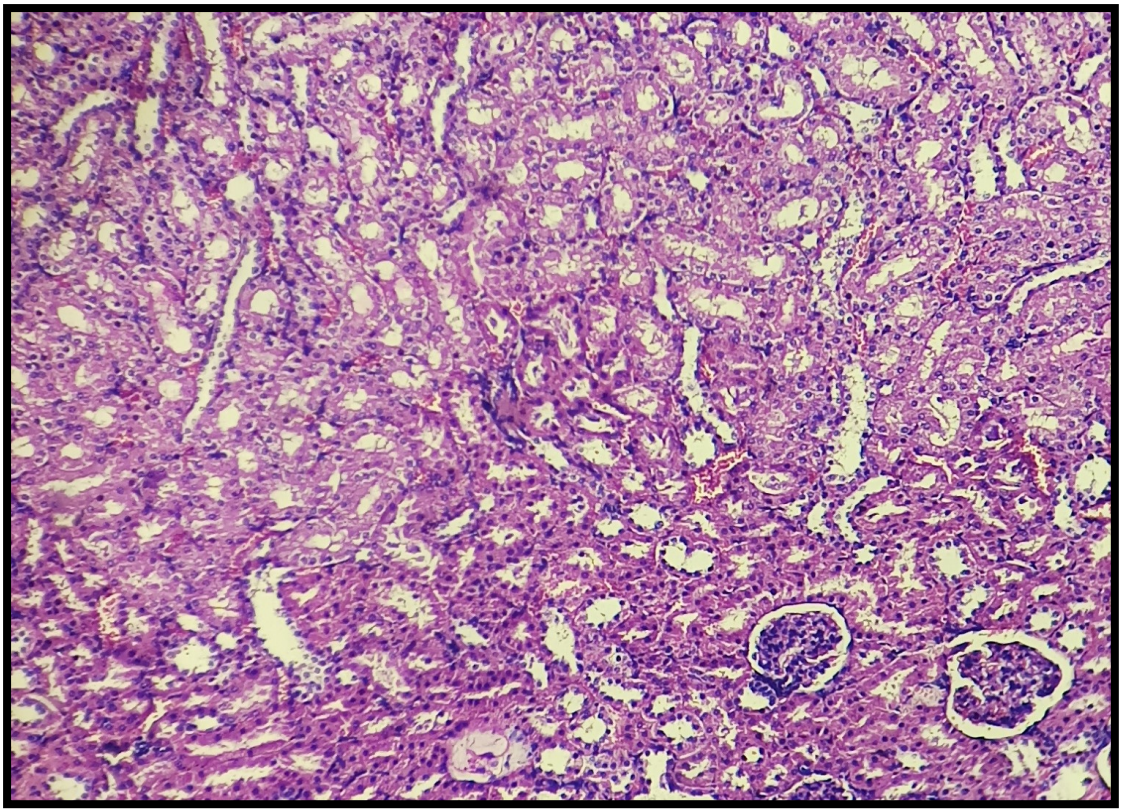

Figure 4.42 : Section of kidney from male control rats (group I) showing normal architecture (H & E stain X 120).

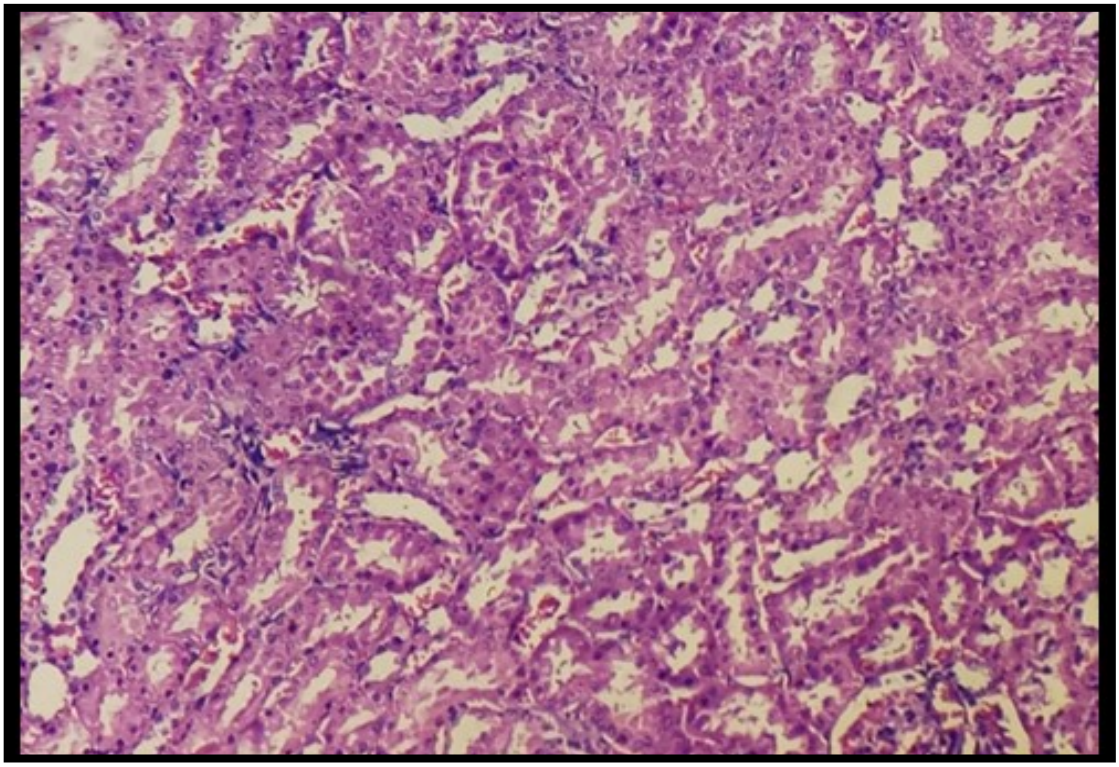

Figure 4.43 : Section of kidney from clove oil treated male rats of group III (200mg/ kg) showing no pathological alterations in architecture (H & E stain X 120).

