A Scientific approach on the Validation of Santha Santhrothaya Mathirai (SSM) a Siddha Herbo-Mineral Preparation for its Safety and Efficacy in the Management of Hepatic disorders

Thesis submitted to

The Tamil Nadu Dr.M.G.R. Medical University

In partial fulfillment for the award of the degree of

Doctor of Philosophy

Faculty of Siddha Medicine

Under the supervision of

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JULY 2016

DECLARATION

I declare that the thesis entitled **"A Scientific approach on the Validation of Santha Santhrothaya Mathirai (SSM) a Siddha Herbo-Mineral Preparation for its Safety and Efficacy in the Management of Hepatic disorders"** submitted by me for the Degree of Doctor of Philosophy is the record of research work carried out by me during the period of July 2011 to June 2016 under the guidance and supervision of **Prof.Dr.M. Murugesan M.D(S)** Former Dean, National Institute of Siddha, Chennai 600 047, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or institution of higher learning.

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CERTIFICATE

I certify that the thesis entitled **" A Scientific approach on the Validation of Santha Santhrothaya Mathirai (SSM) a Siddha Herbo-Mineral Preparation for its Safety and Efficacy in the Management of Hepatic disorders"** submitted for the Degree of Doctor of Philosophy by **Dr. P. Shanmugapriya** is the record of research work carried out by her during the period of July 2011 to June 2016 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or Institution of higher learning.

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(**Prof.Dr.M. Murugesan**)
Guide and Supervisor

ACKNOWLEDGEMENT

I have a great delight in carrying out this research work in my carrier, completing my thesis and presenting in this manner. Throughout the work there have been a lot of guidance, helps and well-wishing hearts who in one way or the other contributed in the completion of my thesis. I hereby wish to owe my gratitude to all of them.

I wish to extend my sincere thanks to The Secretary, Department of Ayush for granting me the permission to pursue the Ph.D degree as a part-time Scholar at Department of Nanju Noolum Maruthuva Neethi noolum, National Institute of Siddha, Tambaram sanatorium, Chennai.

I warmly express my gratitude to The Vice Chancellor, The Tamil Nadu Dr.M.G.R University Guindy for enabling me to proceed this research work.

I express my sincere thanks to the former Director, National Institute of Siddha, Prof. Dr. K. Manickavasagam, M.D(s) . He made this endeavor very easy by his constant motivation and support in facilitating this study.

Words are scarce to express my deep sense of acknowledgement for my respected Guide Prof. Dr. M. Murugesan M.D(s) former Dean, National Institute of Siddha, Tambaram sanatorium, Chennai 47 for suggesting me this precise research topic and for his extensive discussion, dedicated and systemic way of approach, expertise comments and perpetual encouragement. His positive attitude and scholarly concern had been a driving force for the completion of this work.

I wish to convey my sincere and special feeling of gratitude towards Prof. Dr.V. Banumathy M.D(s), Director, National Institute of Siddha, Tambaram sanatorium, Chennai for her kind approach, facilities and support which were vital for the smooth functioning of the project.

I would like to express my sincere thanks to Prof. Dr.V. Mohan M.D(s) former Director i/c,National Institute of Siddha, Tambaram sanatorium, Chennai 47

and Mr. S.M. Murugan , Deputy director (Admin) for their official support for this study.

I extend my sincere thanks to the Doctoral committee members Prof. Dr.Rajavelu Indira Government Ophthalmic hospital, Egmore, Chennai and Prof. Dr.V Suba ,Assistant professor, National Institute of Siddha, Tambaram Sanatorium for their valuable suggestions and kind help in illuminating the sparks of my research so that the idea took the present shape and was completed succesfully.

I extend my sincere thanks to Dr.D. Aravind M.D (s), Asst. Professor, National Institute of Siddha, Tambaram Sanatorium , Prof. Jayaraman, Plant Anatomy Research Centre, West Tambaram, Chennai.for his kind help in herbal drug authentication. I also express my heartfelt thanks to Dr.M.Suresh Gandhi, Asst.Professor, Dept of Geology, University of Madras, Chennai for their valuable support in identification and authentication of mineral samples.

I express my heartfelt gratitude to Dr.L.Sivakumar, General Manager, SKM Pharmaceuticals and their staffs for their kind support in medicine preparation.

My special thanks go to Dr. Rani M.V.Sc, Veterinary pathologist, Animal House, National Institute of Siddha, Tambaram sanatorium for her valuable support and guidance in the successful completion of the animal studies.

I convey my sincere feeling of gratitude towards Dr .R.Murugesan, Senior scientific officer, Grade-1, Indian institute of technology (IIT), Chennai for enabling me to pursue the Sohpisticated instrument analytical part of my study. I express my thanks to Reasearch officer Dr.Ganesan and Dr.Pramodreddy SCRI for their help in animal studies.

I thankfully acknowledge Mr.D. Ramasamy Research Assistant, Regional research Institute of Unani, Royapuram, Chennai, for carrying out the Chromatographic studies.

My sincere thanks to Dr.P.Brindha, Associate Dean, Department of Research and Mrs.K.L.Niraimathi, Assistant professor, Dept of Pharmacy, CARISM, Sastra University, Tanjore for their valuable help in Instrumental Analysis.

I extend my sincere thanks to the Dr.A.Rajasekaran, M.Pharm., Ph.D. ,Director, KMCH College of Pharmacy, Coimbatore and to Mr.Arihara sivakumar, Asst.Professor, Dept of Pharmacology , KMCH for their kind assistance in carrying out the toxicity studies of my research.

I have an immense pleasure to express my deep sense of gratitude towards Dr. D.Sivaraman, Sathyabhama university for enabling me to carry out my pharmacological part of this study and for his constant inspiration and encouraging words that enabled me to improve my research skills. Dr.Mohana Veterinary pathologist for her excellent effort in histopathological studies.

I am sincerely grateful for the moral support of Dr.T.Anandan, Former Director, Siddha cental research Institute, Arumbakkam, Chennai for his invaluable guidance and inspiration that was unlimited to my academic and professional career.

Very special thanks to Dr.Sudhir CARISM, Sastra University, for his very best contribution in interpreting my instrumental analysis study results with his diversified knowledge which enabled me to proceed my research with interest and passion.

It gives me an immense pleasure to acknowledge, Dr.Anoop Austin, Head R&D, Cholaiyil private limited, Chennai for his constant emphasis in enabling me to learn new things and inspired me by his intellect vision, research skills and critical assessment.

I thoughtfully acknowledge Dr.K.Rajalakshmi Ph.D, Associate professor, The TamilNadu Dr.M.G.R Medical university who motivated me right from the beginning of my study with a friendly concern whose thoughtful provocation and criticism for the superficial kindled me to strive for excellence.

I express my thanks to Dr.Kabilan M.D (s) HOD and to all of the Staffs of the Dept of Siddha, The TamilNadu Dr.M.G.R Medical university for their kind guidance and support throughout the study.

My profound heartfelt gratitude towards Dr.K.Thangaraj Prof & HOD, and Dr.Sanjeev, Asst.Professor, Forensic medicine and toxicology, Sri Ramasamy memorial university, Kattankulathur, Kanchipuram for being ever so kind to show their interest in my research work and to give their precious suggestions regarding my study.

I extend my warm thanks to Dr.M.Ramamurthy, Dr.G.J.Christian , Dr.S.Elansekaran Department of Noi Nadal, National Institute of Siddha, Tambaram Sanatorium and Dr. Thanigaivelan, Sai Ram Siddha medical college, Chennai for being around me, helping and supporting me all through my research work and for making my thesis work memorable with their critical comments and for being such wonderful brothers.

I extend my thanks to Dr M.Nijavizhi and Dr.Rangarajan for their help in performing the animal studies.

I thankfully acknowledge Dr.R.Rathinamala and Dr. Sudha revathy for rendering their assistance whenever required.

I also extend my thanks to Dr.R.Madhavan, Dr.S.Murugesan , Dr.V.Manjari, Dr.Rangasundari and all other staffs and PG Scholars, Department of Nanju Noolum Maruthuva Neethi noolum, National Institute of Siddha, Tambaram sanatorium, Chennai.

My appreciation also extends to Dr.Shameem, Lecturer, Dept. of physiology, Government Siddha medical college, Arumbakkam Chennai and Dr.R.Jeeva Gladys, Research Fellow, The Tamil Nadu Dr.M.G.R Medical university for their support in shaping up the presentation of my research with their very best and for the timely and kind assistance.

I express my gratitude with Joyfilled tears to my beloved Parents Late Mr.A. Ponnappan and Mrs.K.Amaravathy for being extremely affectionate and supportive, without whom this would’nt have been possible. I also my heartfelt thanks to Mr.K.Gunasekaran and Mrs.G.Jothi mani who encouraged me a lot in my carrier.

I find no words to express my thanks to my beloved husband Dr.S.Thamodharan Government Siddha medical college, Arumbakkam Chennai and my loving Son T.Lalithaditya who both stood by me and extended their co-operation all through my research and who continue to support me in fulfilling my dreams. I also thank my Brothers A.P.Mageshkumar, G.Vignesh, V.Arun and A.Vasanth for their warm regards and helps in the successful completion of this project.

I extend my thanks to all others who have lended their helping hands whose name is not here but they had been for me whenever I needed.

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ABBREVIATIONS

AAS	-	Atomic Absorption Spectroscopy
ALP	-	Alkaline Phosphate
ALT	-	Alanine Transaminase
AOAC	-	Association of Analytical Communities
AST	-	Aspartate Aminotransferase
b.wt.	-	Body weight
BUN	-	Blood Urea Nitrogen
CAT	-	Catalase
CPCSEA	-	Committee for the Purpose of Control and Supervision of Experiments on Animals
FTIR	-	Fourier Transform Infrared Spectroscopy
GLP	-	Good Laboratory practice
gm	-	Gram
GMP	-	Good Manufacturing Practice
GPR	-	Glutamyl Phosphate reductase
GPX	-	Glutathione peroxidase
HDL	-	High Density Lipoprotein
HPTLC	-	High Performance Thin Layer Chromatography
i.p.	-	Intra Peritoneal
ICP-OES	-	Inductively coupled Plasma Optical Emission Spectroscopy
kg	-	Kilo gram
LDL	-	Low Density Lipoprotein
LPS	-	Lipo Poly Saccharide
MDA	-	Plasma Malonaldehyde
mg	-	Milligram

ml	-	Millilitre
p.o	-	per Oral
SEM	-	Scanning Electron Microscope
SOD	-	Superoxide dismutase
SSM	-	Santha Santhrothaya Mathirai
TGA	-	Thermo Gravimetric Analysis
TGL	-	Triglycerides
VLDL	-	Very Low Density Lipoprotein
WHO	-	World Health Organization
XRD	-	X-ray Diffraction
XRF	-	X-ray Fluorescence

1. INTRODUCTION

The Siddha system of medicine is one among the oldest medical system, indigenous to India. It has greater antiquity and its history can be traced to the remote past. It was nurtured by ancient sages of South India known as Siddhars. This unique system of medicine describes the health of an individual as an ideal perfect state of his or her physical, physiological, social, and spiritual components. According to the Siddha science, the five elemental theories (earth, water, fire, air and space) are the sole constitution of the anatomy and physiology of humans, materials used for the treatment and cure of diseases and also the food which sustains the body[1].

Siddha system also emphasizes the presence of these five elements in the three bio regulating principles Vatham (Space+Air), Pitham (fire) and Kapham (water+earth) that are responsible for the well being of an individual. In each and every cell of human body these three humoursco-exist and function harmoniously.Vatham controls the nervous actions such as movement, sensation etc.,Pitham controls thermo-genesis and metabolic actions of body such as digestion, assimilation, secretion, excretion etc., and Kapham is responsible for the stability. Thus the five elemental and three humoral theory of Siddha system of Medicine forms the basis for disease classification, drug formulation, drug selection and treatment. The derangement of these three humours results in “disease” and they are restored by substituting the drug of same constitution or of opposite nature depending on the humoural imbalance[2].

According to Siddha pathology, liver disorders are caused due to deranged pithahumor (P*ithadosham* - aggravated/stagnated) and may result in symptoms such as giddiness, vomiting, headache, poor digestion and pigmentation problems. These symptoms are best correlated with those of bilious disorders[3].Liver is a major metabolic organ and plays a pivotal role in metabolism of food, provision of nutrients to the blood and excretion of toxins. Liver diseases are caused by toxic chemicals, drugs, viruses (hepatitis A, B, C, D, E), excess alcohol intake etc., and are ranked among the top ten killer diseases in India[4,5]. According to the WHO data

published in April 2011, death due to liver diseases in India has reached to 2.31%. This disease has reached an alarming state of 216,865 of total deaths in the year 2014[6]. With only meagre number ofdependable drugs available for hepatoprotective action, the present scenario has created a stage for scientific evaluation of traditional medicines in the treatment of liver disorders.

The therapeutics of Siddha system consists of herbal, metallic, mineraland animal origin. In the Siddha literature

"NthghUj ioghUk;Qr;dffhy
nkyynkyygwgnre Jhuk ghNu"

The above text describes that when there is a failure in disease response to drugs of herbal origin, metallic and mineral preparations can conquer the illness[6].There are numerous single herbs like Keezhanelli (Phyllanthus niruri), Avuri (Indigofera tinctoria), Nellikai (Phyllanthus amarus), Kadukkai (Terminalia chebula) and also several poly-herbal formulations such as Karisalai chooranam, Elathy chooranam, Seeraga chooram etc that are being used for Liver disorders.

"#jfej;jhJgwgk nrhddehl lhhr;f;rir
Xjh;a %y;a;kkz Zhhr;f;rir–NtjlUk
rjj;u \huhff;d;e;rhrurr;f;rirnadNw
Kjjujjjh Fk nkho;"

The above text highlights the supremacy of metallic and mineral preparations[7].The use of herbo-mineral formulations is one of the distinctive features of Siddha system of medicine from time immemorial and the use of mercurial compounds as catalytic agents occupies a very high place in Siddha therapeutics. Siddhars used five forms of mercury viz., 1.Rasam (Mercury metal) 2.Lingam (Red sulphide of mercury) 3.Veeram (Mercuric chloride) 4.Pooram (Mercurous chloride) 5. Rasa chendooram (Red oxide of mercury)[8].

In recent times, mercury based drugs are cautioned due to its toxic effects and studies by Saper et al., concluded that metallic preparations are toxic by determining the presence of heavy metals using XRF analysis[9]. But these studies have not considered about the physico chemical structural changes of these metals

that has occurred before and after purification process and none of the researches had included toxicological or clinical studies as an evidence.In controversy, there are also studies that declare that the herbo-mineral drugs are comparatively safe when they are properly prepared as per the standard manufacturing process mentioned in the literature.During the purification and preparatory process, intense physico chemical changes are inflicted into the herbo-mineral drugs by using various herbs and diverse components and are made therapeutically effective[10].

The advantages of herbo-mineral preparation over herbal drugs are the virtue of stability over longer duration, lower dosage requirement, easy storability and sustained availability. The metals and minerals which are considered to have less bioavailability are converted to forms that are relatively more bio-compatible by processing them with herbs during the purification and preparatory procedures. Previous study on a mercurial compound called "Kajjalibhasma" by Sathya et al[11] also supports the fact that there was no genotoxicity in terms of mononuclei induction or DNA damage and emphasise the safety of mercurial compounds for human usage.

The available Siddha literature on liver diseases has provided a lot of herbal and herbo- mineral formulations as indications. Several studies have been done on hepatoprotective herbs in recent years but researches on herbo-mineral formulations are very minimal. Therefore this study focuses on *Santha Santhrodhaya Mathirai (SSM)* a classical herbo-mineral formulation mentioned in "Siddha VaidhyaThirattu" and "Kannusamy parambarai vaithiyam" which has been indicated for the treatment of *Pithasuram* and other diseases associated with *pitham* (biliary disease)[12,13].

SSM is in wide clinical usage among Siddha practitioners to treat bilious diseases and jaundice in particular.The ingredients of *SSM* are *Pooram* (Mercurous chloride), *Vengaram* (borax), *Kappu Manjal* (*Curcuma longa*) and *Elumicham pazha saaru* (*Citrus limon*). All these ingredients are said to enhance liver functions[8, 14]. Pooram (mercurous chloride) is said to be toxic in many ways in its raw form. But according to Siddha literature, the preparation of SSM involves the use of organically purified mercurous chloride which is further potentiated

with hepatotonics such as turmeric and lemon juice. Thus it is made perfectly suitable for liver disorders.

According to scientific literature, mercurous compounds are less soluble, less corrosive and less toxic than mercuric salts. Also in-vivo mercury is bound to metallothionein which serves as a protection, as renal damage is caused only by unbound metal[15]. The purification process of mercurial compounds and the processing of drug completely transform the chemical structure. However there is not much scientific data available for SSM regarding its safety and efficacy profile. The reverberating situation of mercurial toxicity and the indications of SSM given in Siddha literature for liver disorders along with its present day clinical usage has urged the investigator to undertake research in this area and to adopt suitable methods for standardization of SSM. It also motivated me to analyze this herbo-mineral formulation both qualitatively and quantitatively using various sophisticated instrumentation analysis.Though the results revealed the presence of mercurial compound, its chemical structure differed from its raw state to purified state and the prepared formulation SSM possessed an organo metallic complex structure which confirms that the structural characterization of the raw material is certainly lost due to the treatment with herbs during purification and preparatory processes.

This drug was extensively studied as per the drug development statergy to ensure quality, efficacy and safety. First the process standardization was carried out to ensure its reproducibility and the other procedures like botanical evaluation chemical evaluation and biological evaluation were carried out.

Under biological evaluation, toxicological analysis was carried out in experimental rats to analyze whether the organo metallic complex structure of the prepared formulation SSM would cause acute, sub-acuteand sub-chronic toxicity. The study results confirmed that SSM is a bio-compatible and safe drug. And the pharmacological studies on hepatoprotective action of SSM re-enforces the indications that have been mentioned in ancient Siddha literature that the drug is therapeutically effectiveand has hepatoprotective action.

2. AIM AND OBJECTIVES

The present research work entitled **"A scientific approach on the validation of Santha Santhrothaya Mathirai (SSM) a Siddha Herbo-mineral preparation for its safety and efficacy in the management of hepatic disorders"**was carried out to verify and validate scientifically for its safety and efficacy of the Siddha herbo-mineral drug, Santha Santhrothaya Mathirai (SSM) in the management of hepatic disorders.

The main objectives that are involved in this study are as follows:

- Collection and authentication of ingredients of SSM.
- To evaluate the scientific rationale behind the purification of SSM.
- To prepare the study drug as per the standard operating procedure (SOP).
- To analyze the physico chemical properties of SSM.
- To generate the safety data by performing the toxicity studies (Acute, subacute, subchronic).
- To evaluate the efficacy and hepato-protective activity in various animal models.

3. REVIEW OF LITERATURE

An in-depth critical analysis of literature has been done, using various ancient Siddha literatures on Santha Santhrothaya Mathirai and Kalleeral noi. Modern literature search has been carried out on liver disorders using modern textbooks and various scientific journals through online sources such as Pub med, Scopus, Science direct, Google scholar etc., in order to establish the credibility of the study.

Siddha –Basic principles

The Siddha system of medicine has its origin in the southeastern part of India, through the ideas and practices of the Tamil sages who were "holy immortals" called as the "Siddhars". The Siddha term originates from "Chit" which means "Consciousness that illumines". It is the first and foremost medical system to describe the health of a human being as a state of an ideal physical, social and spiritual perfection and continues to serve humanity, maintaining physical, psychological and spiritual well being of humans[1, 2].

Sage Thirumoolar Describes Health as follows,

"That which cures physical ailments is medicine,
That which cures psychological ailments is medicine;
That which prevents ailments is medicine;
That which bestows immortality is medicine."

The Siddhars possessed a huge, unique and incomparable knowledge which covers and considers several diseases and ailments generated from varying climatic conditions and changes. This traditional system of medicine has a distinctive store of enormous pharmacopoeia containing herbal, animal and mineral sources.

The Philosophy that lies behind this "Science of life" is

"m z l j j‰Y s sNj g‰z l k
g‰z l j j‰Y s sNj m z l k
m z l Kk g‰z l K nkhdNw
mw‰eJ jhd ghhfFk NghNj."

which means, “What takes place in the macrocosmos holds well in the microcosmos”

According to Siddha system of medicine, ‘Thathuvam’ is considered as a science that deals with basic functions of the human body. Siddhars described 96 principles as the basic constituents of human body for the process of un-manifest energy to come into subtle and a then distinctive gross form which happens on further evolution of thathuvams. The five element theory, humoural theory and six taste theory are the components of the 96 Thathuvam described as below.

The five elemental theory[1]

According to Siddha system, the process of creation and dissolution is individualized as the microcosmos or humanbeings. The creation of microcosmos (Humans) is same as that of macrocosmos (Universe), using the five basic elements namely Aagayam (Space), Kaal (Air), Thee (Fire), Neer (Water), and Mann (Earth). These elements are the building blocks of all the physical and subtle bodies existing in this whole universe. These are called as the ‘Adippadai Boothams’ (Basic Elements) or ‘Panchaboothams’. None of these elements could act independently by themselves. They could act only in co-ordination with the other four elements. This process of evolution of five Mahaboothams from one another in a successive and progressive manner is called as Pancheekaranam (Mutual Intra Inclusion).

e‰yk eh jts‰ t‰Rk Nghileh Jk
fyej kaff Kyfkhjy‰d

“ghuggh G+jikeJ kz eh NjA
gh‰thd thfhaikej‰dhNy
NrugghrlkhrR”.

“jyqfhlb ,ejr rlkhd itkG+jk
e‰yqfhlb ehfhlb e‰dw‰Le j fhlb
tyqfhlb thAthy tsheNj ,Uej
Fyqfhlb thd‰w Fbah a‰UejNj”.

As per the above lines, the universe and the human body are made of five basic elements.

Space

Space has a principle of vacuity or a vast emptiness. It has a special property of sound beyond the array of the other four senses**.**

Air

Air has the principle of motion. It functions as pressure or impact. Air inherits from space so it can be felt and heard. But it is still beyond form, flavor and odour and so it cannot be seen, tasted or smelt.

Fire

Fire has the principle of luminosity. It has the special property of form and carries the general property of touch and sound and is inherited from the above two elements. Fire can be seen, felt and heard but it is beyond flavor or odour.

Water

Water is the principle of liquidity. Its own special property is flavor. It also has the general property of form, touch and sound from its ascendant elements. But it is beyond odour as it cannot be smelt.

Earth

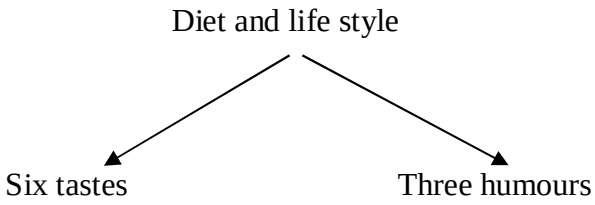
Its function is solidity and special property is odour.It is the only element that has all the five senses of flavor, form, sound, touch and odour.

Taste theory[3]

Man is capable of knowing things only through his five senses ears, skin, eyes, tongue and nose. Therefore it is clear that he has to cognize all the three kinds of objects i.e., minerals, vegetables and animals only through his five senses which are also a combination of five basic elements. Though all the senses can be used to identify these objects, our ancestors have used their sense of “taste” mostly to describe the nature of materials of nature and to analyze the combination of five basic elements in each and every object.

Every taste is the outcome of the mixture of two basic elements. The flavor, colour combination and disparities of touches are the result of varied constitution of five basic elements with different composition.

Relationship between five elements- six taste and three humours



Five Elements	Six tastes	Combination of humours	Three humours	Combination
Space	Sweet	Earth + water	Vatham	Space + Air
Air	Sour	Earth + fire	Pitham	Fire
Fire	Salt	Water + fire	Kapham	Water + Earth
Water	Bitter	Air + Space		
Earth	Pungent	Fire + Air		
	Astringent	Earth+ Air		

Humoural theory

The combination of five elements in different proportion lays the basis for the three life constituents or humours namely the vatham, pitham and kapham which are formed at the embryonic stage of a human being. Hence each individual is born with an inherent constitution and has a predominant influence of any one of the three humours. These three bio-regulating forces are in constant motion and in an ever-shifting dynamic balance with one another for the complete life span of a human being. These humours are neither noticeable nor figurative but can be realized.

Vatham - Movement and propulsion
Pitham - Heat and conversion
Kapham - Form and stability

The harmonious balance of these three humours affects the mental and emotional states of an individual positively and constructively. Imbalance of these humours manifests as negative characteristics, traits and attitudes resulting in diseases.

Imbalances in humours occur in the universe due to natural or unnatural causes which in turn will create changes in human systems. For example the natural disorders like cyclone, heavy rain, mist and scorching sun, diet, lifestyle or impurities of air and water will create changes both in the atmosphere and in the human body. Hence the change in the elementary conditions of external world has its corresponding change in the human organs.

Disease

According to the aggravation or reduction of tastes in the foodstuffs the three humours namely the Vatham, Pitham and Kapham will be affected as per the combination ratio of the five elements. This vitiation of three humours will manifest specific symptoms which will be reflected in the Seven Thathus of the body which is called as Disease.

Seven Dhathus

1. Saaram (Chyle- essence of food)

It gives spirit to the body and mind.

2. Senneer (Blood)

It restores intelligence, skill, complexion, arrogance and voice.

3. Oon (Muscle)

It helps in the bone growth and establishes the part of body according to their functions.

4. Kozhuppu (Fat)

During the functions of each organ of the body it helps for the easy movement of the body.

5. Moolai (Marrow)

It gives strength and softness to the bones by filling the inside of bones.

6. Venneer (Semen)

It stands as the base for reproduction

If the above seven Dhathus increase or decrease from their optimum level their natural functions will be affected.

Siddha diagnostic methods

"k|fDq Fiwa|Dk Nehanra Ak E}Nyhu
ts|Kjyh vz z|a %dW".

According to these lines, the disease diagnosis is made by the physician by seeking the actual state of the three dhoshas. He should also seek for the cause of derangement of dhoshas. Then he should go for setting right the dhoshas with suitable medicines.

The methods of Siddha diagnosis is basically divided into three as follows:

1. Examination through sense organs.
2. Examination through the senses.
3. Examination by interrogation.

The eight fold examination

This is a unique diagnostic tool in Siddha to identify the disease and their causes. This diagnosis is made as follows:

"ehbggh|rk ehe|wk nkho|t|o|
kyk %jj|uk|it kUjJtuhAjk"

1. Reading of pulse.
2. Sensation of patient through touch.
3. Examination of tongue.
4. Examination of colour and complexion.
5. Examination of speech and voice.
6. Examination of eyes.
7. Examination of faeces.
8. Examination of Urine.

Manikadai nool

This is a special method of Siddha diagnosis in which the wrist circumference of a patient is measured by tying a twine above four finger breadth from the wrist. The length of the wrist circumference is correlated with the disease and its symptoms of the patient.

Line of treatment in Siddha

According to Siddha system three different kinds of treatments are given to the patients

1. Divine treatment

This is the superior method of treating diseases with compounds of mercury, sulphur, metal and mineral salts by preparing incredible medicines like kattu, kalangu etc. based on the principles laid down in classical Tamil literature.

2. Rational treatment

Treatment with herbal decoction, powder, pills etc. is called as rational treatment.

3. Surgical treatment

Varieties of surgical procedures are being followed in Siddha system of medicine such as therapy of chuttigai, kaaranool therapy and leech application.

Methods of neutralizing the deranged humours

"Mj‌Kg g‌z‌fs ju mikjj‌lj njho‌yfs ehdf‌y
Ngj‌a‌y jhOk thjk g‌jjkrj j‌ffj jhOk
Xj‌a er‌aj jhNy caufgk jU k‌ff
Xj‌aQ rdjjhy fz z‌y e‌fu‌yh xs‌Azlhfk"

According to these lines Vatham can be neutralized by purgation, Pitham by emetics and Kapham by nasal drops.[16]

Selection of Siddha medicine

The Siddha formulations were based upon Panchabootha theory (five basic elements) and six basic tastes (Arusuvaigal) and oppurai/ethirurai (synergism/antagonism). Roots and leaves of plants are used for minor ailments where as metals and minerals are considered to be higher forms of medicine that can be used when the disease progression is unable to be managed by herbal drugs.

Food as medicine

"c z Nt kUeJ kUeNj c z T"

For good health of the body and mind one should follow the core principle of diet formulated by Siddhars. The Siddha system of medicine refines the process of eating food to make a pleasant thegi (prakrithi). Hence they classified the combinations in food, herbs and metals as Chathru (unfriendly) and Mithru (friendly). As different classes of food require different digestive enzymes, an unfriendly food combination if taken produces unwanted symptoms and diseases. Therefore each thridosham can be neutralized by food, if taken in a particular order and way[1].

Also different geographical regions (Kurinchi, Mullai, Marutham, Neithal and Palai) which pertain to mountain, forest, fertile river beds, coastal and desert leads to various illness. The Siddha system of medicine recommends specific food and medicines for those regions. Similarly each seasonal variations and types of prone diseases are also described in Siddha literature along with suitable diets and remedial measures.

Thus the daily regimens of rules and diets to be followed is mentioned in ancient Siddha system of medicine, as a measure of its preventive care, healthy living and to get rid of physical and mental ailments[1, 2, 3, 7].

Siddha aspects of Liver diseases (Kalleeral Noi)

Based on the Siddha literature, liver diseases are described as follows.

Vathakalleral Noi

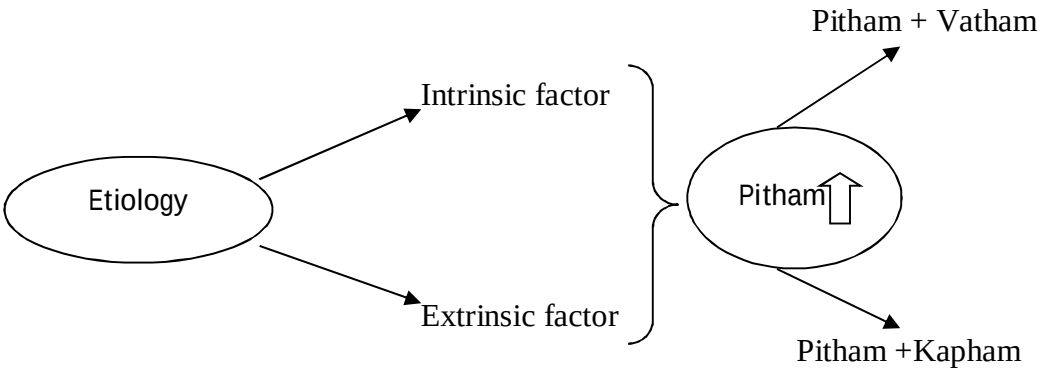
Pithakalleral Noi

Kaphakalleral Noi

The disease may be caused due to following factors:

- Excess dietary intake
- Excessive intake of alcohol
- The disease may also occur in children due to intolerance to food, milk etc.

Pathology of liver diseases



Types of liver diseases

Vatha Liver disease

The aggravated pitham in the body combined with vatham and produces fever, blackish discoloration of skin or face, loss of body strength, abdominal distension, enlargement of lymph nodes in inguinal, cervical and axillary regions. As the disease advances the potency of blood decreases and exhibits the symptoms such as pallor, swelling of upper and lower limbs.

Pitha Liver disease

This disease mainly occurs due to excessive activity of pitha dosha alone. Hence due to excessive pitha dosha the normal function of the liver is lost and the accumulation of bile throughout the body causes the yellowish discolouration of the skin and mucous membrane followed by symptoms such as bitter taste, bilious

vomiting, edema of limbs and anemia. In later stage, abdominal distension also occurs.

Kapha Liver disease

In this type of liver disease excessive activity of pitha dosha will be associated with kapha dosha. Hence there will be gradual enlargement of liver and its size and shape can be palpated. This will be followed by high fever, vomiting, frequent diarrhea, red coloured urine with diminished volume. In addition jaundice and swelling of the body will alsobe present.

Humoural derangements

According to Siddha principles, Mukkutram i.e., Vatham, Pitham and Kapham are the three vital factors which are responsible for the normal physiological condition of the body. If any one of these three humoursdeviate from its standard ratio ie.,1:1/2:1/4 maathirai respectively it gives rise to various pathological changes in the body resulting in diseases.

Thus Kalleral noigal (Liver diseases) are caused by excessive activity of pitha dhosha. This dosha is associated with other two vatham and kapham humours and paravukkaal which is a type of vatham that cause the spreading nature.

Signs and symptom

- Bitter taste in mouth
- Excessive salivary secretion
- Indigestion
- Anorexia
- Bilious vomiting
- Atrophy of muscles of upper and lower limbs
- Abdominal distension
- Frequent fever
- Enlarged liver

According to the ancient Siddha system, several internal as well as external factors continually influence the state of the three Dhoshams- Vatham(wind), Pitham(fire) and Kapham(water) inside our body and they account for the well being of an individual. Alterations in diet, lifestyle and behavioral patterns instantly alter these subtle humours or Dhoshams. The appearance of any symptom of ill health is the first sign of the individual having lost some nature of sensitivity and balance in relation to the nature of his constitution.

According to Siddha literature, Kalleral Noi (Liver disease) will be associated with Jaundice (Kamalai). The following are the salient features of Manjal Kamalai (Jaundice) that are described below:

Kamalai (Jaundice)

Synonyms – Pithu noi, Manjal kamalai, Kaamalla, Kamila.

Manjal kamalai is a pitha disease which is caused due to aggravated Pitham humour. Pitham is the principle which mainly consists of the element fire. It increases due to accumulation of bilious fluid in the blood, muscles, skin, eyes and tongue due to excessive intake of bitter, sour, salty or spicy food, intake of food at irregular intervals and unnatural sexual intercourse. Jaundice is also caused by excessive grief, anger, arguments, excessive physical exercise, very hot weather and excessive exposure to sun.

In T.V.Sambasivam Pillai's Dictionary, (Volume V) Kamalai is defined as follows: A Disease which is characterized by yellowness of the eyes, skin, and urine and by indigestion and loss of appetite.

Symptoms of Jaundice

- Excessive salivation
- Vomiting
- Bitterness
- Loss of appetite
- Indigestion of food
- Dryness of body

- Frog like skin
- Yellowish discolouration of eyes, nail, face and skin
- Pallor of foot, hand, face and eyes
- Fatigue
- Tremors
- Constipation
- Dark coloured stools
- Excessive sleep

Types of Kamalai

According to *Yugi vaidya chindhamani*,

- Vadha Kamalai
- Pitha Kamalai
- Kapha Kamalai
- VadhaKapha Kamalai,
- PithaKapha Kamalai
- Mukkutra Kamalai
- Perumanjal Kamalai
- Azhagu Kamalai
- Sengamala Kamalai
- Kumba Kamalai
- Gunma Kamalai
- Oodhu Kamalai
- Varal Kamalai. [17]

In *Agastiyar 2000*, Jaundice is classified into 8 types - Seven are based on kutra differences, another one is based on the primary disease.

They are

- Vadha Kamalai
- Pitha Kamalai
- Silethuma Kamalai

- Pitha Silethuma Kamalai
- Vadha Silathuma Kamalai
- Sannibatha Kamalai
- Pitha Vatha Kamalai
- Sobai Kamalai.[18]

In *Vaidya Sara Sankiragam* Jaundice is classified into 5 types. They are

- Manjal Kamalai
- Varal Kamalai,
- Vatha Kamalai
- Pitha Kamalai
- Ayya Kamalai.

In *Bala vagadam*, Jaundice is classified into three types. They are

- Oodhu Kamalai
- Manjal Kamalai
- Varal Kamalai.[1]

Pitha Humour and its significance

The nature of Azhal is Atomic. It is sharp and hot. The heat of Azhal is responsible for many actions and their reactions.

Sites of Azhal

According to **Vaithiya Sathagam**, the Pingalai, Urinary bladder, Stomach and Heart are the places where Azhal is sustained. In addition to the above places, the umbilicus, epigastric region, stomach, sweat, saliva, blood, essence of food, eyes and skin are also the places where Azhal sustains. Yugi Muni says that, the Azhal resides in urine and in the places below the neck region.

Character of Azhal

Azhal is responsible for the digestion, vision, maintenance of the body temperature, hunger, thirst, taste etc. Its other characters include thought, knowledge, strength and softness.

Functions of Azhal

1) Maintenance of body temperature.
2) Produces reddish or yellowish colour of the body.
3) Produce heat energy on digestion of food.
4) Produces sweating.
5) Induces giddiness.
6) Produces blood and the excess blood are let out.
7) Gives yellowish colouration to the skin, eyes, faeces and urine.
8) Produce anger, heat, burning sensation, inaction and determination.
9) Gives bitter or sour taste.

Types of Azhal

1. Aakkanal – Anila pitham or Pasaka pitham – The fire of digestion.

It lies between the stomach and the intestine and causes digestion and dries up the moist ingested substance.

2.Vanna eri – Ranjaga pitham – Blood promoting fire

This fire lies in the stomach and gives red colour to the chyle and produces blood. It improves blood.

3. Aatralakkini– Saathaga pitham – The fire of achievement

It gives energy to do the work.

4. Ulloli thee – Prasaka pitham – The fire of brightness.

It gives colour, complexion and lusture to the skin.

5. Nokku Azhal – Alosaga pitham – The fire of vision.

It lies within the eyes and causes the faculty of vision. It helps to visualize things.

Treatment

As the disease mainly develops due to the derangement of pitha dosha, the treatment should be aimed as follows**:**

- Controlling the Dosha and regulating the directional factors which were affected, by inducing Vomiting and diarrhoea.
- Giving appropriate medicines for the disease.

For inducing diarrhoea and vomiting

Sanjeevi mathirai mixed with Euphorbia neriifolia juice (Ilai kalli)

Emetic nut is soaked in lemon juice for two days. It is then soaked in Euphoria neriifolia leaf juice for two days after that it is dried and powdered. The powder is given internally in doses of half to one pinch.

Significant Siddha formulations for Liver diseases

Karkam

- Keezhanelli karkam
- Kaddukkai karkam
- Sivanar vembu karkam
- Nerunjil karkam
- Sarakkondrai karkam
- Avuri karkam

Kudineer

- Kadukkai kudineer
- Mandoorathy kudineer
- Pidangunari kudineer

Thylam

- Kizhanelli thylam
- Sagadevi thylam

Nei

- Puliyarai nei
- Vallarai nei
- Thaneervittan nei
- Kadukkai nei

Parpam

- Ayakantha parpam
- Sangu parpam
- Palagarai parpam

Chendoorum

- Annabedhi chenduram
- Ayakantha chenduram

Mathirai

- Santha sathrothaya mathirai

Modern Literature on Liver Disorders

The liver which is remarkably known as the chemical factory of human body is the largest glandular organ in the body, contributing about 1.5–2.5% of the lean body mass. In the average adult human, it weighs about 1.2 to 1.5 kilograms (3.3 pounds).

It is well recognized that it plays an important role in maintainence of vital body functions. It plays an important role in carbohydrate, protein, and fat metabolism, detoxification, secretion of bile, biotransformation of food, drugs, endogenous and exogenous substances.As liver is receiving a rich supply of blood

and also having exceptional redox systems like cytochromes and several enzymes, it is able to convert these substances into different types of metabolites. These metabolites may either be inert, active or sometimes toxins. This heavy load of metabolism and exposure to harmful chemicals make liver susceptible to multiple disorders, such as, toxin or drug-induced hepatitis, alcoholic liver cirrhosis, viral hepatitis, acute or chronic inflammation of liver etc.,

FUNCTIONAL ANATOMY

Liver lobules which are cylindrical in shape are known as the basic functional unit of liver. They are about 0.8 to 2 millimeters in diameter and few millimeters long. There are approximately 50,000 to 100,000 lobules in a human liver. The liver consists of the right and left lobes, formed by the falciform ligament, ligamentum teres and ligamentum venosum. Each of the right and left lobes are further divided into eight segments and further into many lobules. However, when dealing with physiological aspect and pathological changes of liver, hepatic acinus is considered to be the functional unit. The direction of blood flow into the hepatic acinus is through the portal vein and hepatic artery and after nourishing the liver cells blood drains into the numerous hepatic venous tributaries. Conversely, the bile flows in the direction opposite to that of the blood flow. It flowsinto the inter lobular bile ductsthrough the biliary canaliculi. Liver contains a variety of cells such as hepatocytes, Kupffer cells, Stellate cells (the fat storing cells), endothelial cells, bile ductular cells etc.

LOCATION

Liver is located in the right hypochondriac region of the abdomen beneath the right lower rib cage and just beneath the diaphragm and extends into the left hypochondriac region of the abdomen for a distance which differs from individual to individual. It is attached to the diaphragm, peritoneum, abdominal vessels, and upper gastrointestinal organs by various ligaments and also kept in position by these ligaments.

BLOOD SUPPLY TO THE LIVER

Liver is supplied by both hepatic artery and portal vein and hence it is said to have a dual blood supply. For each minuteabout 1050 milliliters of blood rich in nutrients flow from the portal vein into the liver sinusoids. It constitutes about 80% of total blood supply of liver. In addition to that hepatic artery supplies about 300 milliliters of blood which is rich in oxygen to the sinusoids. It constitutes the remaining 20%. Thus altogether liver receives about 1350 ml/min which is 27 percent of the cardiac output during resting condition. Also under resting conditions liver forms about half of the total lymph formation of the body.

PRINCIPAL FUNCTIONS OF THE LIVER

It carries out a wide range of biochemical and metabolic functions and helps the persons to keep the internal environment of the body healthy.

1. Formation and secretion of bile and its contents.

2. Nutrient and vitamin metabolism

3. Inactivation and detoxification function: Liver is known as the detoxifying factory of the body. It detoxifies and metabolizes various substances including toxins, steroids, and other hormones and thus enables their excretion. Liver cells possess an excellent detoxification system called the *mixed function oxidase* that detoxifies and alters a number of xenobiotics and protects the human from a multitude of potentially dangerous drugs. In certain chronic liver diseases like liver cirrhosis, the above said drug metabolism in liver may be affected by deficient hepatic blood flow or reduced activity of hepatic detoxifying enzymes. This modifies the intensity of therapeutic and toxicological effects.

4. Conjugation of lipophilic compounds like bilirubin, anions and cations so that they can be easily excreted in the bile or urine

5. Synthesis of acute phase proteins, albumin, clotting factors, carrier proteins, steroid binding and hormone binding proteins.

6. It is the largest reticulo endothelial organ in the body. Kupffer cells in liver removes infecting bacteria and bacterial products which enter the body from the gut. Approximately, only less than 1 percent of the bacteria reaching the liver via portal blood from the intestines enter into the systemic circulation. In this way blood is extensively modified chemically during its passage through the hepatic plates

7. Stores vitamins (large amounts of vitamin A, D and B12 and smaller concentrations of vitamin K and folate) and minerals and release them when needed.

8. The Liver Functions as a Blood Reservoir :

Liver can store large quantities of blood in its blood vessels. Normally 450 ml of blood, which is almost about 10 percent of the body's total blood volume, is present in both the hepatic veins and the hepatic sinuses. When the pressure in the right atrium increases it causes backpressure in the liver that leads to liver expansion, and 0.5 to 1 liter of extra blood is stored in the hepatic blood vessels.

9. Regulation of Liver Mass—Regeneration

The liver possesses an exceptional ability to regenerate itself even after a significant tissue loss either due to partial hepatectomy or liver injury, as long as the lesion is uncomplicated by viral infection or any other inflammation. This restoration of liver tissue to normalcy is significantly rapid and it needs only 5 to 7 days in rats.

10. Carbohydrate Metabolism

In carbohydrate metabolism, the liver performs the following functions,

- large amounts of glycogen is stored in liver
- liver converts galactose and fructose into glucose
- Gluconeogenesis

- Liver helps to remove excess glucose from the blood by converting excess glucose into glycogen and stores it. When the blood glucose concentration begins to fall too low the above process reverse i.e., conversion of glycogen into glucose occurs. This ability of liver is called as glucostatic or *glucose buffer function* of the liver.

11. Fat Metabolism

Even though most body cells perform fat metabolism, liver performs some unique parts of fat metabolism and thus play a main role

- It oxidizes fatty acids and provides energy for most of the body functions
- It synthesizes larger amounts of cholesterol, phospholipids and lipoproteins
- Also it synthesises fat from other sources like proteins and carbohydrates

12. Protein Metabolism

The following are functions of the liver in protein metabolism:

- Deamination of amino acids
- Formation of urea and help to remove ammonia from the body fluids
- Liver synthesizes most of the plasma proteins except immune globulins
- It converts one amino acid into another depending on the need and also synthesises other non protein compounds from amino acids

13. The Liver forms the blood substances involved in blood coagulation.

ENTERO HEPATIC CIRCULATION

Enterohepatic circulation refers to the movement of bile salts from the liver to the small intestineandits vice versa. In the small intestines the bile salts help to digest fats and other related substances.

Liver hepatocytes produce bile acids from cholesterol. These bile acids are delivered to the second part of duodenum. There they are conjugated and bile salts are formed. When these bile salts reach proximal and distal ileum they are reabsorbed into portal circulation. As the portal vein enters liver, the hepatocytes extract bile salts very efficiently and only a small amount of bile salts leave the liver and enter into the systemic circulation. Because of entero hepatic circulation each bile salt molecule is reused about 20 times before being excreted. Thus entero hepatic circulation reduces the work load of liver.[20,21]

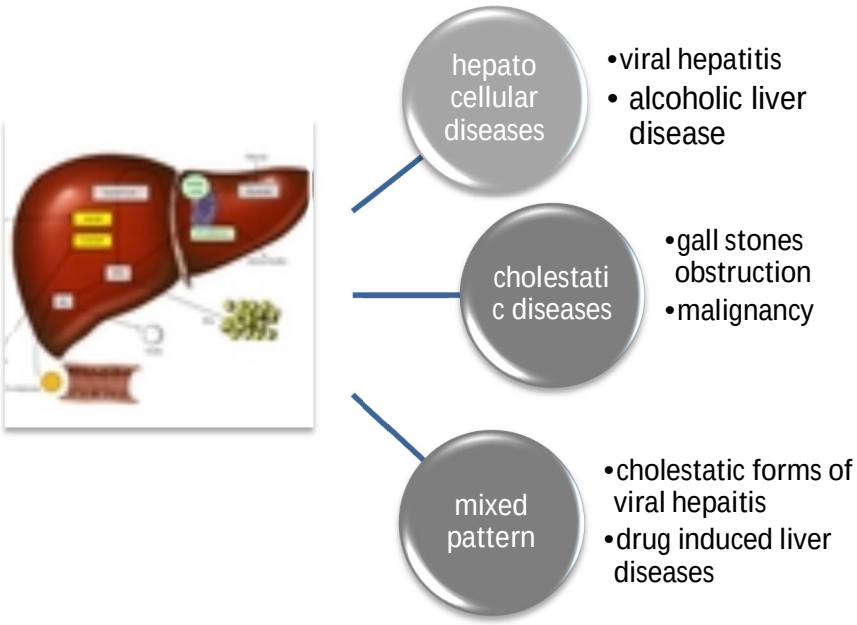
LIVER DISEASES

Liver diseases are generally classified as follows:

- Hepatocellular liver diseases
- Cholestatic (obstructive) liver diseases
- Mixed conditions.

Features of liver injury, inflammation, and necrosis are very much prominent in *hepatocellular diseases*. In *cholestatic diseases,* characteristics reflecting bile flow inhibition predominate. In the mixed pattern, signs and symptoms of both hepatocellular and cholestatic liver diseases are present eg. Cholestatic forms of viral hepatitis and many other drug-induced liver problems.

CLASSIFICATION OF LIVER DISEASES



SYMPTOMS OF LIVER DISEASE

These include jaundice, nausea, poor appetite, fatigue, itching and tenderness in the right upper quadrant, abdominal distention, and intestinal bleeding.

CHRONIC HEPATITIS

It denotes a group of liver disorders developed out of much causesand severity.In chronic hepatitis hepatic inflammation and hence necrosis lasts for at least 6 months. Chronic severe hepatitis will lead to liver cirrhosis. There are several ways through which chronic hepatitis develop. Few of them are as follows

- ❖ Chronic viral infections (Hepatitis A, B, C, D, E)
- ❖ Drugs
- ❖ Auto immune causes

VIRAL HEPATITIS

It denotes inflammatory changes that occur in liver due to viral infections.

PATHOLOGY

The morphologic lesions of viral hepatitis are:

- Pan lobular infiltration with mononuclear cells - primarily of small lymphocytes
- hepatocytic necrosis
- hyperplasia of Kupffer cells
- Different degrees of cholestasis.

PATHOGENESIS OF DRUG AND TOXINS INDUCED HEPATITIS

Hepatotoxicity from drugs and chemicals is the commonest type of hepatitis. Hepatotoxic drugs can damage the liver cells by producing free-radicals or metabolic intermediates. Free radicals cause lipid peroxidation of cell membrane lipids (phospholipids and lipoproteins) and leads to liver cell injury. In contrast, a drug or its metabolite may act indirectly by activating components of the innate or acquired immune system, stimulating apoptotic pathways, or causing damage to biliary pathways. Ultimately bile canalicular pumps are damaged and this results in accumulation of endogenous bile acids, which in turn causes hepatic cellular injury.

AUTOIMMUNE HEPATITIS

When hepatitis occurs without a known or identifiable aetiology, it is referred to as auto immune hepatitis. This type of hepatitis is characterized by prominent extra hepatic features of autoimmunity and sero immunologic abnormalities. This is a chronic disorder characterized by gradually increasing hepatic inflammation and necrosis followed by fibrosis. Ultimately it ends with liver cirrhosis and liver failure.

ALCOHOLIC LIVER DISEASE

Chronic alcohol intake is one of the major causes ofliver diseases. The pathological lesion of alcoholic liver disease consists of threeimportant features viz.,

- ❖ Fatty liver- It is present in more than 90% of daily as well as binge drinkers.
- ❖ Alcoholic hepatitis
- ❖ Cirrhosis.

PATHOGENESIS

Alcohol behaves as a direct hepatotoxin. After intake of alcohol it is metabolized into acetaldehyde which causes an inflammatory cascade that consequently produces multiple metabolic reactions. Lipogenesis, reduction of fatty acid oxidation and abnormal fatty acid synthesis occurs which in turn causes steatosis. Alcohol and its metabolites also activate cell injury and release endotoxin from the injured cells. This initiates a vicious cycle stimulating innate and acquired immunity pathways that release pro inflammatory cytokines like TNF-α and chemokines that ultimately stimulate proliferation of T and B cells. Also alcohol increases the level of harmful protein-aldehyde and increases the oxidative stress which further potentiates the liver injury.

PATHOLOGY

Fatty liver is the main pathologic lesion of alcoholic liver disease.Ingestion of alcohol results in fat accumulation in the entire lobule of hepatocytes. Even though alcohol toxicity leads to a wide range of fatty changes and alters the shape of the hepatocytes with macro vesicular fat, discontinuing alcohol consumption brings back the normal liver cellular arrangement and their fat level. Though alcoholic fatty liver is considered as entirely benign, it is well demarcated by ballooning degeneration, polymorpho nuclear infiltrate and fibrosis in the perivenular and perisinusoidal space of Disse.

LIVER CIRRHOSIS

Liver cirrhosis is a condition that has a variety of clinical manifestations and complications, some of which can be life-threatening. It is usually considered as a non-reversible chronic affliction of the liver which is mostly fatal because of the onset of liver failure or other complications (Jikko *et al* 1984). Thus in the past, it

was usually thought that cirrhosis was an irreversible condition; however, now it is become evident that when the underlying etiological factor that has led to the development of cirrhosis is removed, the fibrotic changes of the liver can be reversed. Regardless of the cause of cirrhosis,

The pathologic features of liver cirrhosis are as follows:

- Hepato cellular fibrosis causing extensive structural distortion.
- Formation of regenerative nodules.
- Decreased hepatocellular mass.
- Reduced liver parenchymal function.
- Disturbances of hepatic blood flow.

CAUSES OF CIRRHOSIS

Causes of liver cirrhosis are many. Most frequent among them are as follows

- Chronic alcohol abuse
- Hemochromatosis
- Inherited metabolic liver disease
- Chronic viral hepatitis
- Autoimmune hepatitis
- Wilson's disease
- Non alcoholic steatohepatitis
- Cystic fibrosis, biliary cirrhosis
- Medications such as methotrexate.

CLINICAL FEATURES OF CIRRHOSIS

These are the result of pathologic changes and exhibit the severity of the liver disease. The complications of cirrhosis are basically the same regardless of the aetiology. Cirrhosis often has no signs or symptoms until liver damage is extensive.Patients with cirrhosis have variable degrees of compensated hepatic function. *Portal hypertension* is one of the significant complicating features of decompensated cirrhosis and is mainly contributing for the development of ascites

and bleeding from esophago gastric varices. Ascites and oesophago gastric bleeding are the two significant complications of decompensated cirrhosis. Liver parenchymal functions are lost that causes jaundice, hypoalbuminemia and coagulation disorders, and ultimately results in porto-systemic encephalopathy.[22]

Jaundice: (Icterus)

Jaundice refers to theyellowish discoloration of tissues especially skin, sclera andmucous membranes. It results fromhyperbilirubinemia **(**increased bilirubin concentration in the body fluids) which leads to the deposition of bilirubin in the susceptible body tissues**.**It isdetectable clinically when theserum bilirubin becomes ≥3 mg/dL. This increased level of serum bilirubin, which is an end product of hemoglobin metabolism leads to jaundice. It occurs when the balance between bilirubin production and removal is disturbed.

Hyperbilirubinemia may result from

- Overproductionof unconjugated bilirubin than the normal liver can excrete (retentionhyperbilirubinemia)
- impaired uptake, conjugation, or excretion of normal amounts of bilirubin by a damaged liver
- Decreased excretion of conjugated bilirubin into the bile ductules andregurgitation or reflux of unconjugated or conjugated bilirubin from diseased liver cells or bile ducts into serum (regurgitation hyperbilirubinemia). Because of the above said causes the excessive bilirubin diffuses into the tissues, which then become yellow.

Thus based on the above aetiology jaundice is divided into three types viz.,

- Pre hepatic jaundice
- Hepatic jaundice
- Post hepatic jaundice

Pre hepatic jaundice due to overproduction of unconjugated bilirubin

Causes for over production

Hemolytic disorders that cause excessive heme production are responsible for pre hepatic jaundice. Both acquired and inherited disorders cause this type of jaundice.

Inherited disorders include

- Spherocytosis
- Thalassemia causing ineffective erythropoiesis
- Sickle cell anemia
- Crigler-Najjar syndrome
- Gilbert's syndrome

Acquired conditions include

- Drug induced hemolysis due to Rifampin, Probenecid etc.,
- Massive blood transfusion
- Hemolysis following certain diseases like malaria

HEPATIC JAUNDICE

In this condition jaundice occurs due to the problems in the liver viz.,

- Various forms of hepatitis
- Liver cancer
- Dubin-Johnson syndrome
- Rotor syndrome

Post hepatic jaundice due to overproduction of conjugated bilirubin

Causes for over production

Obstruction of the biliary tree is commonly due to

- Biliary calculus : Presence of gallstone in the common bile duct
- Cancer of the head of the pancreas.[23]

CHOLURIC AND ACHOLURIC JAUNDICE

Choluric jaundice means presence of bilirubin in urine. Only conjugated bilirubin dissolves in water and hence it can only be present in urine. Hence, choluric jaundice occurs in regurgitation hyperbilirubinemia. Clay coloured stools also results. Acholuric jaundice (absence of bilirubininurine) occurs only in the presence of an excess of unconjugated bilirubin.[24]

SANTHASANTHROTHAYA MATHIRAI (SSM)

Santha Santhrodhaya Mathirai(SSM) is a classical herbo mineral formulation mentioned in Siddha Vaidhya Thirattu and Kannusamy parambarai vaithiyam indicated for the treatment of *Pitha suram* and other diseases associated with *pitham* (biliary disease)[12,13].

"fhej hpggif nghhpA+ uenjhb filahjp
rhej nkhdwiu nahdwiu nahdwiu rkguj
jhaej iujjod kzpnad kzpGhp ajdhkQ
rhej rejpNuh jankd toNdha jhpahNj."

Santha Santhrodhaya Mathirai (SSM) derives its name due to its biological action of alleviating the aggravated pitham humour which is considered as a 'fire' that is responsible for the liver disorders (Santham means alleviate).In the above poem Kantharipagai means Turmeric which acts against the Agni force (Pitham).

Supportive Literature in evidence of SSM for Liver disorders.

S. No.	Ingredients (Tamil Name)	Chemical and botanical name	Pharmacological action
1.	Purified Venkaaram	Sodium biborate (Borax)	Antioxidant, hepatoprotective[25]
2.	Purified Pooram	Mercurous chloride (Calomel)	Antipyretic, Anti-inflammatory[26]
3.	Kappumanjal	Curcuma longa	Antioxidant, antiviral, antifungal, hepatoprotective.[27]
4.	Lemon juice	Citrus limon	Anti-inflammatory, antioxidant, epatoprotective.[28]

Adjuvant: Honey

Indication: Pitha suram (fever associated with liver diseases)

“$whd gpjjRuj jpdF zjijf
$wplNt epjjpiujhd nkjjffhzy
Njwhd ky%juQ rptejp Uejhy
rpjwpNa tapWfope jpLjy rjjp
thwhd thaggpjwwy thajhd ifjjy
kfjjhd %urir nahL jhfq fhzy
jhwhd ntOejpUeJ Nkhly tpffy
jtpjjpLjy rejhgg gLjy fhNz”

The symptoms of *Pitha suram* in Siddha literature mentioned by Sage Yugi which are as follows:

- Excessive sleep
- Reddish urine and stools
- Diarrhoea
- Vomiting
- Bitterness
- Excessive thirst
- Fatigue
- Pallor
- Hiccup

Almost all of the symptoms correlate with most of the symptoms of Liver diseases[4].

Ethno botanical aspects of Herbal ingredients of SSM

1. *Curcuma longa*

Habit and Habitat

Curcuma longa is cultivated extensively all over India. It is an erect perennial herb, It is harvested after 9-10 months when lower leaves turn yellow. It is then handpicked and cured by boiling in the decoction of its own and dried[29]

Sanskrit	:	Haridra
English	:	Turmeric
Botanical name	:	Curcuma longa
Family	:	Zingiberaceae

General Properties

nghdd;wkhk Nkd; Gyhdhww Kk NghFk
kdD GUl tr;akhk - g;dd;naOk
thej; g;jj Njhlikak thjkNghe jgdkhq
$hejkQr s;d f;oqFfF.

Turmeric is used for skin diseases, vomiting, vatham, pitham and kapham related disorders, headache, sinusitis, dropsy.

Phytoconstituents

Tumerones,turmerol,curcuminoids, Curcumin, demethoxycurcumin and bisdemethoxycurcumin, as well as volatile oils limonne,caryophylline and zingiberone α-phellandrene (1%), sabinene (0.6%), cineol zingiberene (25%) and sesquiterpines (53%).6 Curlone, a phytochemical from dried rhizome is used against hepatitis[30]and curcuminoids shows significant antihepatotoxic action.

Action

Carminative, Stimulant, Hepato tonic, Aromatic, Anthelmentic, Anti inflammatory, Febrifuge,Appetizer, haematinic, antiperiodic, expectorant, stomachic,anodyne, diuretic[31]

Uses

- Fresh juice of rhizome is applied to recent wounds, bruises and leech bites.
- Root is usually administered in intermittent fevers
- It is used as ablood purifier and in the treatment of skin diseases both internally and externally.
- Turmeric is used for the treatment of ringworm, itching, eczema and other parasitic skin diseases.
- Inhalation of the fumes of rhizome of the burning turmeric is used for the instant relief of catarrh and coryza.

- The fumes are also used for the relief of hysterical fits.
- Turmeric is mixed with borax and induced for the reduction of swelling.
- Internally turmeric is used for the disorders of Liver such as jaundice and also gall bladder diseases[30]
- A decoction of turmeric is said to relieve the pain of purulent ophthalmia and conjunctivitis[30]
- Curcumin is very effective in treating jaundice and included in the diet of patients with jaundice and infective hepatitis.

Pharmacological studies

Several therapeutic activities have been attributed to Cucuma longa since 1900 BC, for a variety of diseases, including liver disorders. This is due tothe main active ingredient Curcumin, which is obtained from this plant, whose structure was determined as diferuloylmethane in 1910.[32]

- Park et al.2000, studied the antioxidant effect of curcumin and concluded that curcumin causes significant reduction in the lipid peroxidation in CCl_4 treated rats [33]
- A.Ch. Pulla Reddy and B.R. Lokesh carried out an experimental research on Wistar rats which were fed a control diet or the control diet supplemented with 1% (by weight) turmeric for 10 weeks. They establish that the level of superoxide dismutase, catalase and glutathione peroxidase was increased and also decreased the level of lipid peroxidation in liver tissueexplained by enhancing the activities of antioxidant enzymesof rats fed the turmeric-containing diet in comparison with the controls[34].
- I.Dairaku et.al. 2010 demonstrated that curcumin has a wide range of antiviral activity through inhibitory activity against Inosine monophosphate dehydrogenase (IMPDH) which is suggested as a therapeutic target for antiviral and anticancer compounds.Curcumin acts in either noncompetitive or

competitive manner or it is suggested as a potent antiviral compound against different viruses[35].

- R.S.Upendra et.al. 2011, studied that turmeric powder showed that the very goodinhibitory activity against fungal contaminationsin plant tissue culture[36].

- B. K. Prusty et al., 2005 and C.S.Divya et al., 2006 studied that curcumin through apoptosis modulation and also prevention of viral oncogenes and decreasing the transcription of HPVs, and can be a good candidate for the management of highly oncogenic HPV infections[37, 38.]

- H. J. Kim, H. S. Yoo, J. C. Kim et al.,2009., studied the antiviral effect of aqueous extract of Curcuma longa rhizome against HBV in HepG 2.2.15 cells, containing HBV genomes showed repression of HBsAg secretion and suppression of HBV particles production from liver cells without any cytotoxic effect. The Curcuma longa extract enhanced the stability by increasing the rate of p53 protein through as well as transactivating the transcription of p53 gene thereby suppressing the HBV replication.[39]

- Rivera-Espinoza Y and Muriel P 2009., in their research work on curcumin has revealed that it has anti-inflammatory, anti-oxidant, antifungal, antibacterial and anticancer activities. Curcumin possess to restrain nuclear factor-kappaB, thereby given a rational molecular basis to use it in hepatic disorders by modulating several pro-inflammatory and profibrotic cytokines as well as their anti-oxidant properties[32]

Siddha formulations containing Manjal:

S.No	For liver disease	For other indications
1.	Sarabaraasa mathirai	Naga chendooram
2.	Panjalavana chooranam	Sambirani poo pathangam
3.	Mukoottu thylam	Rasagendhi mezhugu
4.	Siddhaathi legium	Kumatti kuzhambu
5.	Vilvaathi legium	Drakshathi chooranam

6.	Karisalai legium	Kadugarokini vadagam
7.	Mandoorathi adai kudineer	Karanthai lehiyam
8.	Maha sinthathi ilagam	Kumattikai thylam
9.	Kanthathi madoorachendoorum	Sangu mathirai
10.	Kadukkai nei	Sirusanthanathi thylam
11.	Thiripalathy gritham	Vatha naasa thylam
12.	Aridrathi chooranam	Neerkovai mathirai

Citrus limon

Habit and Habitat

Citrus limon is a small tree with spreading bushes and grows to a height of 3–6 m (10–20 feet). It has angular branches and sharp thorns at the axils of the leaves. The flowers are solitary or in small clusters. The fruits are round in shapeand are edible. The outer rind or peel is yellow in colour when ripe and is prominently glandular-dotted. It grows all over India.

Sanskrit name : Jambira
English name : Lime
Botanical name : Citrus limon (Linn) Burm.f.
Family : Rutaceae

Phytoconstituents

Lemon fruit contains many important compounds such as phenolic compounds, mainly flavonoids, vitamins (vitamin C), minerals, dietary fiber, essential oil and carotenoids. The peel is richer in flavonoids, neo-eriocitin, neohesperidin and naringin and has minor amount of narirutin.[40]

Action

- Stimulant
- Tonic
- Antiemetic

- Refrigerant
- Anthelmintic
- Astringent
- Appetizer[41]

General Properties

"rjhgyf fd;fhar%yKKdNt
e;jhdkhag ga;jj;ae;eijahafYNk"

kej;u;fFkej;u;aha kddDfFkddndd j
jej;u;fFk;jj;udNghw rhUNk–KejtU
fkgukharruff;d nfz z;akha thfluffr
rkgukhnkYk;rir.

As per the above poem explains lemon greatly pacifying the aggravated pitham therefore prescribed for the treatment of *Pitham* associated disorders. *(Bilious disorders)*

Uses

- Lemon is alkaline in nature and therefore helps to restore the pH balance of our body.
- Lemon is a good stimulant to liver and dissolves uric acid stones and helps in the removal of toxins.
- Lemon is rich in citric acid that helps to liquefy gallstones and calcium deposits
- Lemon also has antibacterial property and prevents hemorrhage because of the presence of the bioflavanoids.
- Rutin present in lemons improve the symptoms diabetic retinopathy.
- Lemon juice is helps in the recovery of scurvy.
- Lemon may be used as an exclusive cool drink in fevers and also a diuretic.
- It is also used in arthritis and can counteract in narcotic poisons.

- It is a good astringent and can be used in throat irritation,itching, uterine bleeding disorder and sunburns.
- It is helpful in Jaundice and also an antiperiodic in reducing the temperature in typhoid and malaria[42]

Pharmacological action

- Oyedepo T.A et al., 2015Studied the Antioxidant and Hepatoprotective Potentials of lemon and concluded that it has the ability to normalize the antioxidant enzymes and peroxidases of liver tissues against paracetamol induced hepatotoxicity.[43]

- Robert Jacob et al., 2000 reported that the phytoconstituents of lemon such as citrus limonoids have potential anticancer activity in mice andit was found to raise the significant amount of detoxifying enzyme. [44]

- Kumar A et al.,2011screened the two citrus fruit peel (Citrus sinensis and Citrus limon) against five pathogenic bacteria .The peel extract of Citrus sinensis and Citrus limon were reported to be as equally potent as the antibiotics, such as metacillin and penicillin.[45]

- Study on Hepatoprotective Effect of Citrus limon Fruit Extract against Carbofuran Induced Toxicity in Wistar Rats by Sunil Kumar Jaiswal et al., 2015 showed that rats pretreated with lemon juice prior to carbofuran exposure, caused significant recovery in the levels of activities of the enzymes (AST, ALT and LDH) both in the tissues and in the serum of rats which confirmed the hepatoprotective activity.[46]

- A. M. Pisoschi et al., 2008 suggested that ascorbic acid (vitamin C) present in lemon has an important role in biosynthesis of collagen and has wound healing property. It also helps in absorption of iron, activation of immune response, and osteogenesis[47]

- Victor Antony Santiago et al.,2012 studied that supplementation with D-limonene has been shown (in rats) to turn around the hepatic fatty acid level besidenon-alcoholic Fatty Liver disease. [48]

- Li et al.,2007 and Sood et al.,2009suggested that extract of citrus limon peel has anti-inflammatory, anti-carcinogenic, anti-viral, anti-oxidant, anti-thrombogenic, and anti-atherogenic effects .[49]

- Lemon has been found to have protective effects on T cell-dependent hepatitis.[50,51]

Siddha formulations contain lemon

S.No	For liver disease	For other indications
1.	Iyappodi ilagam	Asta bairava kuligai
2.	Kaantha chenthooram	Pasana mathirai
3.	Maha suyamaakini chenthooram	Kakkana mathirai
4.	Ashtapaanda vinotha melugu	Neerkovai mathirai
5.	Gunma chooranam	Kandhi kovai
6.	Siru vilvaathi ilagam	Annabedi chenthooram
7.	Maha vilvaathiilagam	Kaantha chenthooram
8.	Maha kodaasuli	Padikara chenthooram
9.	Panja paandu ilagam	Saathi sambira kuzhambu
10.	Anna bethi chenthooram	Ayajambeera karpam
11.	Aya chenthooram	Kirambu pakuva vennai
12.	Aya kaantha chenthooram	Seeraga chooranam
13.	Kool panda legium	Kesari legium
14.	Vilvaathi legium	Malai vambathi thylam
15.	Nelli vadagam	Adathodai nei
16.	Mandoora chenthooram	Bhavana kadukai

B.Ethno chemical aspects of Mineral ingredients of SSM

Borax

Borax is also known as sodium tetraborate. It is an important mineral and a salt of boric acid. It dissolves easily in water and it is white in colour.

Sanskrit name : Tankana, Rasashodhan

English : Sodium borate

Tamil : Vengaram

Source: It occurs as a natural deposit. It is found in masses by evaporation of water, on shores of dried up lakes in India, Tibet and Nepal. In this crude state it is known as Sohagoor or tinkala. It is purified by dissolving it in water, straining through cloth, evaporating to dryness and crystallizing. After the process it is called borax or tankan khar.

General Characters

"ntqfhuf Fzk njdW tjKlD iufff Nfsha
rqfhu khFeNjh\e jdidNa rqfhfF
Kqfd Yjtayyh Tjujjy thAkhwWk
nghqfa ,Ukykhejk NghffL Kzikjhnd."

ntqfhuk ntanjdDk Neha jhfFk.

"nrhwGilnaz Fdkeik Nrhh ahrk
gwfufz fyYhdk gdNdha-newiaj
jlqfzqf gqfUk rhggtlQ reea
alqfzqf yffwNgh nkz."

Action: diuretic, emmenagogue, astringent, antacid, local sedative and anti septic

Uses:

- Borax is used in inflammations and painful piles.
- It is used in cystitis, leucorrhoea, gonorrhea and lithic acid deposits.
- Powdered borax is used as an excellent suitable application in soreness of mouth, urethritis, stomatitis and cracked tongue.
- It is used as an antiseptic lotion in purulent ophthalmia and diphtheria.

- Borax and pepper powder are taken with honey for controlling asthma and cough.
- Borax is given with betel leaf for the prevention of fever and rigor.
- Borax is given with tender coconut water for urinary tract infections.[52]

Pharmacological studies

- Ince et al., 2010 and Nielsen et al., 1987 have claimed that boron limits oxidative damage by enhancing the body stores of glutathione and by inhibiting ROS and acts as a metabolic regulator in enzymatic systems.[53.54]

- Turkez et al. (2007) found that, boron compounds increased erythrocyte antioxidant level in human blood samples [55]

- Pawa & Ali (2006) have suggested that boron modulates oxidative stress parameters and partly normalizes the liver.[56]

- Zafar, H. & Ali 2013, in thiocetamide-induced hepatocellular carcinoma animals, it exposed that boron has beneficial effects on proliferating cell nuclear antigen index and ameliorating the oxidative stress.[57]

- Joseph R. Landolph ., 2007 studied the cytotoxicity and negligible genotoxicity of borax and borax ores to cultured mammalian cells which showed that refined borax did not induce neoplastic transformation in C3H/10T1/2 cells.[58,59]

Siddha formulations contain vengaram

S.No	For liver disease	For other indications
1.	Aya mezhugu	Anandha bairavam
2.	Aya kaantha parpam	Emathanda kuligai
3.	Aarumuga chenthooram	Sulai kudaram
4.	Lavana kuzhambu	Bala sanjeevi
5.	Avvai linga mezhugu	Bramanantha bairavam
6.	Kanda rasa parpam	Meganatha kuligai
7.	Navarasa mezhugu	Vasantha kusumagaram

8.	Gowri sinthamani rasa chenthooram	Virasana boopathi
9.	Sanjeevi mathirai	Vengaara mathirai
10.	Maha vilvaathi legium	Jalotharai mania
11.	Visa baaga ilagam	Kungumapoo mathirai
12.	Agni kumaara mathirai	Maha vasantha kusumagaram
13.	Sowbagya mezhugu	Sivanar amirtham
14.	Rajamani mathirai	Gowri sinthamani
15.	Linga bethi mathirai	Agasthiyar kuzhambu
16.	Karpoora chooranam	Visha kuzhambu

Pooram (Hydrargyrum subchloide)

The calomel (pooram) comes under the panchasootham. It is prepared by the combination of Mercury and salt.

Regional language Names

English : Calomel
Tamil : Pooram
Hindi : Ras kaapoor

Chemical structure

This is mercurous chloride. It is insoluble in water. There are several forms of Mercury which may exist in inorganic state, metallic state, mercury vapor and mercurous mercury (Hg^{+}) or mercuric mercury (Hg^{++}) salts. The Pharmacokinetics, biological behavior and clinical significance of the various forms of mercury may differ depending on its chemical structure.

Chemical Properties

Composition : Mercurous chloride (85% Hg, 15% Cl)
Tests : Completely volatilizes on charcoal, without melting
Crystal description : Tetragonal – Ditetragonal Bipyramidal

Chemical Formula	: Hg_2Cl_2
Mineralogical Name	: Calomel
Colour	: White and Yellowish white
Molar mass	: 472.09 g/mol
Solubility in water	: 0.2 mg/100 ml
Solubility in other Solvents	: Insoluble in ethanol, ether
Hardness	: 1.5 – 2 Talc – Gypsum
Melting Point	: 525° C
Boiling Point	: 383° C

Action[60, 61, 62, 63]

- Laxative
- Tonic
- Antiseptic
- Diuretic

Medicinal properties of Pooram

Pooram possess laxative, tonic, antiseptic, germicide, diuretic, sialagogue, alterative, cholagogue and purgative propertie [64]

Uses[65, 66, 67, 68]

- In 1600, Calomel entered the medical practice as a mild and palatable form of mercury. In 1800 it was widely accepted as an “alterative” as it altered the overall constitution of the body.

- Calomel (mercurous chloride) was thought to stimulate the liver and the gall bladder.

- An extract of calomel, colocynth, jalap and gamboge was used historically according to the *United States Dispensatory* of 1918 and was

found to be a safe cathartic and highly efficient and useful in congestion of the portal circle and torpidity of the liver."(Rush bills pills)

- Mercury was used in the treatment of syphilitic ulcers and also in the treatment of hemiplegia, facial palsy or spasms.

- During the 18th and 19th centuries the British, started using calomel throughout their Empire and very soon it gets the eminence as "valiant medicine", as many practitionersprescribed it for bloodletting, enema and other purgatives for balancing the deranged humours of the body. They believed in Siddha philosophical science that the inflammations of the liver and bilious fevers were caused due to excessive bile in tropical country [69]

- Calomel (mercurous chloride) was used as a purgative and laxative medicine in the form of tablet and injection in the late 19th century.

- It has broad spectrum antimicrobial activity against Gram positive and Gram negative bacteria and also against skin pathogens.[70]

- Mehta N. J. et al Result shows *Rasakarpura* is lesstoxic in comparison to chemically preparedmercuric chloride.[71]

General Properties

"ilthj #iy nah#iy Fdke
njhilthio thjkhQ Nrhz –ailahNjh
nthfFur fhgGu nkhdNw asnthLey
,fF ntyyj NjOeh s."

"rrtdd fUgG ujjy
rhjjj faQR thrk
grfy jhg Nrhgk
gTjjuk gsit F\lk
trjU fuhz NahL
tsujrhu Nkfk
,rjU krT #iy
aitgy Nuhfk NghNk."

“j‰uz lth jqFly thjk
jUQre e‰gj‰d %dW
kUzNl FjJ kiuahgG
kz ilr #iy fghyt‰b
guqf‰r #iy gwf‰uej‰
gffr #iy a‰it KjyNghk
,Uzl Nkd‰ nghdd‰wkhk
,JNtfwgk ,akgNu.”

The above Poem explains that Calomel cures various types of fever, Jaundice, dropsy, hepatomegaly, venereal diseases, throbbing pain, lumbar pain, rheumatism, burning sensation, worm infestation, indigestion, vomiting, chronic ulcers, itching, constipation, scabies etc. It is also effective in the treatment of headache when it is taken along with Jaggery for seven days.

Siddha formulations containing pooram

S.No	For liver disease	For other indications
1.	Iyappodi ilagam	Asta bairava kuligai
2.	Kaantha chenthooram	Pasana mathirai
3.	Maha suyamaakini chenthooram	Kakkana mathirai
4.	Ashtapaanda vinotha melugu	Neerkovai mathirai
5.	Gunma chooranam	Kandhi kovai
6.	Siru vilvaathi ilagam	Annabedi chenthooram
7.	Maha vilvaathiilagam	Kaantha chenthooram
8.	Maha kodaasuli	Padikara chenthooram
9.	Panja paandu ilagam	Saathi sambira kuzhambu
10.	Anna bethi chenthooram	Ayajambeera karpam
11.	Aya chenthooram	Kirambu pakuva vennai
12.	Aya kaantha chenthooram	Seeraga chooranam
13.	Kool panda legium	Kesari legium
14.	Vilvaathi legium	Malai vambathi thylam
15.	Nelli vadagam	Adathodai nei
16.	Mandoora chenthooram	Bhavana kadukai

Toxicologicalaspects

"All medicines are somewhat toxic". Thus Calomel also manifests toxic symptoms if taken in large quantity or drug which are prepared without proper purification and appropriate method of preparation. And also toxic symptoms occur even when consumption procedures are not followed properly. The toxic manifestations are as follows.

Ulcerative gingivitis, Ulcerative stomatitis, Ulcerative gastritis, Ptyalism, Skin eruptions, Lumbago, Ulcerative uvulitis, Ulcerative glossitis, Foul smell in the saliva, Dysphagia, Abscess, Orchitis, Blood stained diarrhoea.

Antidote

1. Oral administration of the juice of Ocimum sanctum (Thulasi) or Momordica charantia (Pagal) or castor oil for 3-5 days.

2. The root bark of Indigofera tinctoria (Avuri) is triturated with hot water and given in the size of 300 mg twice daily.

3. Nilapanai kizhangu Decoction

10 grams of Tuber of Curculigo orchoides (Nilapanai kizhangu), Hydrocotyle asiatica (Vallarai), Alternanthera sessilis (Ponnaankanni),Clerodendron serratum (Kanduparangi) are pounded and boiled in 650 ml of water and a decoction is prepared by reducing to 80 ml. This decoction is given orally twice a day for 2-3 weeks.[72]

4. SCOPE OF THE STUDY

- The medicinal preparations ascribed in Siddha literature are time-tested standard preparations. Hence it is the need of the hour to document standardization procedures based on current analytic techniques to prevent adulteration and to maintain quality control when manufactured in bulk.

- Establishment of suitable methods for analyzing and validating plant based formulations to determine the key bioactive component is a very challenging task for scientists to curtail the batch to batch variation and to access the safety, quality and efficiency of medicinal formulation.

- Siddha literature prescribes purification of the raw ingredients before preparing the medicine to evaluate the structural and chemical changes which occur during the purification process through sophisticated analytical instruments.

- The ingredients of *SSM* include raw drugs having hepato-protective activity (herbal, mineral) which justifies the need to qualitatively and quantitatively determine the contents in this traditional preparation.

- Evaluation of safety and efficacy of SSM will render scientific evidence on the therapeutic armamentarium as a validated internal agent in combating the liver damages induced by various agents' biological or chemical derived toxicants.

5. PLAN OF WORK

PRECLINICAL STUDIES

1. **Standardization of the study drug (SSM)**

- Procurement of raw materials for study drug.
- Identification and Authentication of raw drugs by botanist and geochemist.
- Standardization of purification processes of raw drugs.
- Process standardization of study drug SSM.
- Analytical standardization:
    a. Qualitative analysis
    b. Quantitative analysis

2. **Toxicity studies**

- Acute toxicity study
- Sub acute toxicity study
- Sub chronic toxicity study

3. Pharmacological studies

In vivo models of hepatoprotective screening in rodents

- Ethanol Induced Liver injury in Swiss albino Mice
- Paracetamol - induced liver injury
- LPS +D–galactosamine induced hepatoxicity

6. MATERIALS AND METHODS

The study drug SanthaSanthrothayaMathirai was prepared in triplicates as per Siddha vaidhya thiratu[12] in summer season (June) and the procedures are detailed in the following section.

Process standardization of Santha Santhrothaya Mathirai

6.1 Collection of raw materials

The raw drugVenkaaram (Borax) and Pooram (Mercurous chloride) were procured from reputed raw drug store at Erode. Pepper and betel leaves which are used in the purification of pooram and turmeric and lemon which are used in the preparation of SSM were procured from Erode, Tamil Nadu, India.

6.2 Authentication of raw materials

The herbal ingredients of SSM such as turmeric and lemon were authenticated to confirm the identity of herbal species using organo-leptic characters by Botanist, National Institute of Siddha and pharmacognostical study of herbal ingredients were done in Plant Anatomy Research Centre, West Tambaram, Chennai. The mineral ingredients of this study drug were authenticated at the Dept. of Geology, Anna University campus, Guindy.

6.3 Purification of ingredients of Santha Santhrothaya Mathirai

After authentication, the raw materials were made to undergo purification process because purification process (Suddhi) is the first step in the preparation of Siddha medicines. The word Suddhi means “to get rid of impurities”. Suddhi is a unique process which is employed to purify, detoxify and also to potentiate the effectiveness of drugs used in Siddha medicine[73]. The process of purification of drugs varies according to the drug to be purified and medicine that is to be prepared.

6.3.1 Purification of Pooram (Mercurous chloride)

30 g of black pepper was coarsely powdered and added with 30 g of sliced betel leaves in a black stone mortar and made into karkam (Pasty consistency) by trituration with water. The karkam was mixed with 3.6 L of water in a mud pot. 120 g of Raw Pooram (Sample P) was knotted in a cotton cloth and soaked in the water by hanging in the above pot as ThulaIyanthiram. The mud pot was heated using firewood under low flame until the water was reduced to 900ml. The knotted Pooram was taken out and the cloth over the Pooram was removed. Then washed with water and dried under sunlight. This purified form of Pooram was coded as Sample P1 and observed for loss of weight[8]. The same process was repeated for the purification of another two samples of same raw Pooram and the obtained purified Poorams were coded as Sample P2 and P3. The raw sample P and purified Pooram samples P1, P2 & P3 were both quantitatively and qualitatively analyzed.

$$\text{Loss of weight (gm \%)} = \frac{\text{Wt. of Pooram before purification (gm)} - \text{Wt. of Pooram after purification (gm)}}{\text{Wt. of Pooram before purification (gm)}} \times 100$$

6.3.2 Purification of Vengaram (Borax)

Vengaram –180 g was coarsely powdered using the black stone mortar and roasted over the mud plate until water evaporated out. The fried Vengaram became puffed. Loss of weight was calculated[8]. Thepurified form of Vengaram was coded as sample V1 and observed for loss of weight. The same process was repeated for the purification of another two samples of same raw vengaram and the obtained purified Vengaram were coded as sample V2 and V3.

$$\text{Loss of weight (gm \%)} = \frac{\text{Wt. of Vengaram beforePurification (gm)} - \text{Wt. of Vengaram after purification (gm)}}{\text{Wt. of Vengaram before purification (gm)}} \times 100$$

Coding for Raw drug and raw purified & Prepared medicine

S.No	Drugs	Coding
1.	Raw pooram	P
2.	Purified pooram samples	P1, P2, P3
3.	Raw vengaaram	V
4.	Purified vengaaram	V1, V2, V3
5.	SanthaSanthrothayaMathirai	SSM-A, SSM-B, SSM-C

6.4 Preparation of SanthaSanthrothayaMathirai

The purified Pooram, Vengaram and Kappumanjal were powdered separately and mixed uniformly in a kalvam (Black stone Mortar). To this, lemon juice was added constantly and triturated for 12 hours and made into pill rolling pasty consistency. It is then made into pepper sized pills (60mg) and dried in shade. Three batches of this preparation (SSM-A, SSM-B, SSM-C) were made in a similar manner as per Siddha literature for standardization purpose. The trial drugs were prepared at a GMP certified manufacturing company, Erode, as per the AYUSH GMP guidelines of the Drugs and Cosmetics Act 1947

Ingredients

S. No.	Ingredients	SSM-A (V2,P2)	SSM-B (V3,P3)	SSM-C(V4,P4)
		Quantity		
1.	Purified Vengaram(Borax)	50gm	50gm	50gm
2.	Purified Pooram (Mercurous chloride)	100 gm	100 gm	100 gm
3.	Kappumanjal (Turmeric)	300 gm	300gm	300gm
4.	Elumichaisaaru (Lemon juice)	1350ml	1330 ml	1320ml

6.5 Scientific validation of SanthaSanthrothayaMathirai

After completion of preparation, all three samples of SSM weresubjected to physico-chemical analysis according to standard methods (AOAC, 1980) and also

Ingredients of Santha Santhrothaya Mathirai

Pooram – Before purification

Pooram – After purification

Vengaram – Before purification

Vengaram – After purification

Kappu Manjal

Powdered Kappu Manjal

Process of purification

Ingredients of purification

Vetrilai	Milagu
	
Vetrilai milagu karkam	Pooram purification process
	
Vengaram purification	Preparation of SSM
	

Elumichai

Juice of elumichai

Santha Santhrothaya Mathirai

the qualitative and quantitative analysis of SSM was done by using sophisticated instruments.

6.5.1 Physico-chemical evaluation

The following parameters were tested viz., organoleptic characters, loss on drying, total ash value, uniformity of weight and size, disintegration time, solubility, heavy metal analysis, microbial contamination, aflatoxin and pesticide content.

6.5.1.1 Organoleptic characters

The organoleptic characters of SSM such as colour, odour, and taste were noted by five senses.

Colour Examination

From the three samples (SSM – A, SSM – B, SSM – C) fifteen tablets were taken in clean watch glasses and the colour was observed by naked eye by keeping them against white back ground.

Odour examination

Five tablets of each sample were smelled separately at the interval of two minutes and the odour was noted.

Taste examination

One tablet from each sample was powdered and a pinch of it was tasted. The taste felt was noted.

6.5.1.2 Loss on Drying at 105 ºC

4gm of 3 samples of SSM (SSM-A, SSM-B, SSM-C) were taken in a previously weighed 100 ml beaker and heated in an oven at 105 ^{0}C for 5 hours. The sampleswere cooled in a desiccator and weighed. The procedure isrepeated till constant weight is obtained. The percentage of loss of weight of the sample iscalculated.

Calculation

$$\text{Percentage of loss on drying at } 105\ ^{0}C = \frac{\text{Loss in weight of the sample}}{\text{Weight of the sample taken}} \times 100$$

6.5.1.3 Determination of Ash Value

2 grams of SSM from each sample were taken separately in silica crucible dishes and incinerated at a temperature of 450° C until they became white, indicating the absence of carbon.. Then they were cooled and weighed. Finally the percentage of ash values was calculated.

Calculation

$$\text{Percentage of total ash} = \frac{\text{Weight of the ash}}{\text{Weight of the sample taken}} \times 100$$

6.5.1.4 Acid insoluble ash

Total amount of ash from each sample was added with 25 ml of dilute HCl and boiled for 5 minutes. Insoluble matter was collected in a Gooch crucible, washed with hot water and ignited. The percentage of acid insoluble ash with reference to the air dried drug was calculated.

Calculation

$$\text{Percentage of acid insoluble ash} = \frac{\text{Weight of the acid insoluble ash}}{\text{Weight of the sample taken}} \times 100$$

6.5.1.5 Determination of sulphated ash

2 grams of SSM from each sample were put into the already heated and cooled silica crucible. They were heated, cooled and moistened with 1 ml of sulphuric acid and again heated at 800°C. The procedure is repeated until two successive weights do not differ by more than 0.5 mg[74].

Calculation

$$\text{Percentage of sulphated ash} = \frac{\text{Weight of the sulphated ash}}{\text{Weight of the sample taken}} \times 100$$

6.5.1.6 Estimation of pH

The samples were dissolved in water and pH of the resulting aqueous solutions were calculated using a pH meter at 25°C[75].

6.5.1.7 Disintegration Time

The Disintegration time of SSM (A,B,C) was done by using disintegration apparatus. The SSM tablets were placed in each of the six tubes of the apparatus and the discs were added to each tube. The time in seconds required for the tablets to disintegrate was observed[76].

6.5.1.8 Heavy metal analysis

Heavy metals (lead, cadmium, mercury and arsenic) content of the SSM samples were estimated by using AAS (Atomic absorption spectroscopy) (Thermo Fisher M Series, 650902 V1.27 model) (9). This procedure was done at Regional Research Institute of Unani Medicine, Chennai – 600013.

Instrument details and operating parameters

Thermo Fisher M Series, 650902 V1.27 Model Atomic Absorption Spectrometer (AAS) was used for the analysis.

Lead and Cadmium

Flame technique

Wavelength (Lead) - 217 nm; wavelength (Cadmium) - 228.8 nm; slit width - 0. 5 mm; lamp current (Pb) - 4.0 mA; lamp current (Cd) -. 3.0 mA; carrier gas and flow rate - air and acetylene, 1.1 L/min; sample flow rate - 2 ml/min.

Mercury

Cold vapor technique

Wavelength - 253.7 nm; slit width - 0. 5 mm; lamp current - 3.0 mA; carrier gas and flow rate - argon, 1.1 L/min; sample flow rate - 5ml/min.

Arsenic

Flame vapor technique

Wavelength - 193.7 nm; slit width - 0. 5 mm; lamp current - 6.0 mA; carrier gas and flow rate - acetylene, argon, 1.1 L/min; sample flow rate - 5ml/min. The Hallow cathode lamp for Pb, Cd, Hg and As analysis were used as light source to provide specific wavelength for the elements to be determined[77].

6.5.1.9 Microbial contamination

Total bacterial and fungal counts were done for SSM as per the procedure adapted from a WHO publication in 1998.The physico-chemical studies were done at Regional Research Institute of Unani Medicine (RRIUM), Royapuram, Chennai.

Microbial content determination

1g of SSM was powdered and added in 9 ml of sterile distilled water. Serial dilutions were made and viability was determined using the pour plate method. The plates were incubated for 24hours at 37° C. The following media were used namely Nutrient agar, Cetrimide Nutrient agar, Salt Nutrient agar, MacConkey agar.

For fungal growth detection, Sabouraud dextrose agar was poured into the plate and allowed to set and 1ml aliquot of each sample was spread on the surface and the plates were incubated at 27° C for 72 hours. The plate was placed on a colony counter and the number of colony forming units was observed.The microbial content was taken as the mean of duplicate determinations.

The viable aerobic bacterial count and the viable count for moulds (dry surface method) and the absence (or presence) of Enterobactriacea, Escherichia

coli,Staphylococcus aureus, and Salmonella spp were assessed using well established methods[78,79,80].

6.5.1.10 Analysis of Aflatoxins

The analysis of aflatoxins B1, B2, G1 and G2 of the three samples of SSM was carried out as per Official Analytical Methods of the American Spice Trade Association (ASTA, 1997).

6.5.1.11 Analysis of pesticide residue

The procedure followed for the analysis of pesticide residues was as per AOAC [16], 2005. Pesticide residues were analyzed by Gas Chromatography-Mass Spectra (GC-MS) (Instrument-Agilent, Detector-mass selective detector, Column specification-DB5MS, Carrier gas- Helium, Flow rate-1ml/min, Column length- 30 m, Internal diameter-0.25 mm, Column thickness-0.25μm)[77].

6.5.1.12 Weight variation test

The average weight variation test sample of SSMwas carried out by weighing 20 tablets individually using analytical balance, then calculating the average weight and comparing the individual tablet weights to the average.

6.5.1.13 Size variation test

The average size variation test drug of SSM was carried out by calculating 20 tablets individually using Vernier caliper, then calculating the average size and comparing the individual tablet size to the average[81].

6.5.1.14 UV – Visible spectroscopy

About 500mg of the sample was dissolved in 50ml of methanol with frequent shaking. After filtering; dilute 5ml of the filtrate with 50ml with methanol. Calculate the content of Curcumin at about 426nm using UV-Visible spectrometer. The percentage of curcuminoid was calculated using absorptivity value (E 1%1cm) as 1607 at a wavelength 426nm. The same procedure was carried out for all the three samples.

6.5.1.15 HPTLC (High-Performance Thin Layer Chromatography)

Quantification of Curcumin content was done in all the three samples (SSM-A, SSM-B and SSM-C) by using HPTLC. [CAMAG-Automatic TLC sampler, scanner and visualiser, glass twin trough chamber (20cm×10cm×4cm)]. TLC scanner3 linked to win Cats software (CAMAG). 0.2 cm thickness TLC plate pre-coated with silica gel 60F254 (E Merck) was used in this study.

Standard Curcumin solution - Preparation

A standard curcumin solution of concentration 0.1 mg/ml was prepared by dissolving 1 mg of curcumin in 10 ml of methanol

Preparation of sample solutions

The samples were extracted using methanol with the help of Soxlet apparatus for 4 hours after precisely weighing all the three SSM samples (Sample – A= 0.5415 gm, Sample – B = 0.4807 gm, Sample – C= 0.5007 gm). Separate 25 ml standard flasks were prepared using the filtrate from the extract.

Development of methods

The procedures recommended for the analysis of TLC and HPTLC analysis as per Wagner H and Bladt S, 1996 was followed. Analysis was performed on 20 cm × 10 cm HPTLC silica gel G60 F254 plates (E Merck) of uniform thickness of 0.2 mm. 3 µl of each sample solutions (A, B, C) in duplicates and 1 µl, 2 µl, 3 µl, 4 µl and 5 µl of standard Curcumin solution (Corresponding to 0.1, 0.2, 0.3, 0.4 and 0.5 µg of the standard per spot) were applied separately. For optimization of method, different mobile phase compositions were employed to achieve good separation. Finally the plate was developed using chloroform: methanol 9.5:0.5 (v/v) as a mobile phase in a twin trough chamber to a distance of 8 cm. The plate was air dried and visualized the plate under UV-366 nm.

Calculation of Curcumin in SSM

Curcumin content in SSM samples was calculated using the calibration curve of standard curcumin.

Calibration curve Apply 1 µl, 2 µl, 3 µl, 4 µl and 5 µl of standard Curcumin solution (Corresponding to 0.1, 0.2, 0.3, 0.4 and 0.5 µg of the standard per spot) on the TLC plate. Develop the plate in the solvent system in a twin trough chamber to a distance of 8 cm. Dry and scan the plate densitometrically at UV-366 nm. Record the respective peak areas and preparation of calibration curve by plotting peak area vs concentration of the Curcumin applied.

From the above analytical studies it was seen that the values of all the three samples, SSM A, SSM B and SSM C were approximately similar. Hence only one sample (raw-P, V, purified- P1, V1 and SSM A) was chosen for further analytical procedures.SSM A was used for further studies and is hereafter referred to as SSM.

6.5.1.16Thermo-gravimetry analysis (TGA)

To calculate the organic content of the prepared drug, TGA (Thermo-gravimetry analysis) was performed by placing 2-5 mg of SSM in an alumina cup and heated at the rate of 10°C/min up to 1000°C in a nitrogen atmosphere with a flow rate of 100 ml/min using an SDT Q600 (TA Instruments, USA).

To validate the scientific background of the Raw, purified and prepared drug SSM weresubjected to the following modern techniques.

6.5.1.17Raman spectroscopy

The functional group analyses in SSM was carried out using BRUKER RFS27 Stand alone Raman Spectrometer having scan range from 50 to 4000 cm^{-1}.

6.5.1.18 FTIR (Fourier Transform Infra-Red)

The FTIR spectra was recorded at 400–2000 cm−1 spectral region using a FTIR spectrophotometer (Spectrum RX-I; PerkinElmer). The spectra werecollected at a resolution of 4cm−1.

6.5.1.19 XRD (X-ray diffraction)

The crystallinity of the samples were analyzed using an X-Ray Diffractometer(XRD) (D8 Focus, Bruker, Germany), by irradiating with Cu-kα radiation. The analysis was performed from 10° to 60° (2θ) with a step size of 0.01[82].

6.5.1.20 SEM (Scanning Electron Microscope) analysis

The particle size was determined by using the HR- SEM analysis. Quanta 200 FEG scanning electron microscope (SEM) was used for the analysis.

6.5.1.21 XRF Procedure

The samples were pelletized with boric acid using pelletized machine with high pressure of 22tons.After pellitization the elemental composition of given sample is analyzed through XRF S8 Tiger (Bruker) under vacuum. Elemental composition of Raw, purified and prepared drug were determined by Energy dispersive X-ray Fluorescence (XRF) analysis.

6.5.1.22 Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES)

Perkin-Elmer 5300 DV ICP-OES was used to study the Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) for the determination of elements. 0.1-0.2 g of the sample was mixed with HNO_3: Perchloric acid (2:1).Then, the flask was cooled and the contents were transferred to a beaker and heated .The final solution was diluted to 50 mL with deionized water and taken for analysis.[83]

6.5.1.23 Mercury analysis by titration method:

A well mixed sample of the raw, purified poram and SSM is placed in a kjeldahl standard joint flask of 300 ml capacity and 7 ml conc. $HNO_3$and 15 ml conc. H_2SO_4attached to a standard joint condenser and under reflux gently at first and then more strong for about 30 minutes so that all the organic matters are dissolved. Cool, add 12 ml of conc. HNO_3 and boil. Continue the addition of HNO_3

and boiling until the liquid is colorless or pale yellow on continued boiling. Cool, wash down the condenser with 100 ml of water remove the flask and 1% $KMnO_4$solution drop by drop until a pink colour persists. Add one drop of 6 % hydrogen peroxide solution to remove the excess of permanganate followed by 3 ml of conc. HNO_3 and titrate with N/10 ammonium thio-cynate using ferric alum as indicator.[ref)]

After completion of the above analytical studies, SSM was subjected for further safety and efficacy studies

6.6 TOXICITY STUDIES

To evaluate the safety profile of SSM, Acute, Subacute and Subchronic toxicity studies were carried out as follows.

Experimental Animals

The experimental animals used in the study were Wistar albino rats of both sexes. They were kept in polypropylene cages (3-5/cage) in animal house with air cycles: 15/min and were provided with a temperature of 23±2℃ and 40–65% relative humidity, with a 12-h light/dark artificial light cycle. They were pellet fed (Sai foods, Bangalore, India) and were given purified water ad libitum. Prior to animal study, all the experimental rats were acclimatized at least for 7 days to the conditions of animal house. The animals were identified by cage number and by individual marking on its fur using picric acid. Throughout the study the animal care was strictly followed as per the Guidelines of CPCSEA, Laboratory. This study was carried out with the approval of Institutional Animal Ethics Committee (IAEC), NIS, (IAEC-1248/ac/09/CPCSEA/5 -16/2011)

Test substance and vehicle

The study drug SSM is insoluble in water alone. Therefore to obtain consistency and uniformity in drug distribution the drug is mixed with honey as vehicle. Moreover honey is used as an adjuvant in therapeutic usage of SSM. The

study drug was administered orally as it is given via the same route in clinical practice.

6.6.1 Acute oral toxicity study[85,86]

Methodology

Acute oral toxicity study was carried out by guidelines of Organization for Economic Cooperation and Development (OECD - 423). The study animals were procured from King Institute of preventive medicine, Guindy, Chennai. The Acute and subacute toxicity study was carried out in animal house of National Institute of Siddha, Tambaram.

Selection of Animals

Healthy young adult**,** nulliparous and nonpregnantfemale wistar albino ratsaged 4-6 weeks and 140-160g body weight were used for the study. The preferred rodent species is female since they are generally more sensitive than male rats.

Study Procedure

After acclimatization twelve animals were selected and divided into two groups viz, control and test group. The control group received Honey and water. The test group received the dosage of 2000 mg of SSM that was finely grounded and mixed with 5ml of honey and 5 ml of water (200mg SSM/ml). The test drug SSM was administered respective to their body weight orally by gavage to each animal after an overnight fasting.

S.No	Grouping	No.of animals
1.	Control (Honey + water)	6 Females
2.	Test Group (SSM 2000mg/Kg)	6 Females

Observation

In acute toxicity study, the control and 2000 mg/kg b.wt treated animals were observed for their behavioural signs and mortality at 30 minutes,1,2,4 hours upto 24 hours and then followed for further 14 days.

Behavioural signs

All the experimental animals were observed for behavioural signs such as Piloerection, lacrimation, salivation, diarrhoea, gait, posture, dyspnoea, lethargy, tremors, convulsions, coma and death.

Food and water intake

Food andwater intake was calculated daily**.**

Body weight

Body weights of the animals were recorded once 7 days.

Gross necropsy

At the end of the 14^{th} day,the overnight fasted animals except water were sacrificed by using excessive anaesthesia and subjected to gross pathological examination.Maximum tolerated dose was calculated in accordance with Globally hormonised system of classification.