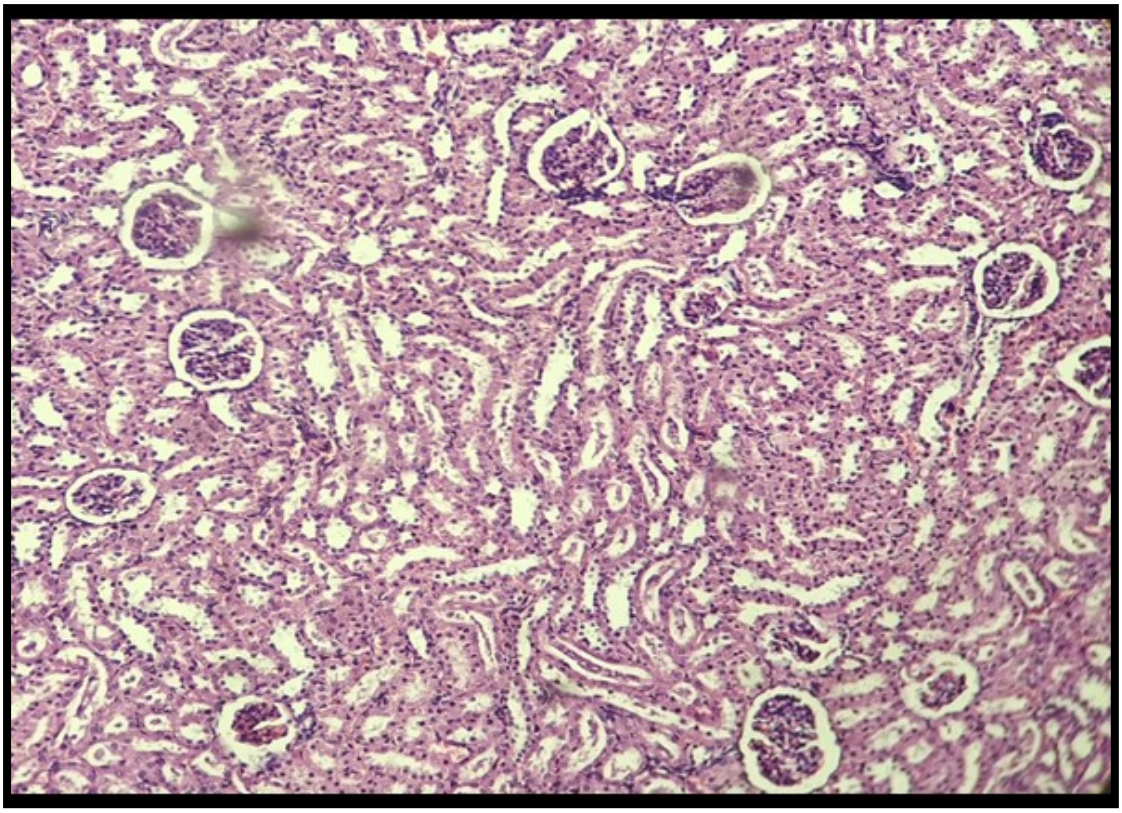

Figure 4.44 : Section of kidney from female control rats (group V) showing normal architecture (H & E stain X 120).

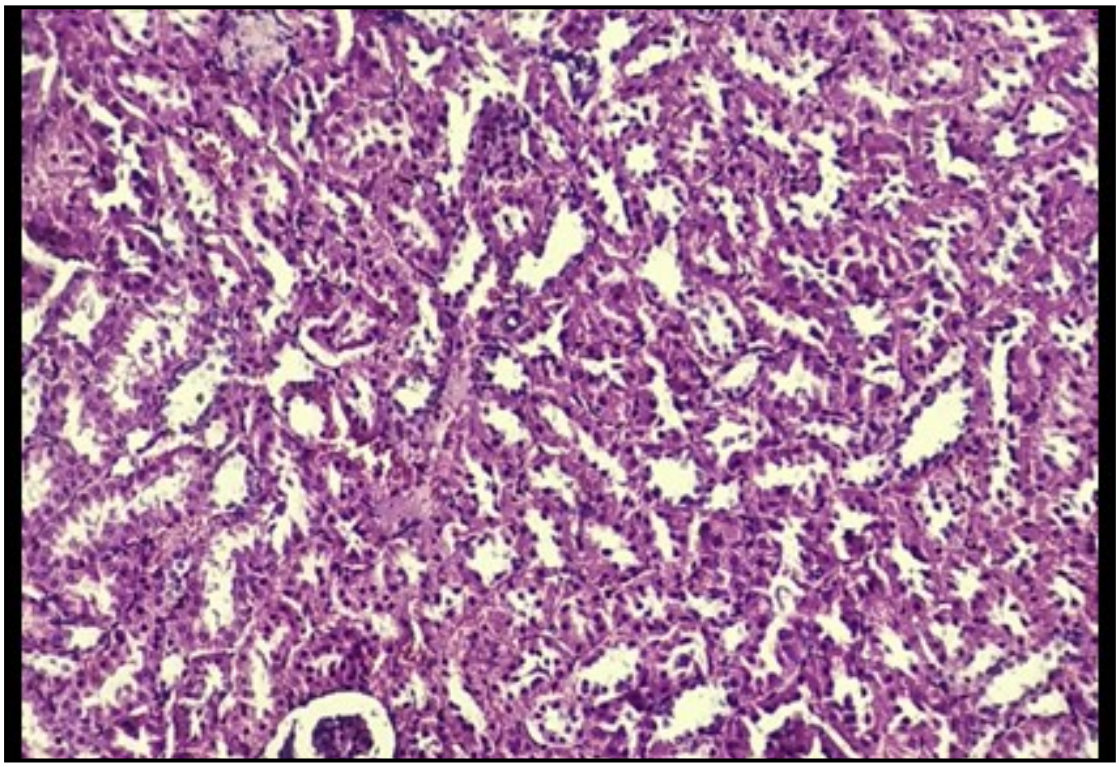

Figure 4.45: Section of kidney from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).

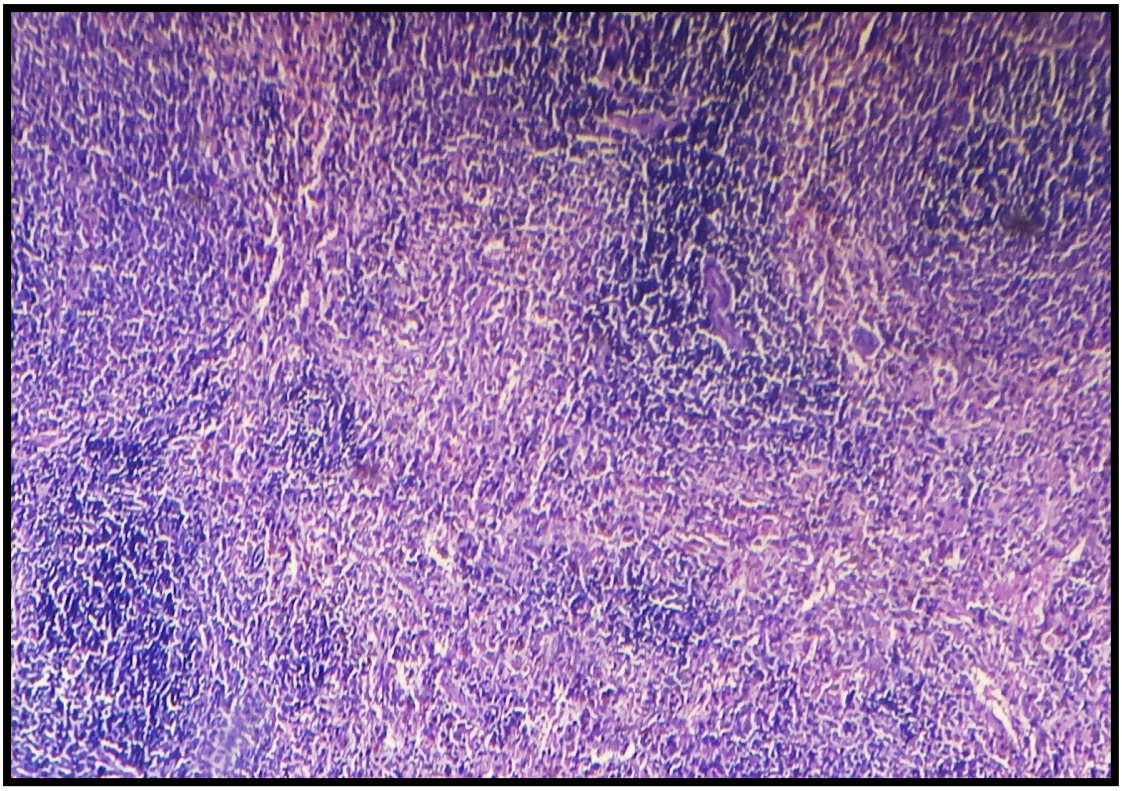

Figure 4.46 : Section of spleen from male control rats (group I) showing normal architecture (H & E stain X 120).

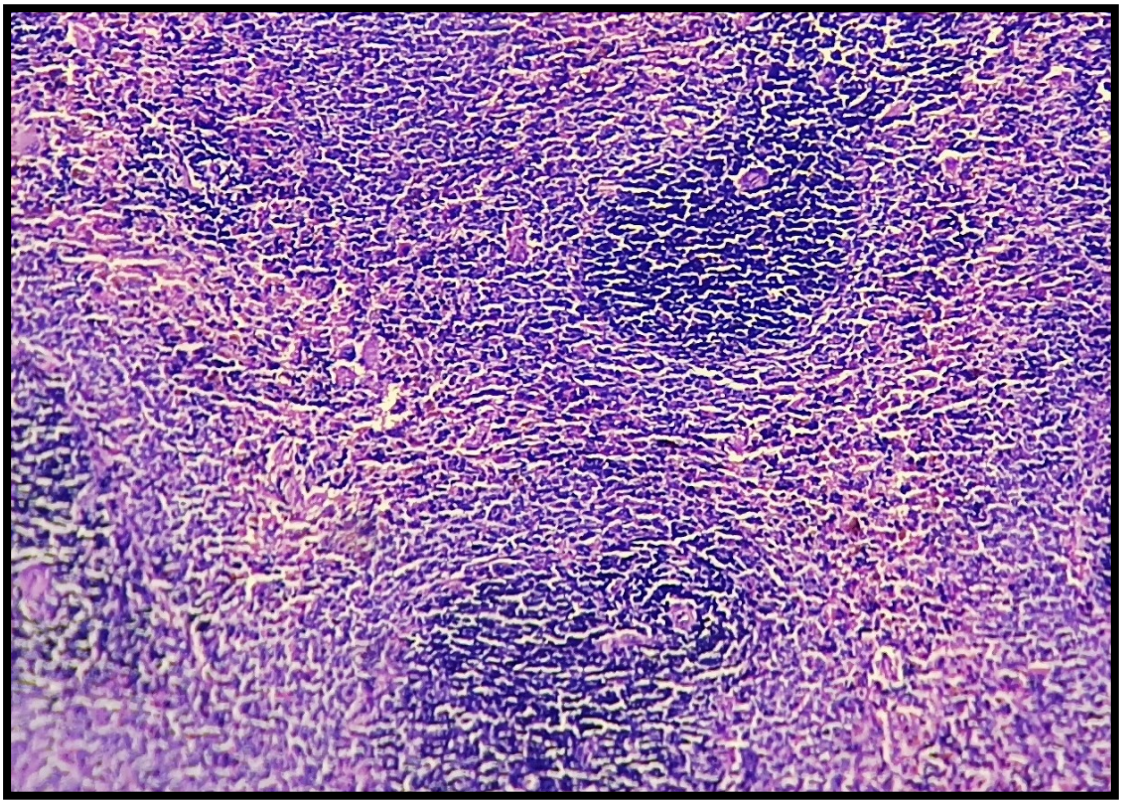

Figure 4.47 : Section of spleen from clove oil treated male rats of group III (200mg/ kg) showing no pathological alterations in architecture (H & E stain X 120).

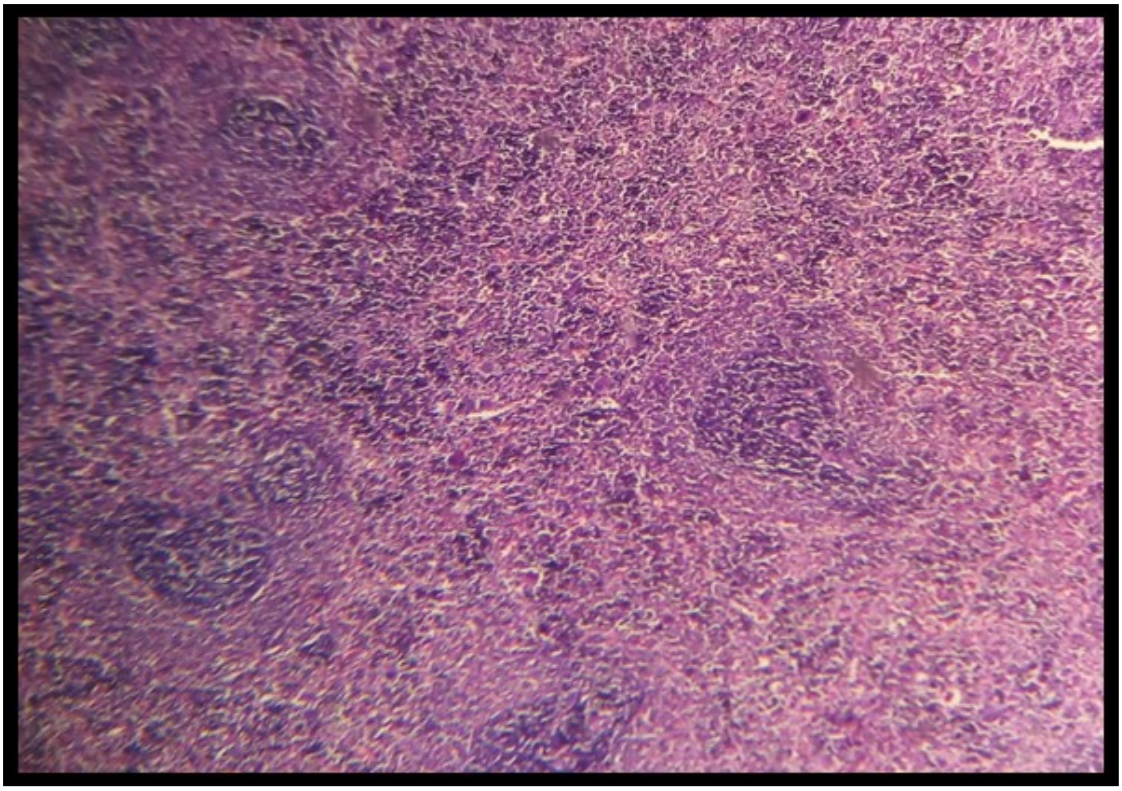

Figure 4.48 : Section of spleen from female control rats (group V) showing normal architecture (H & E stain X 120).

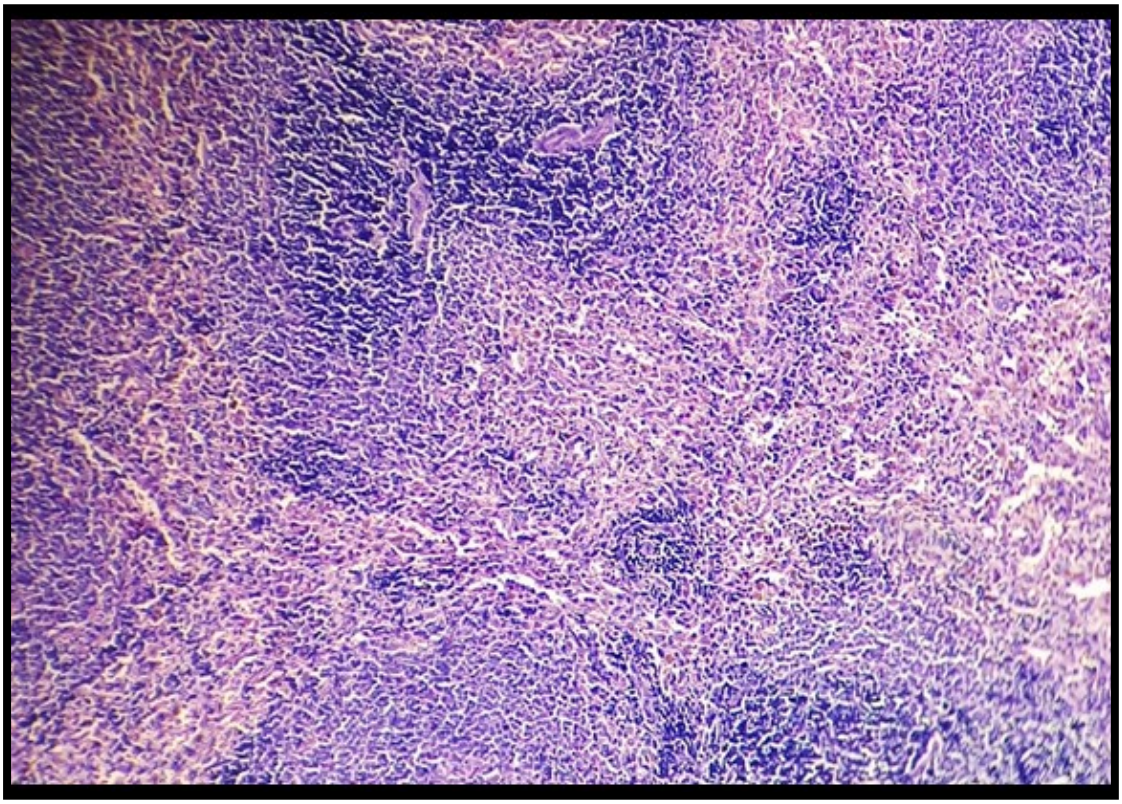

Figure 4.49: Section of spleen from clove oil treated female rats of group VIII (200mg/kg) showing no pathological alterations in architecture (H & E stain X 120).