6.6.2 Repeated dose 28-days oral toxicity study[87]

Methodology

Sub acutetoxicity study was carried out by guidelines of Organization for Economic Cooperation and Development (OECD - 407) with slight modification.

Selection of Animals

Healthy young adultwistar albino rats of both sexes with 140-160g body weight and 6-8 weeks of age were used for the study.

Dosage Calculation

As per the literature for mode of administration of SSM in humans, 2 pills (120 mg) should be mixed with honey and given for 2 times daily. The dose of SSM 240 mg per day was recommended by the Siddha practitioners for the treatment of liver diseases in human being. The animal (rat) dose of 21.6 mg/kg b.wt was arrived from the human dose based on body surface area conversion factor.

Therapeutic dose 240 x 0.018 = 4.3 mg/animal, 21.6 mg/kg b.wt

Study substance preparation

In the present toxicological study, SSM was administered in 3 doses such as low, mid, high 25, 125, 250 mg/kg b.wt respectively.A sufficient quantity of SSM was ground into fine powder in a mortar and stored in air tight sterile container. For mode of administration of SSM, it was suspended in the adjuvant Honey in the following ratios.

Low Dose

For administration of SSM in Low dose group, SSM25 mg was mixed with 2.5ml honey and 2.5 ml of water (SSM 5mg/ml) was prepared.

Mid Dose

For administration of SSM in mid dose group, SSM125 mg was mixed with 2.5ml honey and 2.5 ml of water (SSM 5mg/ml) was prepared.

High Dose

For administration of SSM in high dose group, SSM250 mg was mixed with 2.5ml honey and 2.5 ml of water (SSM 5mg/ml) was prepared.

Study Procedure

Forty Young adult wistar albino rats were selected, which were divided in to 4 groups. Each group consists of 10 animals (n=10, 5males and 5 females) Group I

was treated as vehicle control (honey with water), Group II (n=10, 5males and 5 females) received *SSM* 25 mg/kg, Group III (n=10, 5males and 5 females) received *SSM* 125 mg/kg and group IV (n=10, 5males and 5 females) SSM 250 mg/kg. . All the animals in the control and treatment groups were treated once daily for 28 consecutive days by oral gavage...[86,87]

S. No	Grouping	No.of animals
1.	Control (Honey + Water)	5males and 5 females
2.	Low Dose (*SSM* 25 mg/kg)	5males and 5 females
3.	Mid Dose (*SSM* 125 mg/kg)	5males and 5 females
4.	High Dose (SSM 250 mg/kg)	5males and 5 females

Observation

Behavioural signs

The experimental rats were observed for behavioural changes and signs of mortality and morbidity twice a day, till the completion of treatment. Clinical observations to detect signs of toxicity were made once daily, at the same time ie 1h after vehicle or SSM administration. Food and water intake was calculated daily.Body weights of the animals were recorded once in a week up to 28 days.

Haematalogical Parameters

The animals were sacrificed at the end of study, by using excessive anaesthesia and ed to gross pathological examinationwas performed.Fully automated hematology analyser was used to analyse the Haematological parameters. Enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and other constituents were also estimated .

Plasma was separated and used for the estimation of glucose, triglyceride, cholesterol, creatinine, urea and total protein with standard diagnostic kits using semi-automatic biochemical analyser (Star-21 Plus, India).

Histopathology examinations of organs

Vital organs were observed for gross lesions included brain, heart, liver, kidneys, spleen and stomach and organs and were weighed for relative organ weights. Tissues were fixed in 10% formalin and sections of 5–6 mm were routinely stained with haematoxylin and eosin (H & E) and examined under a light microscope. Any change in the histopathology of the treatment group was analysed and compared with the control group.

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). The test groups were compared with control for testing significance by one way ANOVA followed by Dunnet test using GRAPH PAD INSTAT version 3 software programmes. Values of $p<0.05$ were considered significant.

6.6.3 Repeated Dose 90-days Oral Toxicity study in rats: (sub chronic toxicity study)[88]

Methodology

Sub-chronic toxicity study was carried out as per OECD guidelines guideline-408.This study was conducted after getting the approval of Institutional Animal Ethics Committee (IAEC), KMCH College of pharmacy, Coimbatore, Tamilnadu (KMCRET/MD(S)/02/2014-15).

Selection of animals

Healthy adult wistar albino rats of either sex weighing between 180-200 g were used for the study. The Housing of experimental animals adopted the same procedure as mentioned above.

Procedure

After adaptation of laboratory condition, Fifty animals were randomly divided into control group and drug treated groups for three different doses viz. low dose (25 mg/kg b.w), mid dose (125 mg/kg b.w) and high dose (250 mg/kg b.w).The

animals were administrated with the study drug once daily for 90 days. The animals in group I (control group) received normal saline 5 ml/kg b.w. The animals in group II received test drug SSM25 mg/kg and group III, IV administered with 125 mg/kg and 250 mg/kg respectively. Group V was considered as Satellite groups with SSM high dose (250mg/kg) and was included to determine the delayed occurrence, or persistence of, or recovery from toxic effects.

S.No	Grouping	No.of animals
1.	Control (Honey + Water)	5males and 5 females
2.	Low Dose (*SSM* 25 mg/kg)	5males and 5 females
3.	Mid Dose (*SSM* 125 mg/kg)	5males and 5 females
4.	High Dose (SSM 250 mg/kg)	5males and 5 females
5.	Post retrieval Group High Dose (SSM 250 mg/kg)	5males and 5 females

Observation

During the study, body weight of the animals was calculated weekly once and water intake, food consumption and mortality events were evaluated daily. By the end of 90 days, the animals were fasted for overnight with free access to water. On 91stday the animals were sacrificed with excess anesthesia except recovery group animals.Blood samples were collected and stored in EDTA (ethylenediamine –tetra acetate-K2) for hematological analysis and in clot activator vacutainer for serum separation for biochemical analysis. On 120th day, the Post retrieval group animals were sacrificed with excess anesthesia. After that the following parameters were analysed.

Hematological analysis

Blood samples were analyzed using standard procedure by using hematological analyser Mindray BC 2800. Parameter evaluated includes Packed Cell Volume, Red Blood Cells count, Hemoglobin, Platelet Count, Total and Differential White Blood Cell count, Mean cell Haemoglobin Concentration, Mean Red Cell Volume and Mean Cell Hemoglobin.

Biochemical analysis

Serum samples were analyzed for Creatinine, Urea, Albumin, Total Protein, Total Cholesterol, High Density Lipoprotein (HDL), Low density Lipoprotein (LDL), Triglycerides (TGL), Glucose, Uric acid, Aspartate Transaminase (AST), Alanine amino Transaminase (ALT), using Mindray BS-120 autoanalyzer by standard methods using enzyme assay kits (Mindray Medical International Limited, India).

Histopathological evaluation

Appearances of organs were observed macro and microscopically. These organs included of kidney, liver, brain, heart, lung, spleen, stomach,ovary,uterus and testes. Histological slides of organs were made and observed under the microscope. The pathological observations of these tissues were performed on gross and microscopic bases. Histological examinations were performed on the preserved tissues with particular emphasis on those which showed gross pathological changes.[89]

Statistical analysis

All data were expressed as mean ± standarddeviation (SD). The test groups were compared with control for testing significance by one way ANOVA followed by Dunnet test using GRAPH PAD INSTAT version 3 software programmes. Values of $p<0.05$ were considered significant.

ICP MS Analysis

Inductively Coupled Plasma Mass Spectrometry (Agilent 7700 ICPMS): ICP-MS is a type of mass spectrometry that is highly sensitive and capable of the determination of a range of metals and several non-metals at concentration below one part in 1012 (parts per trillion). Samples are decomposed to neutral elements in high temperature argon plasma and analyzed based on their mass to charge ratios. It is an automated, simple and unique quantitative and qualitative analysis. It measures the elemental content.

Grouping Details

Mercury level was estimated in tissue by Inductively Coupled Plasma Mass Spectrometry (ICP-MS- Agilent 7700) .Rat belongs to control, high dose (SSM 250 mg/kg) and post retrieval group (retrieved from high dose treatment with 30 days retrieval time) was sacrificed and kidney tissues were digested by cold vapors digestion procedure [90].

Procedure

Digestion of sample is carried out by transforming 500 mg of the tissue sample into a closed beaker and 5 ml of concentrated HNO3 was added and digested to near dryness. 16 M nitric acid was further added each time to the sample and digested until the clear solution was obtained. 5ml of 12 M Hydrochloric acid was added to ensure complete digestion .The digested solution was cooled to room temperature and made to the final volume of 100 ml with deionized water. Sample solutions were then filtered through membrane (0.45micron) filter. Finally, the digested samples were used for metal analysis using inductively coupled plasma Mass Spectrometry .Each sample was digested in triplicate. A blank solution was also prepared in a similar manner.

6.7 Pharmacological study ofSanthaSanthrodayaMathirai (SSM)

All studies were conducted after obtaining prior approval (KMCRET/MD(S)/ 02/2014-15) for animal studies from CPCSEA, Government of India through the Institutional Animal Ethics Committee (IAEC), KMCH College of pharmacy, Coimbatore, Tamil Nadu

Hepatoprotective activity

Hepatoprotective activity of santhasandhrodhayamathirai (SSM) will be evaluated in mice by using three different models.

1. Alcohol Induced liver injury
2. Paracetamol Induced liver injury
3. Lipopolysaccharide (LPS)/d-galactosamine (d-GalN)-inducedliver injury

6.7.1 Alcohol induced liver injury

Experimental animals: Healthy adult (6-8 weeks old) Swiss albino male mice weighing between 20-30 g were used for this study. The animals were housed in poly propylene cages and were kept in well ventilated with 100% fresh air by AHU (Air Handling unit). A 12hr light / dark cycle was maintained .Room temperature was maintained between 24 $\pm$ 2^{o} Cand relative humidity 50–65%. They were provided with food (Sai feeds, Bangalore) and water *ad libitum*. All the animals were acclimatized to the laboratory for 7 days prior to experimentation.

Experimental Design Ethanol Induced liver injury Model in Mice.

Animals are divided in to six groups ,each group consists of six animals.

Group I: Administered with normal saline 1 ml/kg , (p.o)

Group II: Administered with Ethanol 50% (5gm/kg),p.o

Group III:Pretreated with Honey (40mg/kg),p.oand Administered with Ethanol 50% (5gm/kg),p.o

Group IV: Pretreated with low dose of test drug SSM in honey (25mg/kg), p.oand Administered with Ethanol 50% (5gm/kg),p.o

Group V: Pretreated with high dose of test drug SSM in honey (125 mg/kg), p.oand Administered with Ethanol 50% (5gm/kg),p.o

Group VI: Pretreated with Liv 52 formulation (2.6ml/kg), p.o and administered with Ethanol 50% (5gm/kg),p.o

Experimental Protocol

Liver damage was induced in all groups except control group. Animal belongs to group I administered with normal saline 1 ml/kg (p.o). No treatment will be given to animals belongs to group II from 0 -7 days, from 8^{th} to 21^{st}day, Ethanol 50% (5gm/kg) was given orally. Animals belongs to group III pretreated with honey (40mg/kg), p.o (0-7 days) and administered with Ethanol 50% (5gm/kg),p.o from day 8^{th} to 21^{st} along with honey one hour after ethanol administration. Animals belongs to group IV and V Pretreated with low (25mg/kg) and high dose

(125mg/kg) of SSM in Honey, p.o (0-7 days) and administered with Ethanol 50% (5gm/kg),p.o from day 8th to 21st along with test drug one hour after ethanol administration. Animals belongs to group VI Pretreated with marketed drug Liv 52at the dose of 2.6ml/kg, p.o (0-7 days) and administered with Ethanol 50% (5gm/kg),p.o from day 8th to 21st along with standard drug one hour after ethanol administration.[92]

6.7.2 Paracetamol -induced liver injury model in mice

Experimental animals: The animals used for the experiment were 6-8 weeks old Swiss albino male mice weighing between 20-30 g . The housing of experimental mice adopted the same procedure as mentioned in above pharmacological study.

Experimental Design Paracetamol - induced liver injury model in mice

Animals are divided in to six groups, each group consists of six animals

Group I: Administered with normal saline 1 ml/kg , (p.o)

Group II: Administered with paracetamol suspension (2gm/kg,bw),p.o

Group III: Pretreated with Honey (40mg/kg),p.oand Administered with paracetamol suspension (2gm/kg,bw),p.o

Group IV: Pretreated with low dose of test drug SSM in honey (25mg/kg), p.oand Administered withparacetamol suspension (2gm/kg,bw),p.o

Group V: Pretreated with high dose of test drug SSM in honey (125 mg/kg), p.oand Administered with paracetamol suspension (2gm/kg,bw),p.o

Group VI: Pretreated with Liv 52 formulation (2.6ml/kg), p.o and Administered with paracetamol suspension (2gm/kg,bw),p.o

Experimental Protocol

Liver damage was induced in all groups except control group. Animal belongs to group I administered with normal saline 1 ml/kg (p.o). No treatment will be given to animals belongs to group II from 0 -7 days, from 8th to 35thday, Paracetamol (2gm/kg) was given orally. Animals belongs to group III pretreated

with honey (40mg/kg), p.o (0-7 days) and administered with Paracetamol (2gm/kg),p.o from day 8th to 35thdayalong with honey one hour after paracetamol administration. Animals belongs to group IV and V Pretreated with low (25mg/kg) and high dose (125mg/kg) of SSM in Honey, p.o (0-7 days) and administered withParacetamol (2gm/kg),p.o from day 8th to 35thalong with test drug one hour after paracetamol administration. Animals belongs to group VI Pretreated with marketed drug Liv 52 at the dose of 2.6ml/kg, p.o (0-7 days) and administered withParacetamol (2gm/kg),p.o from day 8th to 35thdayalong with standard drug one hour after paracetamol administration.[93]

6.7.3 Lipopolysaccharide (LPS)/d-galactosamine (d-GalN)-induced liver injury model in mice

Experimental animals: 6-8 weeks old Swiss albino male mice weighing between 20-30 g were used for this study. The housing of experimental mice adopted the same procedure as mentioned in above pharmacological study.

Experimental Design of LPS /d-GalN-induced liver injury model in mice

Animals are divided in six groups of six animals each

Group I: Administered with normal saline 1 ml/kg , (p.o)

Group II: Injected with LPS (10 μg/kg) in combination with D-GalN (800 mg/kg), i.p.

Group III: Pretreated with Honey (40mg/kg),p.o and injected with LPS (10 μg/kg) in combination with D-GalN (800 mg/kg), i.p.

Group IV: Pretreated with low dose of test drug SSM in honey (25mg/kg), p.o and injected with LPS (10 μg/kg) in combination with D-GalN (800 mg/kg), i.p.

Group V: Pretreated with high dose of test drug SSM in honey (125 mg/kg), p.o and Injected with LPS (10 μg/kg) in combination with D-GalN (800 mg/kg), i.p.

Group VI: Pretreated with Silymarin (200 mg/kg), p.o and injected with LPS (10 μg/kg) in combination with D-GalN (800 mg/kg), i.p.

Experimental Protocol

Liver damage was induced in all groups except control group. Animal belongs to group I administered with normal saline 1 ml/kg (p.o). No treatment will be given to animals belongs to group II from from 0 -7 days, on 8th day received single intra peritoneal injection of LPS (10 μg/kg) in combination with D-GalN (800 mg/kg). Animals belongs to group III pretreated with honey (40mg/kg), p.o (0-7 days) and on 8th day received single intra peritoneal injection of LPS (10 μg/kg) in combination with D-GalN (800 mg/kg) and treated with Honey one hour after LPS challenge. Animals belongs to group IV and V Pretreated with low (25mg/kg) and high dose (125mg/kg) of SSM in Honey, p.o (0-7 days) andon 8th day received single intra peritoneal injection of LPS (10 μg/kg) in combination with D-GalN (800 mg/kg) and treated with SSM one hour after LPS challenge ..Animals belongs to group VI Pretreated with marketed drug Silymarin (200 mg/kg), p.o (0-7 days) and on 8th day received single intra peritoneal injection of LPS (10 μg/kg) in combination with D-GalN (800 mg/kg) and treated with standard drug one hour after LPS challenge. All animals will be sacrificed 24 hours later LPS challenge and were subjected to biochemical and histological studies.[94]

Biochemical investigations: At the end of the experiment mice were fastened overnight and sacrificed after the last day treatment and blood was collected in vacutainer tubes. Tubes were centrifuged at 4,000 RPM for 20 mints. Serum was separated from blood, stored in eppendhroftubes at -4°C and labeled. Serum samples of control and treated mice were analyzed for biochemical investigations including serum glutamate oxaloacetate transaminase (SGOT) or asparate aminotransferase (AST), serum glutamate pyruvate transminase (SGPT) or alanine aminotransferase (ALT), serum alkaline phosphatase (ALP), direct bilirubin (DB), indirect bilirubin (IB), total bilirubin (TB) ,blood urea nitrogen (BUN) and total Protein were determined using Mindray BS-120 autoanalyser by standard methods using enzyme assay kits (Mindray Medical International Limited, India).

Tissue processing for Anti-oxidant Enzyme estimation

After sacrificing the mice at the end of experiment, the whole liver was removed and examined for the clinical investigation. It was immediately transferred into cold phosphate buffer, blotted free of blood and tissue fluids and then weighed on an electronic balance. The tissues were chopped into small pieces with scissors and homogenized in ice-cold phosphate buffer (pH 8) at a concentration of 15% w/v.

They were then centrifuged in cooling centrifuge (Eppendorf, Asia Pacific) at 3000 RPM for 5 min. The supernatant was separated and further centrifuged at 7300 RPM for15 min at 4°C. The final clear supernatant was used for evaluation of SOD, CAT, GPX, GPR, Lipid peroxidation and MDA.

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). The test groups were compared with control for testing significance by one way ANOVA followed by Dunnet test using GRAPH PAD INSTAT version 3 software programmes. Values of $p<0.05$ were considered significant.

Histopathological Analysis

Sample Preparation

The isolated liver specimen was trimmed to small pieces and preserved in 10% neutral buffered formalin solution. The liver specimen was subjected to dehydration with ascending strength of alcohol (isopropanol) of strength 70, 80 and 100% respectively, each for one hour. The infiltration and impregnation was done by treatment with paraffin wax. Paraffin wax was used to prepare paraffin “L” moulds. Specimens were cut into sections of 3-5µ in thickness and were stained with haematoxylin and eosin. Mounting of specimen was done by use of Distrene Phthalate Xylene (D.P.X).Examination of the slides was performed under a light microscope, and digital images were captured using Olympus CX 41 microscope at the magnification ×10 and ×40.

Histomorphometric Grading

Histomorphometric grading analysis will be carried out for investigating significantly important parameters like fibrosis and other inflammatory changes between treatment and control group animals

The degree of inflammation and histopathologic damage with reference to hepatocellular architecture, necrosis, inflammation, mononuclear cell infiltration, Kupffer cells presence, arrangement of sinusoids , vacuolization and vascular congestion was expressed within each liver section classified on a scale of 0–3 (0, absent; 1, mild; 2, moderate; 3, severe) [95.]

Masson's trichrome staining

Masson's trichrome (Sigma, USA) staining is a marker for detecting the degree of fibrosis. 5μm thick sections of liver samples were stained with Masson's trichrome stain and prepared for observing the collagen fibers developed in liver tissues. The slides were examined under a light microscope, and digital images were captured using Olympus CX 41 microscope at the magnification ×40.

In Masson's trichrome stain the fibrous tissue (collagen)is stained blue.The cytoplasm of hepatocytes are stained red. The nuclei can be seen as dark red to black structures within cells.[9]

7. RESULTS

Standardization of SSM involved the selection, identification and authentication of ingredients and maintaining triplicates (SSM-A, SSM-B and SSM-C). The triplicates were subjected to physicochemical evaluation for the identification of right variety, to check better stability and integrity of the product. The following are the results of process standardardization.

7. Process standardization of SSM

Process standardization of SSM involved the identification and authentication of raw drugs, purification process and preparation of study drug.

7.1 Identification and authentication

The herbal ingredients of SSM such as turmeric and lemon were identified by a botanist and authenticated by means of the description of plant anatomy.

7.1.1 Pharmacognacy studies

***Citrus limon* Fruit**

The citrus fruit consists of thick fleshy rind and several spindle shaped juicy vesicle enclosed within the capillary chambers (Fig.1.1, 2).The rind is 750µm thick. It consists of a thin epidermal layer of small, thick walled squarish cells. Along the outer zone of the rind occur wide vertically elongated lysigenous secretary cavities inside which citric acid is stored (Fig.2.1) The ground tissue of the rind is parenchymatous. The cells vary in shape and size. They are thin walled and compact. Along the inner part of the rind these are well defined vascular strands. The strands include a dense cluster of small thick walled and lignified xylem elements. The phloem elements are associated with the xylem along the outer part (Fig.3.1) Calcium oxalate crystals are sparsely distributed in the sub epidermal regions of the rind. (Fig.3.2)The carpellary chambers have distinct epidermal layer of small cubical thick walled cells. The ground tissue consists of small polygonal parenchyma cells with wide air chambers. Vascular strands with circular, radiating

xylem elements are seen in the ground tissues. Phloem surrounds the vascular strand. (Fig.3.2)The juicy follicles are wavy in cross-sectional view. They have thin epidermal layer and juicy content inside (Fig. 1.2; 2.2).

***Curcuma longa* rhizome**

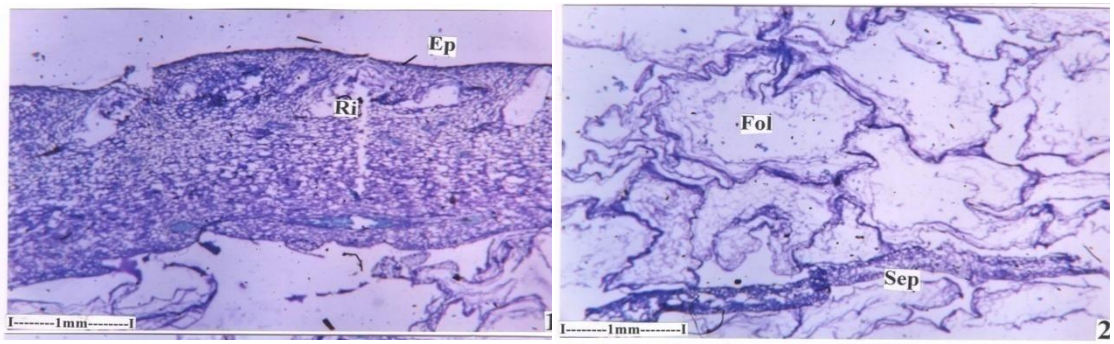
The rhizome is thick, cylindrical with even surface. It consists of uniformly thick wound periderm and large area of parenchymatous ground tissue and numerous scattered collateral vascular bundles (Fig.4.1) The periderm thick and massive measuring 450μm in thickness. It is superficial and consists of about 14 phylem cells, which are horizontally rectangular and are in compact vertical files (Fig. 4.2)

Inner the periderm is wide parenchymatous polygonal, thin walled compact cells. Dispersed in the parenchymatous ground tissues are numerous vascular bundles. The vascular bundles are collateral and closed bundles. (Fig.5.1; 5.2) The outer vascular bundles are slightly smaller than the central bundles. The vascular bundles have wide, angular thick alled metaxylem elements and a few phloem elements. The vascular bundles are ensheathed by sclerenchymatous bundle sheath. The bundlesheath is either complete or incomplete. The metaxylem elements are 40μm wide. (Fig. 6.1, 6.2)Calcium oxalate crystals are fairly common in the ground parenchyma. The crystals are sparsely distributed in the cells (Fig 7.1, 7.2)

The mineral ingredients calomel and borax were analysed for their organolepic structure and were confirmed by a geochemist.

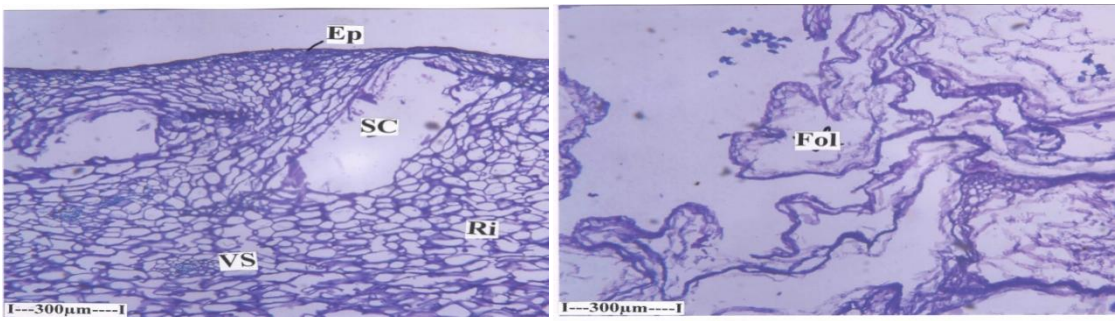
Plant anatomy of citrus fruit

Fig1.1 TS of rind of Citrus fruit **Fig 1.2 Folicles in the fruit**



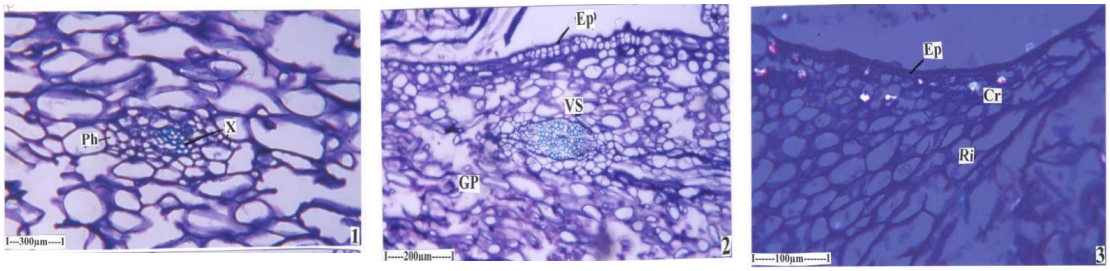
(Ep – Epidermis, Fol – Follicle, Ri – Rind, Sep – Septum)

Fig.2.1 Rind – Outer portion **Fig 2.2 Follicles**



(Ep – Epidermis, Fol –Follicle, Ri – Rind, Sc – Sceretory cavity,VS –Vascular strand)

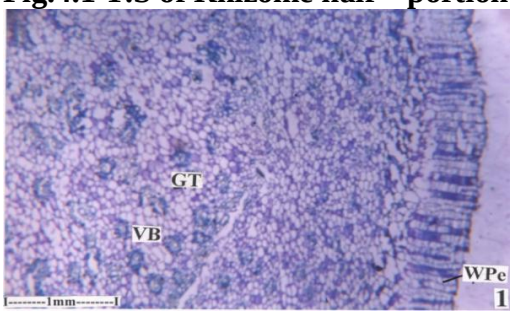
Fig 3.1 Citrus rind – inner vascular bundle **3.2 Septal wall - Vascular bundle** **3.3 Crystals in the Rind**



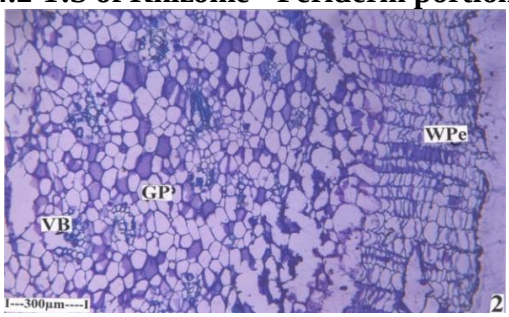
(Cr – Crystals, Ep – Epidermis, GP – Ground Parenchyma, Ri – Rind, Ph – Phloem. VS – Vascular Strand, X – Xylem)

Plant anatomy of curcuma longa

Fig.4.1 T.S of Rhizome half – portion



4.2 T.S of Rhizome - Periderm portion



(GP – Ground Parenchyma, VB – Vascular Bundle, W Pe – Wound Periderm)

Fig 5.1 Periderm zone enlarged

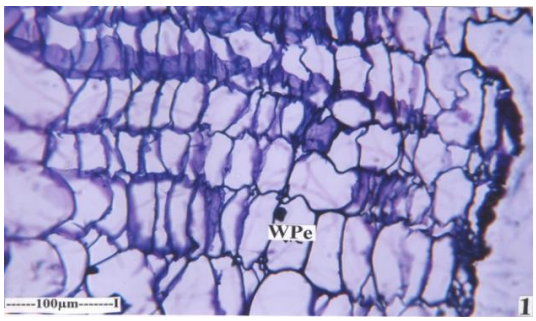
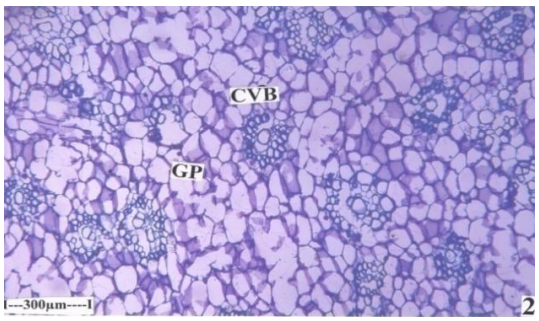


Fig 5.2 Vascular bundles in the rhizome



(GP – Ground Parenchyma, CVB – Central vascular bundle, W Pe – Wound Periderm)

Fig 6.1 Outer Vascular bundle

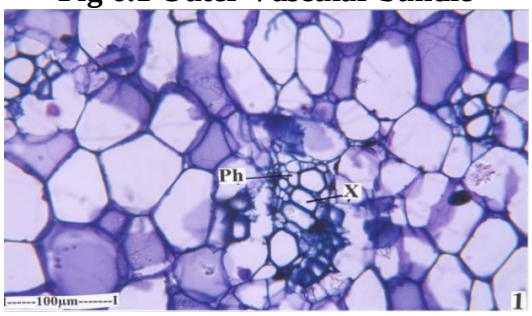
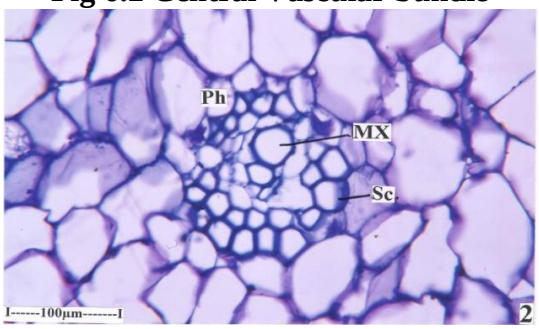


Fig 6.2 Central Vascular bundle



(MX – Metaxylem, Ph-Phloem, Sc- Sclerenchyma, X – Xylem)

Fig 7.1 Crystals in the Rhizomes-(Cr – Crystals)

7.2 Purification of Mineral ingredients

7.2.1 Purification of Pooram

The quantity of ingredients required for standardizing the purification process of Pooram is shown in table 1:

Table 1. Purification of Pooram

Sample	Quantity of piper betal(Vetrilai)	Quantity of piper nigrum (Milagu)	Quantity of water taken	Time taken for the process	Remaining amount of water
Pooram	30 gms	30 gms	3.6 lit	1 hr 40 min	910 ml
	30 gms	30 gms	3.6 lit	1 hr 30 min	905 ml
	30 gms	30 gms	3.6 lit	1 hr 30 min	905 ml

The percentage of weight loss before and after purification of Pooram is shown in Table 2.

Table 2. Purification of Pooram- Weight variation

Sample	Weight of pooram before purification	Weight of pooram after purification	Weight lost on purification	Percentage of weight lost
Raw Pooram (P)	120 gms	105 gms (P1)	15 gms	12.5
	120 gms	103 gms (P2)	17 gms	14.17
	120 gms	103.5 gms (P3)	16.5 gms	13.75

7.2.2 Purification of Vengaram

The percentage of weight loss before and after purification of Vengaram is shown in table 3.

Table 3. Purification of Vengaram

Sample	Weight of vengaram before purification	Weight of vengaram after purification	Weight lost on purification	Percentage of weight lost
Raw Vengaram (P)	180 gms	80 gms (V1)	100 gms	55.6
	180 gms	85 gms (V2)	95 gms	52.8
	180 gms	78 gms (V3)	102 gms	56.7

7.3 Preparation of SSM Samples

The amount of lemon juice that was required for preparing the samples of SSM and the weight of the karkam that was obtained is shown in Table 4.

Table 4. Preparation of SSM

Samples	Quantity of lemon juice added	Weight of karkam
SSM A	1350 ml	1.220 kg
SSM B	1330 ml	1.230 kg
SSM C	1320 ml	1.220 kg

7.4 Physcio-chemical analysis of Santha Santhrothaya Mathirai

The above prepared samples of SSM-A, B and C were subjected to physicochemical analysis and those results are shown in Table-5

Table 5. Physico-chemical analysis of SSM

Sl.No	Test	SSM-A	SSM-B	SSM –C
1	Organoleptic characters: a. Colour b. Odour c. Taste	 Dark Red Turmeric Sour	 Dark Red Turmeric Sour	 Dark Red Turmeric Sour
2	Loss on drying:	3.53%	3.65%	3.62%
3	Total – ash value	11.27%	11.22%	11.32%
4	Acid insoluble ash	8.19%	7.95%	9.50%
5	Sulphated ash	8.12%	7.90%	9.47%
6	Disintegration time	2.32hrs	2.48hrs	2.42hrs
7	Solubility	15.3hrs	15.3hrs	15.3hrs
8	Test of heavy metals a. Lead b. Cadmium c. Mercury d. Arsenic	 0.7686 ppm Not detected 0.9015 ppm Not detected	 0.6069 ppm Not detected 0.9982 ppm Not detected	 0.2447 ppm Not detected 0.8605 ppm Not detected
9	Microbial contamination a. Total Bacterial Count b. Total Fungal Count c. Enterobacteriaceae d. *Escherichia coli* e. Salmonella Spp f. *Staphylococcus aureus*	 $2x10^2$ cfu/g Less than 10cfu/g Absent Absent Absent Absent	 $1x10^2$ cfu/g Less than 10cfu/g Absent Absent Absent Absent	 $1x10^2$ cfu/g Less than10cfu/g Absent Absent Absent Absent
10	Test for aflatoxins (B1,B2,G1,G2)	BDL	BDL	BDL
11	Pesticide residue	Not detected	Not detected	Not detected
12	pH	4.6	4.6	4.8

7.4.1 Estimation of weight and size of SSM

On careful manual preparation of SSM, the wight of the pills varied from 60mg-90mg.But 18 pills were within ±10% weight variation, which is the minimum accepted limit as per Indian Pharmacopeia. Only 2 pills were outside the ±10%. The above results were suited for the acceptance of weight variation as per the criteria designed by Indian Pharmacopeia. So SSM was accepted in the weight variation test. The results of weight variation and size variation of SSM are shown in Table 6 &7.

Table 6. Weight variation of SSM

Sample No.	Weight of pills (mg)	% Weight variation	Within ± 10% variation
1	66	-7.94	Yes
2	70	-2.37	Yes
3	68	-5.16	Yes
4	71	-0.97	Yes
5	64	-10.73	No
6	68	-5.16	Yes
7	75	4.60	Yes
8	72	0.41	Yes
9	70	-2.37	Yes
10	68	-5.16	Yes
11	72	0.41	Yes
12	69	-3.76	Yes
13	65	-9.34	Yes
14	68	-5.16	Yes
15	70	-2.37	Yes
16	75	4.60	Yes
17	82	14.36	No
18	70	-2.37	Yes
19	78	8.78	Yes
20	93	7.39	Yes
Mean	71.7	No. of pills	18
S.D	6.61		

Table 7. Estimation of diameter of the Pill SSM

Sample No.	Absolute reading (mm)
1	4.886
2	5.425
3	5.057
4	5.663
5	4.514
6	4.926
7	5.426
8	5.756
9	5.132
10	4.997
11	5.646
12	4.736
13	4.443
14	4.600
15	5.003
16	4.902
17	5.082
18	5.074
19	5.420
20	5.418
Mean	5.1053
S.D	0.3809

7.4.2 Estimation of size of the SSM

The size of the SSM pills as measured by vernier caliper was found to be 4mm-6mm which is approximately equal to that of pepper size (6mm).

7.4.3 HPTLC analysis of SSM

The finished products of SSM A, B and C were then analysed by using HPTLC method for qualitative and quantitative analysis of major biologically active phyto-constituent which can be used for the crude drug identification.

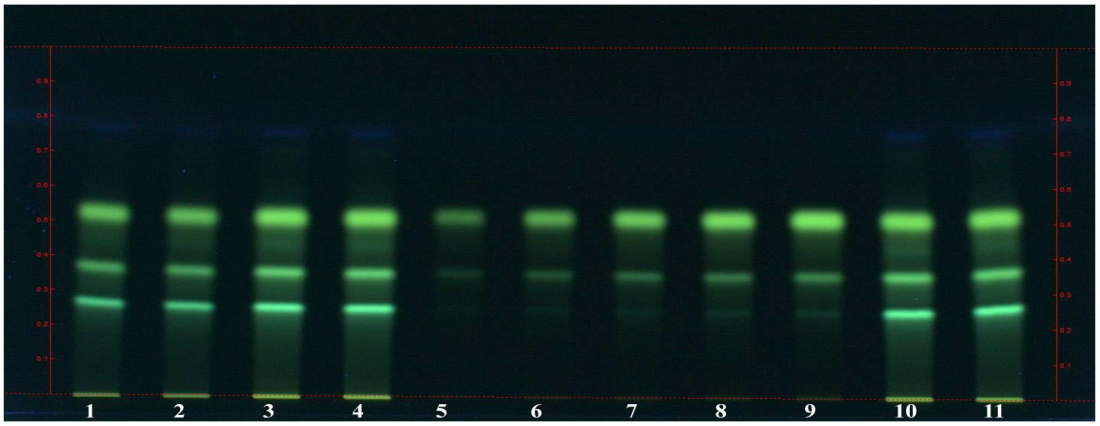
HPTLC method revealed “curcumin” as the major phyto-constituent present in the formulation SSM. Rf values of the bands are visible at 366 nm. These values are presented in Table.8

Table 8. Rf values of the bands visible at 366 nm

Sample –A	Sample -B	Sample –C
0.26 Yellowish green	0.26 Yellowish green	0.26 Yellowish green
0.36Yellowish green	0.36Yellowish green	0.36Yellowish green
0.51Yellowish green (**Curcumin**)	0.51Yellowish green (**Curcumin**)	0.51Yellowish green (**Curcumin**)
0.76Blue	0.76Blue	0.76Blue

Standard Curcumin showedyellowish green spot at Rf. 0.51. TLC plate and HPTLC chromatogram is shown in figure 8.

Fig. 8. TLC profile of test solutions –A, B and C with Standard Curcumin

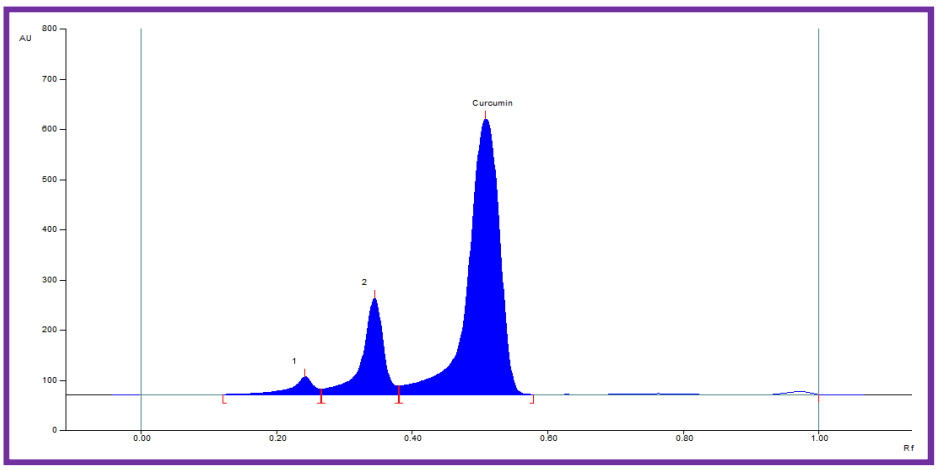


Track : 1 & 2 – Sample A; 2 & 3 – Sample B; 5 to 9 – Standard Curcumin and 10 & 11 – Sample C.

Finger print images of standard curcumin and SSM samples A, B and C and their corresponding Rf values are shown in figure 9,10,11and 12.

The standard curcumin was present in the three samples of SSM was confirmed by the HPTLC densitometry chromatogram as shown in Fig. 13.

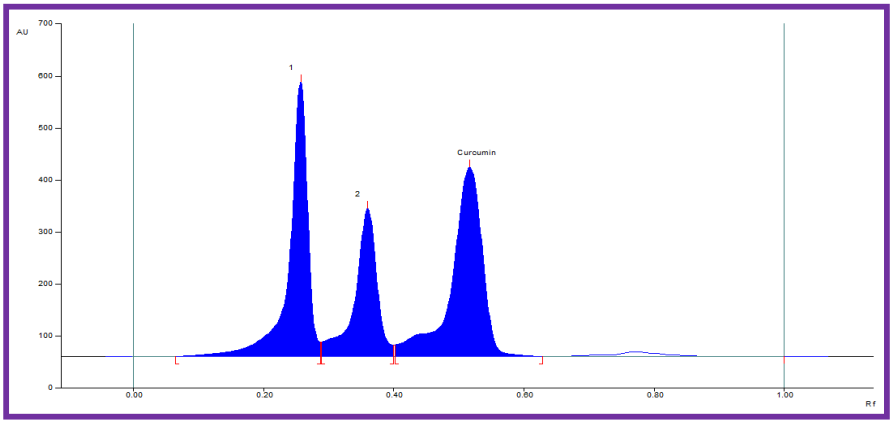
Fig.9. HPTLC finger print of the standard solution of Curcumin



Rf values of the HPTLC finger print of the standard solution of Curcumin

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.18 Rf	1.9 AU	0.25 Rf	17.3 AU	3.76 %	0.27 Rf	6.2 AU	496.6 AU	3.27 %
2	0.27 Rf	6.3 AU	0.35 Rf	91.7 AU	19.97 %	0.38 Rf	9.4 AU	2416.3 AU	15.90 %
3	0.38 Rf	9.4 AU	0.51 Rf	350.3 AU	76.26 %	0.58 Rf	0.2 AU	12280.3 AU	80.83 %

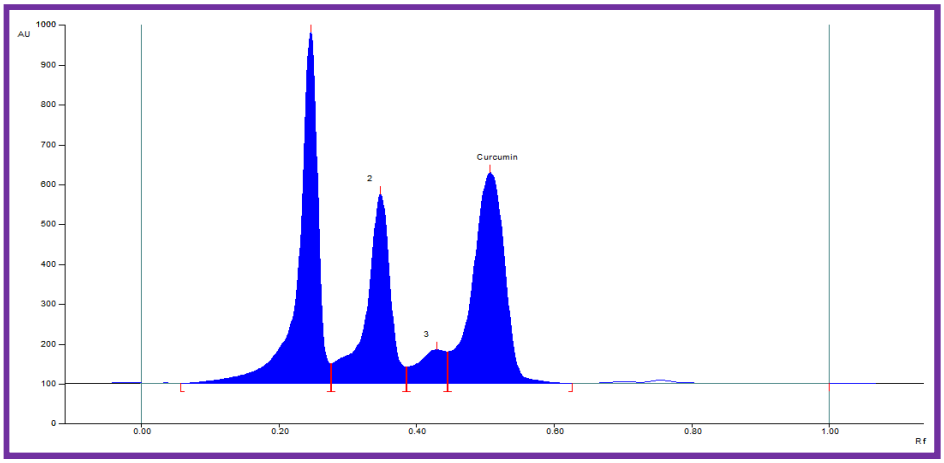
Figure-10. HPTLC finger print of the test solution of sample –A



Rf values of the HPTLC finger print of the test solution of sample –A

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.07 Rf	0.0 AU	0.26 Rf	528.5 AU	44.89 %	0.29 Rf	27.5 AU	12439.3 AU	35.70 %
2	0.29 Rf	27.6 AU	0.36 Rf	284.9 AU	24.20 %	0.40 Rf	22.1 AU	8043.3 AU	23.08 %
3	0.40 Rf	22.2 AU	0.52 Rf	363.9 AU	30.91 %	0.63 Rf	0.1 AU	14364.2 AU	41.22 %

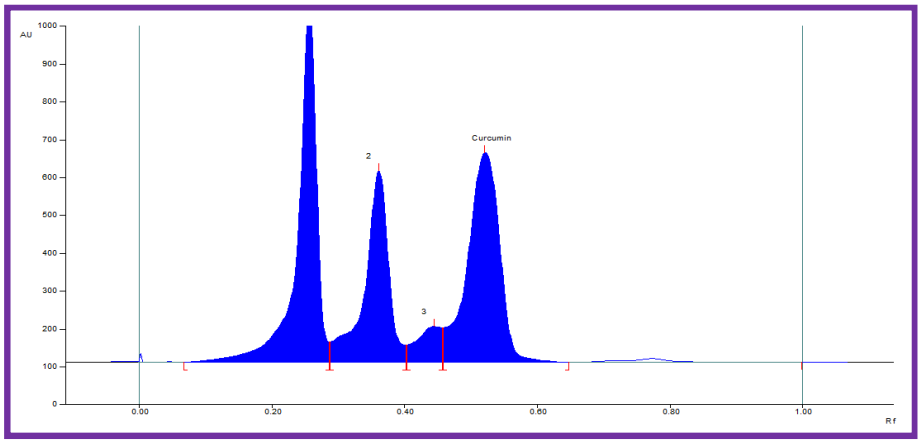
Fig.11. HPTLC finger print of the test solution of SSM –B



Rf values of the HPTLC finger print of the test solution of SSM –B

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.06 Rf	0.0 AU	0.25 Rf	882.8 AU	44.76 %	0.28 Rf	49.0 AU	20410.9 AU	35.68 %
2	0.28 Rf	49.4 AU	0.35 Rf	476.2 AU	24.15 %	0.39 Rf	41.3 AU	13568.0 AU	23.72 %
3	0.39 Rf	41.3 AU	0.43 Rf	84.4 AU	4.28 %	0.45 Rf	79.2 AU	2856.0 AU	4.99 %
4	0.45 Rf	79.3 AU	0.51 Rf	528.7 AU	26.81 %	0.63 Rf	0.0 AU	20366.0 AU	35.60 %

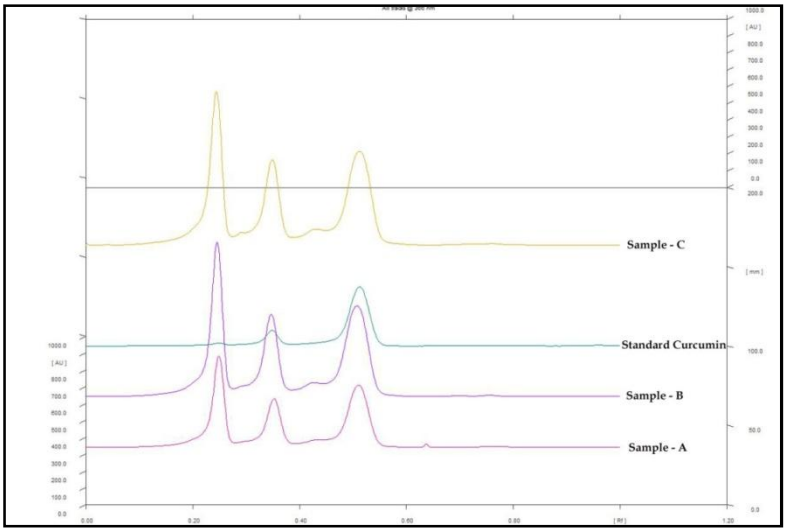
Fig.12. HPTLC finger print of the test solution of SSM – C



Rf values of the HPTLC finger print of the test solution of sample –C

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.07 Rf	0.1 AU	0.26 Rf	935.5 AU	44.76 %	0.29 Rf	53.1 AU	22529.4 AU	35.79 %
2	0.29 Rf	53.4 AU	0.36 Rf	506.6 AU	24.24 %	0.40 Rf	45.9 AU	15112.3 AU	24.01 %
3	0.40 Rf	46.1 AU	0.45 Rf	94.4 AU	4.52 %	0.46 Rf	91.3 AU	2978.0 AU	4.73 %
4	0.46 Rf	91.5 AU	0.52 Rf	553.5 AU	26.48 %	0.65 Rf	0.1 AU	22322.8 AU	35.47 %

Fig. 13. HPTLC densitometry chromatogram of SSM samples with standard curcumin



The superimposability of UV spectra λ max. at 424 nm (Fig. 14) confirmed the presence of the marker compound curcumin in all three samples of SSM.

Fig.14. UV-Superimposable spectra curcumin in Sample - A, B, C with curcumin

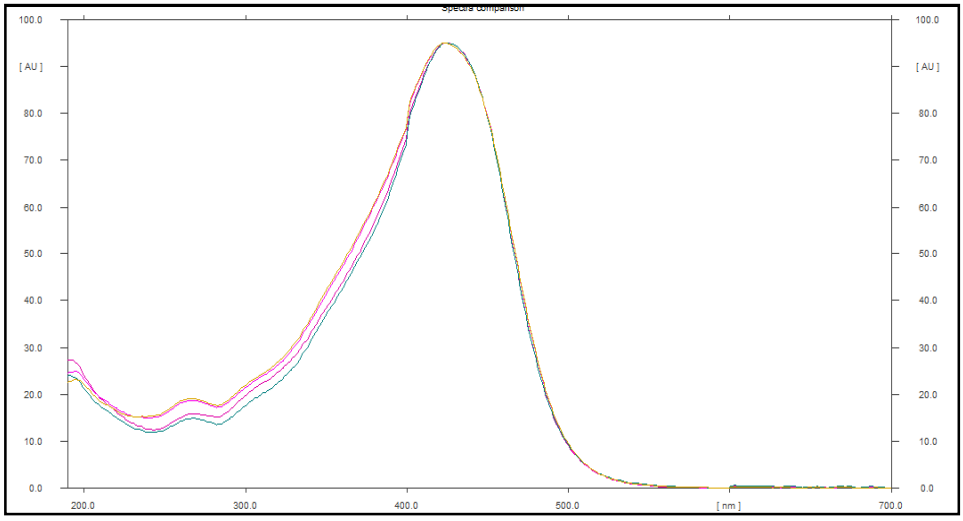
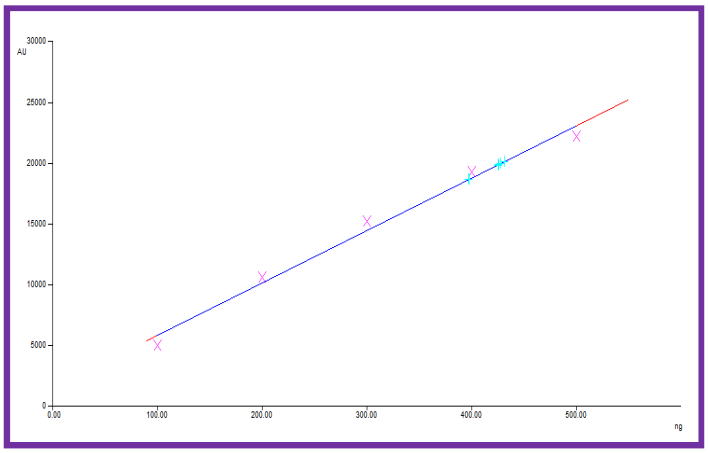


Fig.15. Calibration curve of the standard Curcumin



Percentage of Curcumin present

With the help of the calibration curve (Fig. 15) the percentage of Curcumin content present in the SSM samples A, B and C was **0.6589 %,0.6884 %**and**0.7104 %**respectively.