CHAPTER – V

SUMMARY AND CONCLUSIONS

5.1 SUMMARY

The present study was conducted to evaluate *in-vitro* antibacterial, *in-vivo* anti-inflammatory (100, 250 and 500 mg/kg) effects and safety of clove oil (50, 100 and 200 mg/kg) following repeated oral administration in wistar rats.

5.2 *IN-VITRO* ANTIBACTERIAL ACTIVITY OF CLOVE OIL

Screening of clove oil for antibacterial activity was done by the disc diffusion method. It was performed using an 18 h culture at 37°C in 10 ml of Muller Hinton agar (for *S. agalactiae* 5% defibrinated sheep blood was added). The test suspension was standardized to match 0.5 McFarland turbidity standard. The clove oil was suspended in 10% dimethylsulfoxide (DMSO) with tween 80 and sterilized by filtration through 0.45 µm membrane filter. Under aseptic condition, empty sterilized discs were impregnated with 50 µl of different concentrations (1:1, 1:2, 1:5, 1:10 and 1:20) of the respective clove oil and placed on the agar surface. Paper disc moistened with aqueous DMSO was placed on the seeded petriplate as a vehicle control. Standard discs containing antibacterial drug (cefotaxime, ampicillin, tetracycline and gentamicin) was used as reference control. The petri plates were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured.

The results revealed that the clove oil showed antibacterial activity with varying magnitudes. The zone of inhibition above 10 mm in diameter was taken as positive results. Both gram positive (*Staphylococcus aureus, Listeria monocytogenes* and *Streptococcus agalactiae*) and gram negative (*Salmonella typhimurium, Pseudomonas aeruginosa* and *Escherichia coli*) bacteria were sensitive to the clove oil. There was no inhibition in growth of bacteria with the vehicle control (10% DMSO with 0.5% v/v tween 80). Four antibacterial drugs (cefotaxime, ampicillin, tetracycline and gentamicin) were also tested against all six organisms and were found active against test bacteria.

5.3 *IN-VIVO* ANTI-INFLAMMATORY ACTIVITY OF CLOVE OIL

The *in-vivo* anti-inflammatory assay of clove oil (*Syzygium aromaticum*) was carried out using rat paw edema method. Fifty rats were divided randomly into 10 groups each group of 5 males and 5 females. All rats were injected subcutaneously with 0.1 ml of a 10% w/v carrageenan suspension s/c as a local acute edema inducer after 30 minutes subsequent to oral administration of clove oil. Rats of control male and female groups were kept untreated. Rats of male and female standard control group were treated orally with indomethacin @ 10 mg/kg b.wt. as a reference drug, respectively. Clove oil was given orally to male and female rats at three different dose rate 100, 250 and 500 mg/kg b.wt., respectively. Edema was expressed as the increase in paw volume (ml). The paw volume was measured up to the tibiotarsal articulation. Volume of edematous paw was measured by using plethysmometer (PLM-01 plus, Orchid Scientific Instrument, India) at 0 hr (before treatment), 1, 2, 3, 4, 6 and 24 hours after treatments. Increase in paw thickness was measured by using digital plethysmometer and percent inhibition were calculated.

The results revealed that the clove oil showed anti-inflammatory effect with varying magnitudes at various doses in both male and female wistar rats. The anti-inflammatory effect of indomethacin was highest at 3h (41.75% in male, 42.99% in female) as compare to other doses of clove oil treated rats. The anti-inflammatory effect of clove oil was highest at 3h (35.77% in male, 35.46% in female) at the dose rate of 500 mg/kg. At 3h all doses gave higher anti-inflammatory effect. Clove oil showed dose dependent anti-inflammatory activity in male and female rats.

5.4 SAFETY STUDY OF CLOVE OIL IN RATS

The safety study of clove oil was conducted on forty (40) wistar rats divided into eight groups, each group contains 5 males and 5 females. Group I and V served as control. Clove oil administered orally at dose of 50, 100 and 200 mg/kg body weight once daily for 28 days in male rats of group II, III and IV as well as in female rats of group VI, VII and VIII, respectively.

The animals of all the groups were observed daily for clinical signs and mortality. Body weight and feed consumption of animals were monitored at weekly interval. At the end of experiment on 29^{th} day, blood sample were collected for the

hematological and serum biochemical investigations. All the male and female rats were sacrificed at the end of experiment and subjected to post mortem examination. Organs (kidney, liver, spleen and heart) were collected for gross and histopathological examination.

In the present study, the utilized test at 50, 100 and 200 mg/kg body weight were found to be safe. There was no significant difference observed in body weight of rats of control groups and no significant difference was observed in body weight of group II, III & IV of male rats and V, VII & VIII of female rats as compared to control group on day 7, 14, 21 and 28 days. No significant difference was observed in feed consumption of groups II, III and IV of male rats as compared to group I (male control) and also no significant difference was observed in female rats of group VI, VII and VIII as compared to group VI (female control) on 7^{th}, 14^{th}, 21^{st} and 28^{th} day of study.

No significant changes have been observed in Hb, RBCs, PCV, TLC, MCV, MCH and MCHC in clove oil treated male rats of group II, III and IV and female rats of group VI, VII and VIII at the end of experiment as compared to control male & female rats, respectively. No significant change have been observed in serum creatinine, BUN, bilirubin, AST, ALT, total cholesterol, total protein and albumin in clove oil treated male rats of group II, III and IV and in female rats of group VI, VII and VIII at the end of experiment on 28^{th} day as compared to male and female control rats, respectively.

Histopathology of organs like kidney, liver, spleen and heart from vehicle control rats (group I and V) did not showed any gross or microscopic changes. All organs collected showed normal microscopic structure. Whereas histopathology of kidney, liver, spleen and heart from clove oil treated male rats of group II, III and IV and in female rats of group VI, VII and VIII did not showed any marked gross or histopathological changes at the end of experiment on 28^{th} day as compared to male and female control groups, respectively.

5.3 CONCLUSIONS

Following conclusions could be drawn based on the results of the present study:

1. Clove oil has antibacterial activity against test organisms like *Salmonella typhimurium, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes* and *Streptococcus agalactiae.*
2. Clove oil showed dose dependent anti-inflammatory activity @ 100, 250 and 500 mg/kg b.wt. in male and female wistar rats.
3. The highest anti-inflammatory activity was observed at 3 hour post oral administration of clove oil in both male & female wistar rats.
4. Clove oil was found safe following repeated oral administration @ 50, 100 and 200 mg/kg b.wt. for 28 days.