7.4.4 UV-Visible spectroscopy of SSM

The curcumin content of three samples of SSM results represented in Table.9

Table 9. Curcumin content of SSM by UV

Sl. No	Samples	Result
1	SSM A	1.15%
2	SSM B	1.19%
3	SSM C	1.23%

The physico chemical and chromatographic study reveals that all the 3 samples of SSM did not show much variation in process standardization. Hence one of the sample (SSM-A) was chosen for further scientific validation which was performed on raw, purified and prepared medicine SSM by using sophisticated instruments.

The SSM mentioned in further studies hereafter refers to SSM-A.

7.4.5 Thermogravimetric Analysis (TGA) of SSM

One of the objectives of this study was to understand the science behind the preparation of SSM pills by using contemporary methods. Hence, the final product of the preparation (i.e. SSM pills) along with the precursors and intermediates of preparation were systematically analyzed for its composition and speciation. As the preparation of SSM includes both organic and inorganic substances, initially, in order to know the composition of organic and inorganic substances present in the final drug, TGA analysis was carried out and the results are shown in Fig.16 and table.10

Fig.16: TGA analysis of SSM

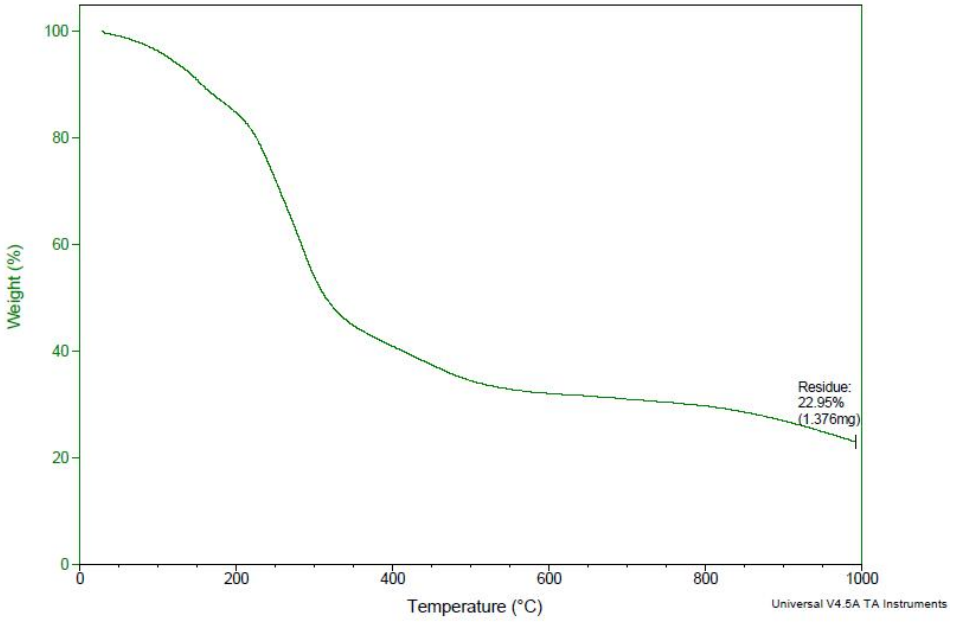


Table 10. TGA analysis of SSM

Sl. No.	Test Parameters	Result	Limit of Detection or Range or Specification
1.	Weight Loss	77.05 %	NA

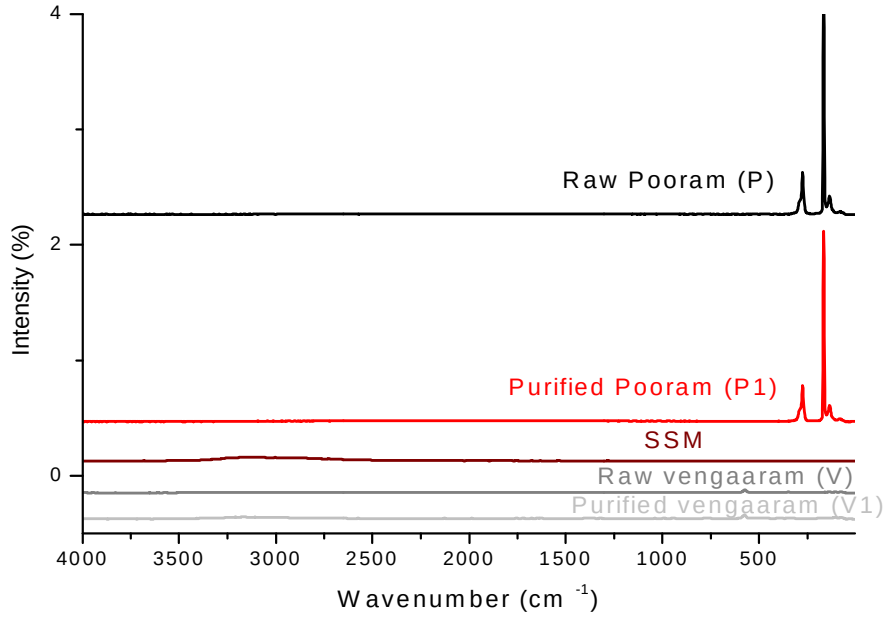
As the TGA analysis showed that major content in final product is organic, vibrational spectroscopy was employed to understand the physical and chemical modifications involved in preparation process using Raman spectroscopy and FTIR.

7.4.6 Raman spectroscopy of raw, purified (pooram, vengaram) and SSM

Raman spectra of raw pooram (P), purified pooram (P1), raw venkaaram (V), purified venkaaram (V1) and SSM A pill 1 (SSM) with spectral signatures from 4000 – 0 cm^{-1} are shown in Fig. 17.

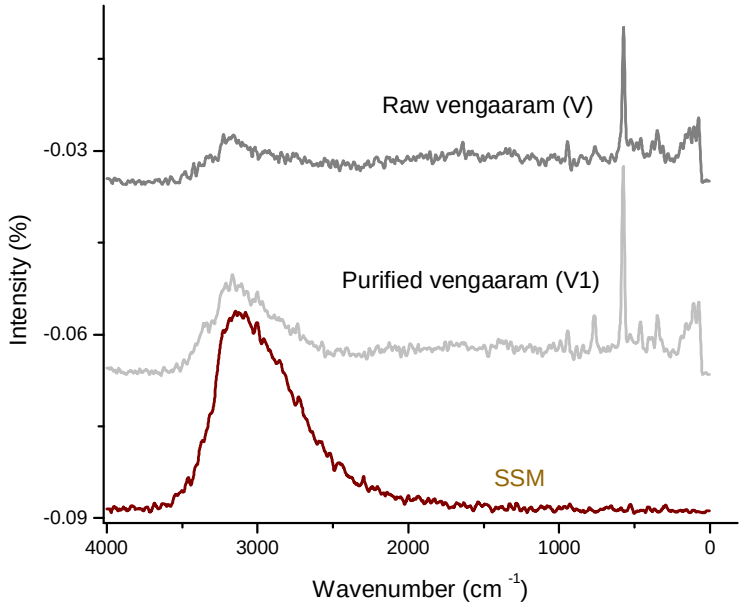
From this figure 17 it is clear that P and P1 have similar spectral signatures that represent the presence of mercurous chloride (274cm^{-1}, 166cm^{-1} and 135.82cm^{-1}). Absence of organic peaks indicates that theorganic contents of SSM could not be identified by Raman spectroscopy. As the intensity range of SSM, V and V1 are very low; they are separately studied under different magnification.

Fig. 17. Raman spectra of raw, purified and prepared samples



Raman spectra of V, V1 and SSM with spectral signatures from 4000 – 0 cm^{-1} are shown in Fig. 18. Peaks at 349 cm^{-1}, 573 cm^{-1} and 945 cm^{-1} represent the presence of $Na_2B_4O_7$. Same peaks were consistent in V1, indicating no detectable change in chemical composition during purification. Presence of O-H peak at 3200 indicates the presence of hydroxyl group in V, V1 and SSM.

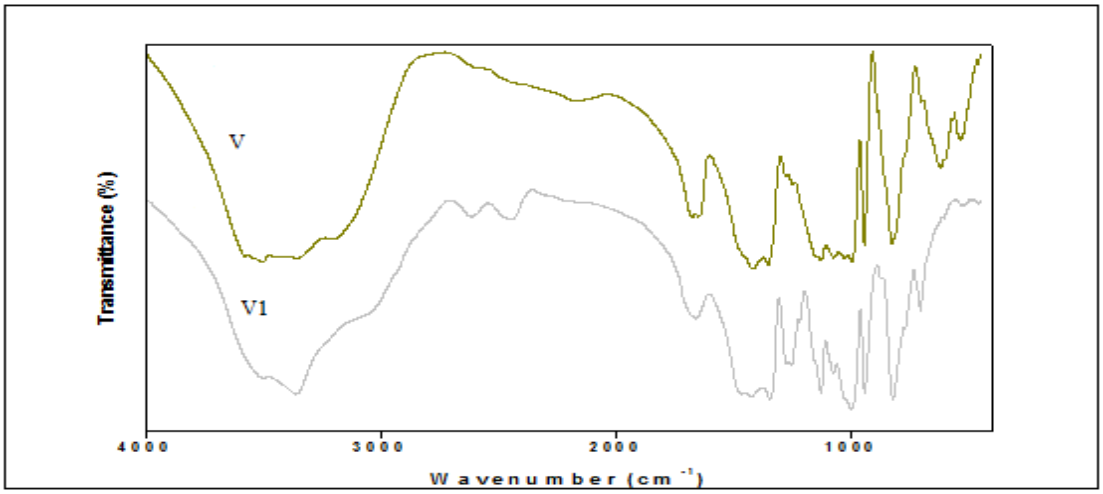
Fig 18: Raman spectra of raw, purified vengaram and SSM



However, Raman spectra of SSM did not show any peaks of notable intensity. So, no possible conclusion related with its physico-chemical nature could be drawn out of it. Hence we performed IR spectroscopy and results are shown in Fig 19.

7.4.7 Fourier transform infrared spectroscopy (FTIR)of raw, purified (pooram, vengaram) and SSM

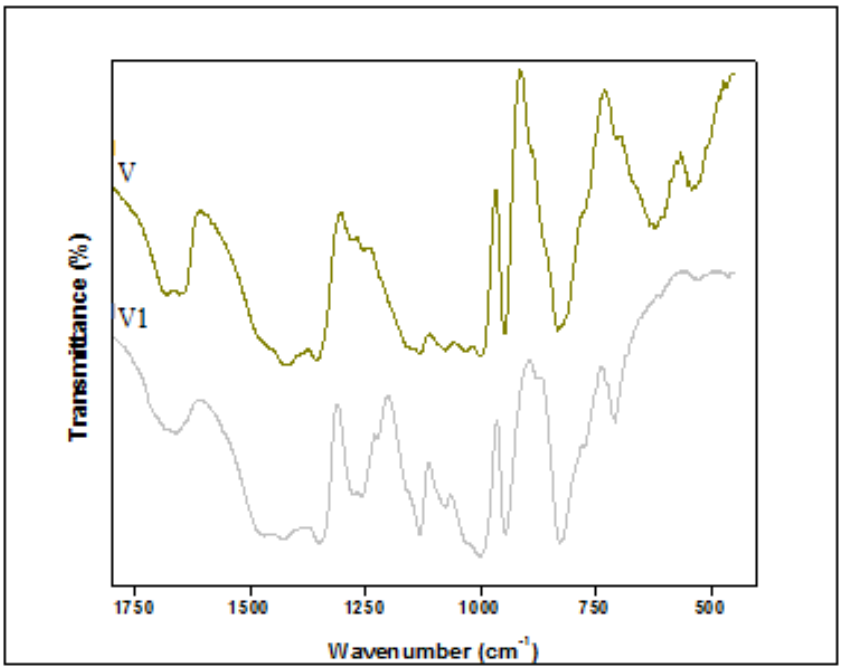
Fig. 19: FTIR spectra of raw and purified vengaram



FTIR spectrum of V shown in Fig.19- A broad peak around 3100-3500 with sharp tips at 3582, 3502, and 3350 region and a shoulder at 3190 represents the OH – stretching from H_2O or from the specific molecule. A wide shallow peak was found at 2175. Sharp bifid peaks present in 1679 and 1651 are attributed to OH-bending. The other characteristic bands observed between 1400 and 400 represents the vibrations from bonds involved in boron-oxygen coordination. A wide peak appearing in 1350-1500 regions was assigned to asymmetrical stretching of trigonal boran. A broad peak around 1150-1100 with tips at 1132, 1077, 1032 represents OH – in plane bending vibrations. A sharp peak appearing in 948 may be either due to symmetrical stretching of trigonal boran or asymmetrical stretching of tetrahedral boran. A sharp peak at 832 with shoulder at 891 and 774 was assigned to symmetrical stretching of tetrahedral boran. A peak at 621 with shoulder at 572 and 550, 540 also represents symmetrical stretching of tetra hedral boran.

However, this spectrum did not differ much from the spectra of V1. Slight shift of peaks in 3100-3500 regions indicate that OH groups are bonded to the main borate chain differently. This may be due to the effect of heat during purification. Apart from this there is not much difference noted in the V1 spectra compared with V as seen in Fig.20

Fig. 20: FTIR spectra of raw and purified vengaram (wavelength-500-2000)



FTIR spectrum of P and P1 did not show any difference. A broad peak around 3100-3500 represents the OH stretching which should be from the KBr used for measurement. The results are presented in Fig.21.

Fig. 21: FTIR spectra of raw and purified pooram

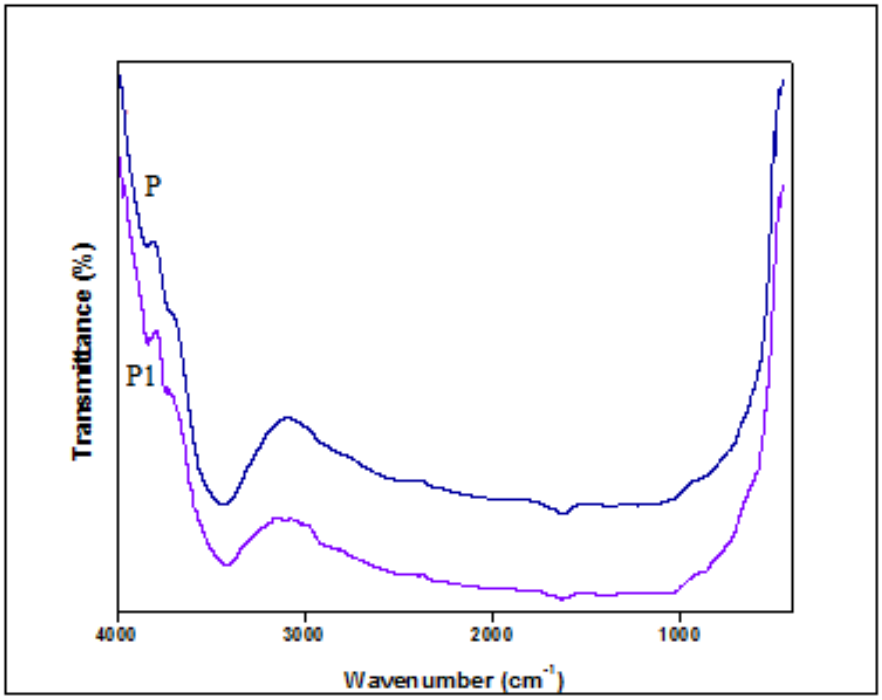
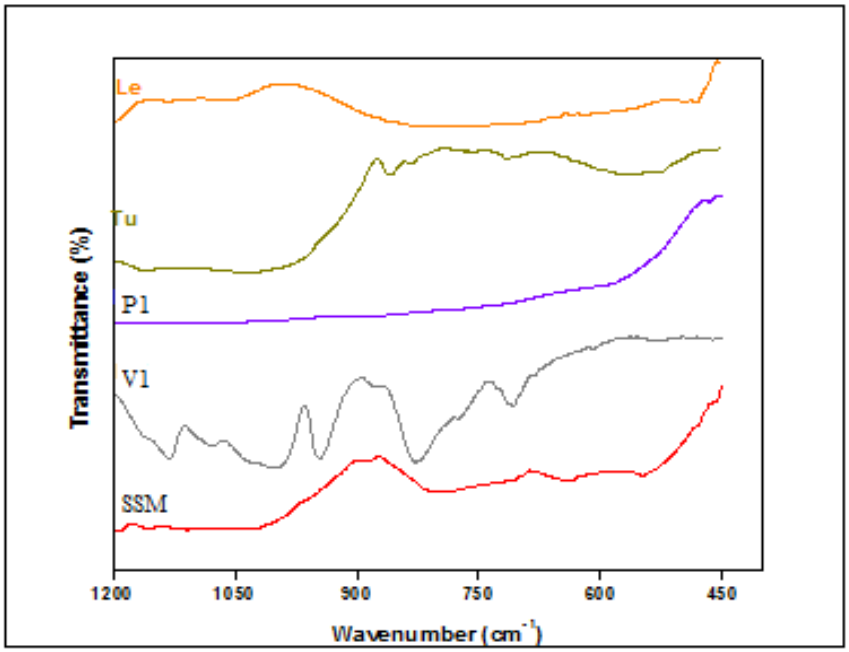


Fig. 22. FTIR spectra of lemon,turmeric, purified poorm.vengaram, SSM



Le-Lemon

Tu - Turmeric

Fig. 23. FTIR spectra of lemon,turmeric, purified poorm, vengaram, SSM (Wavelength 500-4000)

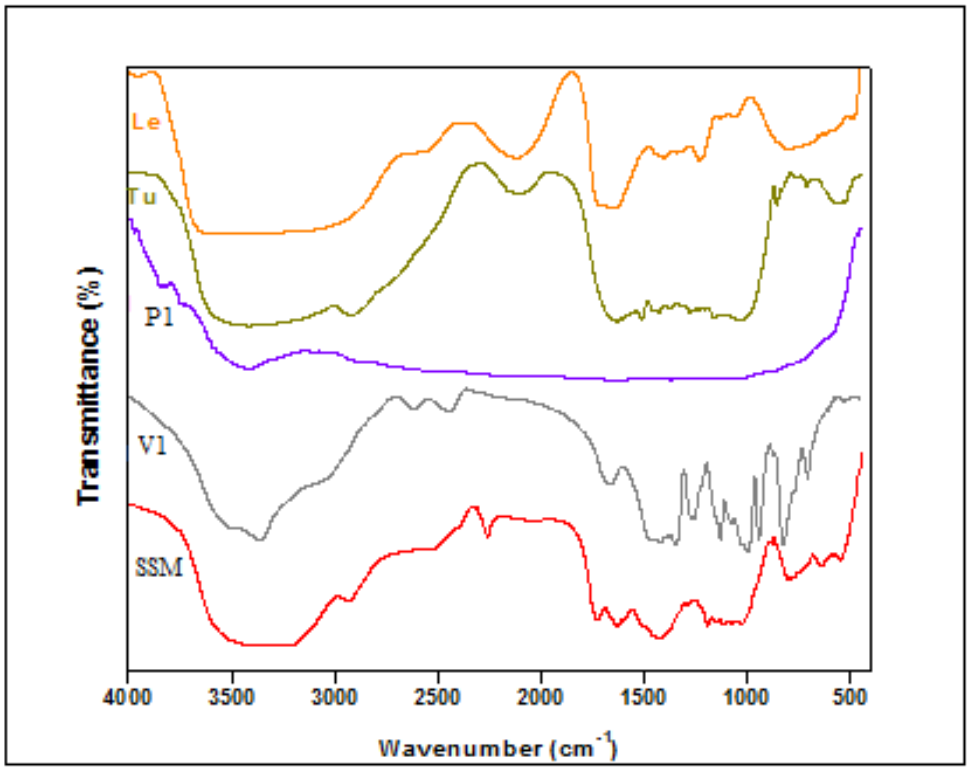


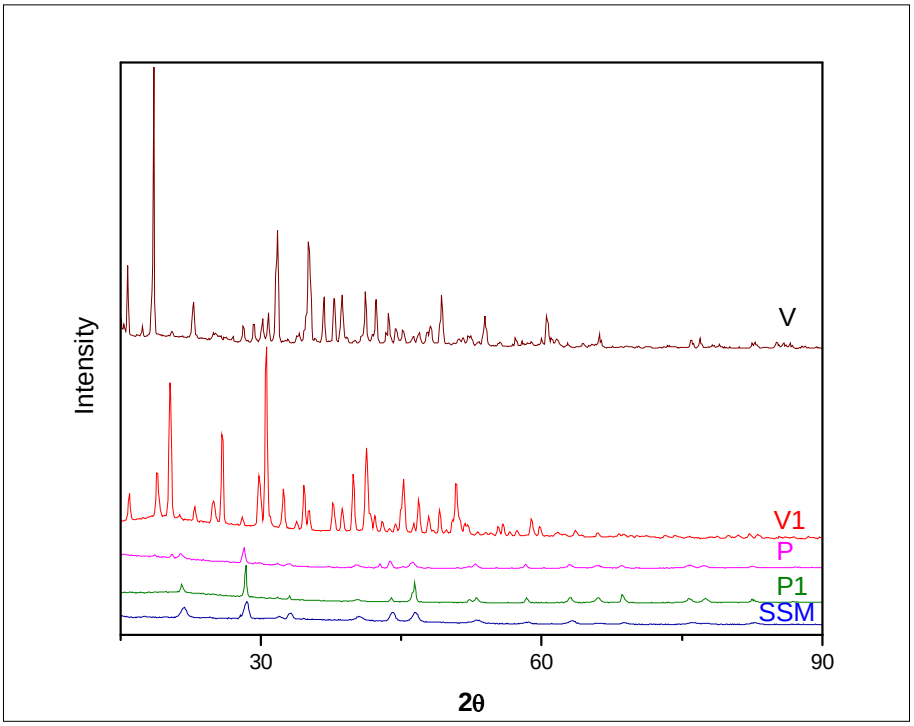
Fig.22,23 shows that the FTIR spectra of turmeric and lemon showed number of vibrations from organic substances. These includes C-H stretching (~2920), C=O stretching (~1633), C=C stretching (~1600),CC=O in plane vibration (~1510) , in plane bending of CH3 (~1450), Enolic bending or in plane C-H bending (~1380), C-O stretching (~1280), CH overtones (~1160), C-O-C stretching (~1030), C-H out of plane bending (~850) and C-C stretching (~760). Apart from these,spectra also showed the signatures of O-H stretching (3100-3500).

SSM showed the signatures of OH- stretching (3500- 3200, 2261), CH stretching (2926), C-O stretching (1731), C=O stretching (1627) andC=C stretching (1600). This also showed the presence of trigonalboran1427, 1298and tetrahedral boran (883, 808, 792, 780, 640, 574 and 549).However, when compared with the spectra of raw materials, peak at 1633, that represents C=O stretching gets shifted to 1627 in SSM. Moreover, peaks at 1510, 1380 disappears in SSM. The peak for O-H stretching in the SSM is shifted to higher wave number and also sharpening of the peak is observed at the region 3200-3500 cm^{-1}. This is an evidence of increased inter

molecular H bonding in SSM. Shift of peak in the range of 1000-4000cm-1 supports the formation of organo-mineral complex. The complex formation may be formed due to the interaction occurring between C=C, C=O, -OH, aromatic ring, etc. present in curcumin which is supported by shift in the C=O stretching of the complex.

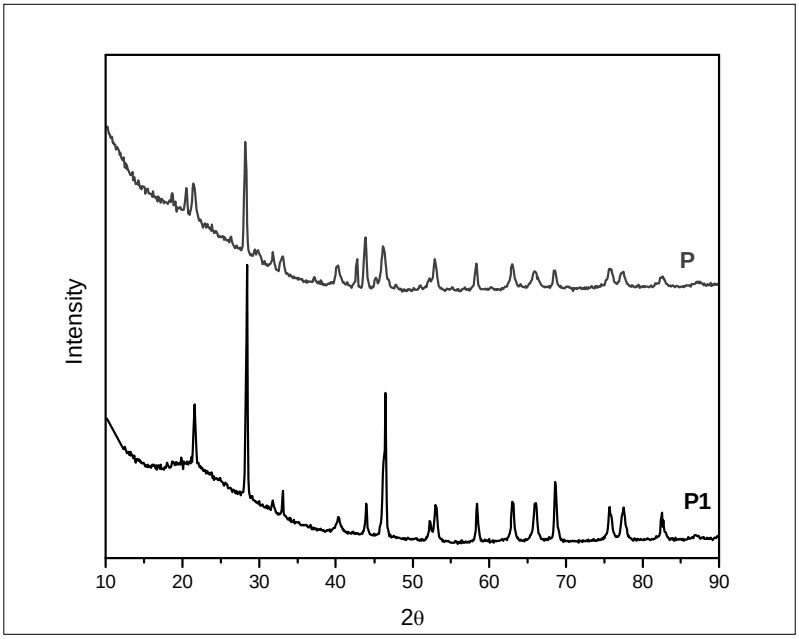
Though FTIR and Raman analysis showed the presence of organo-mineral complex, exact form of organo-mineral complex is still unknown. Based on the raw materials added there is a chance for formation of 2 forms of complexes. Either compounds of turmeric can form complex with boron or it can form complex with mercury in P1. There is also chance for formation of both types of complexes also depending on the environmental conditions.Inorder to determine the presence/ absence of organo-metallic complex, XRD was performed.

Fig. 24. XRD Graph of raw, purified samples and prepared SSM



From fig.24 graph,V showed the presence of rhombohedral form of $Na_2B_4O_7$ $10H_2O$– 15.7, 18.5, 22.8, 31.8, 35.09, 36.7, 37.7, 38.6, 41.1, 42.3, 43.6, 49.3, 53.9, 604. Whereas, V1showed the presence of orthorhombic form of $Na_2B_4O_7$ $5H_2O$ – 15.9, 18.8, 20.2, 25.8, 30.6, 32.4, 34.5, 37.6, 38.6, 39.8, 41.2, 45.2, 46.8, 47.9, 49.1 and 50.82.

Fig. 25: XRD graph of raw and purified pooram



P and P1showed (fig-25) the presence of Hg_2Cl_2(2theta: 21.54, 28.12, 33.00, 40.56, 44.25, 46.44, 53.01) with tetragonal shape. The increase in the intensity of 46.44 peak in PP signifies the change in crystalline nature, but the exact change could not be established. SSM showed a spectrum similar to that of P1. There was absence of organometallic complex, increase in the intensity of 44.5 peak and decrease in intensity of 46.44 peak in SSM compared with P1. Signatures of V1 are completely absent in SSM.

7.4.8 Scanning Electron Microscopy imaging of raw, purified (pooram, vengaram) and SSM

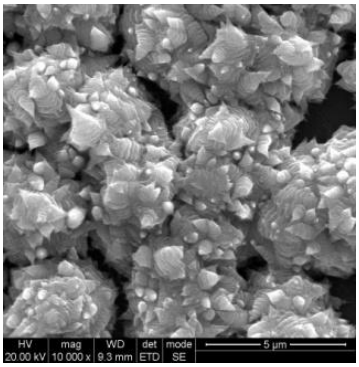
Fig 26.1 shows SEM image of Raw pooram with a particle size of 5 µm at a magnification of 10000. Fig 26.2 and Fig 26.3 shows that at a magnification of 10000 the particle size of purified pooram is 1µm. Both raw and purified pooram did not show any significant variation in SEM images. Both the samples showed approximately spherical shaped particles with lot of pointed projections from its surfaces.

Fig 26.4 shows SEM image of Raw vengaram with a particle size of 1µm at a magnification of 30000. Fig 26.5 and Fig 26.6 shows that at a magnification of 30000 and 60000 the particle size of purified vengaram is 1µm and 500 nm respectively. Both raw and purified venkaaram did not show any change in morphology of particles. They are approximately polygonal in shape.

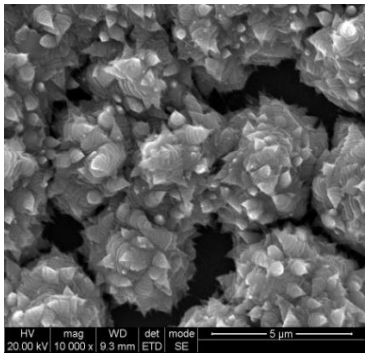
Fig 26.7,Fig 26.8 and Fig 26.9 shows SEM images of SSM with a particle size of 10 µm, 5 µm and 2 µm at a magnification of 5000, 10000 and 20000 respectively. Both raw and purified pooram did not show any significant variation in SEM images. Both the samples showed approximately spherical shaped particles of around 4-5 micros with lot of pointed projections from its surfaces. The SEM imageing was done on powdered SSM. All the three showed difference in morphology in powdered samples. SSM showed small tube like structures placed over a lump of sample. Specific internal morphology of the samples could not be determined by using SEM, due to the high aggregation of particles present in the sample.

Fig. 26: SEM imaging of raw, purified (pooram, vengaram) and SSM

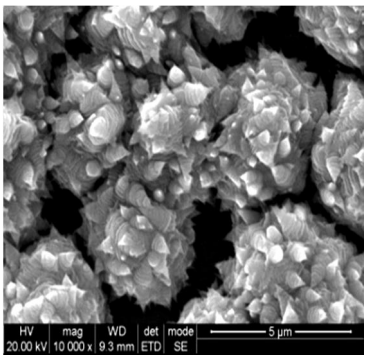
26.1 Raw pooram



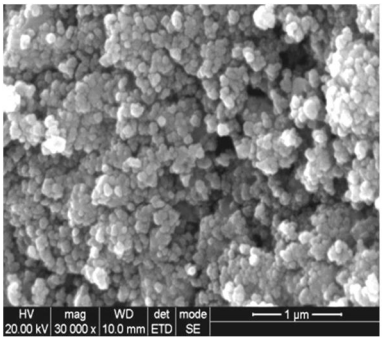
26.2 Purified pooram



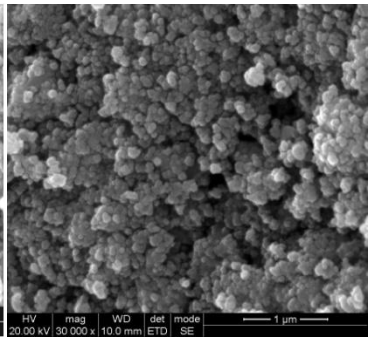
26.3 Purified pooram



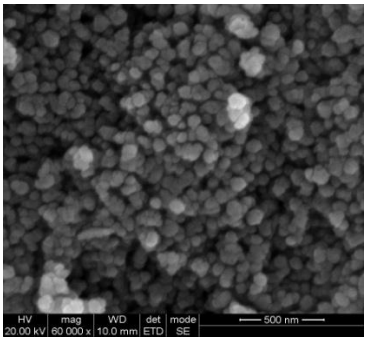
26.4 Raw Vengaram



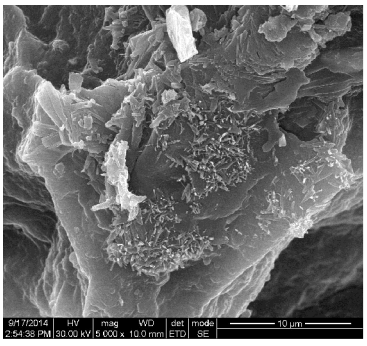
26.5 Purified vengaram



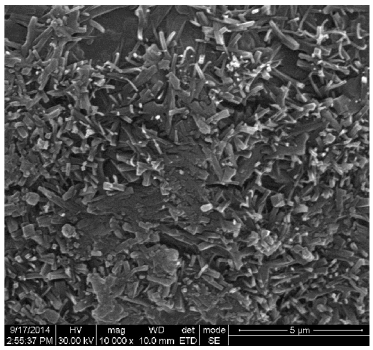
26.6 Purified vengaram



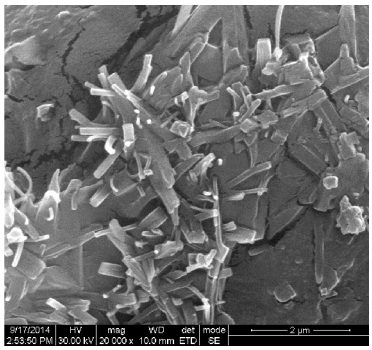
26.7 SSM



26.8 SSM



26.9 SSM



Further studies on elemental analysis of raw, purified and prepared sample of SSM were carried out using XRF, ICP-OES. Mecury content was analysed by titration method.

7.4.9 X-ray fluorescence (XRF) analysis of raw, purified (pooram, vengaram) and SSM

XRF analysis of raw pooram qualitates the presence of Hg(87.23%), Cl, MnO, Tl, Na_2O, SeO_2, SiO_2, CuO. Purified pooram contains the additional trace elements CaO, K_2O, Na_2O, SiO_2, P_2O_5, SO_3, MgO, Fe_2O_3 and Al_2O_3along with Hg(59.59%).

The XRF analysis reveals that the trace elements CuO and Tl were not detected in the purified pooram as well as in prepared medicine SSM. It has also shown that SSM contains newer trace elements such as AS_2O_3, MoO_3, ZnO in addition to CaO, K_2O, Na_2O, SiO_2, P_2O_5, SO_3, MgO, Fe_2O_3, Al_2O_3,AS_2O_3, MoO_3, ZnO along with Hg(58.82%), MnO and Cl. (Table 11 & Fig-27)

Table 11: X-ray fluorescence (XRF) analysis

S.NO	Formula	RP (%)	PP3 (%)	SSM (%)
1	Hg	87.23	59.59	58.82
2	Cl	11.94	6.45	6.55
3	Mno	0.30	0.19	0.10
4	Tl	0.19	-	0.08
5	Na_2O	0.13	6.13	4.83
6	Seo_2	0.10	-	-
7	Sio_2	0.07	2.63	2.33
8	CuO	0.03	0.05	-
9	K_2O	-	17.03	15.96
10	CaO	-	2.31	5.54
11	SO_3	-	1.66	1.68
12	P_2O_5	-	1.85	1.89
13	MgO	-	0.95	0.93
14	Fe_2O_3	-	0.54	0.62
15	PbO	-	0.39	-
16	Al_2O_3	-	0.22	0.20
17	Au	-	-	-
18	As_2O_3	-	-	0.16
19	MoO_3	-	-	0.13
20	ZnO	-	-	0.10

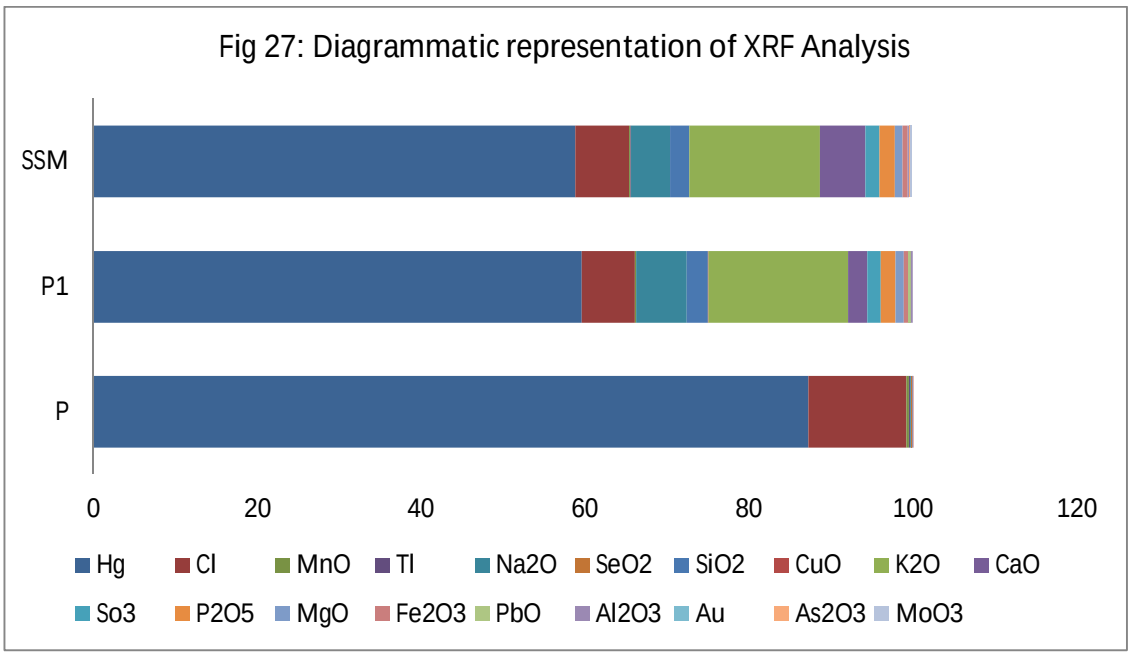


Fig 27: Diagrammatic representation of XRF Analysis

7.4.10 Inductively coupled plasma optical emission spectrometry (*ICP-OES*) analysis of SSM

The results of the ICP-OES analysis of raw, purified puram,vengaram and SSM are tabulated in table 12 which shows that the quantity of mercury and sodium have considerably reduced in SSM when compared to raw and purified samples. In addition there is presence of trace elements such as Ca, K, Fe, Mg., Mn and P in purified and prepared samples.

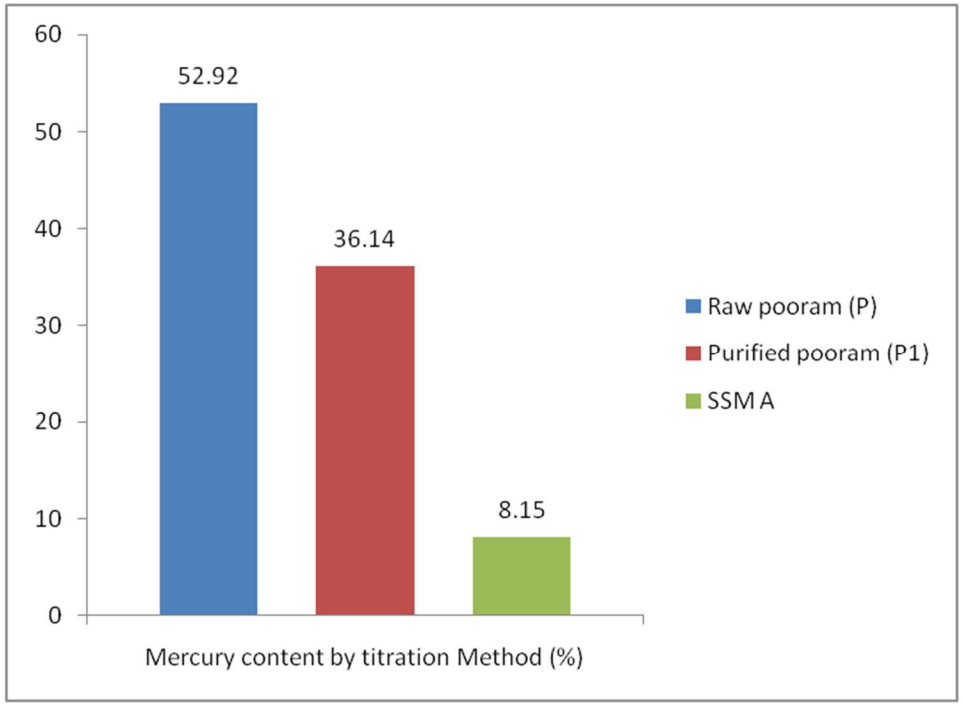
Table 12. ICP-OES analysis

Sl No	Element	Raw Pooram (ppm)	Purified Pooram(ppm)	Raw vengaram (ppm)	Purified vengaram(ppm)	SSM (ppm)
1	Hg	123.241	55.874	BDL	BDL	3.154
2	As	BDL	BDL	BDL	BDL	BDL
3	Cd	BDL	BDL	BDL	BDL	BDL
4	Pb	BDL	BDL	BDL	BDL	BDL
5	Na		23.110	545.862	203.110	125.383
6	Ca		14.150			4.150
7	K		70.821			10.821
8	Fe		2.38			0.380
9	Mg					1.020
10	Mn					1.321
11	P					8.541
12	Si					1.234
13	Zn					1.587

7.4.11 Mercury content of SSM by titration Method

The mercury content in the SSM has reduced considerebly from the raw and purified poram and prepared SSM which are seen in fig 28 where the values are 52.92%, 36.14%, 8.15% respectively.

Fig. 28. Mercury content by titration Method (%)



7.5 Toxicity Studies of SSM

The toxicity studies were carried out in experimental animal models to determine the safety potential of SSM. The results of acute, sub acute and subchronic toxicity are as follows:

7.5.1 Acute toxicity study of SSM

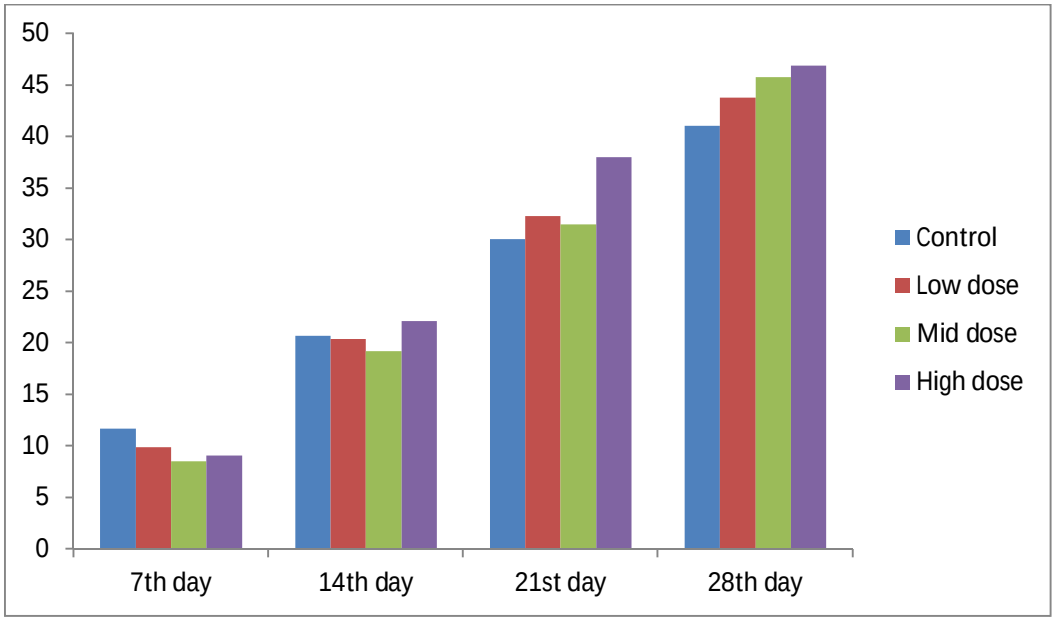
Acute toxicity of *SSM* does not produce any toxicity signs and mortality at the dose level of 2000mg/kg, b.wt in the animals during 14 days of the study.The results were shown in table.13. Further, the gross necropsy revealed no abnormalities in the internal organs of the experimental animals.

7.5.2 Repeated dose 28 days study of SSM (Sub acute)

In Sub acute toxicity study the animals were carefully monitored for 28 days.There was no behavioural abnormality and mortality throughout the study period. Body weight was gradually gained in *SSM* administered rats when compared with the vehicle treated rats but it is not statistically significant (Figure .29). No significant difference in food and water consumption was observed between the vehicle and *SSM* treated animals throughout the study.

7.5.2.1 Effect of SSM in body weight of rats – 28 days repeated oral toxicity study

Fig. 29: Effect of SSM in increase of body wt.in Wistar albino rats- 28 days repeated oral toxicity study



Values were expressed as mean±SEM. for n=10 rats in each group, Oneway ANOVA followed by Dunnet"s test.

7.5.2.2 Measurement of haematological, serum electrolytes, biochemical parameters and relative organ weights.

Haematological results were shown in fig.30.a, b& 31c,d. There was no change in any of the tested haematological parameters between the control and the SSM treated rats. No significant changes were seen in the serum Na, K, Ca**,** Cl, and pH between the control and SSM treated animals (fig.32.a, b). No significant

changes in the plasma biochemical parameters such as glucose, cholesterol, triglyceride, bilirubin and liver marker enzymes was observed during the administration of SSM for a period of 28 days . Renal parameters such as urea, creatinine, albumin and total protein did not exhibit any significant change in when compared to the vehicle control (fig.34.a, b).

Effects of SSM on relative organ weights were shown in fig 33. No statistically significant changes in the relative organ weights were observed between the vehicle and SSM treated rats.

7.5.2.3 Histopathology findings of SSM in 28 days repeated oral toxicity study

The gross pathological examination of the sub acute toxicity in organs did not reveal any abnormal changes. Histopathological results of brain, heart, liver, kidneys and spleen of control, mid dose, high dose SSM treated are as follows. (Fig. 35.c)

Fig.35.c.1 - Histopathology of Liver –control, mid dose and high dose SSM shows no abnormal deficits . Mild mononuclear infiltration seen in high dose group.

Fig.35.c.2 - Histopathology of brain(cerebrum) reveals no abnormal changes in mid dose and high dose group treated with SSM.

Fig.35.c.3- No histopathological change was observed in Kidney treated with mid and high dose of SSM

Fig.35.c.4 -Histopathology of stomach shows normal architecture with no changes in Control, mid dose and high dose of SSM treated rats.

Fig.35.c.5 –Normal histology was observed in spleen of SSM treated rats in both mid dose and high dose groups when compared with the control.

Fig.35.c.6 -Histopathology of lung shows no abnormal feature with normal histology in both mid dose and high dose treated groups.

Fig.34.g- No histopathological change was observed in heart treated with mid and high dose of SSM

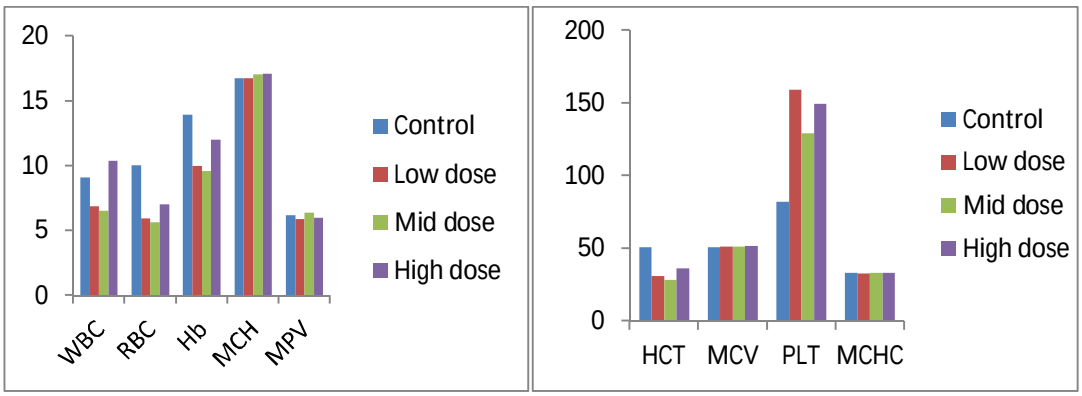
Table 13: Effect of SSM on behavioral Signs of rats in acute Toxicity Study

Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Control	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
SSM 2000mg/kg	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

X- Absent

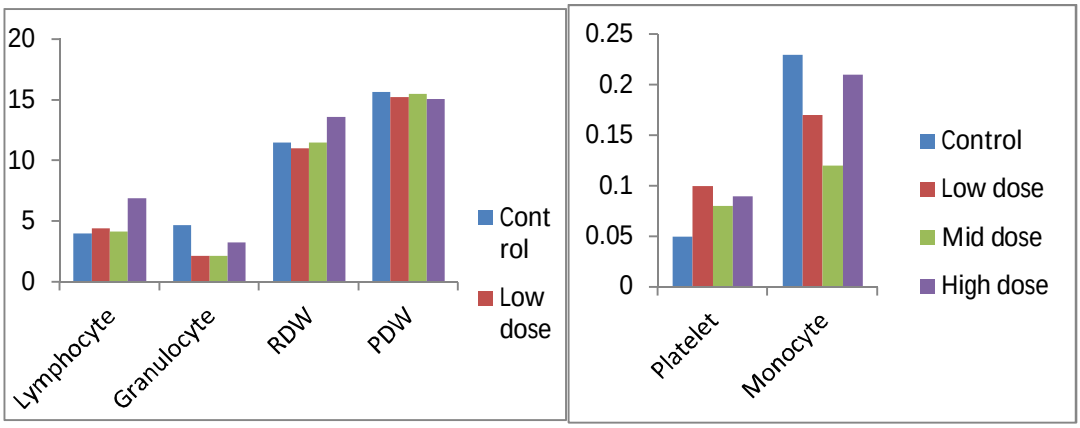
1. Lethality 2. Convulsion 3.Tremor 4. Sedation 5. Excitation 6.Abnormal gait 7.Motor coordination 8.Piloerection9. Head movements 10. Reactivity to touch 11.Diarrhoea 12. Salivation 13. Lacrimation 14.posture 15.dyspnea 16.coma.

Fig. 30a,b. Effect of SSM in haematological parameters in Wistar albino rats – 28 days repeated oral toxicity study



Values were expressed as mean±SEM. for n=10 rats in each group, Oneway ANOVA followed by Dunnet"s test.

Fig.31c,d. Effect of SSM in haematological parameters in Wistar albino rats – 28 days repeated oral toxicity study



Values were expressed as mean±SEM. for n=10 rats in each group, One way ANOVA followed by Dunnet"s test.

Fig.32a,b. Effect of SSMon serum electrolyte in Wistar albino rats

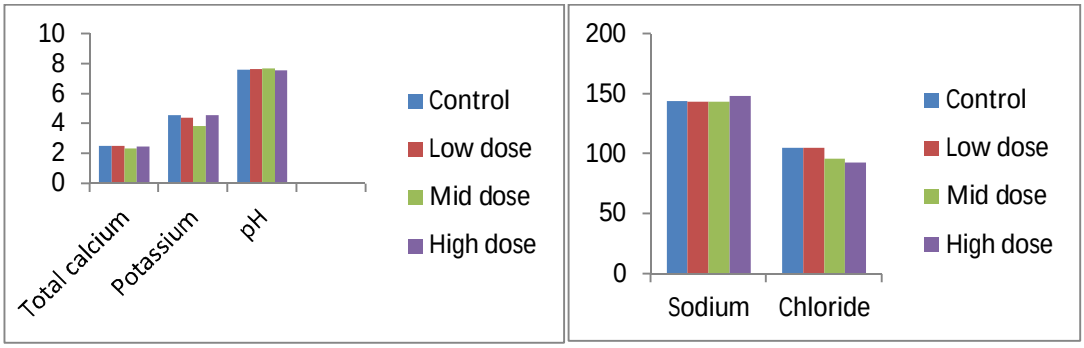
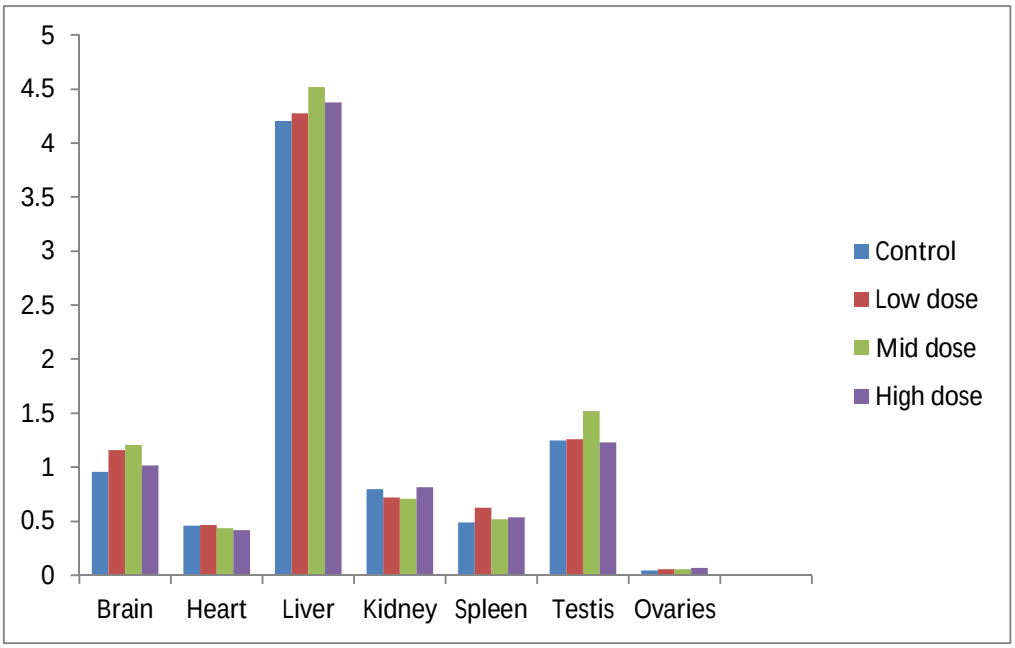


Fig.33. Effect of SSMon relative organ weights in Wistar albino rats



Values were expressed as mean±SEM. for n=10 rats in each group, Oneway ANOVA followed by Dunnet"s test.

Fig.34a. Effect of SSM on biochemical parameters in Wistar albino rats

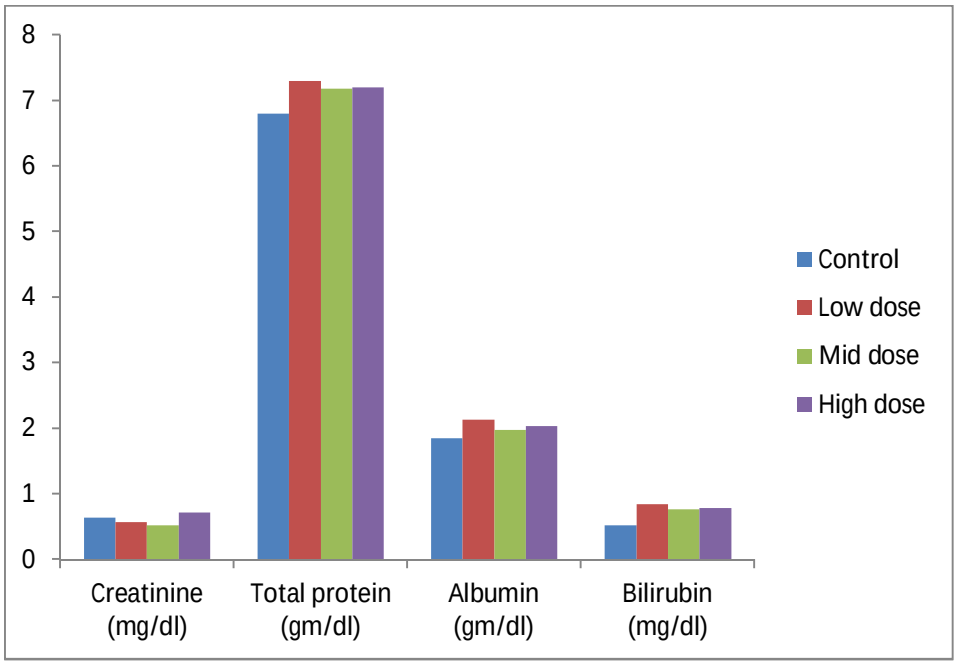
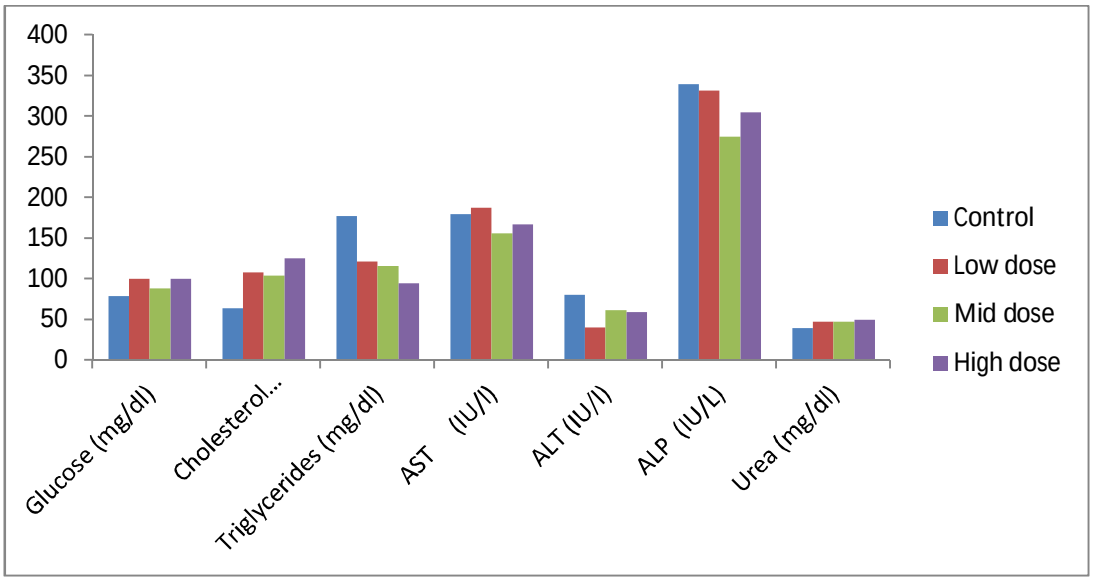


Fig.34b. Effect of SSM on biochemical parameters in Wistar albino rats



Values were expressed as mean±SEM. for n=10 rats in each group, Oneway ANOVA followed by Dunnet"s test.

7.5.3 Sub Chronic Toxicity Study of SSM

After completion of acute and subacute toxicity studies,the subchronic toxicity study was performed to determine the potential safety profile on long term administration of SSM. The following parameters were observed in this study.

7.5.3.1 Observation of behavioural signs in SSM treated rats

Table.14. showed the clinical observation of all the animals during the 90 days study period, no behavioural abnormality and mortality was noted. Mild sedative and moderate laxative action with fluffy faecal matter was noted in SSM low dose , high dose group including the post retrieval group without any significant weight loss**.**

Table 14. Effect of SSM on clinical Signs of rats in Sub-Chronic Toxicity Study

Clinical Signs Parameters	Group I Honey Control	Group II SSM 25 mg/kg Low dose	Group III SSM 125mg/kg Mid dose	Group IV SSM 250 mg/kg High dose	Group V SSM 250 mg/kg Post retrieval
Number of animals observed	5 Male 5 Female	5 Male 5 Female	5 Male 5 Female	5 Male 5 Female	5 Male 5 Female
Lacrimation	Absence	Absence	Absence	Absence	Absence
Salivation	Absence	Absence	Absence	Absence	Absence
Animal appearance	Normal	Normal	Normal	Normal	Normal
Tonic Movement	Absence	Absence	Absence	Absence	Absence
Clonic Movement	Absence	Absence	Absence	Absence	Absence
Laxative action	Absence	Mild	Moderate	Moderate	Moderate
Touch Response	Normal	Normal	Normal	Normal	Normal
Respiratory Distress	Nil	Nil	Nil	Nil	Nil
Skin Color	Normal	Normal	Normal	Normal	Normal
Stereotype behaviour	Absence	Absence	Absence	Absence	Absence
Piloerection	Absence	Absence	Absence	Absence	Absence
Limb Paralysis	Absence	Absence	Absence	Absence	Absence
Posture	Normal	Normal	Normal	Normal	Normal
Open field behaviour	Normal	Normal	Normal	Normal	Normal
Muscular coordination	Normal	Normal	Normal	Normal	Normal
Muscle grip	Normal	Normal	Normal	Normal	Normal
Sedation	Absence	Absence	Absence	Very mild	Mild
Social Behaviour	Normal	Normal	Normal	Normal	Normal
Urine Analysis	No Abnormality	No Abnormality	No Abnormality	No Abnormality	No Abnormality
Fecal Pellet consistency	Normal	Soft blobs	Soft blobs	Fluffy	Soft blobs
Mortality	Nil	Nil	Nil	Nil	Nil

7.5.3.2 Observation of food and water intake in SSM treated rats

There was significant increase in food and water intake in SSM treated male and female rats both in mid dose (125mg/kg), high dose (250mg/kg) and post retrieval group when compared with the control **(Table-15 &16)**.

Table 15. Effect of SSM on Food intake of Male and Female Rats in Sub-Chronic Toxicity Study

Days	Control (Honey 2ml)	*SSM*			
		Low dose	Mid dose	High dose	Post retrieval
Week 2	17 ± 1	18.67 ± 1.52	16.67 ± 1.15	22 ± 2.64**	16.33 ± 1.53
Week 4	15.86 ± 3.72	21.43 ± 1.27	19 ± 2.23**	18.57 ± 0.57	18.43 ± 2.37
Week 6	18.57 ± 4.9	21.86 ± 1.06	19.14 ± 1.34	22.71 ± 0.75	18.57 ± 2.76
Week 8	18.14 ± 4.9	19.57 ± 0.97	19.86 ± 1.34	21.57 ± 1.25	19.86 ± 2.55
Week 10	20 ± 3.16	19.86 ± 1.21	21.29 ± 1.79	21.71 ± 1.25	20.43 ± 2.51
Week 12	17.57 ± 4.58	20.71 ± 0.75	23.86 ± 0.89**	16 ± 1.41	21.43 ± 2.07
Week 13	19.67 ± 4.16	20.33 ± 2.51	20.67 ± 0.89	17.67 ± 0.57	22.43 ± 1.90
Week 15	-	-	-	-	23 ± 1.63
Week 17	-	-	-	-	23 ± 0.81
Effect of SSM on Food intake of Female Rats in Sub-Chronic Toxicity Study					
Week 2	21.23 ± 2.08	18.67 ± 1.52	16.67 ± 1.15	22 ± 2.64**	19.33 ± 1.53
Week 4	21.57 ± 1.81	21.43 ± 1.27	19 ± 2.23	18.57 ± 1.51*	20.14 ± 1.35
Week 6	20.86 ± 3.38	21.86 ± 1.06	19.14 ± 1.34	22.71 ± 0.75	20.57 ± 0.78
Week 8	19.43 ± 1.81	19.57 ± 0.97	19.86 ± 1.34	21.57 ± 0.97	22 ± 0.81
Week 10	22.43 ± 1.81	19.86 ± 1.21*	21.29 ± 1.79	21.71 ± 1.25	22.71 ± 0.76
Week 12	20.86 ± 1.46	19.71 ± 0.71	23.86 ± 0.89	16 ± 1.41**	23.71 ± 1.38
Week 13	16.67 ± 1.15	20.33 ± 2.51**	20.87 ± 0.57**	17.67 ± 0.57	24.14 ± 1.07
Week 15	-	-	-	-	24.29 ± 1.11
Week 17	-	-	-	-	24.75 ± 0.96

Values were expressed as mean ± S.D (n = 5). P value was calculated using one way ANOVA followed by Dunnett test. Significance was indicated as *p < 0.05 and **p < 0.01 on compared with control group.

Table 16. Effect of SSM on Water intake of Male and Female Rats in Sub-Chronic Toxicity Study

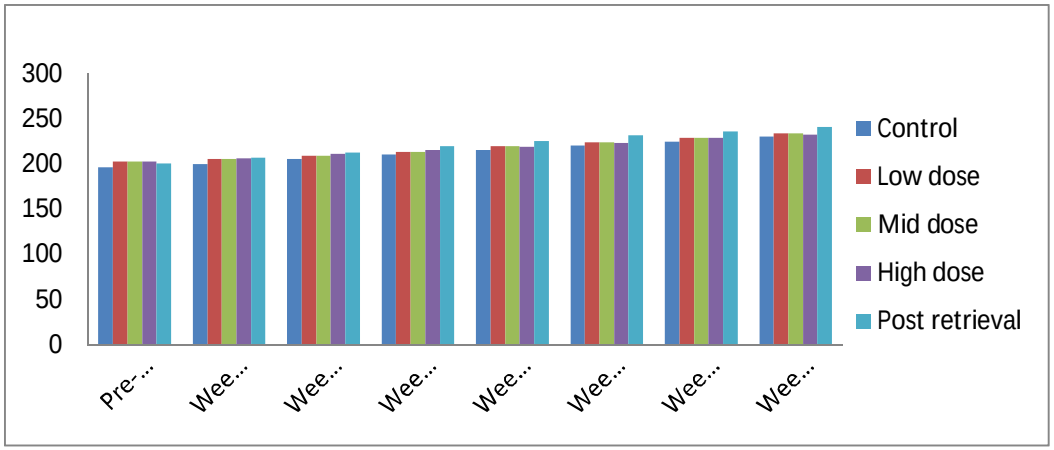
Days	Control (Honey 2ml)	*SSM*			
		Low dose	**Mid dose**	**High dose**	**Post retrieval**
Week 2	32 ± 3.6	33.67 ± 2.51	30 ± 2	30.33 ± 2.08	31 ± 1.15
Week 4	34.29 ± 4.07	37.71 ± 1.89	38.71 ± 3.90	35.57 ± 5.19	35.29 ± 0.76
Week 6	35.43 ± 4.89	36.86 ± 3.33	37.29 ± 5.52	32.43 ± 2.07	36.43 ± 2.23
Week 8	34.29 ± 2.43	34 ± 2.16	40.29 ± 3.59**	33 ± 2.58	38.14 ± 2.12*
Week 10	33.71 ± 2.43	41 ± 3.33**	37 ± 3.23	36.86 ± 2.26	39 ± 1.16**
Week 12	36.43 ± 3.04	40.71 ± 2.75	36.57 ± 2.07	40.71 ± 3.03	39.29 ± 2.06
Week 13	36.67 ± 1.5	40.67 ± 3.05*	36.33 ± 1.52	41.33 ± 1.52**	40.71 ± 2.93*
Week 15	-	-	-	-	42.57 ± 1.51
Week 17	-	-	-	-	41 ± 1.15
Effect of SSMon Water intake of Female Rats in Sub-Chronic Toxicity Study					
Week 2	33 ± 1.73	28.67 ± 2.51**	32 ± 2	30.67 ± 2.51*	32 ± 1.73
Week 4	34.14 ± 5.27	32.14 ± 3.02	28.57 ± 4.37	33.86 ± 2.61	32.14 ± 1.95
Week 6	41.86 ± 3.07	33 ± 3.05 **	30.14 ± 3.33**	39.71 ± 4.38	33 ± 2.83**
Week 8	37.57 ± 3.2	37.71 ± 4.751	27.71 ± 2.56**	42.57 ± 2.37	35 ± 2.04
Week 10	35.14 ± 2.96	39.29 ± 2.81	27.43 ± 2.99**	38.86 ± 2.61	36.29 ±2.93
Week 12	39.97 ± 3.59	39.57 ± 1.71	34.7 ± 2.81*	36.43 ± 2.07	38.14 ± 2.55
Week 13	32.67 ± 2.08	38.33 ± 1.52**	35.67 ± 2.08	29.67 ± 2.51*	39.57 ± 2.23
Week 15	-	-	-	-	41.29 ± 1.80
Week 17	-	-	-	-	42.75 ± 2.63

Values were expressed as mean ± s.d (n = 5). p value was calculated using one way anova followed by dunnett test. significance was indicated as *p < 0.05 and **p < 0.01 on compared with control group

7.5.3.3a. Body weight changes in SSM treated Male rats

There was gradual increase in body weight in low dose,mid dose, high dose and post retrieval group when compared with control. The results are expressed in fig.35a.

Fig.35a. Effect of SSM in body weight of Male rats

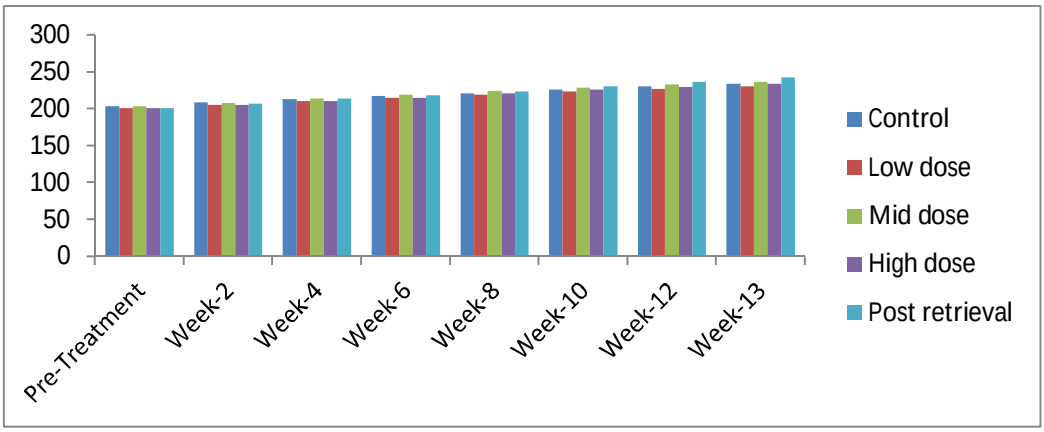


Values were expressed as mean±SEM. for n=10 rats in each group, Oneway ANOVA followed by Dunnet"s test.

7.5.3.3b Body weight changes in SSM treated female rats

There was gradual increase in body weight in low dose,mid dose, high dose and post retrieval group when compared with control. The results are shown in fig.35b.

Fig. 35b.Effect of SSM in body weight of Female rats



Values were expressed as mean±SEM. for n=10 rats in each group, Oneway ANOVA followed by Dunnet"s test.

7.5.3.4 Haematological parameters of SSM treated animals in Subchronic toxicity study

The results of haematological parameters are summarised in the **Table 17,18**. There was significant increase in packed cell volume and neutrophil count in both male and female rats in mid and high doses of SSM when compared with the control. However the RBC count was significantly increased only in male rats on high dose of SSM when compared with the control. No significant change was observed in any of the haematological parameters between the control and the SSM treated rats.The post retrieval group shows a closer resemblance with the control group parameters.

Table 17. Effect of SSM on Haematology profile of Male Rats in Sub-Chronic Toxicity Study

Parameter	Control (Honey 2ml)	*SSM*			
		Low dose	Mid dose	High dose	Post retrieval
WBC count ($\times 10^3$ µl)	9.26 ± 1.21	11.06 ± 2.50	10.58 ± 1.74	12.42 ± 3.9	9.04 ± 2.22
RBC ($\times 10^6$ µl)	7.14 ± 1.42	7.63 ± 0.91	7.16 ± 2.02	18.60 ± 1.19**	7.12 ± 0.68
PLT ($\times 10^3$ µl)	564 ± 2.37	797 ± 1.42**	559.4 ± 6.23	621.4 ± 8.78**	542 ± 1.00**
MCV (fl)	55.14 ± 3.91	61.52 ± 7.01	59.68 ± 9.30	57.08 ± 6.66	55.96 ± 2.46
MCH (pg)	18.88 ± 1.74	20.54 ± 3.09	19.22 ± 4.26	19.2 ± 2.77	19.36 ± 2.73
MCHC (g/dl)	32.48 ± 2.08	34.02 ± 2.56	31.86 ± 2.33	31.08 ± 3.26	30.08 ± 1.79
HGB (g/dl)	14.04 ± 1.60	14.02 ± 2.94	14.37 ± 3.60	15.4 ± 1.65	12.9 ± 0.95
Lymph (%)	71.92 ± 6.15	76.2 ± 7.25	72.22 ± 18.8	66.72 ± 5.63	71 .62 ± 4.33
Mon (%)	4.74 ± 2.04	3.44 ± 1.30	2.22 ± 0.85*	4.04 ± 1.44	3.46 ± 0.94
Gran (%)	25.62 ± 6.13	10.8 ± 6.48**	26.47 ± 6.03	27.94 ± 4.34	25.84 ± 3.89
MPV (fl)	5.2 ± 1.06	5.12 ± 1.46	6.38 ± 1.52	4.94 ± 0.47	4.38 ± 1.26
RDW (%)	13.26 ± 1.90	12.98 ± 3.44	12.24 ± 1.67	14.38 ± 3.19	10.6 ± 1.54
HCT (%)	42.56 ± 7.34	42.56 ± 6.85	38.44 ± 9.38	39.39 ± 6.67	40.22 ± 4.79
PDW	13.9 ± 3.99	13.28 ± 1.96	14.44 ± 2.50	16.72 ± 1.66	13.48 ± 1.65
Packed cell volume(%)	46.33 ± 4	56.46 ± 6.55	69.82 ± 8.43**	69.78 ± 8.30**	55.48 ± 6.25
Neutrophils (%)	70 ± 7.77	68.66 ± 3.83	70 ± 9.10	70.74 ± 9.15	70.84 ± 9.61

Values were expressed as mean ± S.D (n = 5). P value was calculated using one way ANOVA followed by Dunnett test. Significance was indicated as *p < 0.05 and **p < 0.01 on compared with control group.

Table 18. Effect of SSM on Haematology profile of Female Rats in Sub-Chronic Toxicity Study

Parameter	Control (Honey 2ml)	*SSM*			
		Low dose	**Mid dose**	**High dose**	**Post retrieval**
WBC count ($\times 10^3$ µl)	8.82 ± 1.53	11.16 ± 4.62	11.82 ± 2.27	14.54 ± 1.96*	8.84 ± 1.11
RBC ($\times 10^6$ µl)	7.64 ± 1.13	6.58 ± 1.3	7.12 ± 1.47	7.56 ± 1.25	6.86 ± 1.06
PLT ($\times 10^3$ µl)	550.8 ± 2.26	592.2 ± 2.08**	560 ± 1.24**	617.6 ± 3.09**	657.2 ± 3.63**
MCV (fl)	54.83 ± 3.48	49.6 ± 4.14	60.26 ± 5.51	54.4 ± 3.75	57.36 ± 4.09
MCH (pg)	20.32 ± 2.88	17.02 ± 1.84	18.77 ± 3.15	16.36 ± 1.62	21.06 ± 2.13
MCHC (g/dl)	32.58 ± 1.80	30.82 ± 3.69	33.21 ± 2.52	31.82 ± 2.03	31.82 ± 3.82
HGB (g/dl)	13.08 ± 2.30	13.1 ± 2.18	13.96 ± 1.91	14.36 ± 3.16	13.18 ± 1.29
Lymph (%)	71.14 ± 3.87	75.68 ± 6.39	72.14 ± 7.10	66.02 ±13.45	71.1 ± 5.78
Mon (%)	4.08 ± 0.80	4.2 ± 1.21	3.06 ± 1.66	3.48 ± 1.62	3.7 ± 0.98
Gran (%)	22.18 ± 7.07	24.3 ± 6.55	23.22 ± 5.96	22.96 ± 4.66	27.88 ± 2.42
MPV (fl)	5.42 ± 1.08	5.16 ± 1.23	5.4 ± 1.33	5.28 ± 1.07	5.56 ± 1.42
RDW (%)	12.2 ± 1.37	11.61 ± 1.93	12 ± 1.26	11.92 ± 1.21	12.9 ± 2.22
HCT (%)	42.42 ± 6.17	43.6 ± 5.07	36.52 ± 6.14	40.74 ± 7.7	39.48 ± 5.03
PDW	15.2 ± 2.96	12.27 ± 2.83	16.26 ± 1.35	14.24 ± 3.06	15.68 ± 1.45
Packed cell volume(%)	47.42 ± 2.23	48.28 ± 5.03	54.25 ± 6.75	61.5 ± 12.63*	63.66 ± 5.05*
Neutrophils (%)	70.14 ± 6.42	74.06 ± 4.56	56.66 ± 8.23*	69.98 ± 7.49	67.7 ± 2.79

Values were expressed as mean ± S.D (n = 5). P value was calculated using one way ANOVA followed by Dunnett test. Significance was indicated as *$p < 0.05$ and **$p < 0.01$ on compared with control group.

7.5.3.5 Biochemical parameters of SSM treated animals in Subchronic toxicity study

Biochemical parameters of SSM treated animals in Subchronic toxicity study showed no significant changes. The liver marker enzymes were decreased on SSM treatment in both male and female groups of which AST showed a significant decrease on SSM treated male rats on high dose. Post retrieval group in both male and female group showed significant decrease in liver parameter ALT and there was increase in total protein when compared to the control. No significant changes were observed in any of the renal parameter BUN and serum creatinine. Total protein showed an increase in low dose of SSM (within physiological limits) in female rats when compared with the control but no such increase was observed in mid and high doses of SSM (**Table 19& 20**).

Table 19. Effect of SSM on Bio-chemical profile of Male Rats in Sub-Chronic Toxicity Study

Parameter	Control (Honey 2ml)	SSM			
		Low	Mid	High	Post retrieval
B.sugar ® (mg/dl)	88.8 ± 12.4	90 ± 21.11	92.87 ± 23.47	88.6 ± 13.94	96.6 ± 13.35
BUN (mg/dl)	12.6 ± 3.20	18.2 ± 3.49	17 ± 5.61	10.6 ± 2.68	14 ± 2.74
S.creatinine (mg/dl)	1.18 ± 0.5	1.04 ± 0.27	0.62 ± 0.44	0.78 ± 0.38	0.58 ± 0.16
T.cholesterol(mg/dl)	97.2 ± 10.94	114.4 ±19.77	87.6 ± 21.49	123.4 ± 24.75	107.2± 11.73
triglycerides (mg/dl)	96 ± 12.41	88 ± 13.66	75.8 ± 23.08	84.6 ± 17.247	9.04 ± 4.04
HDL (mg/dl)	62.8 ± 15.69	66.4 ± 11.1	53.6 ± 9.83	62.4 ± 10.45	69.2 ± 5.72
LDL (mg/dl)	45.8 ± 18.91	69.6 ± 13.59*	43.6 ± 9.55	45.6 ± 14.43	55.6 ± 5.59
VLDL (mg/dl)	17.8 ± 4.20	17.4 ± 3.05	16.64 ± 3.84	17 ± 3.93	13.6 ± 2.41
T.protein (g/dl)	6.76 ± 1.75	5.76 ± 2.05	5.1 ± 0.96	5.34 ± 1.67	5.8 ± 0.33
albumin (g/dl)	3.68 ± 1.26	4.34 ± 11.26	4.04 ± 0.52	4.1 ± 0.78	3.72 ± 0.55
(AST) (IU/ml)	106 ± 28.83	128.2 ± 11.26	101 ± 18.18	72.4 ± 8.38**	120 ± 4.06
(ALT) (IU/L)	28.2 ± 3.49	23.8 ± 7.53	26.4 ± 7.64	29.4 ± 7.98	18.2 ± 1.64*
(ALP) (IU/L)	109.6 ± 16.8	113.2 ± 18.36	101.2 ± 19.87	95 ± 29.18	119.4 ± 14.4

Values were expressed as mean ± S.D (n = 5). P value was calculated using one way ANOVA followed by Dunnett test. Significance was indicated as *p < 0.05on compared with control group.

Table.20.Effect of SSM on Bio-chemical profile of Female Ratsin Sub-Chronic Toxicity Study

Parameter	Control (Honey 2ml)	SSM			
		Low	Mid	High	Post retrieval
B.sugar ® (mg/dl)	71.6 ± 7.50	96 ± 26.62	85.6 ± 18.39	99 ± 17.13*	90 ± 21.11
BUN (mg/dl)	18.4 ± 5.81	16.4 ± 7.86	19.6 ± 6.80	17.2 ± 4.97	13.4 ± 1.94
S.creatinine (mg/dl)	0.82 ± 0.33	1.22 ± 0.39	0.68 ± 0.22	0.62 ± 0.37	0.68 ± 0.16
T.cholesterol(mg/dl)	115.4 ± 21.34	106.2 ± 21.52	106.2 ± 12.4	118.4 ± 12.88	101.2± 7.4
triglycerides (mg/dl)	96.8 ± 18.7	76.4 ± 21.62	67 ± 24.4	85.2 ± 15.19	79.6 ± 9.86
HDL (mg/dl)	52.6 ± 13.63	67.6 ± 8.62	59.2 ± 11.45	58.4 ± 8.76	65.8 ± 6.22
LDL (mg/dl)	36.2 ± 21.88	52.2 ± 10.8	52 ± 13.55	37.2 ± 12.07	56.2 ± 7.22
VLDL (mg/dl)	19.2 ± 4.02	194 ± 5.22	15.22 ± 2.28	17.2 ± 2.78	15.4 ± 2.70
T.protein (g/dl)	3.7 ± 0.62	5.16 ± 1.19	5.62 ± 0.96*	4.62 ± 1.20	5.18 ± 0.76*
albumin (g/dl)	3.58 ± 1.08	3.9 ± 0.75	4.16 ± 1.06	4.12 ± 0.72	3.76 ± 0.36
(AST) (IU/ml)	101.4 ± 13.65	117.4 ± 8.50	86.8 ± 19.79	99.6 ± 28.29	120.4 ± 6.35
(ALT) (IU/L)	35.4 ± 9.78	34.6 ± 10.09	22.4 ± 5.64	27.2 ± 6.83	20.6 ± 2.97*
(ALP) (IU/L)	129 ± 51.51	82 ± 17.42	100.2 ± 21.32	102.6 ± 28.1	122 ± 4.95

Values were expressed as mean ± S.D (n = 5). P value was calculated using one way ANOVA followed by Dunnett test. Significance was indicated as *p < 0.05 on compared with control group.

7.5.3.6 Individual organ weight of SSM treated animals in subchronic toxicity study

No statistically significant changes were observed in the individual organ weights were observed between the vehicle and all group of SSM treated rats. The values showed in Table.21,22.

Table 21. Quantitative data on absolute organ weight of Female Rats in Sub-Chronic Toxicity Study

Organs	Control (Honey 2ml)	SSM			
		Low	Mid	High	Post retrieval
Heart (gms)	0.69 ± 0.08	0.61 ± 0.08	0.58 ± 0.10	0.58 ± 0.097	0.66 ± 0.11
Liver (gms)	5.74 ± 0.54	5.44 ± 0.59	4.38 ± 0.72**	5.38 ± 0.57	5.9 ± 0.31
Kidneys (gms)	1.174 ± 0.05	1.21 ± 0.04	1.21 ± 0.04	1.20 ± 0.03	1.14 ± 0.03
Spleen (gms)	0.41 ± 0.07	0.42 ± 0.07	0.4 ± 0.05	0.39 ± 0.05	0.33 ± 0.04
Brain (gms)	1.88 ± 0.18	1.64 ± 0.18	1.98 ± 0.11	1.91 ± 0.19	1.72 ± 0.15
Lung (gms)	1.07 ± 0.14	1.08 ± 0.09	1.07 ± 0.09	1.08 ± 0.14	1.03 ± 0.07
Stomach (gms)	0.81 ± 0.08	0.81 ± 0.07	0.81 ± 0.08	0.78 ± 0.08	0.76 ± 0.04
Uterus + ovary (gms)	0.74 ± 0.12	0.72 ± 0.10	0.72 ± 0.08	0.79 ± 0.05	0.84 ± 0.23

Table.22.Quantitative data on absolute organ weight of Male Rats in Sub-Chronic Toxicity Study

Organs	Control (Honey 2ml)	SSM			
		Low	Mid	High	Post retrieval
Heart (gms)	0.71 ± 0.08	0.60 ± 0.06	0.59 ± 0.10	0.55 ± 0.10*	0.61 ± 0.07
Liver (gms)	5.02 ± 0.73	4.82 ± 0.51	4.96 ± 0.45	5 ± 0.97	6.16 ± 0.78
Kidneys (gms)	1.14 ± 0.11	1.21 ± 0.05	1.22 ± 0.08	1.28 ± 0.15	1.15 ± 0.03
Spleen (gms)	0.38 ± 0.07	0.38 ± 0.08	0.41 ± 0.07	0.40 ± 0.08	0.35± 0.03
Brain (gms)	1.82 ± 0.25	1.73 ± 0.10	1.97 ± 0.22	1.93 ± 0.24	1.82 ± 0.18
Lung (gms)	1.09 ± 0.15	1.07 ± 0.08	1.10 ± 0.09	1.05 ± 0.04	1.01 ± 0.06
Stomach (gms)	0.78 ± 0.05	0.84 ± 0.03	0.84 ± 0.07	0.77 ± 0.08	0.80 ± 0.05
Testes (gms)	2.17 ± 0.19	2.07 ± 0.33	2.03 ± 0.14	1.97 ± 0.20	1.67 ± 0.05

Values were expressed as mean ± S.D (n = 5). P value was calculated using one way ANOVA followed by Dunnett test. Significance was indicated as *p < 0.05 and **p < 0.01 on compared with control group.

7.5.3.7 Histopathological findings of SSM in subchronic toxicity study

After sacrificing the animal, gross pathological changes were observed in all the animals. No abnormal findings were observed. Male and female rats belonging to Group I(control), IV(SSM High dose) and Group V(Post retrieval) were subjected to histopathological analysis for recording the organo toxicity pertaining to the usage of high dose of test drug SSM 250mg/kg.

Table 23. Histopathological examination of control and SSM treated groups

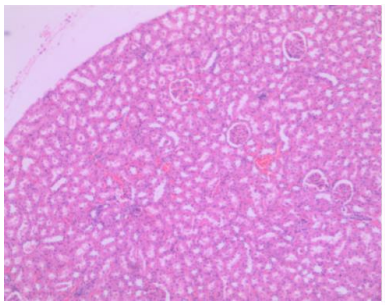
Group	Treatment	Gender	Organs subjected to Histological Examination
GROUP I	Honey 5 ml/kg b.w	Male	kidney, liver, brain, heart, lung, spleen, stomach and testes
GROUP I	Honey 5 ml/kg b.w	Female	kidney, liver, brain, heart, lung, spleen, stomach, ovary and uterus
GROUP IV	SSM 250 mg/kg b.w	Male 1	kidney, liver, brain, heart, lung, spleen, stomach and testes
GROUP IV	SSM 250 mg/kg b.w	Male 2	kidney, liver, brain, heart, lung, spleen, stomach and testes
GROUP IV	SSM 250 mg/kg b.w	Female 1	kidney, liver, brain, heart, lung, spleen, stomach, ovary and uterus
GROUP IV	SSM 250 mg/kg b.w	Female 2	kidney, liver, brain, heart, lung, spleen, stomach, ovary and uterus
GROUP V	SSM 250 mg/kg b.w	Male 1	kidney, liver, brain, heart, lung, spleen, stomach and testes
GROUP V	SSM 250 mg/kg b.w	Female 1	kidney, liver, brain, heart, lung, spleen, stomach, ovary and uterus

Histopathological changes in Kidney

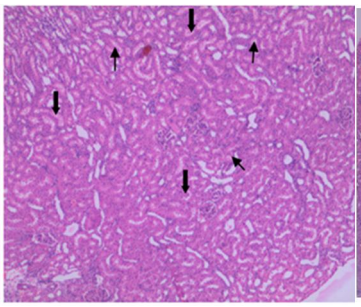
The light microscopy examinations of section of the kidney belonging to group IV treated with 250 mg/kg of test drug revealed the presence of minimal renal tubular degeneration followed by the presence of renal tubular cast when compared to that of the control group I.The changes are marked as black arrow marks in the fig-36. Group VI were treated with the standard drugs LIV 52/silymarin In post retrieval group, interstitial connective tissue and glomeruli appears normal.

Fig. 36. Histopathology of Kidney

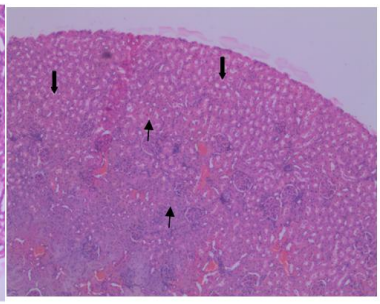
GROUP I (Male) 10X



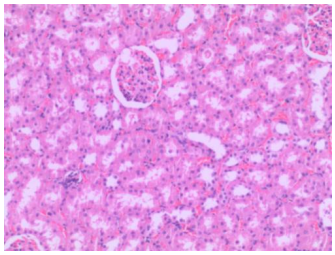
GROUP IV (Male 1) 10X



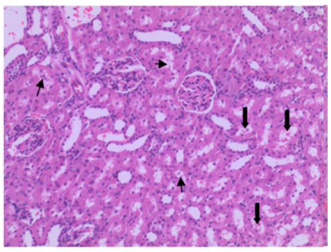
GROUP IV (Male 2) 10X



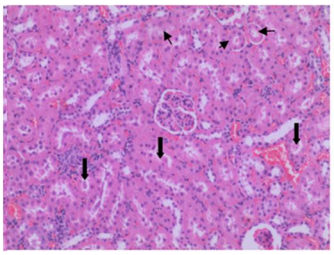
GROUP I (Male) 40X



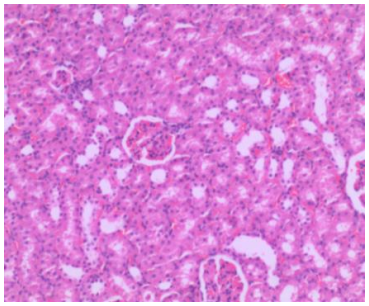
GROUP IV (MALE 1) 40X



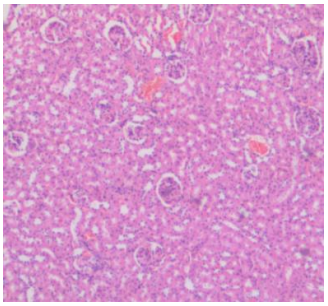
GROUP IV (MALE 2) 40X



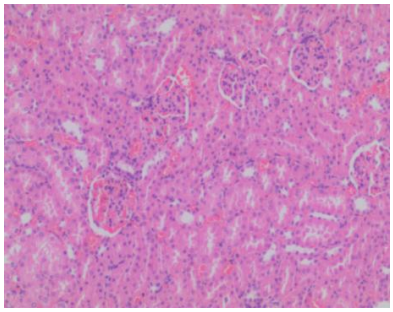
GROUP I (Male) 40X



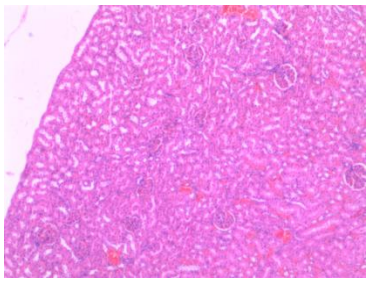
GROUP V (MALE) 10X



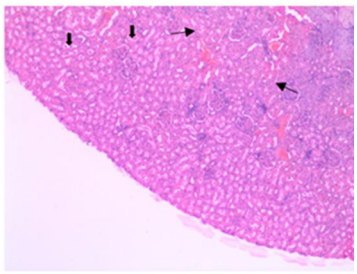
GROUP V (MALE) 40X



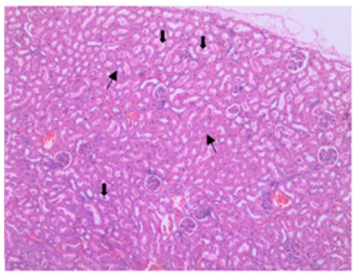
GROUP I (Female)
10X



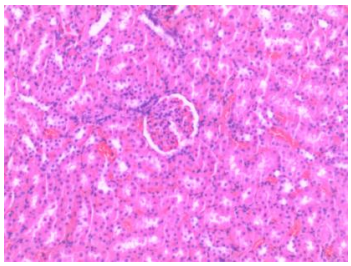
GROUP IV (Female 1)
10X



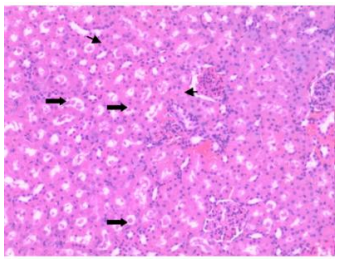
GROUP IV (Female 2)
10X



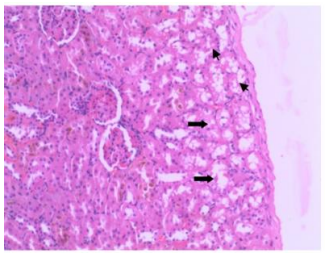
GROUP I (Female)
40X



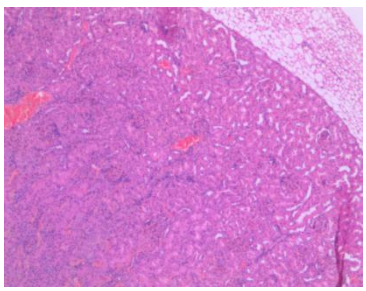
GROUP IV (Female 1)
40X



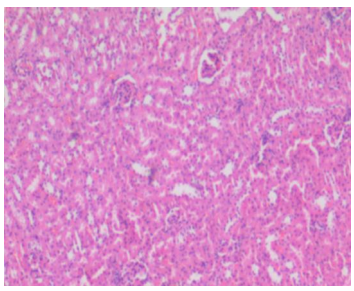
GROUP IV (Female 2)
40X



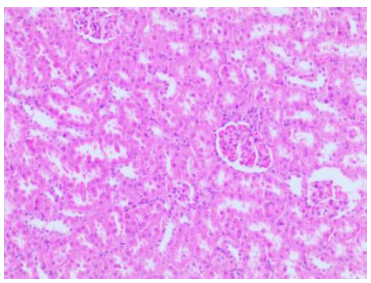
GROUP I (Female)
10X



GROUP V (Female)
10X



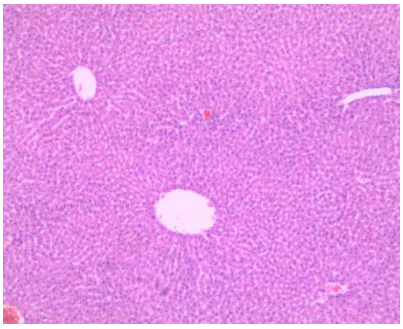
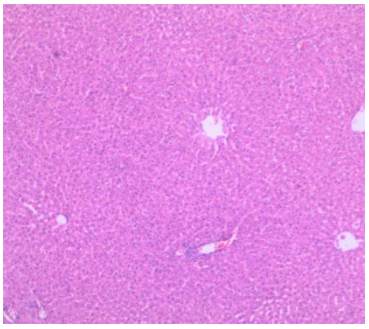
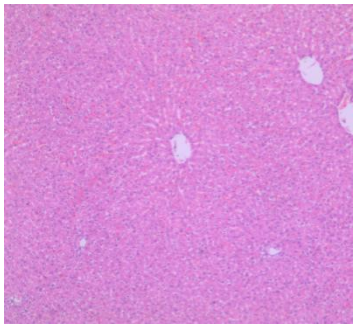
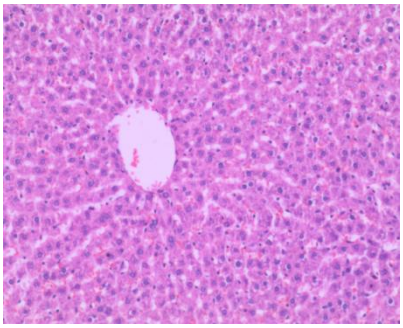
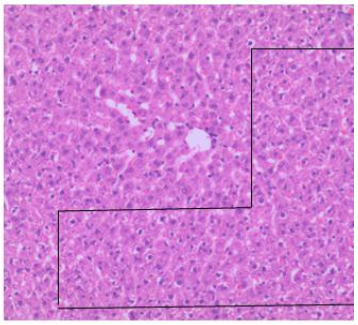
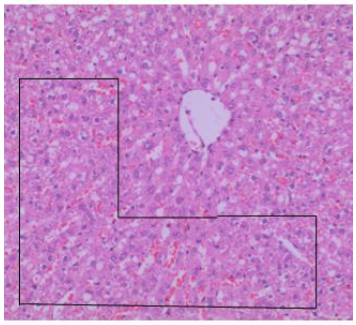
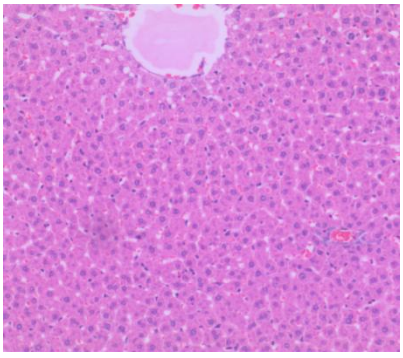
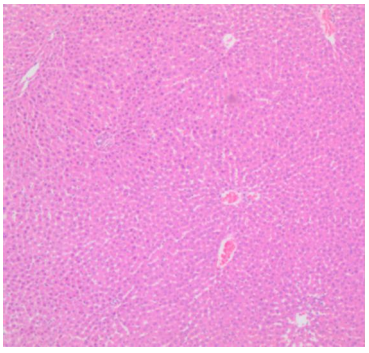
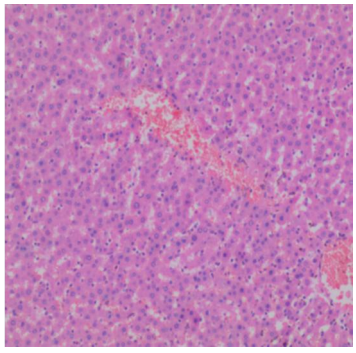
GROUP V (Female)
40X



Histopathological changes in Liver

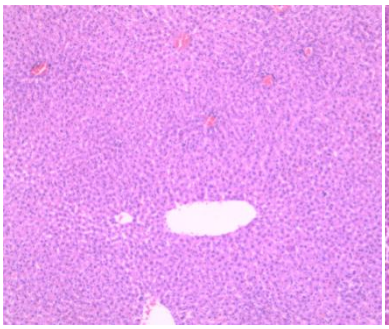
Microscopic section of liver samples belonging to group IV treated with 250 mg/kg of SSM revealed the presence of mild centrilobular necrosis associated with dilation of sinusoidal space when compare to that of the control group I**(Fig 37).** In post retrieval group, Hepatocellular architecture, including hepatic sinusoid and hepatic cord were normal in both male and female samples

Fig. 37. Histopathology of Liver

GROUP I (Male) 10X	GROUP IV (Male 1) 10X	GROUP IV (Male 2) 10X
		
GROUP I (Male) 40X	**GROUP IV (MALE 1) 40X**	**GROUP IV (MALE 2) 40X**
		
GROUP I (Male) 40X	**GROUP V (MALE) 10X**	**GROUP V (MALE) 40X**
		

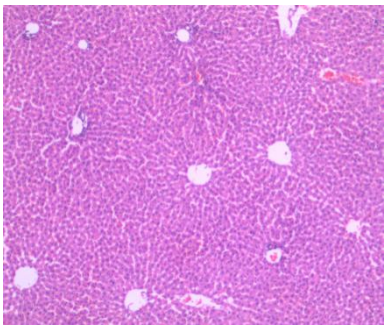
GROUP I (Female)

10X



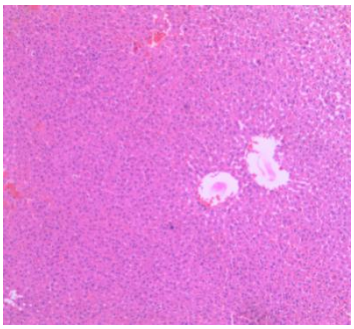
GROUP IV (Female 1)

10X



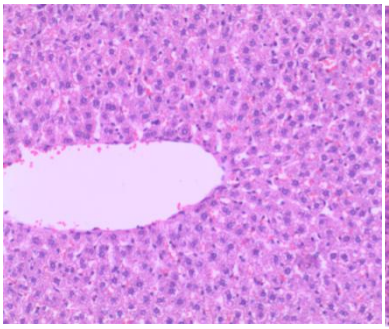
GROUP IV (Female 2)

10X



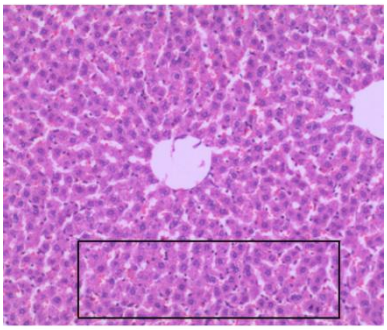
GROUP I (Female)

40X



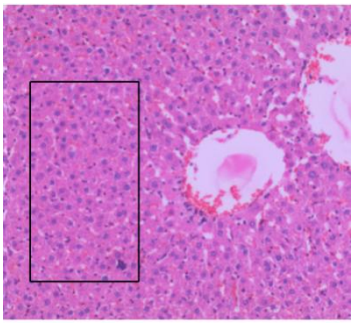
GROUP IV (Female 1)

40X



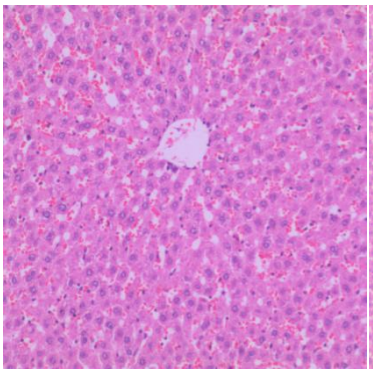
GROUP IV (Female 2)

40X



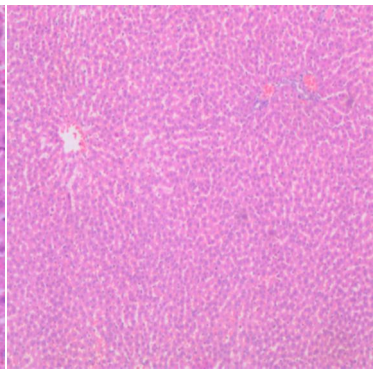
GROUP I (Female)

40X



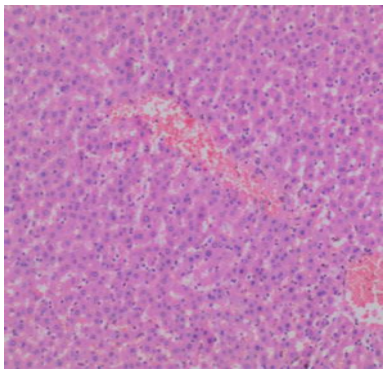
GROUP V (Female)

10X



GROUP V (Female)

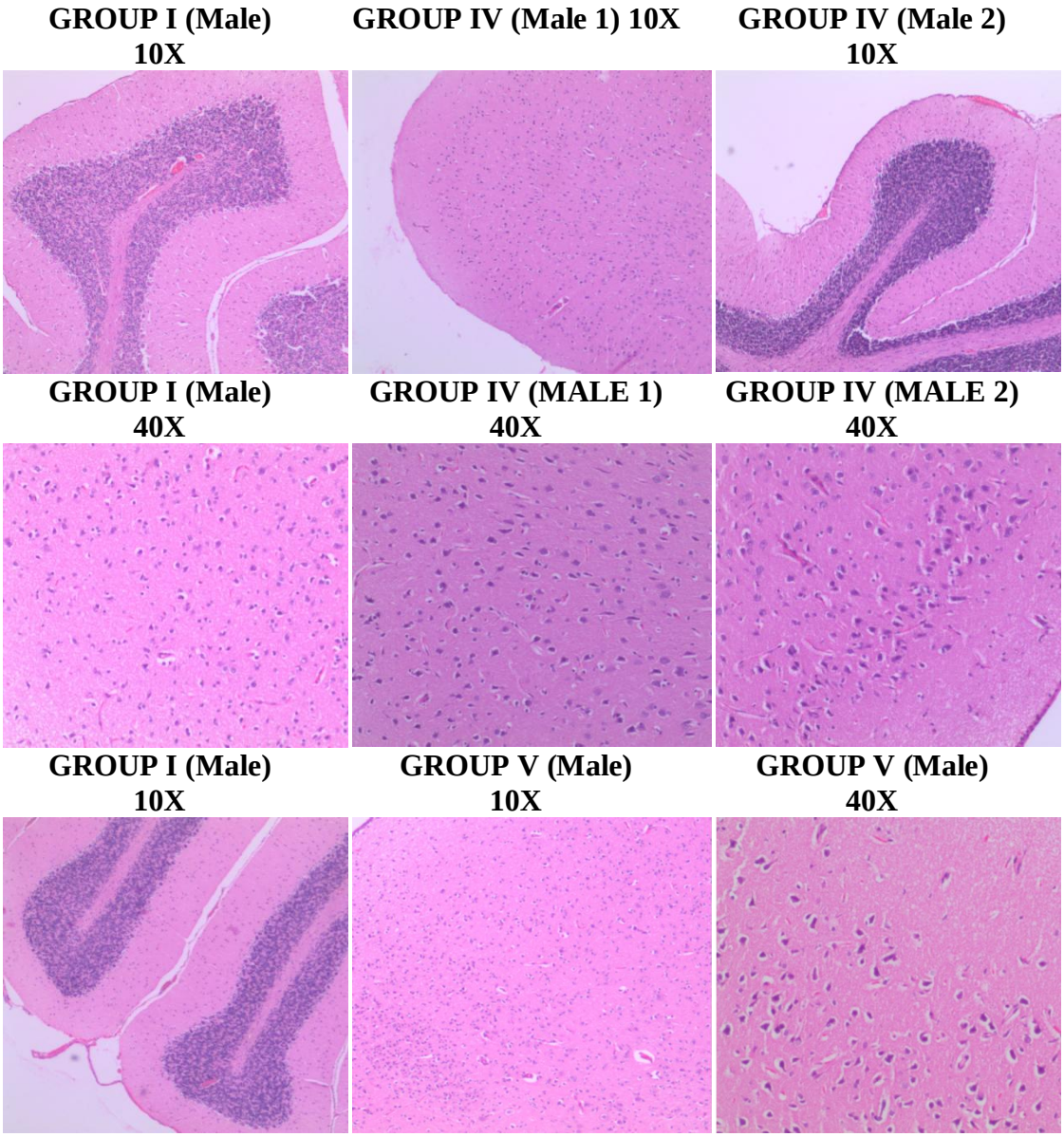
40X



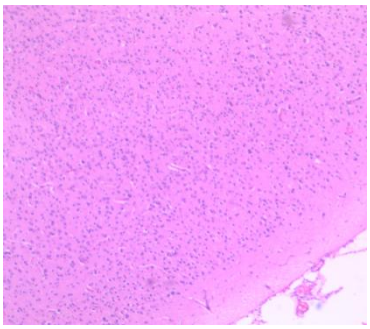
Histopathological changes inBrain

Fig 38 shows regular marginal alignment on the neurons with promising histology . Neurons are very intact and there were no signs of edema or degeneration in brain sample belongs to group IV treated with 250 mg/kg of test drug when compared to that of the control group I**.** Whereas the arrangement of the neurons appears intact with no sings of degeneration or apoptotic changes in both the samples of post retrieval group with normal neurons and dense cytoplasm in cortex region.