REFERENCES

Abdelrahman, M. T., Maina, E. N., & Elshemy, H. A. (2018). Clove (*Syzygium aromaticum*) and honey extracts significantly reduce inflammatory cytokines and liver function enzymes in experimental rats fed on carbon tetrachloride (CCl4). *Journal of Radiation Research and Applied Sciences*, *11*(4), 416-422.

Abdel-Wahhab, M. A., & Aly, S. E. (2005). Antioxidant property of *Nigella sativa* (black cumin) and *Syzygium aromaticum* (clove) in rats during aflatoxicosis. *Journal of Applied Toxicology: An International Journal*, *25*(3), 218-223.

Abdullah, B. H., Hatem, S. F., & Jumaa, W. (2015). A comparative study of the antibacterial activity of clove and rosemary essential oils on multidrug resistant bacteria. *UK Journal of Pharmaceutical and Biosciences*, *3*(1), 18-22.

Adam, S. I., Mohamed, S. B., & Abdelgadir, W. S. (2013). Effects of the aqueous extract of clove (*Syzygium aromaticum*) on Wistar Rats. *British Journal of Pharmacology and Toxicology*, *4*(6), 262-266.

Ahmad, T., Shinkafi, T. S., Routray, I., Mahmood, A., & Ali, S. (2012). Aqueous extract of dried flower buds of *Syzygium aromaticum* inhibits inflammation and oxidative stress. *Journal of Basic and Clinical Pharmacy*, *3*(3), 323.

Anderson, W. G., McKinley, R. S., & Colavecchia, M. (1997). The use of clove oil as an anesthetic for rainbow trout and its effects on swimming performance. *North American Journal of Fisheries Management*, *17*(2), 301-307.

Apel, M.A.; Lima, M.E.L.; Sobral, M.; Young, M.C.M.; Cordeiro, I.; Schapoval, E.E.S.; Henriques, A.T.; Moreno, P.R.H. (2010). Anti-inflammatory activity of essential oils

from leaves of *Myrciaria tenella* and *Calycorectes sellowianus*. *Pharmaceutical Biology*, *48*(4), 433-438.

Arung, E. T., Matsubara, E., Kusuma, I. W., Sukaton, E., Shimizu, K., & Kondo, R. (2011). Inhibitory components from the buds of clove (*Syzygium aromaticum*) on melanin formation in B16 melanoma cells. *Fitoterapia*, *82*(2), 198-202.

Ayoola, G. A., Lawore, F. M., Adelowotan, T., Aibinu, I. E., Adenipekun, E., Coker, H. A. B., & Odugbemi, T. O. (2008). Chemical analysis and antimicrobial activity of the essential oil of *Syzigium aromaticum* (clove). *African Journal of Microbiology Research*, *2*(7), 162-166.

Bachiega, T. F., de Sousa, J. P. B., Bastos, J. K., & Sforcin, J. M. (2012). Clove and eugenol in noncytotoxic concentrations exert immunomodulatory/anti-inflammatory action on cytokine production by murine macrophages. *Journal of Pharmacy and Pharmacology*, *64*(4), 610-616.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils–a review. *Food and Chemical Toxicology*, *46*(2), 446-475.

Bhat, S. K., & Kempraj, V. (2009). Biocidal potential of clove oils against Aedes albopictus–A comparative study. *African Journal of biotechnology*, *8*(24), 6933-6937.

Bhowmik, D., Kumar, K. S., Yadav, A., Srivastava, S., Paswan, S., & Dutta, A. S. (2012). Recent trends in Indian traditional herbs *Syzygium aromaticum* and its health benefits. *Journal of Pharmacognosy and Phytochemistry*, *1*(1), 13-23.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*(3), 223-253.

Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L., & Ferret, A. (2007). Invited

review: essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science*, *90*(6), 2580-2595.

Chaieb, K., Zmantar, T., Ksouri, R., Hajlaoui, H., Mahdouani, K., Abdelly, C., & Bakhrouf, A. (2007). Antioxidant properties of the essential oil of *Eugenia caryophyllata* and its antifungal activity against a large number of clinical Candida species. *Mycoses*, *50*(5), 403-406.

Chami, F., Chami, N., Bennis, S., Trouillas, J., & Remmal, A. (2004). Evaluation of carvacrol and eugenol as prophylaxis and treatment of vaginal candidiasis in an immunosuppressed rat model. *Journal of Antimicrobial Chemotherapy*, *54*(5), 909-914.

Charles, D. J. (2013). Saffron. In antioxidant properties of spices, herbs and other sources, pp. 509–520.

Chaves, A. V., He, M. L., Yang, W. Z., Hristov, A. N., McAllister, T. A., & Benchaar, C. (2008). Effects of essential oils on proteolytic, deaminative and methanogenic activities of mixed ruminal bacteria. *Canadian Journal of Animal Science*, *88*(1), 117-122.

Chorianopoulos, N., Kalpoutzakis, E., Aligiannis, N., Mitaku, S., Nychas, G. J., & Haroutounian, S. A. (2004). Essential oils of Satureja, Origanum, and Thymus species: chemical composition and antibacterial activities against foodborne pathogens. *Journal of Agricultural and Food Chemistry*, *52*(26), 8261-8267.

Cimanga, K., Kambu, K., Tona, L., Apers, S., De Bruyne, T., Hermans, N., & Vlietinck, A. J. (2002). Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic

of Congo. *Journal of Ethnopharmacology*, *79*(2), 213-220.

Cortés-Rojas, D. F., de Souza, C. R. F., & Oliveira, W. P. (2014). Clove (*Syzygium aromaticum*): a precious spice. *Asian Pacific Journal of Tropical Biomedicine*, *4*(2), 90-96.

Daniel, A. N., Sartoretto, S. M., Schmidt, G., Caparroz-Assef, S. M., Bersani-Amado, C. A., & Cuman, R. K. N. (2009). Anti-inflammatory and antinociceptive activities A of eugenol essential oil in experimental animal models. *Revista Brasileira de Farmacognosia*, *19*(1B), 212-217.

Darshan, S., & Doreswamy, R. (2004). Patented antiinflammatory plant drug development from traditional medicine. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *18*(5), 343-357.

Diaz, M. R., & Sembrano, J. M. (1985). A comparative study of the efficacy of garlic and eugenol as palliative agents against dental pain of pulpal origin. *The Journal of the Philippine Dental Association*, *35*(1), 3-10.

Djilani, A., Dicko, A., 2012. The therapeutic benefits of essential oils. In: Bouayed, J., Bohn, T. (Eds.), Nutrition, Well-being and Health. InTech, Croatia, pp. 155–178.

Dorman, H. J. D., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, *88*(2), 308-316.

Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., & Merillon, J. M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agricultural and Food Chemistry*, *57*(5), 1768-1774.

Erasto, P., Bojase-Moleta, G., & Majinda, R. R. (2004). Antimicrobial and antioxidant flavonoids from the root wood of Bolusanthus speciosus. *Phytochemistry, 65*(7), 875-880.

Evans, W. C. (2001). Trease and Evans pharmacognosy. 14th Ed. London.

Friedly, E. C., Crandall, P. G., Ricke, S. C., Roman, M., O'bryan, C., & Chalova, V. I. (2009). In vitro antilisterial effects of citrus oil fractions in combination with organic acids. *Journal of food science*, *74*(2), 67-72.

Fu, Y., Zu, Y., Chen, L., Shi, X., Wang, Z., Sun, S., & Efferth, T. (2007). Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytotherapy Research, 21*(10), 989-994.

Gashlan and Beladi (2016). Effects of clove oil on blood glucose level, lipid profile, lipid peroxidation, and kidney function on diabetic rats. *International Journal of Development Research, 06*(10), 9657-9664.

Ghosh, R., Nadiminty, N., Fitzpatrick, J. E., Alworth, W. L., Slaga, T. J., & Kumar, A. P. (2005). Eugenol causes melanoma growth suppression through inhibition of E2F1 transcriptional activity. *Journal of Biological Chemistry*, *280*(7), 5812-5819.

Gora, J., & Lis, A. (2005). *The most valuable essential oils.* Torun: Publisher of the Nicolaus Copernicus University.

Grespan, R., Paludo, M., de Paula Lemos, H., Barbosa, C. P., Bersani-Amado, C. A., de Oliveira Dalalio, M. M., & Cuman, R. K. N. (2012). Anti-arthritic effect of eugenol on collagen-induced arthritis experimental model. *Biological and Pharmaceutical Bulletin*, *35*(10), 1818-1820.

Guan, W., Li, S., Yan, R., Tang, S., & Quan, C. (2007). Comparison of essential oils of clove

buds extracted with supercritical carbon dioxide and other three traditional extraction methods. Food Chemistry, *101*(4), 1558-1564.

Gülçin, İ. (2011). Antioxidant activity of eugenol: A structure–activity relationship study. *Journal of Medicinal Food*, *14*(9), 975-985.

Hajhashemi, V., Ghannadi, A., & Jafarabadi, H. (2004). Black cumin seed essential oil, as a potent analgesic and antiinflammatory drug. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *18*(3), 195-199.

Hamini-Kadar, N., Hamdane, F., Boutoutaou, R., Kihal, M., & Henni, J. E. (2014). Antifungal activity of clove (*Syzygium aromaticum* L.) essential oil against phytopathogenic fungi of tomato (*Solanum lycopersicum* L) in Algeria. *Journal of Experimental Biology and Agricultural Sciences*, *2*(5), 447-454.

Hammer, K. A., Carson, C. F., Riley, T. V., & Nielsen, J. B. (2006). A review of the toxicity of *Melaleuca alternifolia* (tea tree) oil. *Food and Chemical Toxicology*, *44*(5), 616-625.

Han, X., & Parker, T. L. (2017). Anti-inflammatory activity of clove (*Eugenia caryophyllata*) essential oil in human dermal fibroblasts. *Pharmaceutical biology*, *55*(1), 1619-1622.

Hosseini, M., Kamkar Asl, M., & Rakhshandeh, H. (2011). Analgesic effect of clove essential oil in mice. *Avicenna Journal of Phytomedicine*, *1*(1), 1-6.

Işcan, G.; Kirimer, N.; Kürkçüoglu, M.; Arabaci, T.; Küpeli, E.; Başer, K.H.C. (2006) Biological activity and composition of the essential oils of *Achillea schischkinii* Sosn. and *Achillea aleppica* D.C. subsp. *aleppica*. *Journal of agricultural and food*

chemistry, *54*(1), 170-173.

Issac, A., Gopakumar, G., Kuttan, R., Maliakel, B., & Krishnakumar, I. M. (2015). Safety and anti-ulcerogenic activity of a novel polyphenol-rich extract of clove buds (*Syzygium aromaticum* L). *Food & function*, *6*(3), 842-852.

Juhas, Š., Bujnakova, D., Rehak, P., Cikos, S., Czikkova, S., Vesela, J., & Koppel, J. (2008). Anti-inflammatory effects of thyme essential oil in mice. *Acta Veterinaria Brno*, *77*(3), 327-334.

Kamatou, G. P., Vermaak, I., & Viljoen, A. M. (2012). Eugenol—from the remote Maluku Islands to the international market place: a review of a remarkable and versatile molecule. *Molecules*, *17*(6), 6953-6981.

Kamatou, G.P.P.; Viljoen, A.M. (2010). A review of the application and pharmacological properties of α-bisabolol and α-bisabolol-rich oils. *Journal of the American Oil Chemists' Society*, *87*(1), 1-7.

Kim, S. Y., Kang, D. H., Kim, J. K., Ha, Y. G., Hwang, J. Y., Kim, T., & Lee, S. H. (2011). Antimicrobial activity of plant extracts against *Salmonella typhimurium*, *Escherichia coli* O157: H7, and *Listeria monocytogenes* on fresh lettuce. *Journal of Food Science*, *76*(1), 41-46.

Kumar, Y., Agarwal, S., Srivastava, A., Kumar, S., Agarwal, G., & Khan, M. Z. A. (2014). Antibacterial activity of Clove (Syzygium aromaticum) and Garlic (Allium sativum) on different pathogenic bacteria. *International Journal of Pure & Applied Bioscience*, *2*(3), 305-311.

Lino, C. S., Gomes, P. B., Lucetti, D. L., Diógenes, J. P. L., Sousa, F. C. F., Silva, M. G. V., & Viana, G. S. B. (2005). Evaluation of antinociceptive and antiinflammatory

activities of the essential oil (EO) of *Ocimum micranthum* Willd. from Northeastern Brazil. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *19*(8), 708-712.

Marchese, A., Barbieri, R., Coppo, E., Orhan, I. E., Daglia, M., Nabavi, S. F., & Ajami, M. (2017). Antimicrobial activity of eugenol and essential oils containing eugenol: A mechanistic viewpoint. *Critical Reviews in Microbiology*, *43*(6), 668-689.

Mendes, S.S.; Bomfim, R.R.; Jesus, H.C.R.; Alves, P.B.; Blank, A.F.; Estevam, C.S.; Antoniolli, A.R.; Thomazzi, S.M. (2010). Evaluation of the analgesic and anti-inflammatory effects of the essential oil of *Lippia gracilis* leaves. *Journal Ethnopharmacology*, *129*, 391-397.

Mishra, R. K., & Singh, S. K. (2008). Safety assessment of Syzygium aromaticum flower bud (clove) extract with respect to testicular function in mice. *Food and Chemical Toxicology*, *46*(10), 3333-3338.

Mitsch, P., Zitterl-Eglseer, K., Kohler, B., Gabler, C., Losa, R., & Zimpernik, I. (2004). The effect of two different blends of essential oil components on the proliferation of Clostridium perfringens in the intestines of broiler chickens. *Poultry Science*, *83*(4), 669-675.

Moon, S. E., Kim, H. Y., & Cha, J. D. (2011). Synergistic effect between clove oil and its major compounds and antibiotics against oral bacteria. *Archives of Oral Biology*, *56*(9), 907-916.

Najafi, P., & Torki, M. (2010). Performance, Blood Metabolites and Immunocompetaence of Broiler. *Journal of Animal and Veterinary Advances*, *9*(7), 1164-1168.

Nakatsu, T., Lupo Jr, A. T., Chinn Jr, J. W., & Kang, R. K. (2000). Biological activity of essential oils and their constituents. *Studies in Natural Products Chemistry, 21*, 571-631.

Nam, H., & Kim, M. M. (2013). Eugenol with antioxidant activity inhibits MMP-9 related to metastasis in human fibrosarcoma cells. *Food and Chemical Toxicology*, *55*, 106-112.

Neveu, V., Perez-Jimenez, J., Vos, F., Crespy, V., Du Chaffaut, L., Mennen, L. & Scalbert, A. (2010). Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. *Database-2010*.

Nikoui, V., Ostadhadi, S., Bakhtiarian, A., Abbasi-Goujani, E., Habibian-Dehkordi, S., Rezaei-Roshan, M. & Giorgi, M. (2017). The anti-inflammatory and antipyretic effects of clove oil in healthy dogs after surgery. *Pharma Nutrition*, *5*(2), 52-57.

Nowak, K., Ogonowski, J., Jaworska, M., & Grzesik, K. (2012). Clove Oil-Properties And Applications. *Chemik*, *66*(2), 145-152.

Nurdjanah, N., & Hardja, S. (1991). Distillation method influences the yield and quality of cloves leaves oil. *Industrial Crops Research Journal (Indonesia)*, *3*(2), 18-26.