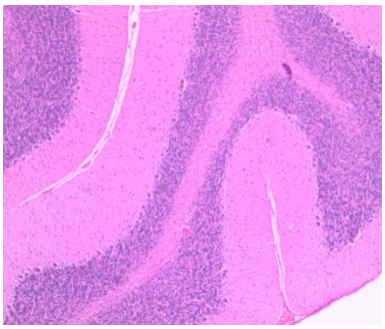
Fig. 38. Histopathology of Brain



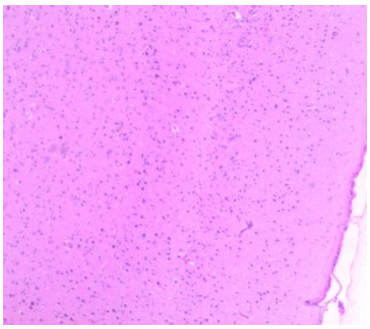
GROUP I (Female)
10X



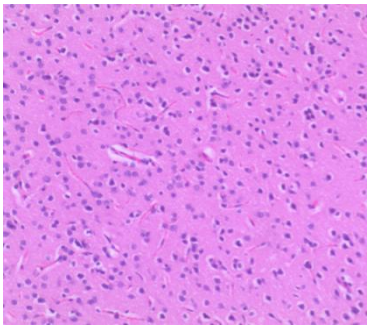
GROUP IV (Female 1)
10X



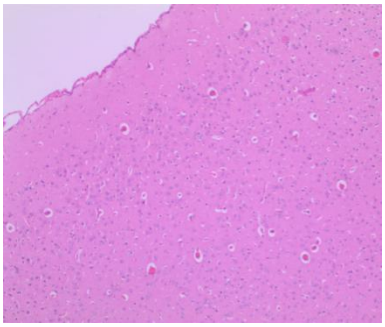
GROUP IV (Female 2)
10X



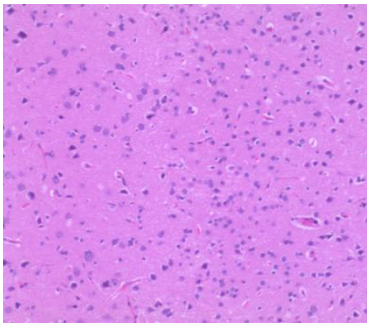
GROUP I (Female)
40X



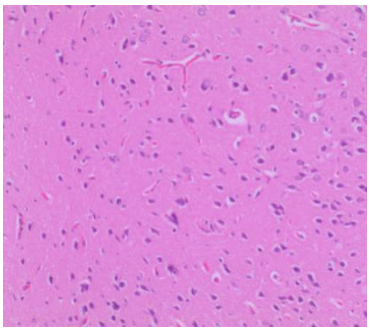
GROUP IV (Female 1)
40X



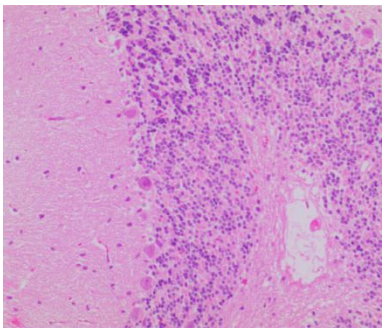
GROUP IV (Female 2)
40X



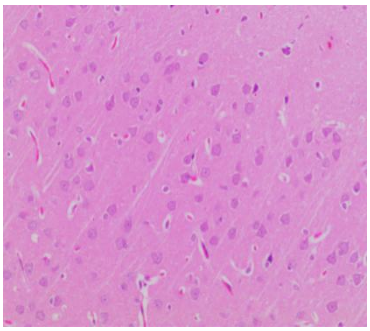
GROUP I (Female)
40X



GROUP V (Female)
10X



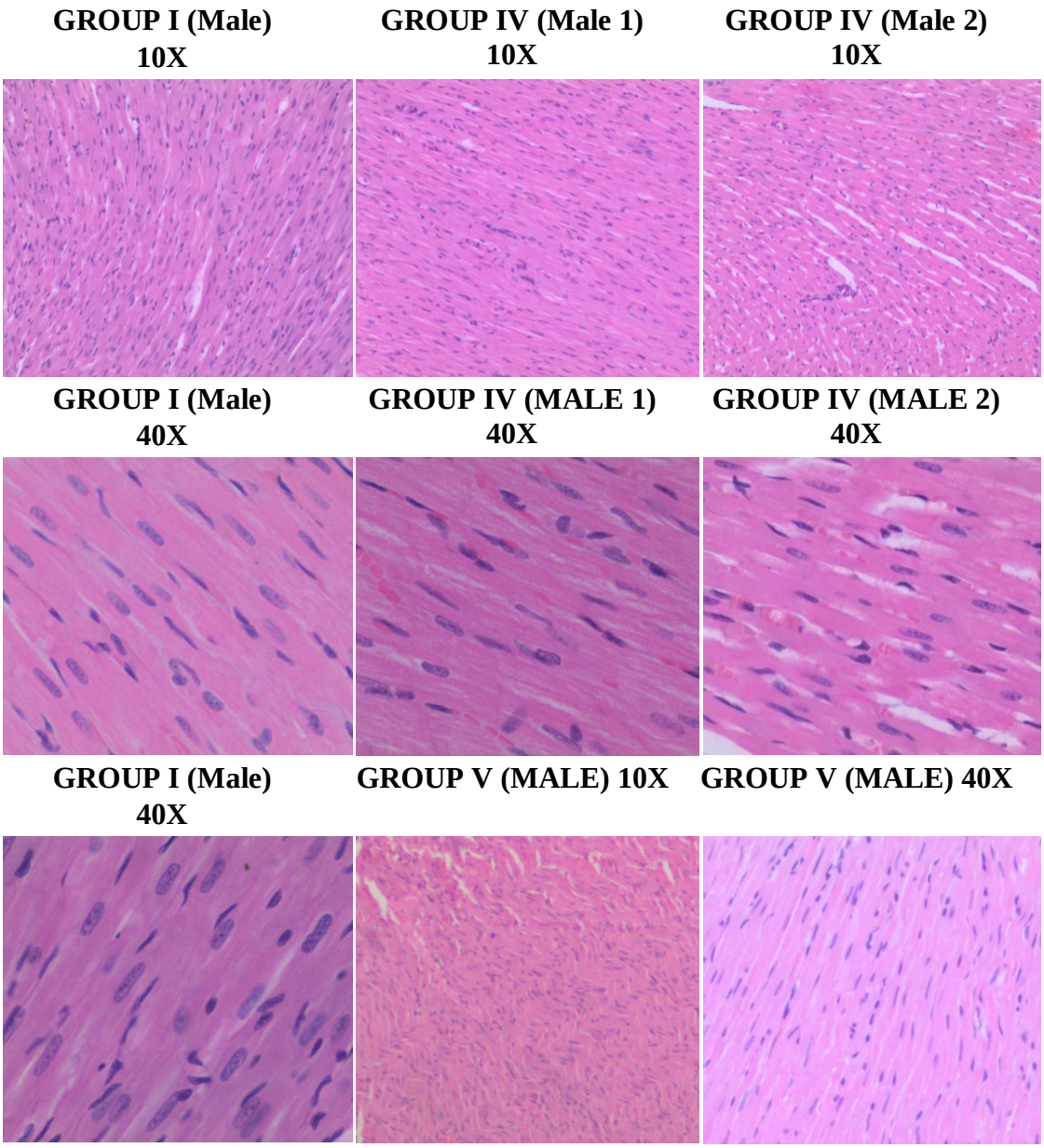
GROUP V (Female)
40X



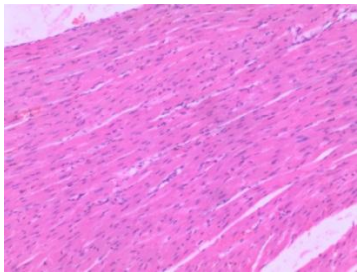
Histopathological changes in Heart

Fig 39 explains the left ventricular and atrial wall samples appear normal in group IV treated with 250 mg/kg of test drug when compared to that of the control group I. Further Sample reveals the presence of normal cardiac fiber with myofibrillar striations. No sign of lesion and infarction. In post retrieval group, arrangement of cardiac muscle fibers was normal in both male and female heart sample. Myocardial tissue appears normal with orderly striated heart muscle fibers.

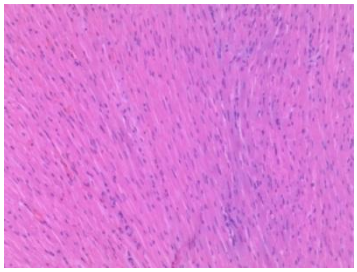
Fig. 39. Histopathology of Heart



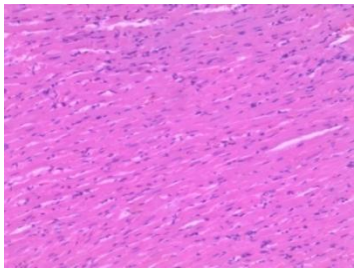
GROUP I (Female)
10X



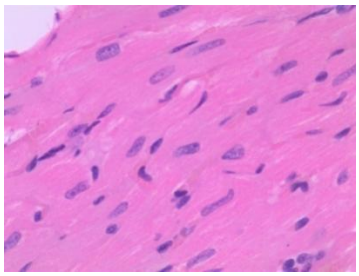
GROUP IV (Female 1)
10X



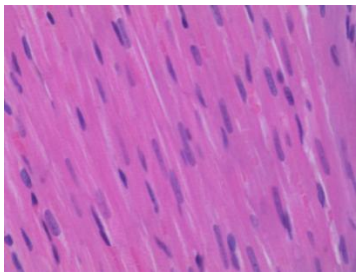
GROUP IV (Female 2)
10X



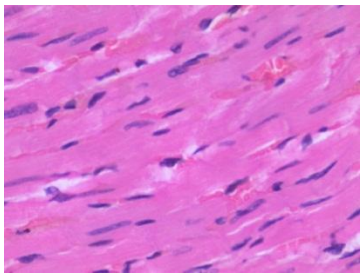
GROUP I (Female)
40X



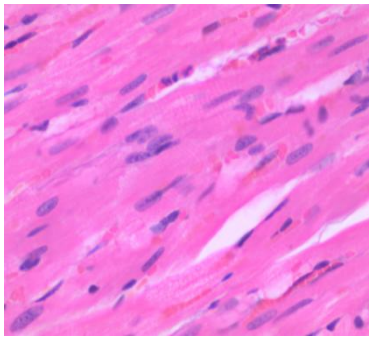
GROUP IV (Female 1)
40X



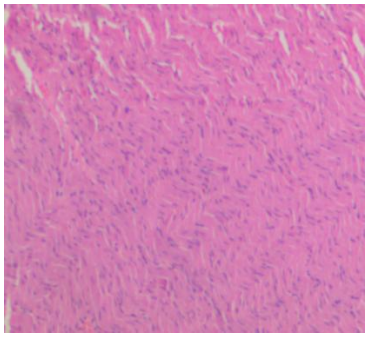
GROUP IV (Female 2)
40X



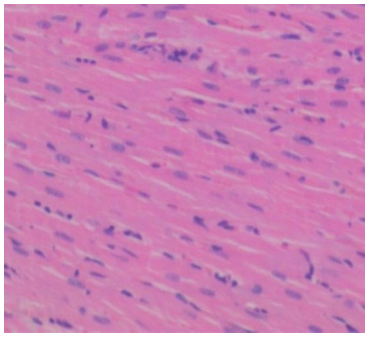
GROUP I (Female)
40X



GROUP V (Female)
10X



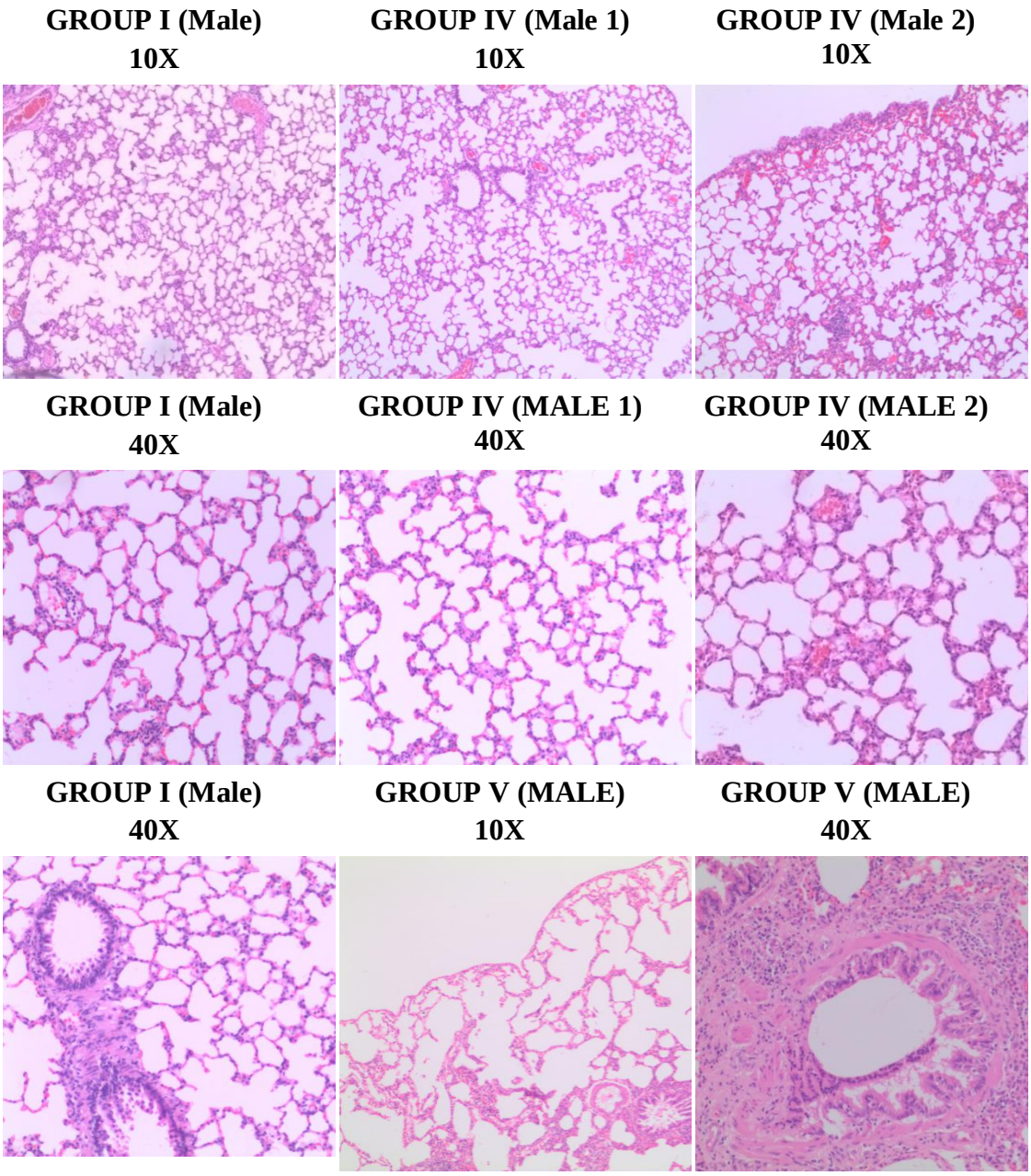
GROUP V (Female)
40X



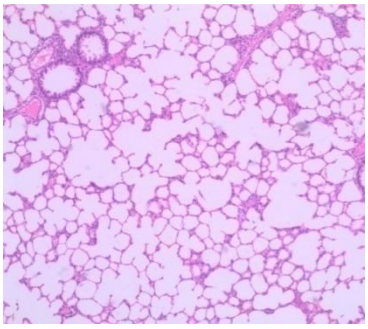
Histopathological changes inLung

Fig 40 shows the light microscopic examination of lung of group IV sample treated with 250 mg/kg of test drug revealed normal alveoli and alveolar sac with no signs of infiltration when compare to that of the group I honey treated rats**.** In post retrieval group, perivascular region appears normal, alveolar septa and wall appeared widen and normal. No signs of lymphocyte cuffing

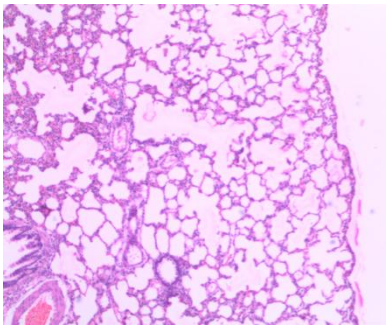
Fig. 40. Histopathology of Lung



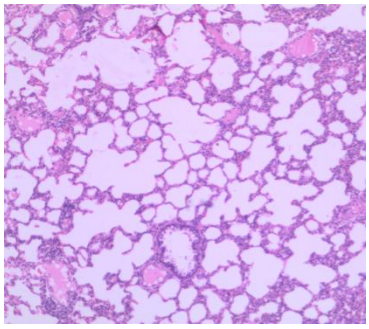
GROUP I (Female)
10X



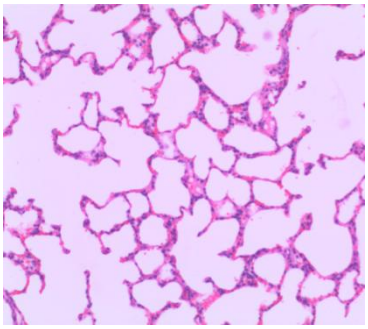
GROUP IV (Female 1)
10X



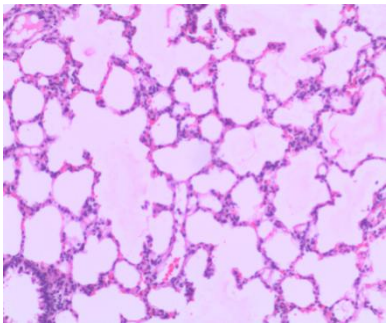
GROUP IV (Female 2)
10X



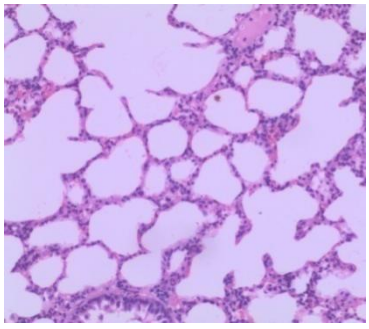
GROUP I (Female)
40X



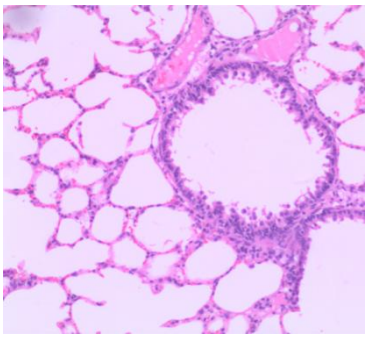
GROUP IV (Female 1)
40X



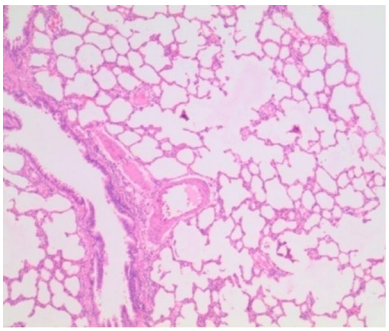
GROUP IV (Female 2)
40X



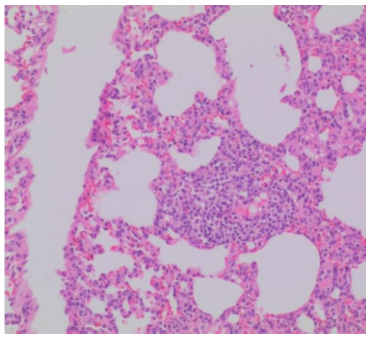
GROUP I (Female)
40X



GROUP V (Female)
10X



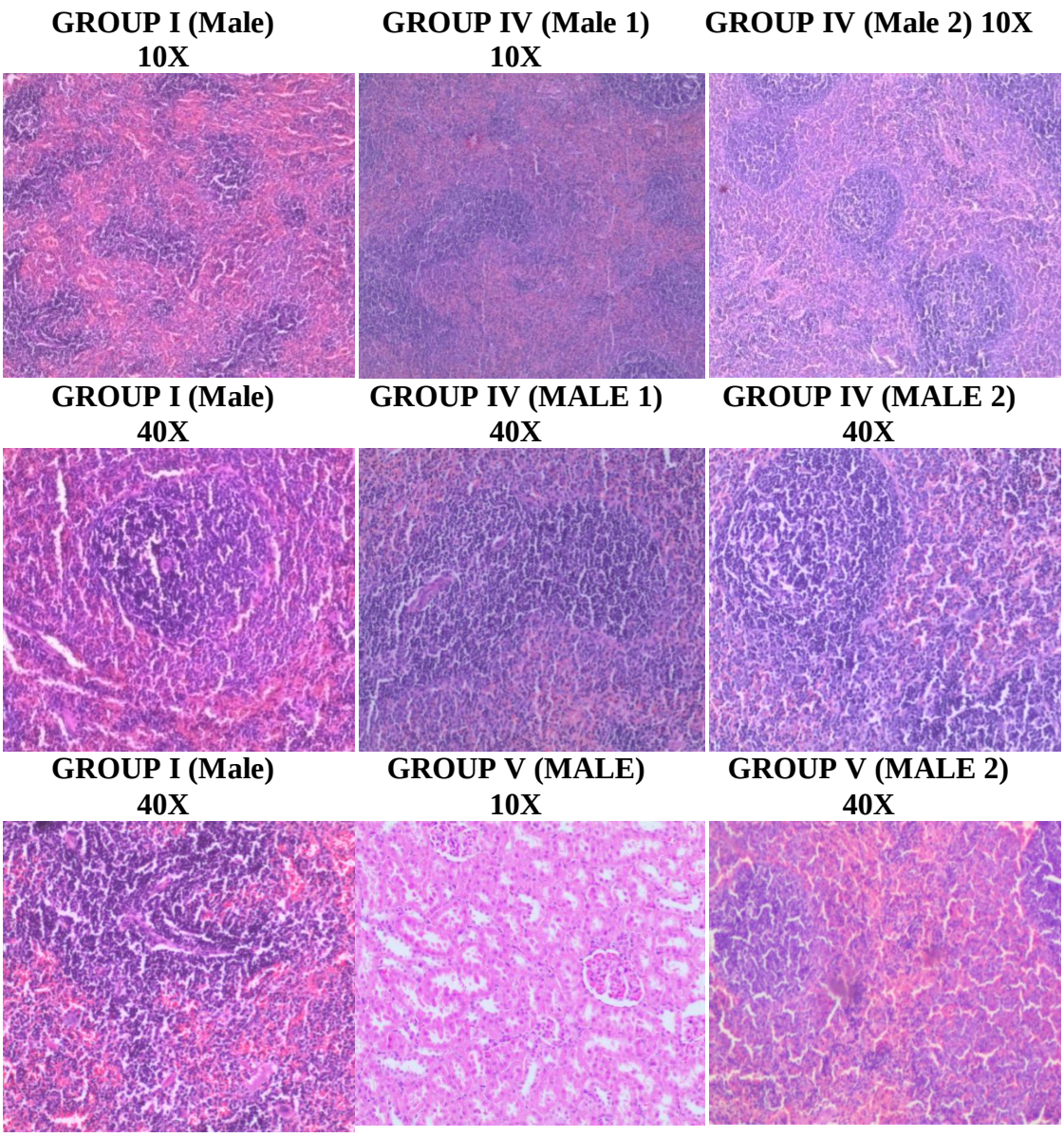
GROUP V (Female)
40X



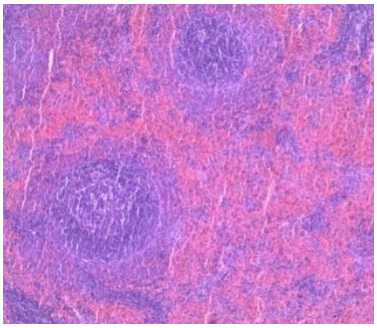
Histopathological changes in Spleen

Histopathology of spleen sample belongs to group IV treated with 250 mg/kg of test drug revealed the presence of normal lymphoid follicles, PALS region of the spleen surrounding the central artery with typical red pulp and white pulp. Further no signs of follicular abnormalities were observed in group IV when compare to that of the control group I **(Fig 41).** In post retrieval group, Appearance of LF – lymphoid follicle; PALS – periarterial lymphoid sheath was normal with no significant signs of enlargement in both the samples

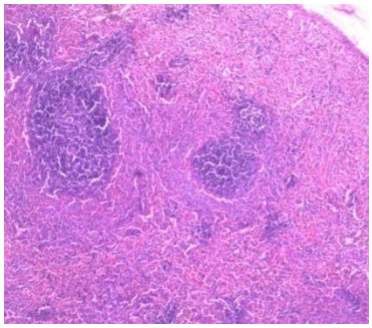
Fig 41. Histopathology of Spleen



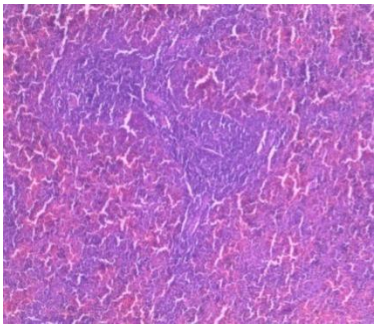
GROUP I (Female)
10X



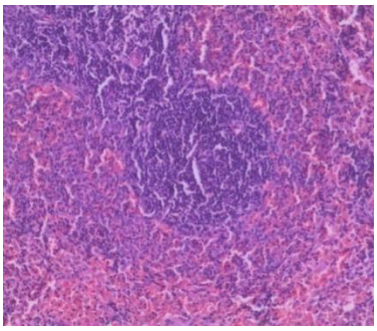
GROUP IV (Female 1)
10X



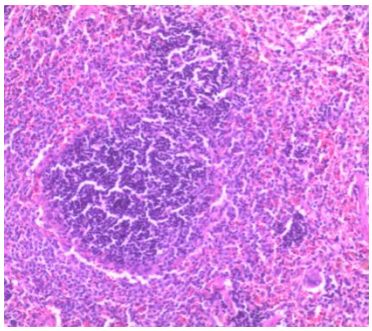
GROUP IV (Female 2)
10X



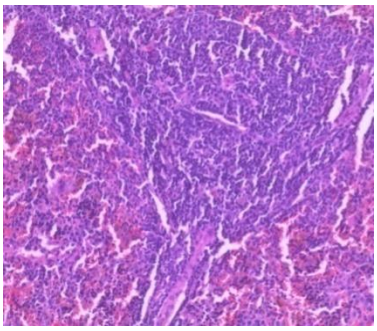
GROUP I (Female)
40X



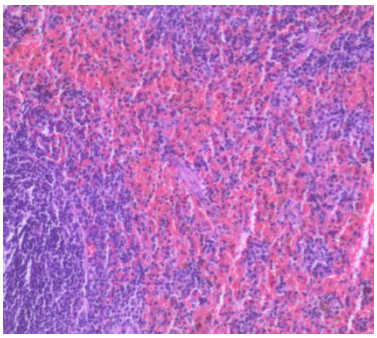
GROUP IV (Female 1)
40X



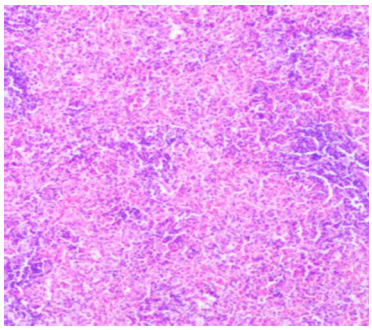
GROUP IV (Female 2)
40X



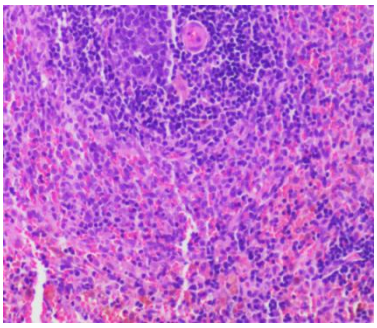
GROUP I (Female)
40X



GROUP IV (Female)
10X



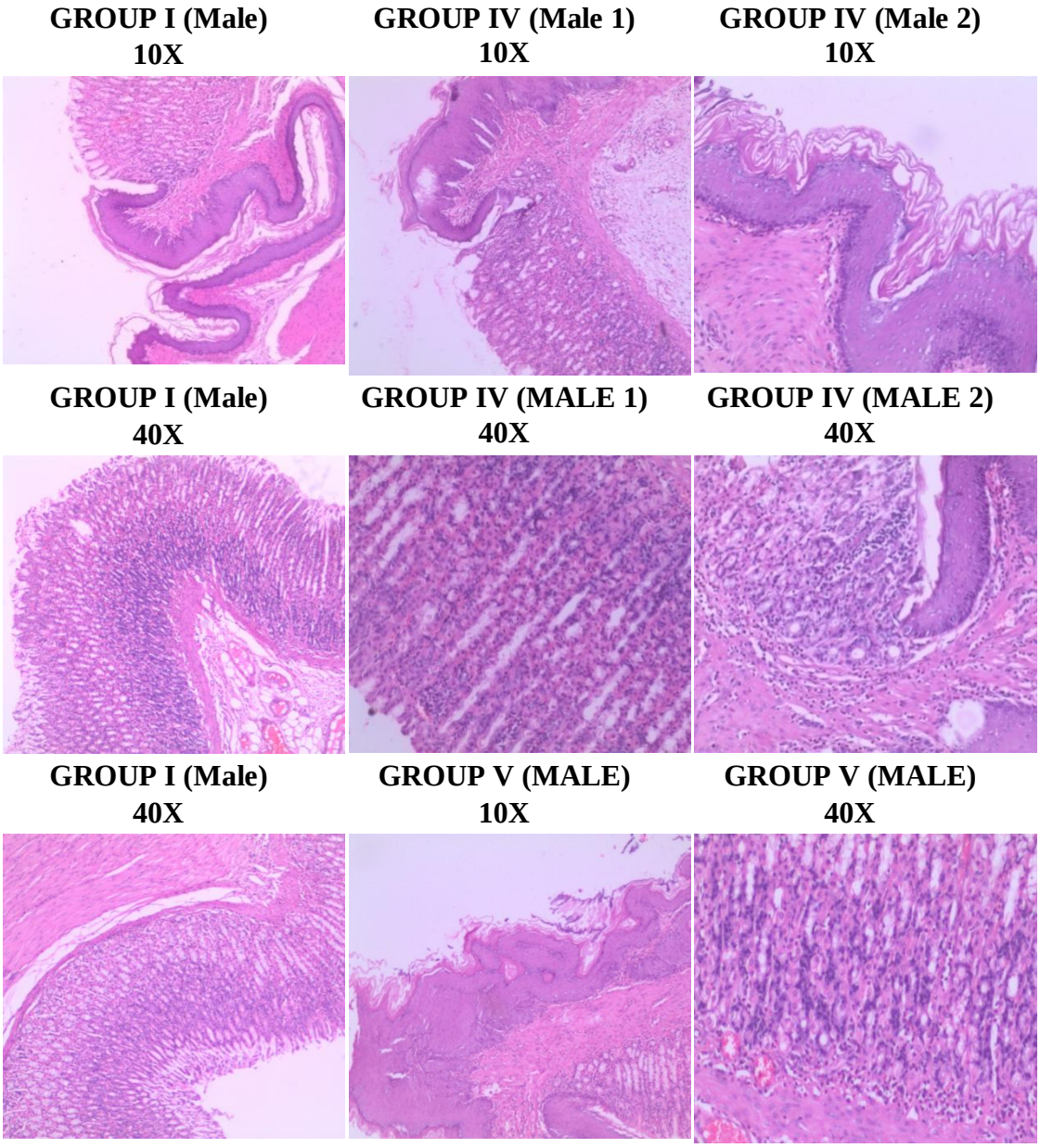
GROUP IV (Female)
40X



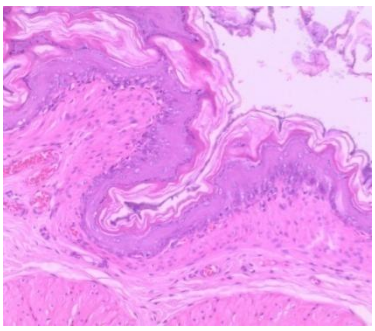
Histopathological changes inStomach

Microscopic analysis of stomach samples belongs to group IV treated with 250 mg/kg of SSM revealed normal anatomy of muscular stomach with epithelial layer keratinized stratified squamous epithelium, Lamina propria and Sub-mucosa when compare to that of the group I rats **(Fig 42).** In post retrieval group, Microscopic analysis of stomach of both the sample reveals normal anatomy of muscular stomach with epithelial layer keratinized stratified squamous epithelium, Lamina propria and Sub-mucosa

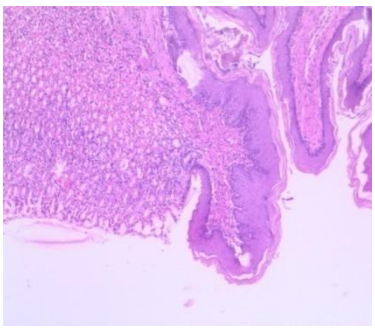
Fig 42. Histopathology of Stomach



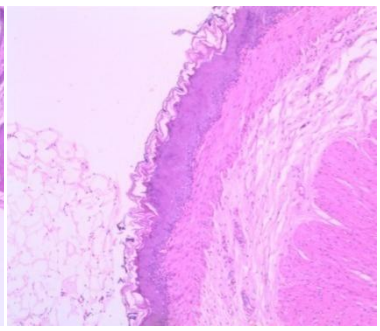
GROUP I (Female)
10X



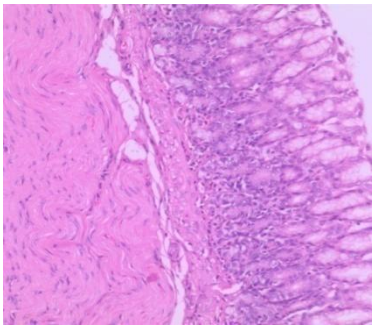
GROUP IV (Female 1)
10X



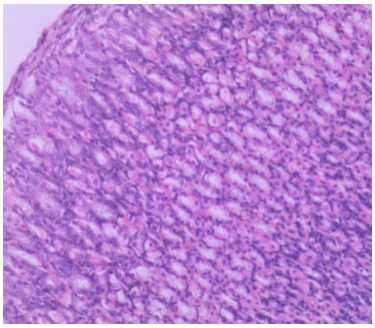
GROUP IV (Female 2)
10X



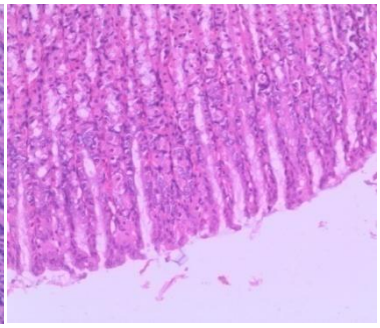
GROUP I (Female)
40X



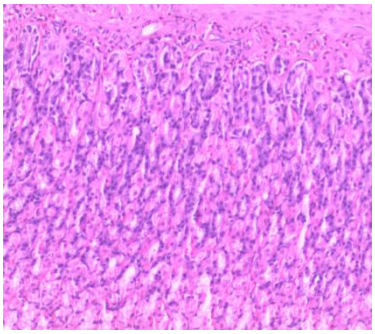
GROUP IV (Female 1)
40X



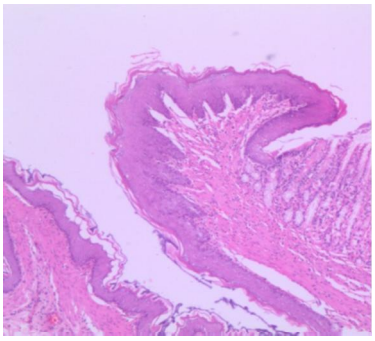
GROUP IV (Female 2)
40X



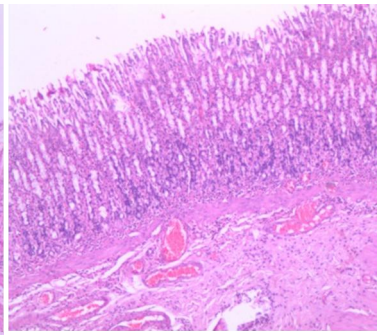
GROUP I (Female)
40X



GROUP IV (Female)
10X



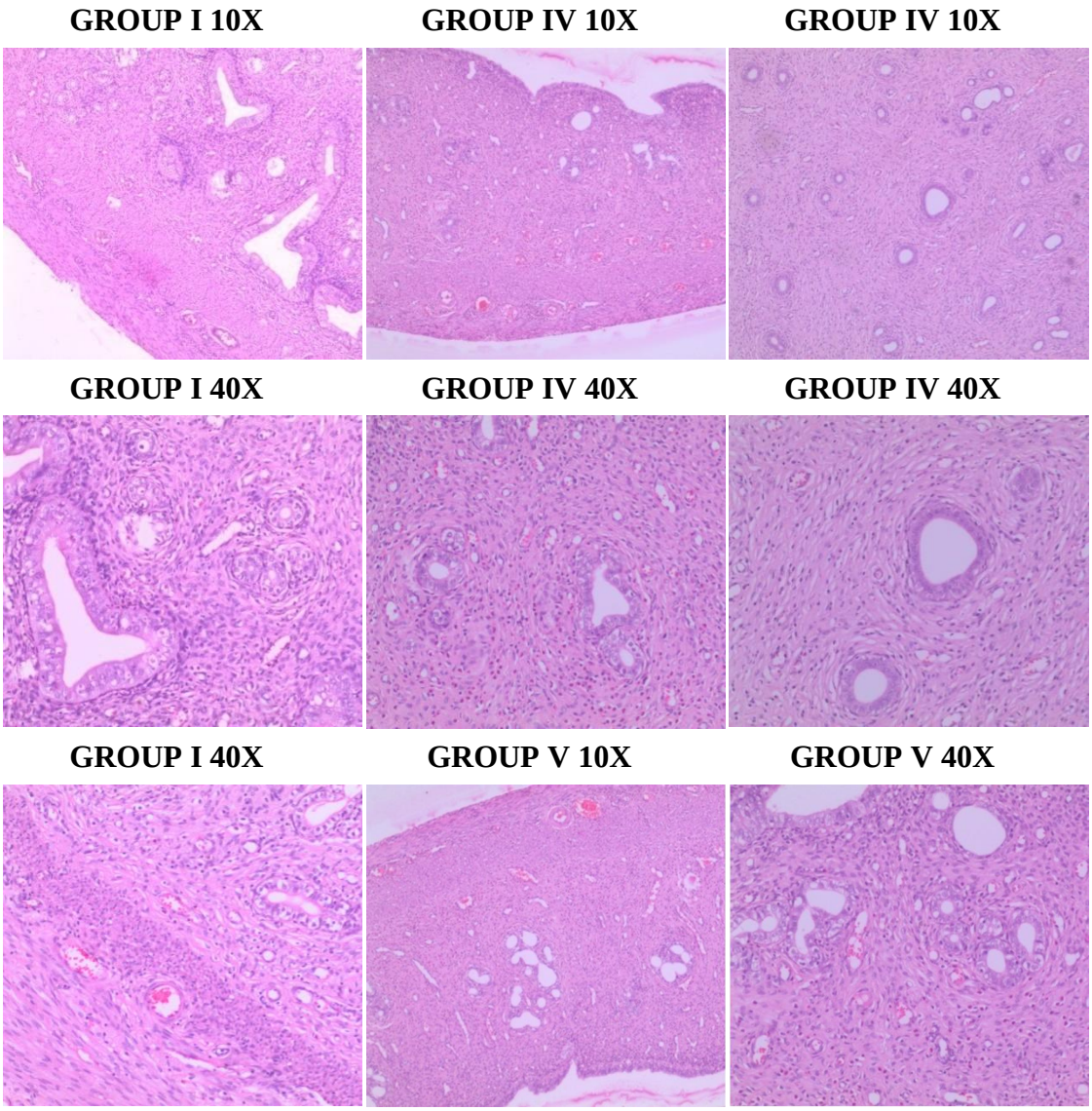
GROUP IV (Female)
40X



Histopathological changes inUterus

Microscopic observation of uterus sample belongs to group IV treated with 250 mg/kg of SSM, shown normal lumen with various layers of uterus such as endometrium, myometrium, and perimetrium. Inner and outer myometrial layer appears normal. Histology of uterus sample belongs to group IV appears normal and no significant changes when compare to that of the group I rats **(Fig 43).** Appearance of uterine layers endometrium, myometrium, and perimetrium was normal and no signs of degeneration was observed in the recovery group.

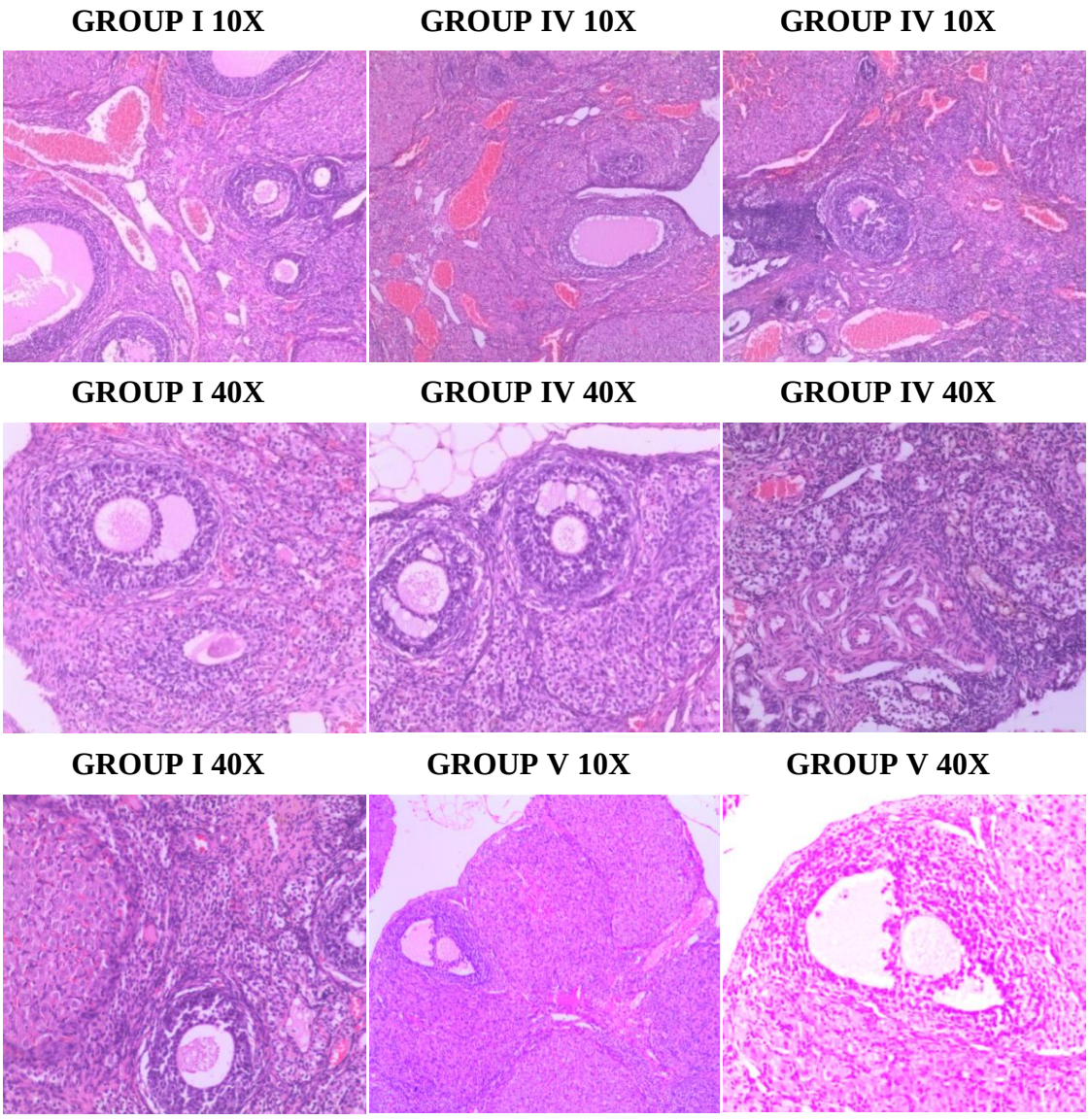
Fig 43. Histopathology of Uterus



Histopathological changes inOvary

Histopathology of ovary samples belongs group IV treated with 250 mg/kg of SSM revealed normal corpus luteum (CL) and few mature ovarian follicles with no signs of abnormality when compare to that of the group I treated rats **(Fig 44).** **N**ormal ovarian cortex and primordial follicles with no signs of follicular degeneration was observed in the post retrieval group.

Fig 44. Histopathology of Ovary

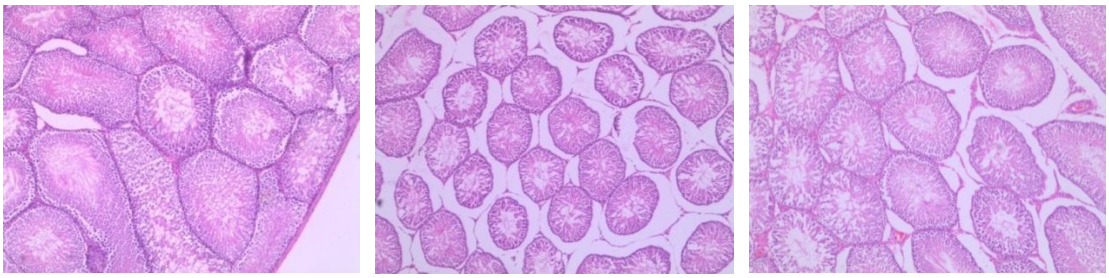


Histopathological changes inTestes

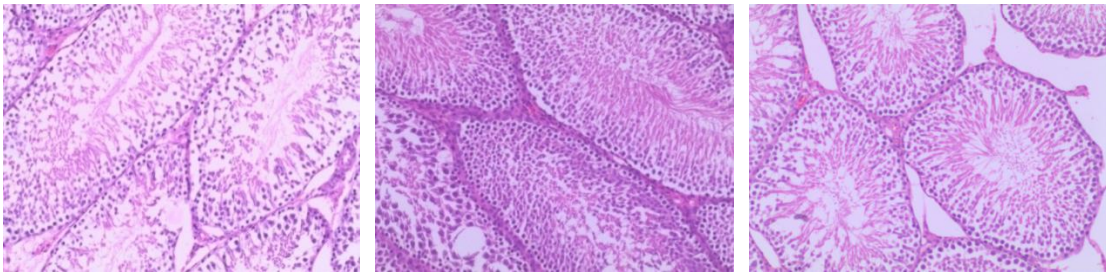
Histocytological observation of testicular tissue of male rats belongs to group IV treated with 250 mg/kg of test drug shown well differentiated germ cells with respect of spermatogonia includes spermatid and sperm. It was observed that presence of mature somatic cells project the perfect histomorphology of testicular cells in this group. Primary spermatocytes with large centered nucleus and dense chromatin were observed in this group. Hence the histology of testes appears normal and no significant abnormality was observed in group IV when compare to that of group I rats **(Fig 45).** Well differentiated germ cells with no signs of cellular hypertrophy was observed in the satellite (Post retrieval) group.

Fig 45. Histopathology of Testis

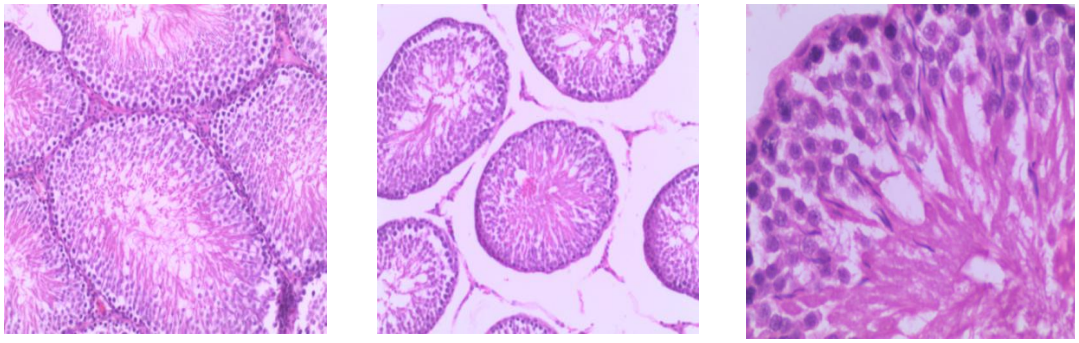
GROUP I 10X **GROUP IV 10X** **GROUP IV 10X**



GROUP I 40X **GROUP IV 40X** **GROUP IV 40X**



GROUP I 40X **GROUP V 10X** **GROUP V 40X**



From the above results minimal changes were observed in the liver and kidney. Therefore ICP-MS analysis was performed to determine the content of mercury in the renal tissue.

7.5.3.8 ICP-MS analysis of renal tissue

The mercury content of renal tissue in SSM treated rats were shown in table.24. Minimal concentration of mercury was found in the high dose group. But at the same time, Mercury concentration was found to be below the detectable level in both post retrieval and control group.

Table 24. Determination of mercury concentration of SSM treated rats.

S.No	Group	Element	Concentration (mg/L)
1.	High dose treated rats (SSM 250 mg/kg)- Animal 1	Mercury (Hg)	0.017±0.006
2.	Post Retrieval(SSM 250 mg/kg)	Mercury (Hg)	BDL
3.	control	Mercury (Hg)	BDL

BDL- Below detective Level

7.6 Pharmacological studies

Following the safety confirmation of SSM, the following pharmacological studies were carried out to check the efficacy of the test drug. The results are as follows.

7.6.1 Alcohol induced liver injury

Group I animals received the saline and served as normal control. Group II(Ethanol induced) animals showed significant increase in the hepatic marker enzymes AST, ALT, ALP , Bilirubin, urea and liver weight and decrease in Total proteins indicating liver damage. Group III (Honey + Ethanol) shows increased level of all the above marker enzymes almost equal to that of Group II. Effects of SSM at dose levels (25mg/Kg, 125mg/kg) were shown in Group 1V and Group V.Group VI

animals were treated with Liv 52 a standard drug. Group IV showed moderate decrease in the marker enzymes ALT, ALP, Urea and weight of the liver. Group V animals showed noteworthy decrease in all the marker enzymes AST, ALT, ALP, Bilirubin, urea and liver weight and decrease in Total proteins which were markedly elevated in the induced group (Group II). The results shown in Table-25 reveal that the effect of SSM in High dose (125mg/kg) Group was almost equal to that of the standard drug Liv 52 as indicated in Group VI.

Table.25. Effect of SSM on Serum Liver enzyme level inAlcohol Induced liver injury in mice

PARAMETER	Group I	Group II	Group III	Group IV	Group V	Group VI
AST (IU/ml)	71.33±4.02	126.2±1.96	120.5±1.2	121.7±1.76	112.3±2.11**	115.8±1.78**
ALT (IU/L)	22.17±3.43	69.17±4.4	52.17±1.2**	43.67±1.31**	34.33±1.5**	41±2.85**
ALP (IU/L)	124.3±3.18	241.8±3.36	226±2.0**	212.3±3.25**	194.7±2.31**	154.8±2.46**
Total bilirubin (mg/dl)	0.4±0.02	0.93±0.07	0.88±0.0	0.84±0.03	0.71±0.06*	0.68±0.05**
Direct bilirubin (mg/dl)	0.27±0.02	0.57±0.06	0.53±0.01	0.46±0.02	0.43±0.03*	0.46±0.04
Indirectbilirubin (mg/dl)	0.13±0.01	0.36±0.03	0.35±0.0	0.37±0.02	0.28±0.03	0.21±0.02**
Total Protein (gm/dl)	5.73±0.26	3.78±0.11	4.05±0.17	4.27±0.24	4.7±0.18**	5.2±0.11**
Serum Urea (mg/dl)	19.17±1.3	81±2.94	76±2.2	66.33±1.65**	52.67±1.73**	37.17±2.02**
Weight of liver(gm)	1.78±0.19	3.37±0.13	3.15±0.16	2.8±0.13*	2.35±0.16**	2.13±0.10**

Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg. Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

The animals of Group I (Saline) served as normal control. Group II(Ethanol induced) animals showed significant Decrease in hepatic antioxidant levels and enhanced activity of lipid peroxidation .Group III (Honey + Ethanol) shows increased level of all the above marker enzymes almost equal to that of Group II. Group IV (25mg/Kg) showed mild increase in the antioxidant enzyme with moderate reduction in the LPO. whereas and Group V (125mg/kg) and the Group VI (Liv 52) treated groups showed significant increase in antioxidant levels, with reduction in lipid peroxidation level (LPO) when compared with Ethanol induced animals (Table - 26).

Table 26. Effect ofSSM on Liver Anti-oxidant enzyme level in Alcohol Induced liver injury in mice

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI
SOD Units/mg protein	9.9±0.49	4.9±0.16	5.21±0.15	5.91±0.24*	6.3±0.19**	7.57±0.33**
CAT Units/mg protein	3.12±0.19	1.28±0.1	1.31±0.11	1.73±0.09	1.93±0.15**	2.42±0.15**
GR (nano moles / min / mg protein)	45.83±2.39	17.83±2.12	18.5±0.85	25.17±2.04*	31.67±1.76**	37.33±2.10**
GSH-Px (nmoles of NADPH oxidized/ min/ mg protein)	52.5±2.36	26.5±1.96	28.83±1.4	34±1.34*	38±1.84**	47.67±2.89**
LPO (nano mole of MDA/mg protein)	2.9±0.16	18.9±0.59	17.38±0.76	10.75±0.40**	9.17±067**	7.2±0.22**

Values are expressed as mean ± S.E.M. (n=6), comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg). Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Histopathology of Liver

To assess the changes in the liver tissues of all the 6 groups Morphometric analysis was carried out based on the below scoring system.

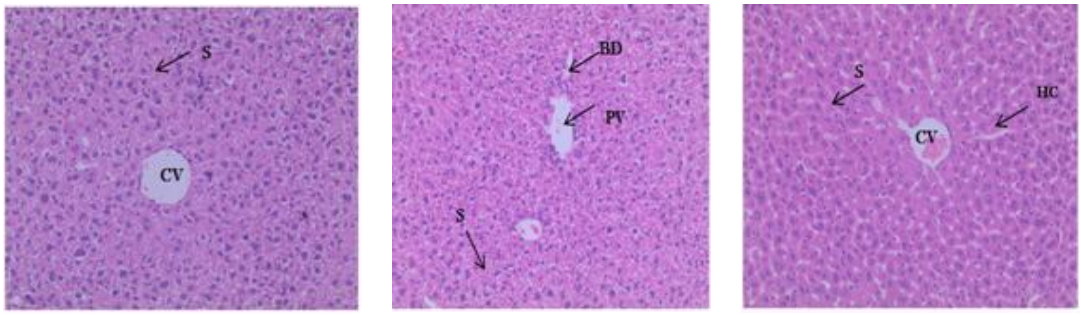
The degree of inflammation and histopathologic damage with reference to hepatocellular architecture, necrosis, inflammation, mononuclear cell infiltration, Kupffer cells presence, arrangement of sinusoids, vacuolization and vascular congestion was expressed within each liver section classified on a scale of 0–3 (0, absent; 1, mild; 2, moderate; 3, severe) .

Percentage Change	Observation	Score
No change	No change	0
Less than 30%	Mild	1
More than 30 and less than 50%	Moderate	2
Greater than 50%	severe changes	3

Fig. 46. Histomorphometric Analysis on H&E staining of Alcohol induced liver injury in mice

Fig. 46a. Histomorphometric Analysis ofGroup I animals

Sample 1- score 0 **Sample 2- score 0** **Sample 3- score 0**



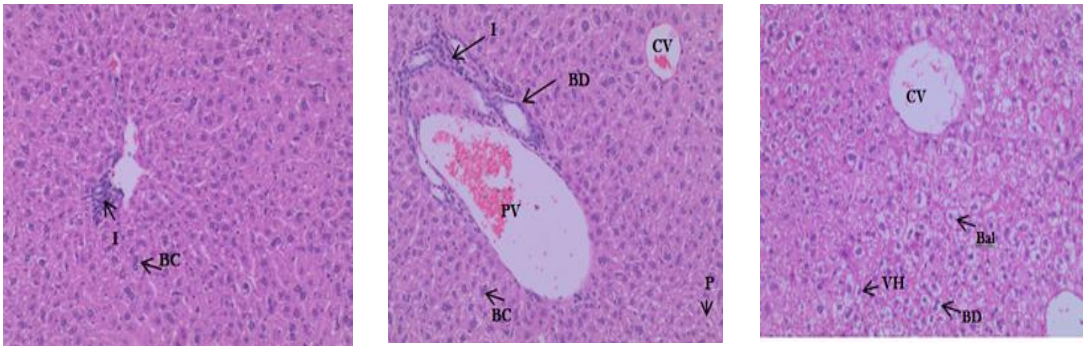
CV-Central vein, S-Hepatic sinusoid, HC-Hepatic Cord, BD-Bile duct, PV-Portal vein

Figure 46.a shows the histological picture of the control group to which only saline was given

- Normal central vein with well projected hepatocytes and prominent intact nucleus and cytoplasm
- Appearance of portal vein, bile duct and hepatic artery was normal
- Appearance of hepatic sinusoid and hepatic cord was normal

Fig 46 b. Histomorphometric Analysis ofGroup II animals

Sample 1- score 3 **Sample 2- score 3** **Sample 3- score 3**



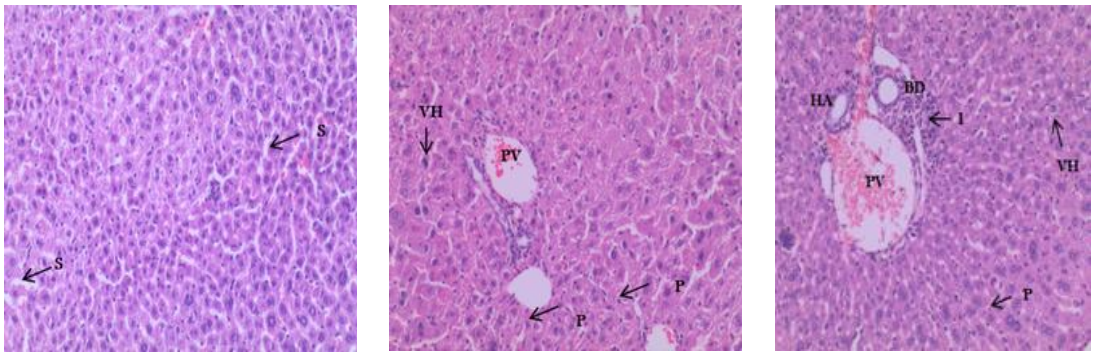
HA-Hepatic Artery, I –Infilteration with mononuclear inflammatory cells, BD-Bile duct, S-Hepatic sinusoid, PV-Portal vein, VH-Vacuolated hepatocytes, BC-Binucleated cells

Figure 46.b. shows the following histological changes in the liver tissues of Group II (Ethanol induced) animals

- Presence of ballooning degeneration with more binucleated cells was observed.
- Portal area showed lymphocytic infiltration as a sign of inflammation induced by inducing agent
- Marginal Necrotic changes were observed and foci of necrosis, mainly in mid zonal and periportal region.
- Central vein dilatation was observed

Fig. 46c. Histomorphometric Analysis ofGroup III animals

Sample 1- score 3 **Sample 2- score 2** **Sample 3- score 1**



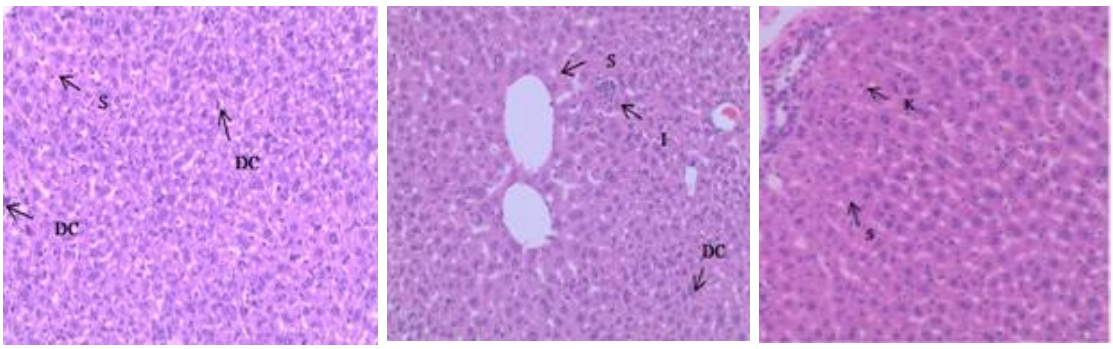
HA-Hepatic Artery, I –Infilteration with mononuclear inflammatory cells, BD-Bile duct, S-Hepatic sinusoid, PV-Portal vein, VH-Vacuolated hepatocytes.

Figure 46.c. depicts the following liver tissue changes in group III(ethanol+honey) animals

- Increased sinusoidal space with infiltration of inflammatory cells in between the bile duct and hepatic artery.
- Inflammatory cell infiltration was numerous around pelvic vein and bile duct
- Derangement in hepatic cord projection with distant arrangement was observed.
- Presence of pyknotic nucleus denotes the sign of oxidative stress

Fig. 46. dHistomorphometric Analysis ofGroup IV animals

Sample 1- score 2 **Sample 2- score 2** **Sample 3- score 2**



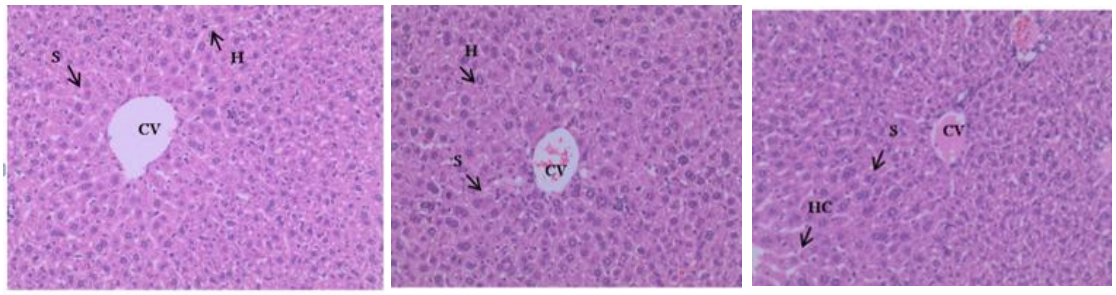
I –Infilteration with mononuclear inflammatory cells, BD-Bile duct, S-Hepatic sinusoid, DC-Dark condensed chromatin.

Figure 46.d. shows the histological picture of group IV (SSM low dose) animals as follows

- Numerous hepatocytes with dark condensed chromatin was observed
- Increased marginal sinusoidal space in centri lobular, mid zonal and periportal area
- Presence of Kupffer cells and inflammatory cells was observed

Fig. 46e. Histomorphometric Analysis of Group V animals

Sample 1- score 2 **Sample 2- score 1** **Sample 3- score 1**



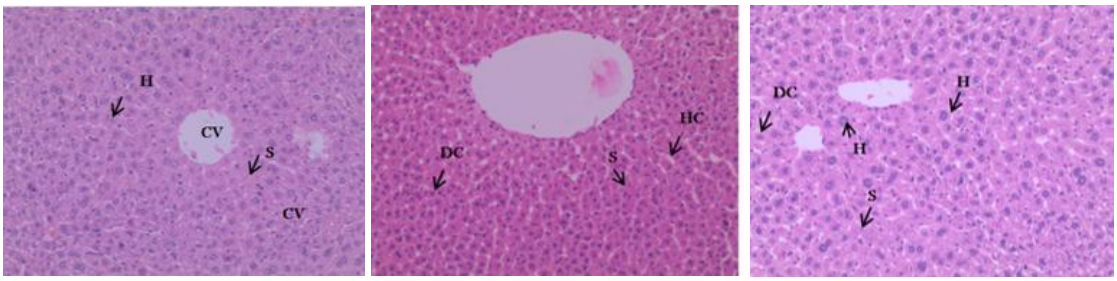
CV-Central Vein, S-Hepatic sinusoid, HC-Hepatic cord

Figure 46.e. illustrates the histology of group V (SSM high dose) animals, which is

- Restoration of hepatocyte structure back to the normal with occasional derangement was observed
- Increased number of nucleated hepatocyte with prominent chromatin was observed.

Fig. 46f. Histomorphometric Analysis of Group VI animals

Sample 1- score 1 **Sample 2- score 1** **Sample 3- score 1**



CV-Central Vein, S-Hepatic sinusoid, HC-Hepatic cord, DC-Dark condensed chromatin

Figure 46 f. explains the histological changes of group VI (Liv 52) animals, which are

- Appearance of hepatocyte was normal with well project cytoplasm and nucleus. No signs of nuclear inflammation
- Occasional presence of hepatocytes with condensed nucleus was observed

Table 27: Morphometric score of Alcohol Induced Hepatotoxicity

S.No	Groups	Score (Mean+SEM)
1	Group I	0
2	Group II	3 ± 0
3	Group III	2 ± 0.57
4	Group IV	2 ± 0
5	Group V	1.33 ± 0.33
6	Group VI	1 ± 0

Table 27 shows the morphometric scoring of all the groups that revealed severe hepatic injury was reversed back to near normal in the SSM high dose and Liv 52 treated animals.

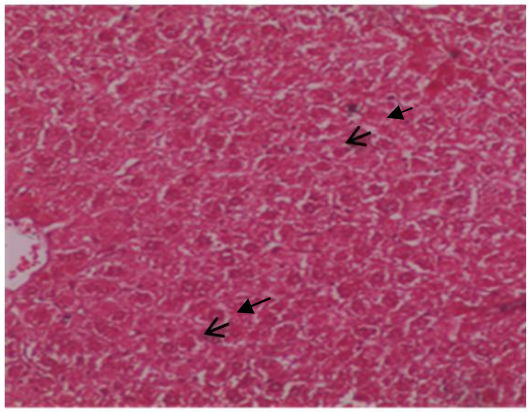
Masson's trichrome staining

Masson's trichrome staining were adapted to notice the degree of fibrosis and observe the collagen fibers of the liver. The cytoplasm of hepatocytes are stained red and fibrous tissue (collagen) is stained blue. The nuclei can be seen as dark red to black structures within cells.

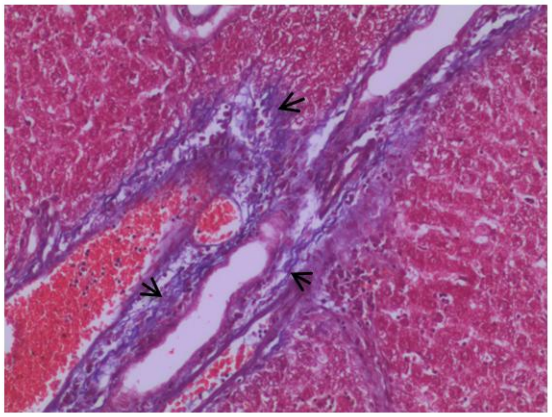
In figure 47 the trichrome stained liver belonging to control group mice shows normal architecture of hepatocyte with intact arrangement of prominent nucleus. Sample belonging to group II shows characteristic deposition of collagen fibrous tissue in central vein zone. Group III shows minimal collagen deposition indicating minimal fibrosis. Liver section of group IV, V treated with low (25mg/kg) and high dose (125mg/kg) of SSM shown reduced level of fibrosis with restored hepato cellular architecture in treated mice.Normal hepatocytes with integrated cytoplasm was observed in group VI mice treated with standard drug Liv 52.

Fig. 47. Masson's trichrome staining of Alcohol Induced liver injury in mice

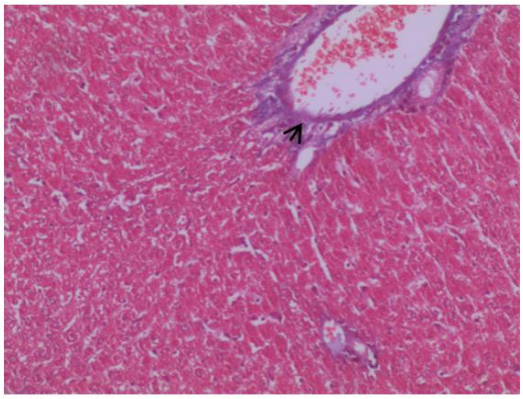
Group I- Control



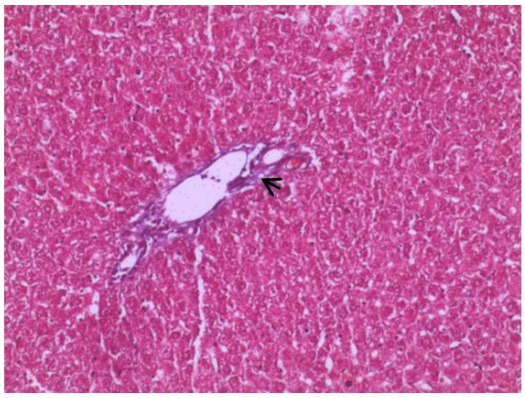
Group II- Ethanol Induced



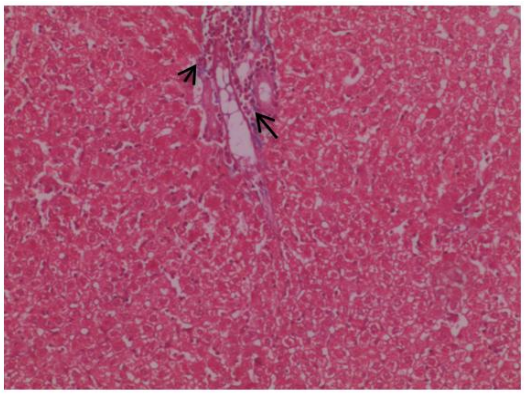
Group III- Ethanol+ Honey



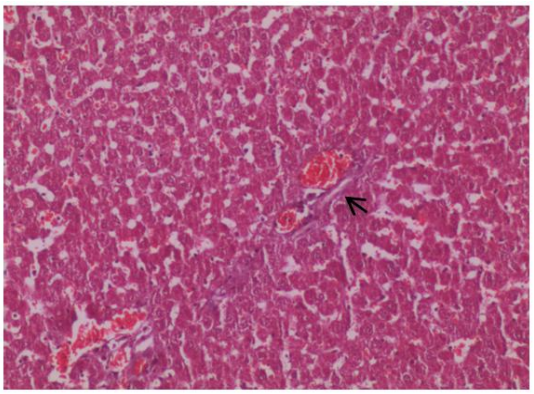
Group IV- SSM Low Dose



Group V-SSM High Dose



Group VI- Liv 52



7.6.2 Paracetamol induced liver injury

In this study Group I animals received the saline and served as normal control. Group II(Paracetamol induced) animals showed significant increase in the hepatic marker enzymes AST, ALT, ALP , Bilirubin, urea and liver weight and decrease in total proteins signifying hepatic injury. Group III (Honey + paracetamol) shows considerable increase of all the hepatic marker enzymes nearly equivalent to Group II. Effects of SSM at dose levels (25mg/Kg, 125mg/kg) showed highly significant decrease in all the hepatic biomarkers AST, ALT, ALP, Bilirubin, urea and liver weight and decrease in Total proteins when compared to in the Induced group (Group II) in which they were obviously elevated. Though the standard drug treated group VI shows the complete reduction of all the liver enzymes to near normalcy, the results shown in Table-28 reveal that the effect of SSM in both Low (25mg/Kg) and High dose (125mg/kg) Group was almost equal to that of the standard drug Liv 52(2.6ml/Kg).

Table 28. Effect ofSSM on Serum Liver enzyme level inParacetamol Induced liver in mice

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI
AST (IU/ml)	84±5.09	142.2±4.41	136.8±1.58	126.3±1.8**	118.3±1.96**	92±1.44**
ALT (IU/L)	21±2.6	66.33±4.77	52±1.61**	51.5±2.05**	44±2.1**	32±1.71**
ALP (IU/L)	121.5±3.09	288.2±7.61	260.8±2.02**	220.5±3.91**	198.2±4.47**	145.3±2.91**
Total bilirubin (mg/dl)	0.49±0.03	1.2±0.05	1.13±0.05	1.02±0.03*	0.81±0.03**	0.64±0.03**
Direct bilirubin (mg/dl)	0.34±0.02	0.83±0.04	0.8±0.04	0.69±0.03*	0.51±0.02**	0.41±0.03**
Indirectbilirubin (mg/dl)	0.15±0.02	0.37±0.03	0.33±0.04	0.32±0.05	0.29±0.02	0.22±0.03*
Total Protein (gm/dl)	6.93±0.23	3.43±0.16	3.58±0.29	5.35±0.18**	5.91±0.22**	6.4±0.2**
Serum Urea (mg/dl)	19±1.18	81±.94	71±2.15	52.83±2.24**	32.67±1.5**	29.33±2.04**
Weight of Liver(gm)	1.55±0.09	3.55±0.22	3.05±0.11	2.51±0.18**	2.23±0.15**	2.05±0.17**

Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

The animals of Group I(Saline) served as normal control. Group II(Paracetamol induced) animals showed significant decrease in hepatic antioxidant levels SOD, CAT, GR,GSH-Px and enhanced activity of lipid peroxidation(LPO). Group III (Honey +Paracetamol) also shows considerable increased in these hepatic antioxidants almost equal to that of Group II. Group IV (25mg/Kg) and Group V (125mg/kg) showed highly significant increase in the antioxidant enzyme with significant decline in the LPO as almost equal to that of the standard drug Liv52.when compared with paracetamol induced animals (Table-29).

Table 29. Effect of SSM on Liver Anti-oxidant enzyme level inParacetamol induced hepatotoxicity

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI
SOD Units/mg protein	9.03±0.51	2.07±0.13	3±0.12	5.47±0.22*	6.4±0.26**	8.55±0.40**
CAT Units/mg protein	2.77±0.21	1.36±0.14	1.58±0.11	2.11±0.18*	2.33±0.14**	2.58±0.26**
GR (nano moles / min / mg protein)	41.17±1.99	14.33±1.54	19±1.59	27.5±3.33**	33.83±1.30**	36.5±2.11**
GSH-Px (nmoles of NADPH oxidized/min/ mg protein)	50.83±1.74	12.67±1.80	18±0.97	26.33±1.20**	34.5±1.11**	43.17±1.58**
LPO (nano mole of MDAMDA/mg protein)	2.17±0.26	16.23±2.09	14.1±0.43	10.48±0.33**	7.25±0.14**	5.33±0.20**

Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

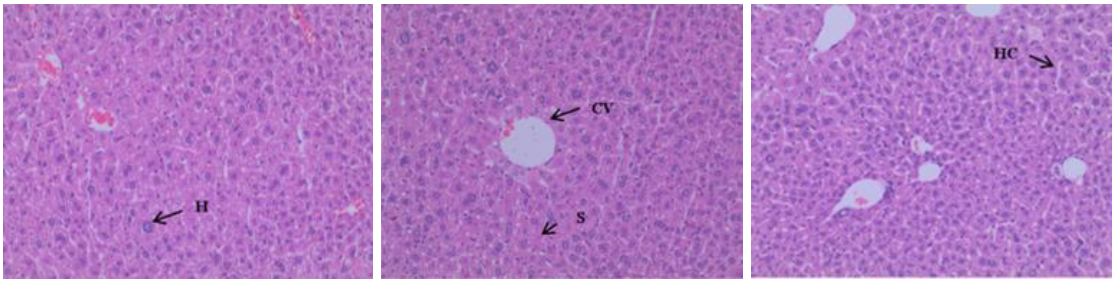
Histopathology of liver

To evaluate the changes in the liver tissues of all the 6 groups morphometric analysis was carried out based on the scoring system mentioned in alcohol induced liver injury.

Fig.48. Histomorphometric Analysis on H&E staining of Paracetamol Induced liver injury in mice

Fig 48.a. Histomorphometric Analysis of Group I animals

Sample 1- score 0 **Sample 2- score 0** **Sample 3- score 0**



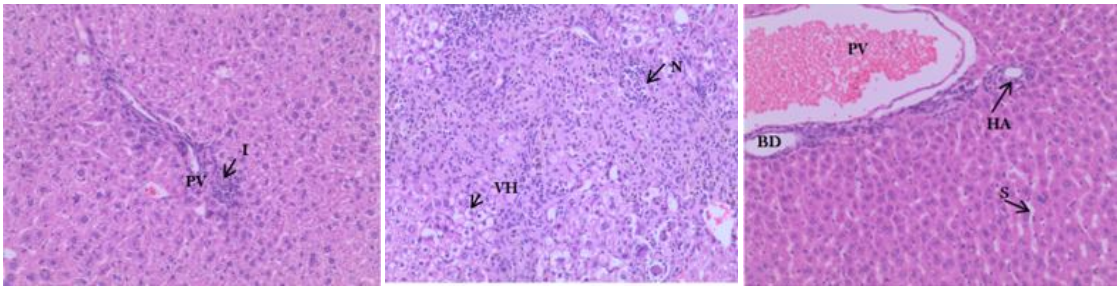
CV-Central vein, S-Hepatic sinusoid, HC-Hepatic Cord

Figure 48 a. shows the histological picture of the control group to which only saline was given

- Appearance of hepatic cord was normal and radial in nature, no signs of cellular degeneration

Fig 48.b. Histomorphometric Analysis of Group II animals

Sample 1- score 3 **Sample 2- score 3** **Sample 3- score 2**



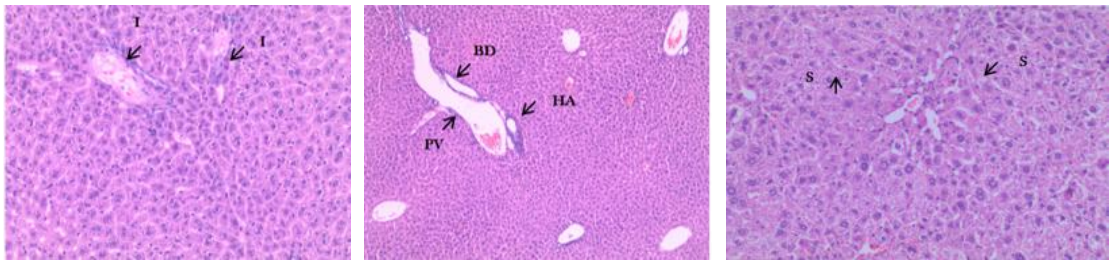
HA-Hepatic Artery, I –Infilteration with mononuclear inflammatory cells, BD-Bile duct, S-Hepatic sinusoid, PV-Portal vein, VH-Vacuolated hepatocytes.

Figure 48b. shows the following histological changes in the liver tissues of Group II (paracetamol induced) animals, which are

- Photo micrograph shown more necrotic changes characterized by shrinkage of hepatocytes
- More number of inflammatory cells present near portal vein region
- Increased sinusoidal space and vacuolated hepatocytes was observed
- Presence of nuclear chromatin condensation which is characterized by pyknosis

Fig 48.c. Histomorphometric Analysis of Group III animals

Sample 1- score 3 **Sample 2- score 2** **Sample 3- score 2**



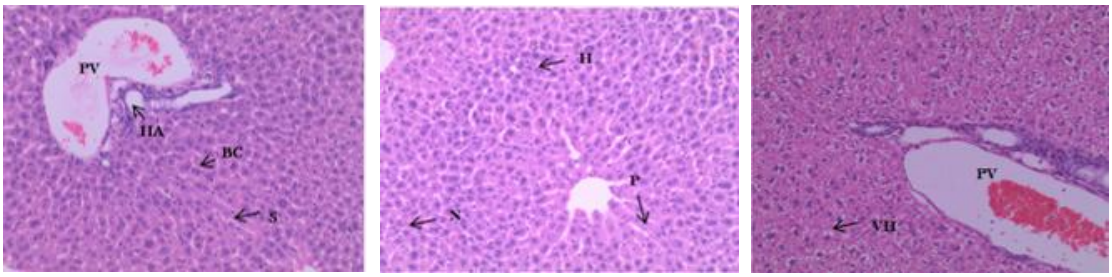
I- Infilteration with mononucleate inflammatory cells, BD- Bile Duct, HA- Hepatic Artery, S-Hepatic Sinusoid, PV-Portal vein

Figure 48c depicts the following liver tissue changes in group III (paracetomol + honey) animals, as follows

- Portal vein appears stretched with elongated bile duct. Hepatocytes appears clustered with condensed chromatin. Marginal migration of inflammatory cells was observed
- Arrangement of parenchymal cells appears radial with increased sinusoidal space.
- Derangement in the appearance of portal triad was observed in this group

Fig 48.d.. Histomorphometric Analysis ofGroup IV animals

Sample 1- score 3 **Sample 2- score 2** **Sample 3- score 1**



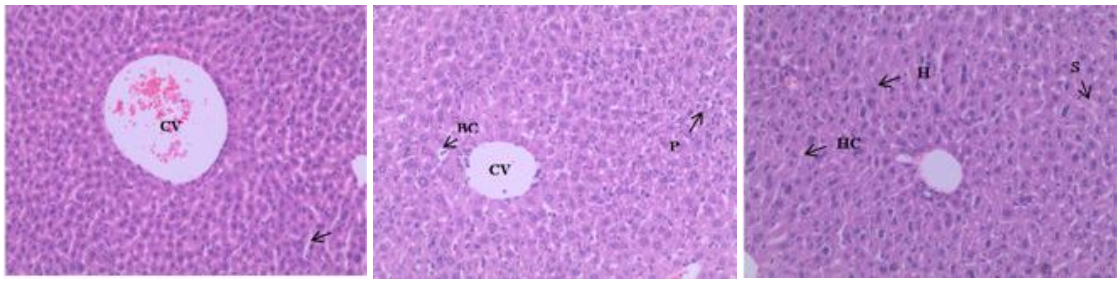
N- Necrotic Change, PV-Portal vein, HA-Hepatic artery, BC-Binucleated cells, HC- Hepatic Cord, S-Hepatic Sinusoid, VH-Vacuolated hepatocytes.

Figure 48d. shows the histological picture of group IV (SSM low dose) animals as follows

- Dilated portal vein with moderate inflammatory changes was observed
- Appearance of hepato cellular architecture was clustered with normal hepatic artery
- Rejuvenated hepatocytes emerging from the necrotic zone
- Increased binucleated cells appeared in centrilobular and mid zonal area.
- Rare appearance of vacuolated hepatocytes

Fig 48,e. Histomorphometric Analysis ofGroup V animals

Sample 1- score 2 **Sample 2- score 1** **Sample 3- score 2**



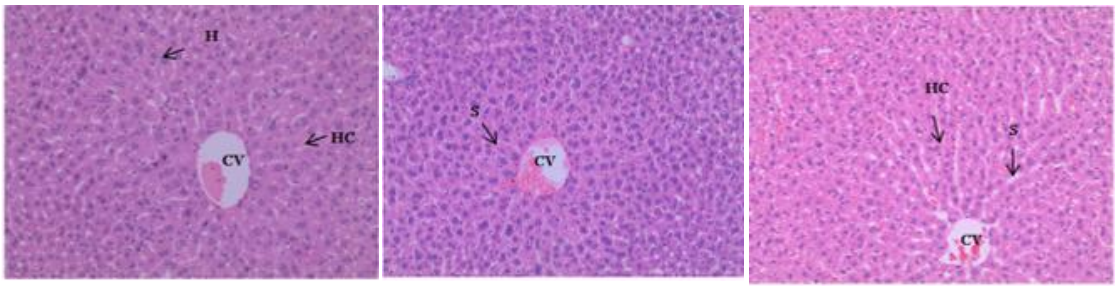
P-Pyknotic nuclei , CV- Central Vein, HC- Hepatic Cord, S-Hepatic Sinusoid, BC-Binucleated cells.

Figure 48e. illustrates the histology of group V (SSM high dose) animals, which are

- Restoration of hepatocytes was clearly observed with few loci of necrotic areas
- central vein was apperas dialted
- Sinusoidal space apperas distanct in some areas of periportal zone.
- Signs of inflammaion seems to be reduced with few projection of pyknotic cells
- Appearance of hepatic cord was regullar with linear and dense arrangment of hepatocytes

Fig 48.f. Histomorphometric Analysis ofGroup VI animals

Sample 1- score 1 **Sample 2- score 1** **Sample 3- score 0**



CV- Central Vein, HC- Hepatic Cord, S-Hepatic Sinusoid

Figure 48f. explains the histological changes of group VI (Liv 52) animals, which are

- Appearance of central vein was normal with few RBC
- Hepatocyte architecture was almost restored back to the normal
- Signs of inflammation and necrosis was greatly reduced

Table 30. Morphometric score of Paracetamol Induced Hepatotoxicity

S.No	Groups	Score
1	Group I	0
2	Group II	2.67 ± 0.33
3	Group III	2.33 ± 0.33
4	Group IV	2 ± 0.58
5	Group V	1.67 ± 0.33
6	Group VI	0.67 ± 0.33

Table 30 confirm that the morphometric scoring of all the groups revealed severe hepatic injury was repaired back to almost normal in the SSM high dose and Liv 52 treated animals when compared to other groups.

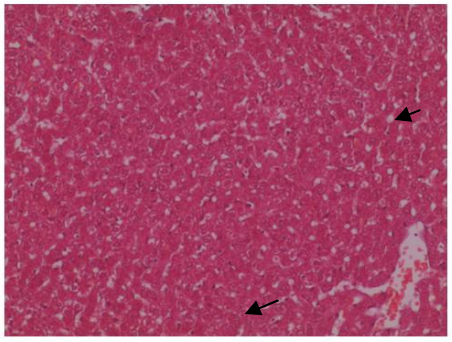
Masson's trichrome staining

Figure 46 represent that the Liver sections of control group mice appeared normal without signs of collagen deposition. Microscopic observation of liver sample belongs to paracetamol treated group revealed increased deposition of

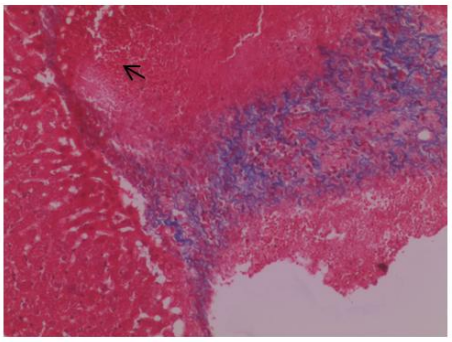
collagen fibers around the congested central vein indicating severe fibrosis.Sample belongs to group III shown moderate collagen deposition on portal track.The level of fibrosis and deposition of collagen was greatly controlled in trichrome stained liver of group IV, V treated with low (25mg/kg) and high dose (125mg/kg) of SSM. Marginal restoration of hepato architecture with trace deposition of fibrous tissue was observed in group VI treated with standard drug Liv 52.

Fig. 49. Masson's trichrome staining of Paracetamol Induced liver injury in mice

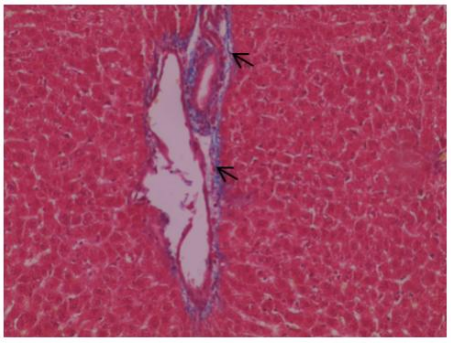
Group I- Control



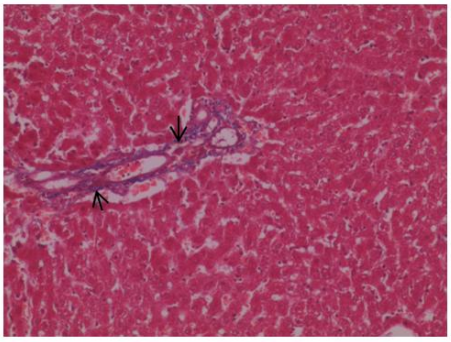
Group II- Paracetamol Induced



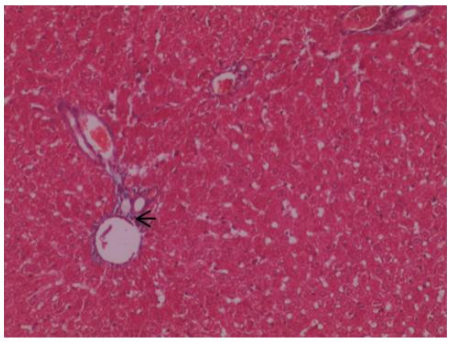
Group III- Paracetamol + Honey



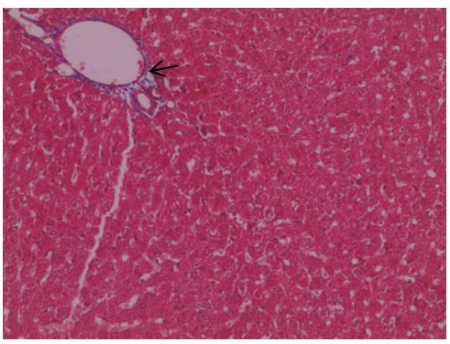
Group IV- SSM Low Dose



Group V- SSM High Dose



Group VI- Liv 52



7.6.3 (LPS)/d-galactosamine induced liver injury

The animals which received saline(Group I) served as normal control. Group II(d-galactosamineinduced) animals showed significant increase in the hepatic marker enzymes AST, ALT, ALP , bilirubin, urea and liver weight and decrease in Total proteins suggestive of liver cell injury. Group III (Honey + d-galactosamine) shows a great increase in all the hepatic enzymes almost equivalent to Group II. SSM treated Groups IV at dose levels (25mg/Kg), showed significant decrease in all the hepatic biomarkers AST, ALP and urea. Group V(125mg/kg) and Group VI(Silymarin 200mg/Kg) showed highly significant reduction in all the hepatic biomarkers AST, ALT, ALP, bilirubin, urea and liver weight and decrease in Total proteins when compared to in the Induced group (Group II) in which they were noticeably elevated. The results indicated in the Table-31 shows that the test drug SSM at high dose (125mg/kg) enhanced complete reduction of all the liver enzymes to near normalcy, and had equivalent effect as that of the standard drug Silymarin 200mg/Kg.

Table 31. Effect of SSM on Serum Liver enzyme level ind-galactosamine induced liver injury

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI
AST (IU/ml)	84.67±3.45	133.3±2.08	124.5±1.72	120.7±1.54**	107.7±2.43**	89.33±3.87**
ALT (IU/L)	24.83±1.92	53.83±4.25	46.5±2.28	41.5±3.27*	31.67±1.53**	28.5±2.26**
ALP (IU/L)	124.8 ±3.4	260.5±4.72	253.3±2.6	234.5±2.5**	190.8±2.39**	130.3±1.41**
Total bilirubin (mg/dl)	0.43±0.03	1.11±0.05	1.03±0.03	0.95±0.05*	0.88±0.05**	0.6±0.02**
Direct bilirubin (mg/dl)	0.29±0.02	0.7±0.04	0.63±0.04	0.6±0.03	0.51±0.03**	0.39±0.02**
Indirect bilirubin (mg/dl)	0.13±0.02	0.41±0.02	0.41±0.03	0.34±0.03	0.37±0.03	0.2±0.02**
Total Protein (gm/dl)	6.98±0.24	4.87±0.38	5.03±0.12	5.57±0.22	5.87±0.33	5.33±0.16
Serum Urea (mg/dl)	15±1.24	77.83±2.57	70.5±1.18	49.33±2.22**	40.5±1.9**	24±1.53**
Weight of Liver(gm)	1.81±0.21	3±0.17	2.9±0.17	2.48±0.14	2.23±0.12**	2±0.18**

Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

The normal hepatic antioxidant parameters are shown in of Group I (Saline) which represented normal control. Group II(d-Galactosamine induced) animals showed significant reduction in hepatic antioxidant levels SOD, CAT, GR,GSH-Px and increase in lipid peroxidation(LPO). Group III (Honey + d-Galactosamine) also shows increase in these hepatic antioxidants as that of d-Galactosamine induced Group II.Group IV(25mg/Kg) and Group V (125mg/kg) showed highly significant increase in the antioxidant enzyme with significant decline in the LPO as almost equal to that of the standard drug Silymarin(200mg/Kg) when compared with paracetamol induced animals (Table-32).

Table 32. Effect of SSM on Liver Anti-oxidant enzyme level in d-galactosamine induced liver injury

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI
SOD Units/mg protein	10.92±0.81	4.27±0.32	5.85±0.35	5.92±0.3**	6.79±0.17**	7.23±0.35**
CAT Units/mg protein	3.02±0.27	1.55±0.11	1.65±0.12	1.9±0.13*	2.25±0.21*	2.33±0.15**
GR (nano moles / min / mg protein)	44.17±2.55	22±1.79	25.5±0.85	31.17±1.30**	34.33±1.41**	39.5±2.14**
GSH-Px (nmoles of NADPH oxidized/min/ mg protein)	51.67±2.19	29.5±1.56	31.33±2.32	35.67±1.76	40±1.24**	44±2.37**
LPO (nano mole of MDA/mg protein)	2.27±0.28	15±1.04	12.32±0.55	9.97±0.35**	7.03±0.08**	5.08±0.19**

Values are expressed as mean ± S.E.M. (n=6), comparisons were made between: a Group II (negative control) vs Group III (Honey 40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

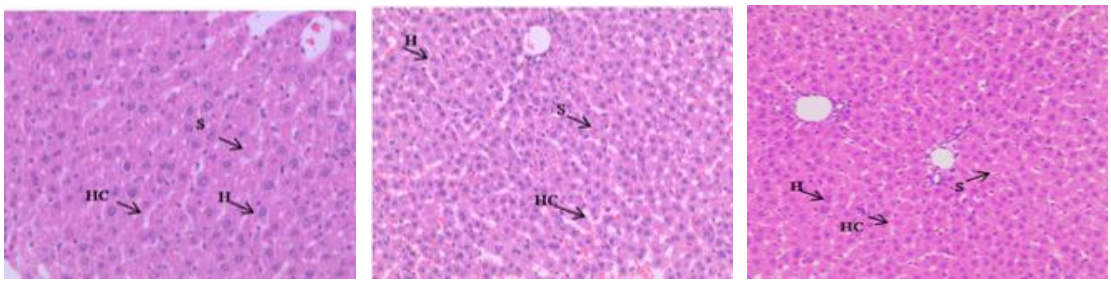
Histopathology of liver

To evaluate the changes in the liver tissues of all the 6 groups morphometric analysis was carried out based on the scoring system mentioned in alcohol induced liver injury.