Nurdjanah, N., Rusli, S., & Vianna, A. (1990). Effect of weight and time of refining clove stalks on the quality and yield of the oil produced. *Pembri Litri*, *15*(4), 153-157.

Omidbeygi, M., Barzegar, M., Hamidi, Z., & Naghdibadi, H. (2007). Antifungal activity of thyme, summer savory and clove essential oils against Aspergillus flavus in liquid medium and tomato paste. *Food Control*, *18*(12), 1518-1523.

Oyemitan, I. A., Iwalewa, E. O., Akanmu, M. A., & Olugbade, T. A. (2008). Antinociceptive and antiinflammatory effects of essential oil of Dennettia tripetala G. Baker (Annonaceae) in rodents. *African Journal of Traditional, Complementary and*

Alternative Medicines, *5*(4), 355-362.

Öztürk, A., & Özbek, H. (2005). The anti-inflammatory activity of *Eugenia caryophyllata* essential oil: an animal model of anti-inflammatory activity. *European Journal of General Medicine*, *2*(4), 159-163.

Park, M. J., Gwak, K. S., Yang, I., Choi, W. S., Jo, H. J., Chang, J. W., ... & Choi, I. G. (2007). Antifungal activities of the essential oils in *Syzygium aromaticum* (L.) Merr. Et Perry and *Leptospermum petersonii* Bailey and their constituents against various dermatophytes. *The Journal of Microbiology*, *45*(5), 460-465.

Perez-Conesa, D., McLandsborough, L., & Weiss, J. (2006). Inhibition and inactivation of *Listeria monocytogenes* and *Escherichia coli* O157: H7 colony biofilms by micellar-encapsulated eugenol and carvacrol. *Journal of Food Protection*, *69*(12), 2947-2954.

Pourgholami, M. H., Kamalinejad, M., Javadi, M., Majzoob, S., & Sayyah, M. (1999). Evaluation of the anticonvulsant activity of the essential oil of *Eugenia caryophyllata* in male mice. *Journal of Ethnopharmacology*, *64*(2), 167-171.

Rahman, M. M., & Gray, A. I. (2002). Antimicrobial constituents from the stem bark of *Feronia limonia*. *Phytochemistry*, *59*(1), 73-77.

Rana, I. S., Rana, A. S., & Rajak, R. C. (2011). Evaluation of antifungal activity in essential oil of the *Syzygium aromaticum* (L.) by extraction, purification and analysis of its main component eugenol. *Brazilian Journal of Microbiology*, *42*(4), 1269-1277.

Rodrigues, T. G., Fernandes Jr, A., Sousa, J. P. B., Bastos, J. K., & Sforcin, J. M. (2009). In vitro and in vivo effects of clove on pro-inflammatory cytokines production by macrophages. *Natural Product Research*, *23*(4), 319-326.

Rutkowski, A., Gwiazda, S., & Dąbrowski, K. (2003). *Compendium of food additives.*

Hortimex.

Saeed, S., & Tariq, P. (2008). *In vitro* antibacterial activity of clove against Gram negative bacteria. *Pakistan Journal of Botany*, *40*(5), 2157-2160.

Saeed, T. A., Osman, O. A., Amin, A. E., & El Badwi, S. M. (2017). Safety Assessment and Potential Anti-Inflammatory Effect of Ethanolic Extract of *Syzygium aromaticum* in Albino Rats. *Advances in Bioscience and Biotechnology*, *8*(11), 411.

Shahavi, M. H., Hosseini, M., Jahanshahi, M., Meyer, R. L., & Darzi, G. N. (2016). Clove oil nanoemulsion as an effective antibacterial agent: Taguchi optimization method. *Desalination and Water Treatment*, *57*(39), 18379-18390.

Shalaby, S. E., El-Din, M. M., Abo-Donia, S. A., Mettwally, M., & Attia, Z. A. (2011). Toxicological affects of essential oils from *Eucalyptus globules* and Clove *Eugenia caryophyllus* on albino rats. *Polish Journal of Environmental Studies*, *20*(2), 429-434.

Shan, B., Cai, Y. Z., Sun, M., & Corke, H. (2005). Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *Journal of Agricultural and Food Chemistry*, *53*(20), 7749-7759.

Singh, A. K., Dhamanigi, S. S., & Asad, M. (2009). Anti-stress activity of hydro-alcoholic extract of *Eugenia caryophyllus* buds (clove). *Indian Journal of Pharmacology*, *41*(1), 28.

Singh, V., Pahuja, C., Ali, M., & Sultana, S. (2018). Analysis and bioactivities of essential oil of the flower buds of *Syzygium aromaticum* (L.) Merr. et LM Perry. *Journal of Medicinal Plants*, *6*(6), 79-83.

Sofia, P. K., Prasad, R., Vijay, V. K., & Srivastava, A. K. (2007). Evaluation of antibacterial activity of Indian spices against common foodborne pathogens. *International Journal*

of Food Science & Technology, *42*(8), 910-915.

Stevenson, D. E., & Hurst, R. D. (2007). Polyphenolic phytochemicals–just antioxidants or much more. *Cellular and Molecular Life Sciences*, *64*(22), 2900-2916.

Taher, Y. A., Samud, A. M., El-Taher, F. E., ben-Hussin, G., Elmezogi, J. S., Al-Mehdawi, B. F., & Salem, H. A. (2015). Experimental evaluation of anti-inflammatory, antinociceptive and antipyretic activities of clove oil in mice. *Libyan Journal of Medicine*, *10*(1), 285-86.

Tanko, Y., Mohammed, A., Okasha, M. A., Umah, A., & Magaji, R. (2008). Anti-nociceptive and anti-inflammatory activities of ethanol extract of Syzygium aromaticum flower bud in wistar rats and mice. *African Journal of Traditional, Complementary and Alternative Medicines*, *5*(2), 209-212.

Tsai, T. H., Huang, W. C., Lien, T. J., Huang, Y. H., Chang, H., Yu, C. H., & Tsai, P. J. (2017). Clove extract and eugenol suppress inflammatory responses elicited by *Propionibacterium acnes in vitro* and *in vivo*. *Food and Agricultural Immunology*, *28*(5), 916-931.

Tyler, V. E., Brady, L. R., & Robbers, J. E. (1988). *Pharmacognosy Lea & Febriger (9th ed.)*, Philadelpia *USA*, pp 103-155.

Vanin, A. B., Orlando, T., Piazza, S. P., Puton, B. M., Cansian, R. L., Oliveira, D., & Paroul, N. (2014). Antimicrobial and antioxidant activities of clove essential oil and eugenyl acetate produced by enzymatic esterification. *Applied biochemistry and biotechnology*, *174*(4), 1286-1298.

Vijayasteltar, L., Nair, G. G., Maliakel, B., Kuttan, R., & Krishnakumar, I. M. (2016). Safety assessment of a standardized polyphenolic extract of clove buds: Subchronic toxicity

and mutagenicity studies. *Toxicology Reports, 3*, 439-449.

Wayne, P. A. (2002). National committee for clinical laboratory standards. *Performance standards for antimicrobial disc susceptibility testing*, *12*, 01-53.

Winter, C. A., Risley, E. A., & Nuss, G. W. (1962). Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. *Proceedings of the Society for Experimental Biology and Medicine*, *111*(3), 544-547.

Wybenga, D. R., Di Giorgio, J., & Pileggi, V. J. (1971). Manual and automated methods for urea nitrogen measurement in whole serum. *Clinical Chemistry*, *17*(9), 891-895.

Xu, J. G., Liu, T., Hu, Q. P., & Cao, X. M. (2016). Chemical composition, antibacterial properties and mechanism of action of essential oil from clove buds against *Staphylococcus aureus*. *Molecules*, *21*(9), 1194.

Yadav, M. K., Chae, S. W., Im, G. J., Chung, J. W., & Song, J. J. (2015). Eugenol: a phyto-compound effective against methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* clinical strain biofilms. *Plos One*, *10*(3), 1-21.

Zhu, X., Zhang, H., & Lo, R. (2004). Phenolic compounds from the leaf extract of artichoke (*Cynara scolymus* L.) and their antimicrobial activities. *Journal of Agricultural and Food Chemistry*, *52*(24), 7272-7278.

APPENDICES

Appendix – I: One-way-analysis of variance (ANOVA) of clove oil against *S. typhimurium, P. aeruginosa, E. coli, S. aureus, L. monocytogenes* and *S. agalactiae* using antibacterial sensitivity test

		d.f.	Mean Square	F	Significance
Salmonella typhimurium	Between Groups	8	226.250	28.951	0.000*
	Within Groups	18	7.815		
	Total	26			
Pseudomonas *aeruginosa*	Between Groups	8	324.833	27.069	0.000*
	Within Groups	18	12.000		
	Total	26			
Escherichia coli	Between Groups	8	104.750	12.856	0.000*
	Within Groups	18	8.148		
	Total	26			
Staphylococcus aureus	Between Groups	8	127.231	17.799	0.000*
	Within Groups	18	7.148		
	Total	26			
Listeria monocytogenes	Between Groups	8	155.676	7.901	0.000*
	Within Groups	18	19.704		
	Total	26			
Streptococcus agalactiae	Between Groups	8	254.926	35.849	0.000*
	Within Groups	18	7.111		
	Total	26			

NS: Non Significant, *- Significant ($p<0.05$).

Appendix – II: One-way-analysis of variance (ANOVA) of clove oil on carrageenan-induced rat paw edema (ml) in male wistar rats

		d.f.	Mean Square	F	Significance
0h	Between Groups	4	0.000	0.265	0.897^{NS}
	Within Groups	20	0.002		
	Total	24			
1h	Between Groups	4	0.013	12.041	0.000*
	Within Groups	20	0.001		
	Total	24			
2h	Between Groups	4	0.049	25.870	0.000*
	Within Groups	20	0.002		
	Total	24			
3h	Between Groups	4	0.423	124.665	0.000*
	Within Groups	20	0.003		
	Total	24			
4h	Between Groups	4	0.283	260.116	0.000*
	Within Groups	20	0.001		
	Total	24			
6h	Between Groups	4	0.117	40.707	0.000*
	Within Groups	20	0.003		
	Total	24			
24h	Between Groups	4	0.010	2.466	0.078*
	Within Groups	20	0.004		
	Total	24			

NS: Non Significant, *- Significant ($p<0.05$).

Appendix – III: One-way-analysis of variance (ANOVA) of clove oil on carrageenan-induced rat paw edema (ml) in female wistar rats

		d.f.	Mean Square	F	Significance
0h	Between Groups	4	0.000	0.218	0.925^{NS}
	Within Groups	20	0.001		
	Total	24			
1h	Between Groups	4	0.008	5.372	0.004*
	Within Groups	20	0.002		
	Total	24			
2h	Between Groups	4	0.024	4.805	0.007*
	Within Groups	20	0.005		
	Total	24			
3h	Between Groups	4	0.293	51.994	0.000*
	Within Groups	20	0.006		
	Total	24			
4h	Between Groups	4	0.180	38.064	0.000*
	Within Groups	20	0.005		
	Total	24			
6h	Between Groups	4	0.073	26.457	0.000*
	Within Groups	20	0.003		
	Total	24			
24h	Between Groups	4	0.006	2.064	0.124*
	Within Groups	20	0.003		
	Total	24			

NS: Non Significant, *- Significant ($p<0.05$).

Appendix – IV: One-way-analysis of variance (ANOVA) of clove oil on body weight of male (I-IV) and female (V-VIII) rats

		d.f.	Mean Square	F	Significance
0 Day	Between Groups	7	19148.796	66.022	0.000^{NS}
	Within Groups	32	290.037		
	Total	39			
7 Day	Between Groups	7	21785.625	44.347	0.000^{NS}
	Within Groups	32	491.250		
	Total	39			
14 Day	Between Groups	7	25183.929	41.951	0.000^{NS}
	Within Groups	32	600.313		
	Total	39			
21 Day	Between Groups	7	27713.929	57.105	0.000^{NS}
	Within Groups	32	485.312		
	Total	39			
28 Day	Between Groups	7	28876.339	82.800	0.000^{NS}
	Within Groups	32	348.750		
	Total	39			

NS: Non Significant, *- Significant ($p<0.05$).

Appendix – V: One-way-analysis of variance (ANOVA) of clove oil on feed consumption of male (I-IV) and female (V-VIII) rats

		d.f.	Mean Square	F	Significance
Week 1	Between Groups	7	1.211	0.427	0.861^{NS}
	Within Groups	8	2.838		
	Total	15			
Week 2	Between Groups	7	3.560	1.414	0.318^{NS}
	Within Groups	8	2.518		
	Total	15			
Week 3	Between Groups	7	1.801	0.945	0.523^{NS}
	Within Groups	8	1.905		
	Total	15			
Week 4	Between Groups	7	3.506	2.157	0.152^{NS}
	Within Groups	8	1.626		
	Total	15			

NS: Non Significant, *- Significant ($p<0.05$).

Appendix –VI: One-way-analysis of variance (ANOVA) of clove oil on hematological parameters of male (I-IV) and female (V-VIII) rats

		d.f.	Mean Square	F	Significance
Hb	Between Groups	7	2.039	1.680	0.149^{NS}
	Within Groups	32	1.214		
	Total	39			
RBCs	Between Groups	7	2.290	1.405	0.237^{NS}
	Within Groups	32	1.629		
	Total	39			
PCV	Between Groups	7	38.195	1.715	0.141^{NS}
	Within Groups	32	22.267		
	Total	39			
TLCs	Between Groups	7	0.264	0.603	0.749^{NS}
	Within Groups	32	0.438		
	Total	39			
MCV	Between Groups	7	10.744	0.215	0.979^{NS}
	Within Groups	32	49.870		
	Total	39			
MCH	Between Groups	7	2.260	0.725	0.652^{NS}
	Within Groups	32	3.116		
	Total	39			
MCHC	Between Groups	7	8.218	0.567	0.777^{NS}
	Within Groups	32	14.484		
	Total	39			

NS: Non Significant, *- Significant ($p<0.05$).

Appendix –VII: One-way-analysis of variance (ANOVA) of clove oil on serum biochemical parameters of male (I-IV) and female (V-VIII) rats

		df	Mean Square	F	Sig.
ALT	Between Groups	7	197.821	1.025	0.433^{NS}
	Within Groups	32	193.027		
	Total	39			
AST	Between Groups	7	102.510	0.553	0.788^{NS}
	Within Groups	32	185.459		
	Total	39			
TC	Between Groups	7	268.090	1.206	0.328^{NS}
	Within Groups	32	222.221		
	Total	39			
TP	Between Groups	7	0.371	0.912	0.510^{NS}
	Within Groups	32	0.407		
	Total	39			
Albumin	Between Groups	7	0.134	1.066	0.407^{NS}
	Within Groups	32	0.126		
	Total	39			
BUN	Between Groups	7	6.877	1.232	0.315^{NS}
	Within Groups	32	5.583		
	Total	39			
Creatinine	Between Groups	7	0.021	1.155	0.355^{NS}
	Within Groups	32	0.019		
	Total	39			
Bilirubin	Between Groups	7	0.049	1.192	0.336^{NS}
	Within Groups	32	0.041		
	Total	39			

NS: Non Significant, *- Significant ($p<0.05$).