Fig: 50 Histomorphometric Analysis on H&E staining of LPS)/d-GalN-induced liver injury

Fig. 50a. Histomorphometric Analysis ofGroup I animals

Sample 1- score 0 **Sample 2- score 0** **Sample 3- score 0**



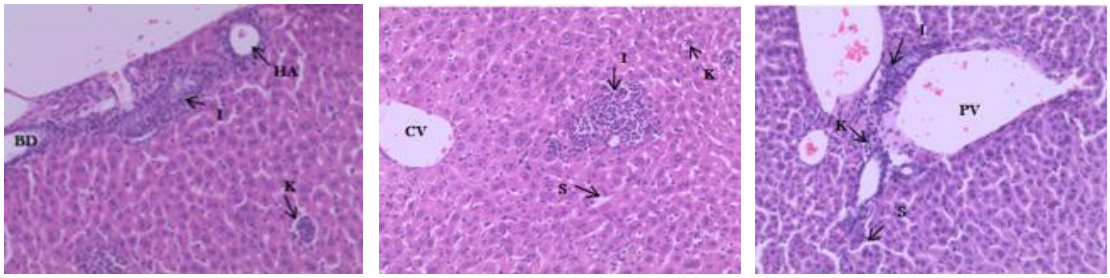
HC- Hepatic Cord, S-Hepatic Sinusoid

Figure 50.a shows the histological picture of the control group to which only saline was given

- Cytoarchitecture of hepatocytes appears normal, Arrangement of cells are at regular interval with equidistant sinusoidal space.

Fig. 50b. Histomorphometric Analysis ofGroup II animals

Sample 1- score 3 **Sample 2- score 3** **Sample 3- score 3**



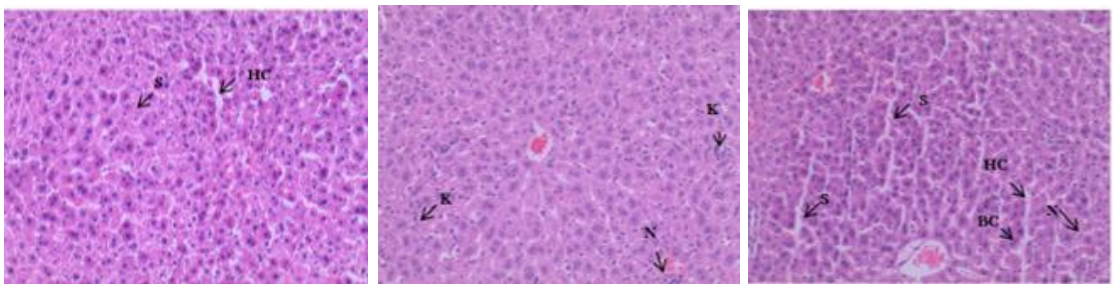
N- Necrotic Change, CV- Central Vein, BD- Bile Duct, HC- Hepatic Cord, S-Hepatic Sinusoid, K- kupffer cell, I – Infilteration with mononuclear inflammatory cells, HA-Hepatic Artery

Figure 50 b. shows the following histological changes in the liver tissues of Group II d-GalN-induced animals, which are

- Clustered infiltration of mono nuclear inflammatory cells was observed
- Derangement in hepatocellular architecture with increased sinusoidal space
- Increased number of kupffer cells radiated around central zone along with inflammatory cells was observed

Fig. 50c. Histomorphometric Analysis ofGroup III animals

Sample 1- score 3 **Sample 2- score 3** **Sample 3- score 2**



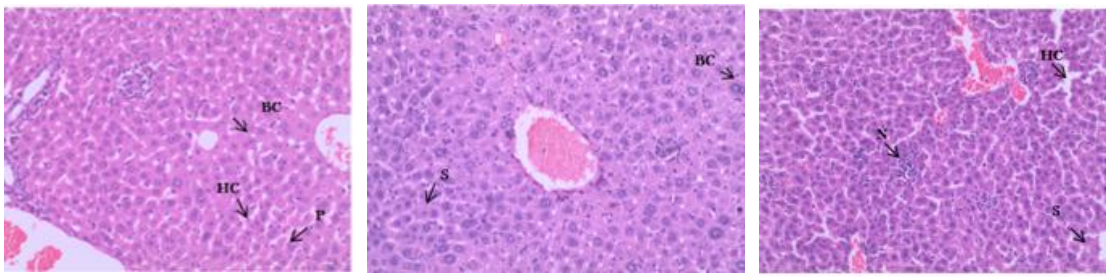
N- Necrotic Change, BC-Binucleated cells, CV- Central Vein, BD- Bile Duct, HC-Hepatic Cord, S-Hepatic Sinusoid, K- kupffer cell,

Figure 50c. depicts the following liver tissue changes in group III(d-GalN-induced) animals, as follows

- Increased level of binucleated cells and sinusoidal space was observed
- Few loci of necrotic changes was observed
- Derangement in hepatic cord was observed
- Constricted appearance of central vein was observed.

Fig 50.d Histomorphometric Analysis ofGroup IV animals

Sample 1- score 2 **Sample 2- score 2** **Sample 3- score 3**



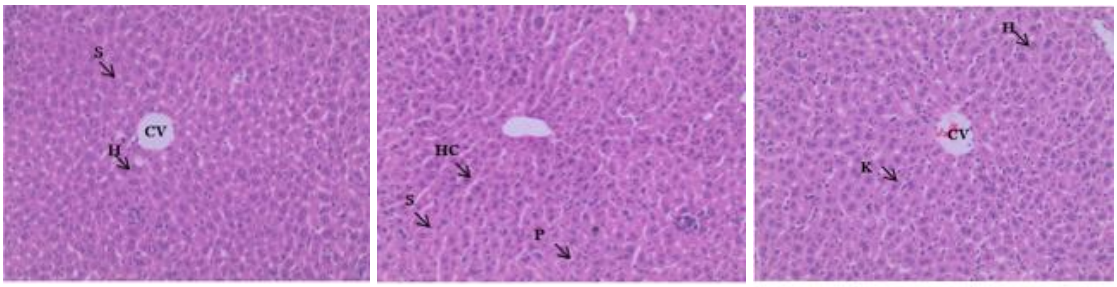
N- Necrotic Change, BC-Binucleated cells, CV- Central Vein, BD- Bile Duct, HC-Hepatic Cord, S-Hepatic Sinusoid, K- kupffer cell,

Figure 50d. shows the histological picture of group IV (SSM low dose) animals as follows

- Increased sinusoidal Space with mild derangement in hepatic cord arrangement was observed
- Cells undergoing marginal necrosis was clearly observed in periportal zone

Fig. 50e. Histomorphometric Analysis ofGroup V animals

Sample 1- score 2 **Sample 2- score 1** **Sample 3- score 1**



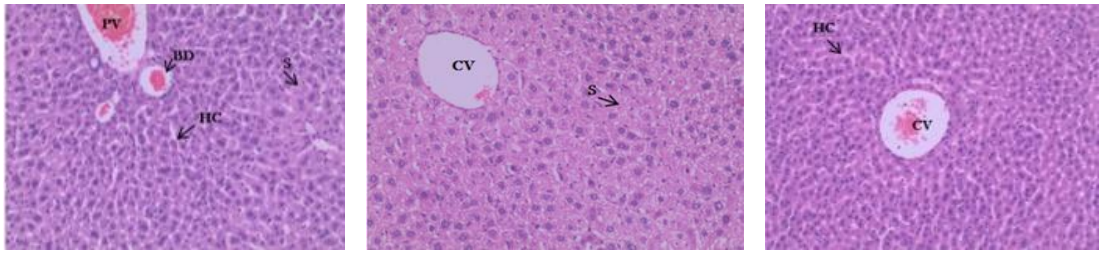
P-Pyknotic nuclei, CV- Central Vein, BD- Bile Duct, HC- Hepatic Cord, S-Hepatic Sinusoid, K- kupffer cell

Figure 50 e. illustrates the histology of group V (SSM high dose) animals, which are

- Appearance of central vein was normal with few RBC preset in it,
- Signs of inflammation was greatly reduced but signs of necrosis still presist occasionally
- Appearance of hepatic cord and sinusoidal space was normal

Fig 50.f.. Histomorphometric Analysis of Group VI animals

Sample 1- score 0 **Sample 2- score 0** **Sample 3- score 1**



PV-Portal Vein, CV- Central Vein, BD- Bile Duct, HC- Hepatic Cord, S-Hepatic Sinusoid

Figure 50 f. explains the histological changes of group VI (Silymarin) animals, which are

- Well projected radial arrangement of hepatocyte on to the cord was observed
- Appearance of portal vein, bile duct and hepatic artery was normal with some RBC cells filled in

Table 33. Morphometric score of LPS induced Hepatotoxicity

S.No	Groups	Score
1	Group I	0
2	Group II	3 ± 0
3	Group III	2.5 ± 0.22
4	Group IV	2.33 ± 0.21
5	Group V	1.33 ± 0.33
6	Group VI	0.33 ± 0.33

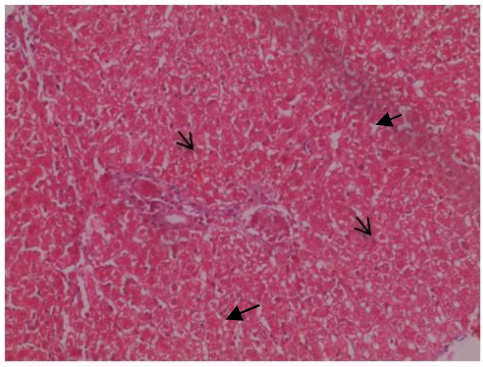
Table 33 confirmthat the morphometric scoring of all the groups revealed severe hepatic injury was repaired back to almost normal in the SSM high dose and Silymarin treated animals when compared to other groups.

Masson's trichrome staining

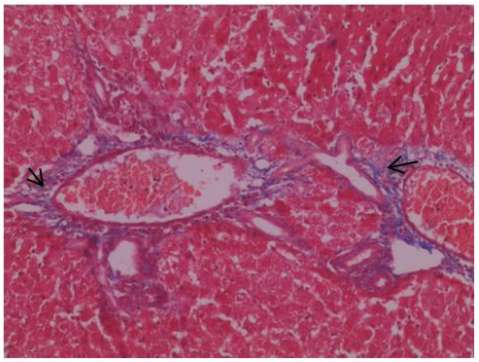
In figure 51 the liver section of control group mice shows normal cytoplasm of hepatocytes that are stained red and nuclei as dark red color. Significant level of fibrous tissue stained blue present in periportal region with diffused pattern of radiating in between the cords of the hepatocytes was observed in group II mice. Moderate accumulation of extracellular matrix proteins around central vein was observed in group III mice. The level of liver fibrosis and deposition of EMP was greatly reduced in group IV, V treated with low (25mg/kg) and high dose (125mg/kg) of SSM. Hepatocytic architecture and fibrous tissue formation was restored back to the normal in the group VI treated with standard drug Silymarin.

Fig. 51. Masson's trichrome staining of Lipopolysaccharide (LPS)/d-galactosamine (d-GalN)-induced liver injuryin mice

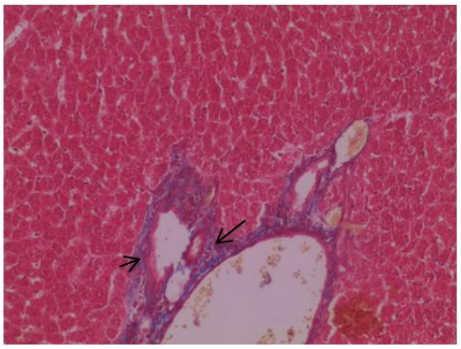
Group I- Control



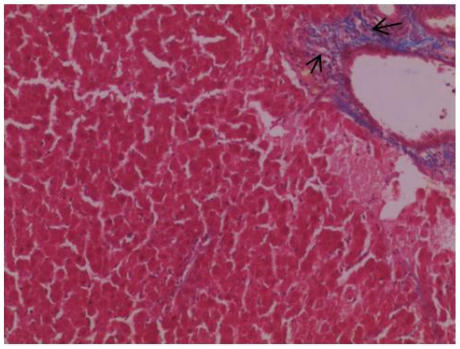
Group II- d-galactosamine Induced



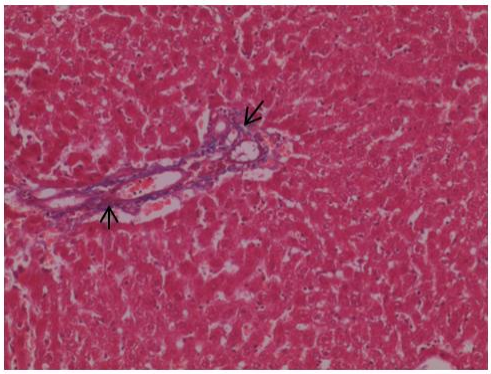
Group III- d-GalN+ Honey



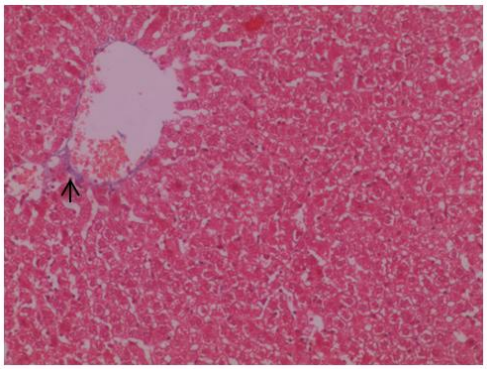
Group IV- SSM Low Dose



Group V- SSM High Dose



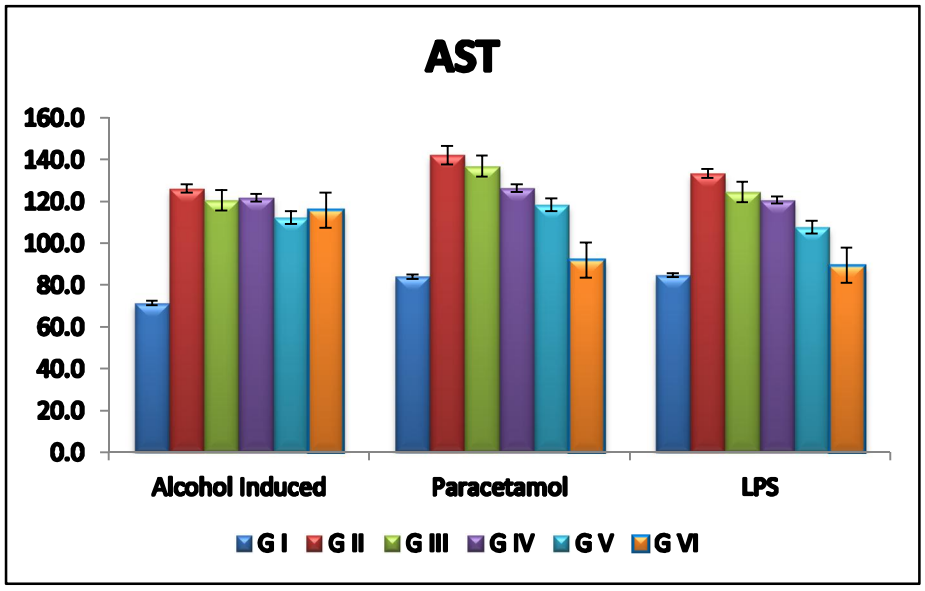
Group VI- Silymarin



Further graphical representation of the above pharmacological study results of each hepatic parameter are shown from fig 52 to fig 65

Figure 52: Effect of SSM on estimation of AST in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

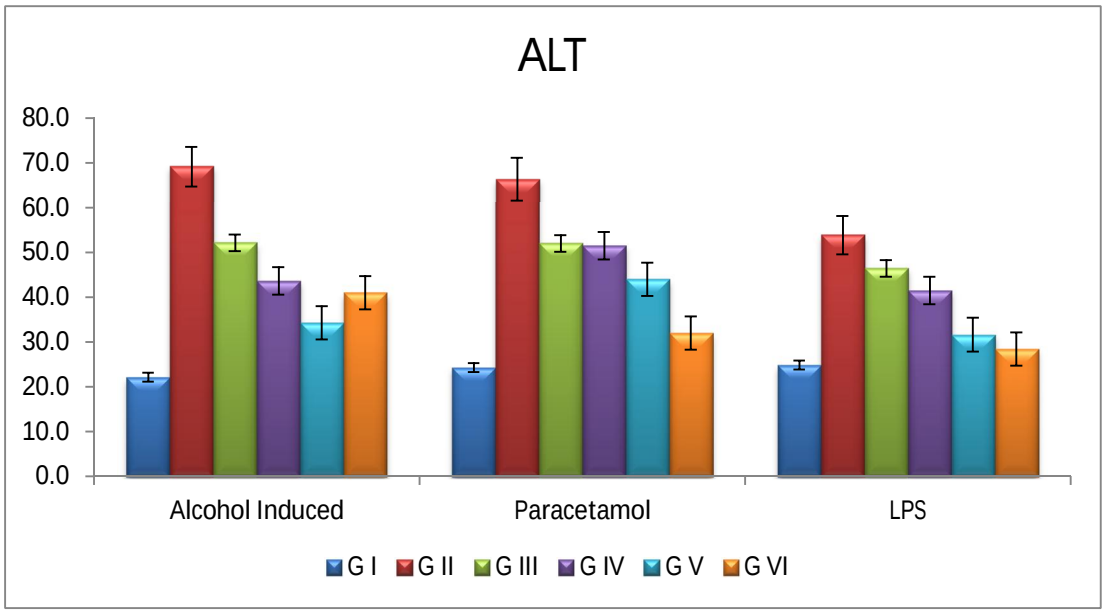
Groups	Total AST/(IU/ml)		
	52.a Alcohol Model	52.b Paracetamol Model	52.c LPS- d Galn Model
Group I	71.33±4.02	84±5.09	84.67 ± 3.45
GroupII	126.2± 1.96	142.2± 4.41	133.3 ± 2.08
Group III	120.5±1.2	136.8 ± 1.58	124.5 ± 1.72
Group IV	121.7±1.76	126.3 ± 1.8**	120.7 ± 1.54**
Group V	112.3±2.11**	118.3 ± 1.96**	107.7 ± 2.43**
Group VI	115.8±1.78**	92± 1.44**	89.33 ± 3.87**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test.

Figure 53: Effect of SSM on estimation of ALT in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

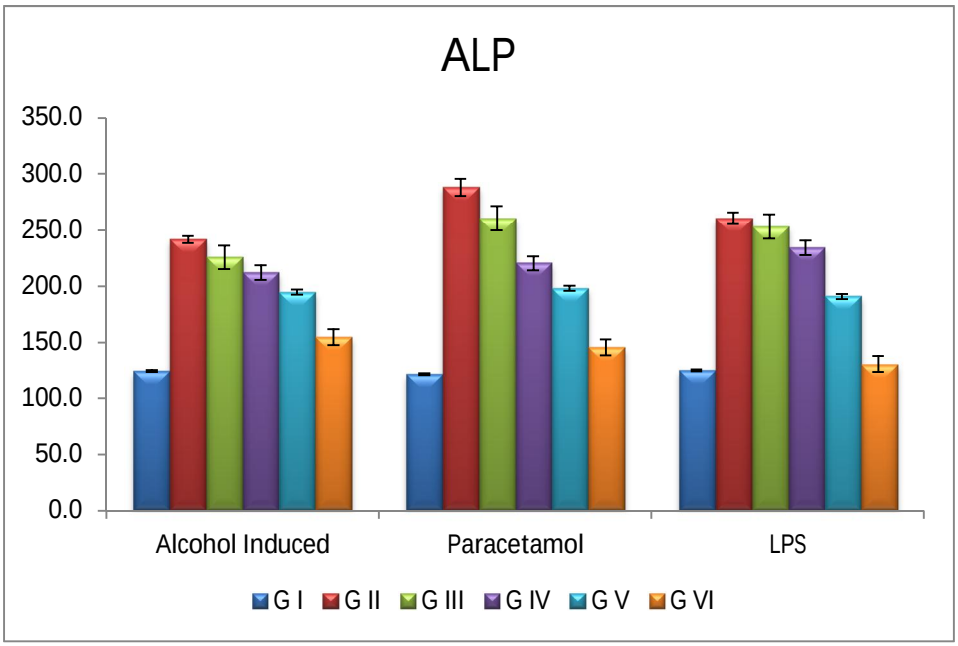
Groups	ALT		
	53.a Alcohol Model	53.b Paracetamol Model	53.c LPS- d Galn Model
Group I	22.17± 3.43	21± 2.6	24.83 ± 1.92
GroupII	69.17± 4.4	66.33± 4.77	53.83 ± 4.25
Group III	52.17± 1.2**	52 ± 1.61**	46.5 ± 2.28
Group IV	43.67± 1.31**	51.5 ± 2.05**	41.5 ± 3.27*
Group V	34.33± 1.5**	44± 2.1**	31.67 ± 1.53**
Group VI	41± 2.85 **	32± 1.71**	28.5 ± 2.26**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 54: Effect of SSM on estimation of ALP in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

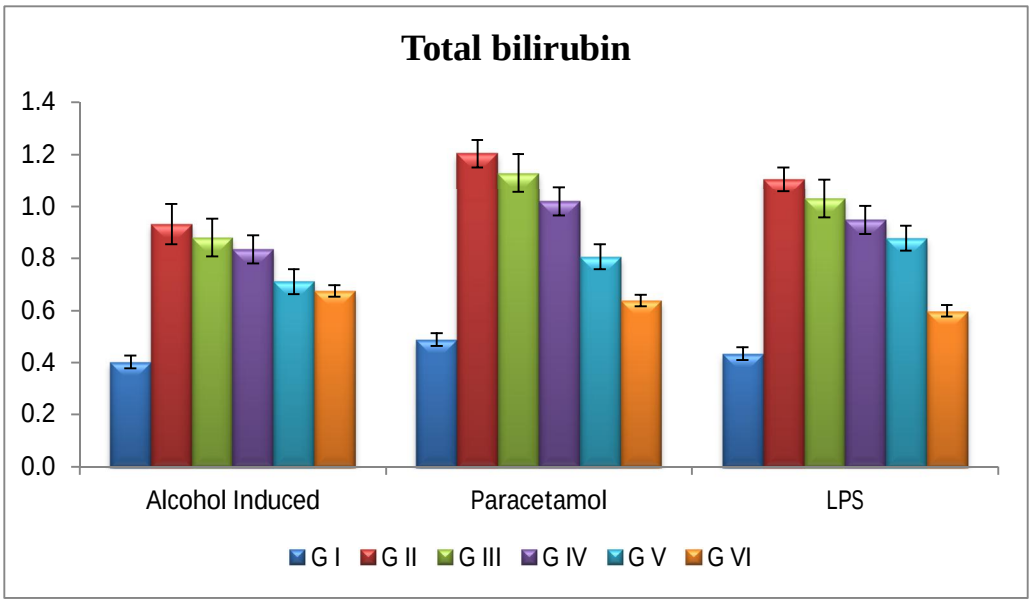
Groups	ALP		
	54.a Alcohol Model	54.b Paracetamol Model	54.c LPS- d Galn Model
Group I	124.3± 3.18	121.5± 3.09	124.8± 3.4
GroupII	241.8± 3.36	288.2± 7.61	260.5± 4.72
Group III	226 ± 2.0**	260.8± 2.02**	253.3± 2.6
Group IV	212.3 ± 3.25**	220.5± 3.91**	234.5± 2.5**
Group V	194.7± 2.31**	198.2± 4.47**	190.8± 2.39**
Group VI	154.8± 2.46**	145.3± 2.91**	130.3± 1.41**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 55: Effect of SSM on estimation of TOTAL BILIRUBIN in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

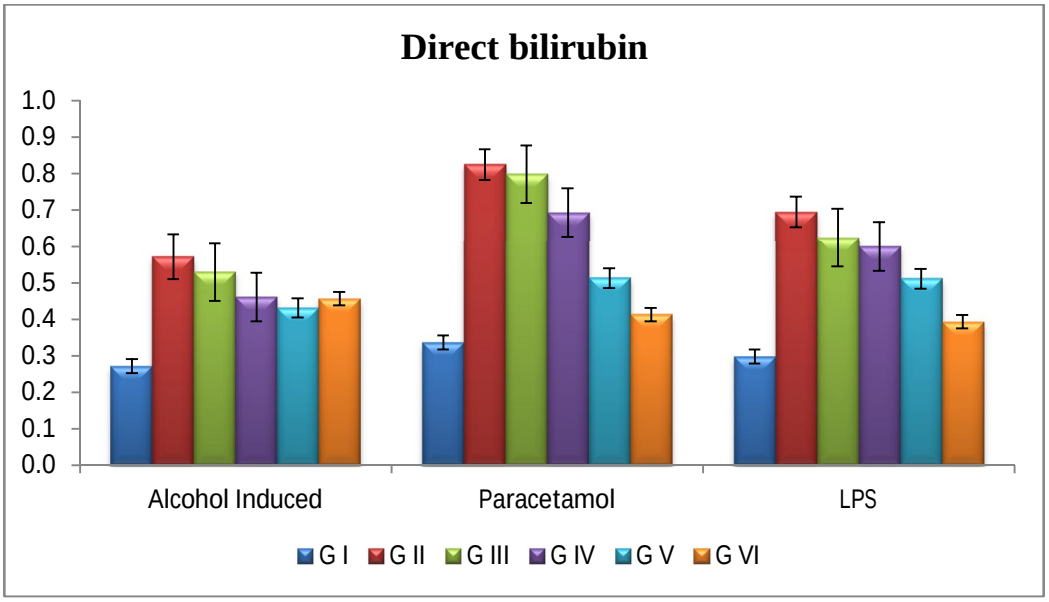
Groups	TOTAL BILIRUBIN		
	55.a Alcohol Model	55.b Paracetamol Model	55.c LPS- d Galn Model
Group I	0.4 ± 0.02	0.49 ± 0.03	0.43 ± 0.03
GroupII	0.93 ± 0.07	1.2 ± 0.05	1.11 ± 0.05
Group III	0.88 ± 0.02	1.13 ± 0.05	1.03 ± 0.03
Group IV	0.84 ± 0.03	1.02 ± 0.03*	0.95 ± 0.05*
Group V	0.71 ± 0.06*	0.81 ± 0.03**	0.88 ± 0.05 **
Group VI	0.68 ± 0.05**	0.64 ± 0.03**	0.6 ± 0.02**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 56: Effect of SSM on estimation of Direct bilirubin in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

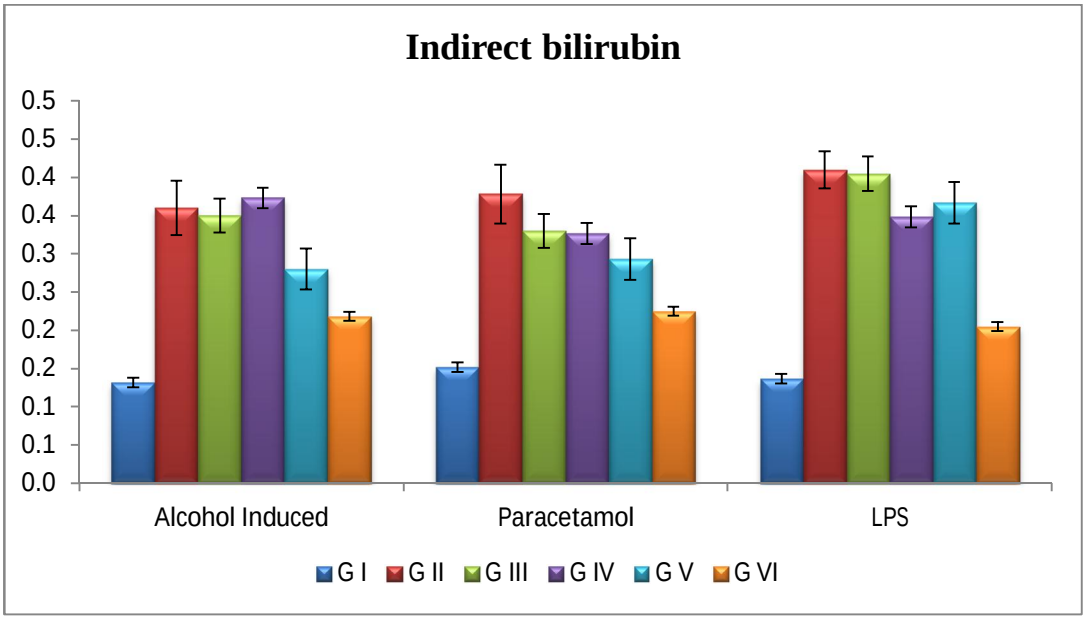
Groups	Direct bilirubin mg/dl		
	56.a Alcohol Model	56.b Paracetamol Model	56.c LPS- d Galn Model
Group I	0.27 ± 0.02	0.34 ± 0.02	0.29 ± 0.02
GroupII	0.57 ± 0.06	0.83 ± 0.04	0.7 ± 0.04
Group III	0.53 ± 0.01	0.8 ± 0.04	0.63 ± 0.04
Group IV	0.46 ± 0.02	0.69 ± 0.03*	0.6 ± 0.03
Group V	0.43 ± 0.03*	0.51 ± 0.02**	0.51 ± 0.03**
Group VI	0.46 ± 0.04	0.41 ± 0.03**	0.39 ± 0.04**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 57. Effect of SSM on estimation of Indirect bilirubin in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

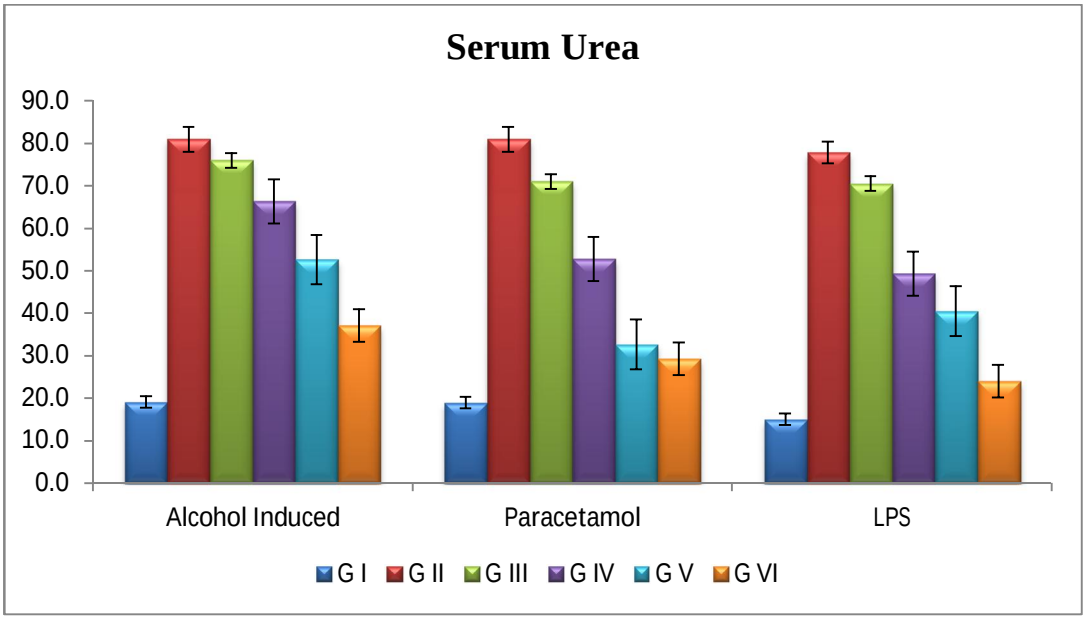
Groups	Indirect bilirubin mg/dl		
	57.a Alcohol Model	57.b Paracetamol Model	57.c LPS- d Galn Model
Group I	0.13 ± 0.01	0.15 ± 0.02	0.13 ± 0.02
GroupII	0.36 ± 0.03	0.37 ± 0.03	0.41 ± 0.02
Group III	0.35 ± 0.0	0.33 ± 0.04	0.41 ± 0.03
Group IV	0.37 ± 0.02	0.32 ± 0.05	0.34 ± 0.03
Group V	0.28 ± 0.03	0.29 ± 0.02	0.37 ± 0.03
Group VI	0.21 ± 0.02**	0.22 ± 0.03*	0.2 ± 0.02**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 58: Effect of SSM on estimation of Serum Urea in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

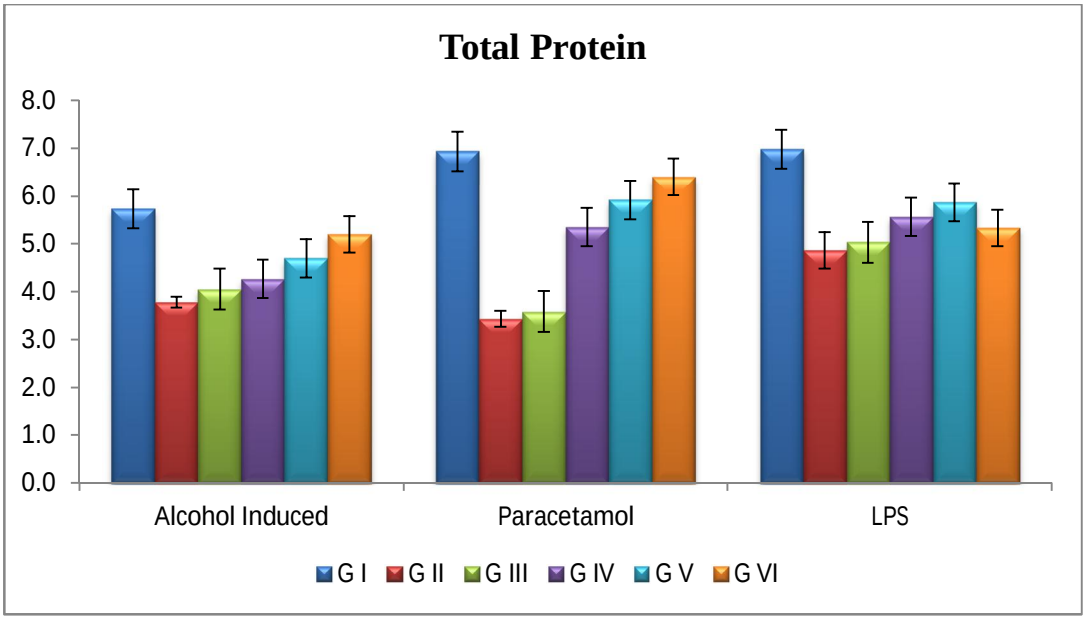
Groups	Serum Urea mg/dl		
	58.a Alcohol Model	58.b Paracetamol Model	58.c LPS- d Galn Model
Group I	19.17 ± 1.3	19 ± 1.18	15 ± 1.24
GroupII	81 ± 2.94	81 ± .94	77.83 ± 2.57
Group III	76 ± 2.2	71 ± 2.15	70.5 ± 1.18
Group IV	66.33 ± 1.65**	52.83 ± 2.24**	49.33 ± 2.22**
Group V	52.67 ± 1.73**	32.67 ± 1.5**	40.5 ± 1.9**
Group VI	37.17 ± 2.02 **	29.33 ± 2.04 **	24 ± 1.53**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 59: Effect of SSM on estimation of Total Protein in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

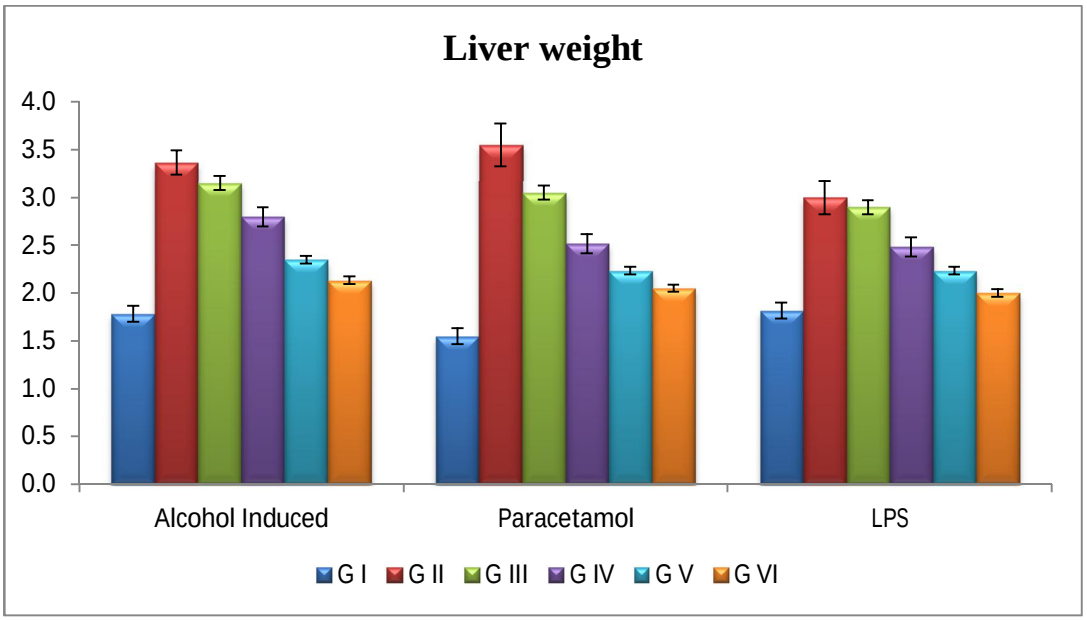
Groups	Total Protein mg/dl		
	59.a Alcohol Model	59.b Paracetamol Model	59.c LPS- d Galn Model
Group I	5.73±0.26	6.93± 0.23	6.98± 0.24
GroupII	3.78±0.11	3.43± 0.16	4.86± 0.38
Group III	4.05±0.17	3.58± 0.29	5.03± 0.12
Group IV	4.26±0.24	5.35± 0.18**	5.56± 0.22
Group V	4.7±0.17**	5.91± 0.22**	5.86± 0.33
Group VI	5.2±0.11**	6.4± 0.21**	5.33± 0.16



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 60: Effect of SSM on estimation of Weight of Liver in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

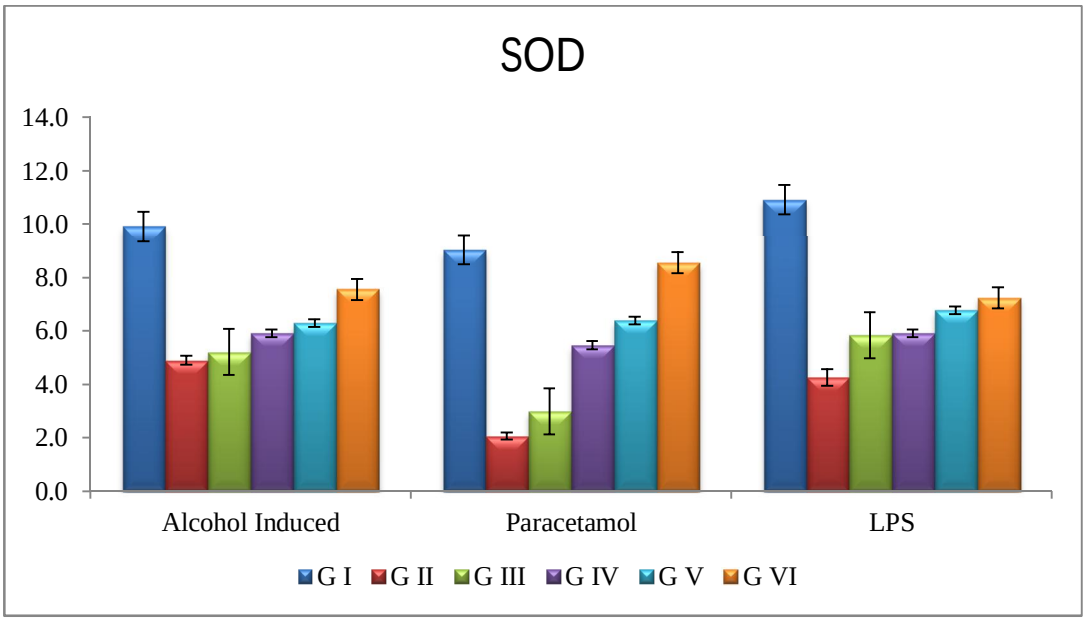
Groups	Weight of Liver mg/dl		
	60.a Alcohol Model	60.b Paracetamol Model	60.c LPS- d Galn Model
Group I	1.78 ± 0.19	1.55 ± 0.09	1.81 ± 0.21
GroupII	3.37 ± 0.13	3.55 ± 0.22	3 ± 0.17
Group III	3.15 ± 0.16	3.05 ± 0.11	2.9 ± 0.17
Group IV	2.8 ± 0.13 *	2.51 ± 0.18**	2.48 ± 0.14
Group V	2.35 ± 0.16**	2.23 ± 0.15**	2.23 ± 0.12 **
Group VI	2.13 ± 0.10 **	2.05 ± 0.17**	2 ± 0.18 **



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 61: Effect of SSM on Estimation of SOD in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

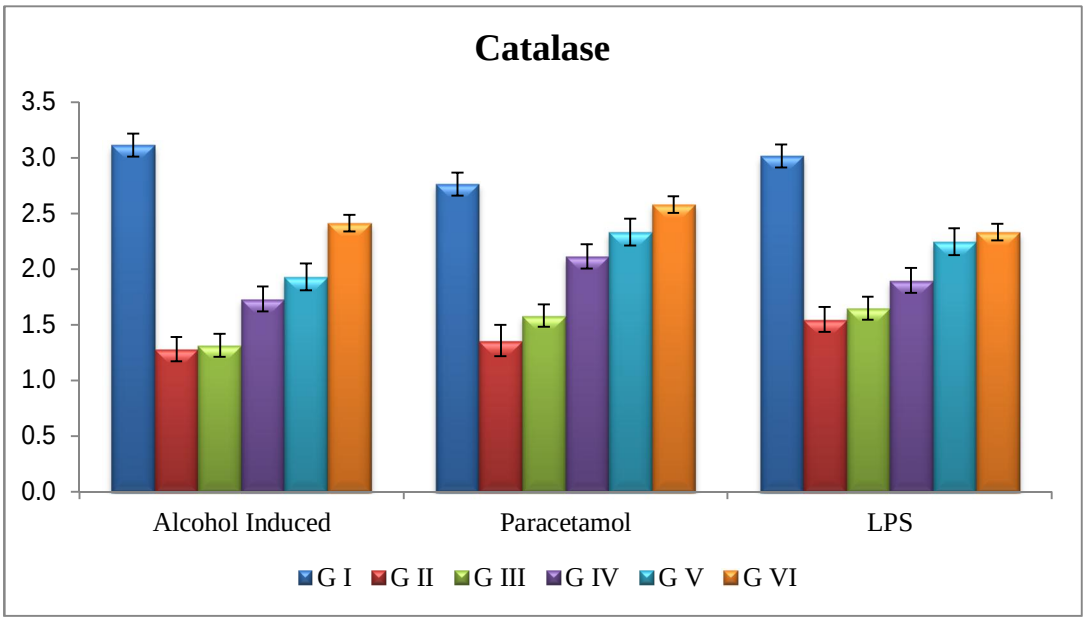
Groups	Estimation of SOD mg/dl		
	61.a Alcohol Model	61.b Paracetamol Model	61.c LPS- d Galn Model
Group I	9.9 ±0.49	9.03 ±0.51	10.92 ±0.81
Group II	4.9 ±0.16	2.07 ±0.13	4.27 ±0.32
Group III	5.21 ±0.15	3 ±0.12	5.85 ±0.35
Group IV	5.91 ±0.24*	5.47 ±0.22**	5.92 ±0.3**
Group V	6.3 ±0.19**	6.4 ±0.26**	6.79 ±0.17**
Group VI	7.57 ±0.33**	8.55 ±0.40**	7.23 ±0.35**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 62: Effect of SSM on Estimation of CATALASE in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

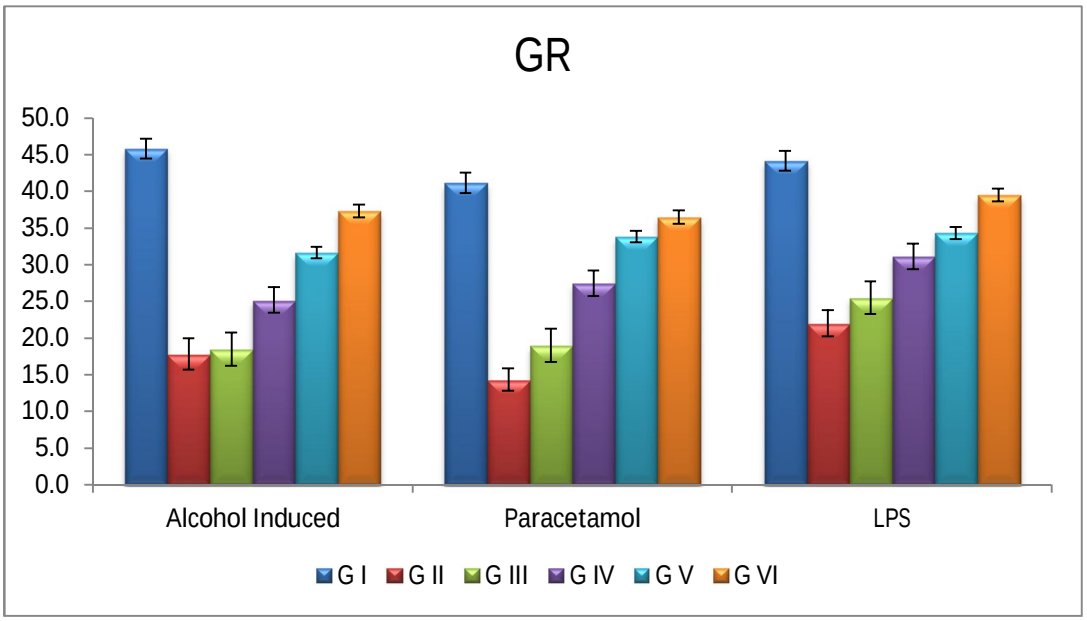
Groups	Estimation of CATALASE mg/dl		
	62.a Alcohol Model	62.b Paracetamol Model	62.c LPS- d Galn Model
Group I	3.12 ±0.19	2.77 ±0.21	3.02 ±0.27
Group II	1.28 ±0.1	1.36 ±0.14	1.55 ±0.11
Group III	1.31 ±0.11	1.58 ±0.11	1.65 ±0.12
Group IV	1.73 ±0.09	2.11 ±0.18*	1.9 ±0.13
Group V	1.93 ±0.15**	2.33 ±0.14**	2.25 ±0.21*
Group VI	2.42 ±0.15**	2.58 ±0.26**	2.33 ±0.15**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 63: Effect of SSM on Estimation of GR in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

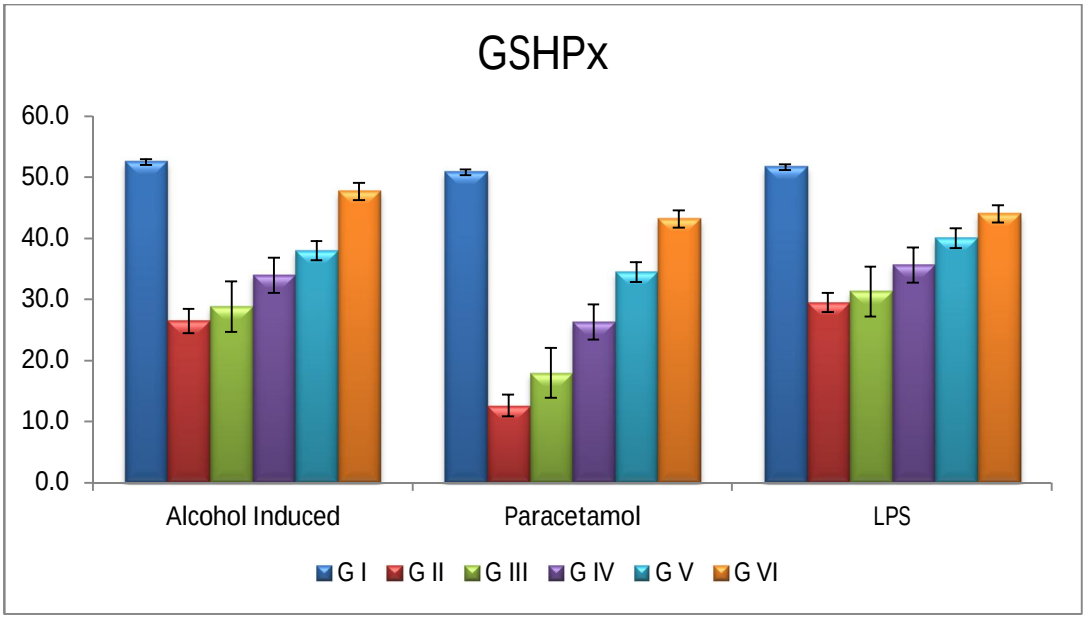
Groups	Estimation of GR mg/dl		
	63.a Alcohol Model	63.b Paracetamol Model	63.c LPS- d Galn Model
Group I	45.83 ±2.39	41.17 ±1.99	44.17 ±2.55
GroupII	17.83 ±2.12	14.33 ±1.59	22 ±1.79
Group III	18.5 ±0.85	19 ±1.59	25.5±0.85
Group IV	25.17 ±2.04*	27.5 ±3.33**	31.17 ±1.30**
Group V	31.67 ±1.76**	33.83 ±1.30**	34.33 ±1.41**
Group VI	37.33 ±2.10**	36.5 ±2.11**	39.5±2.14**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 64: Effect of SSM on Estimation of GSH in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

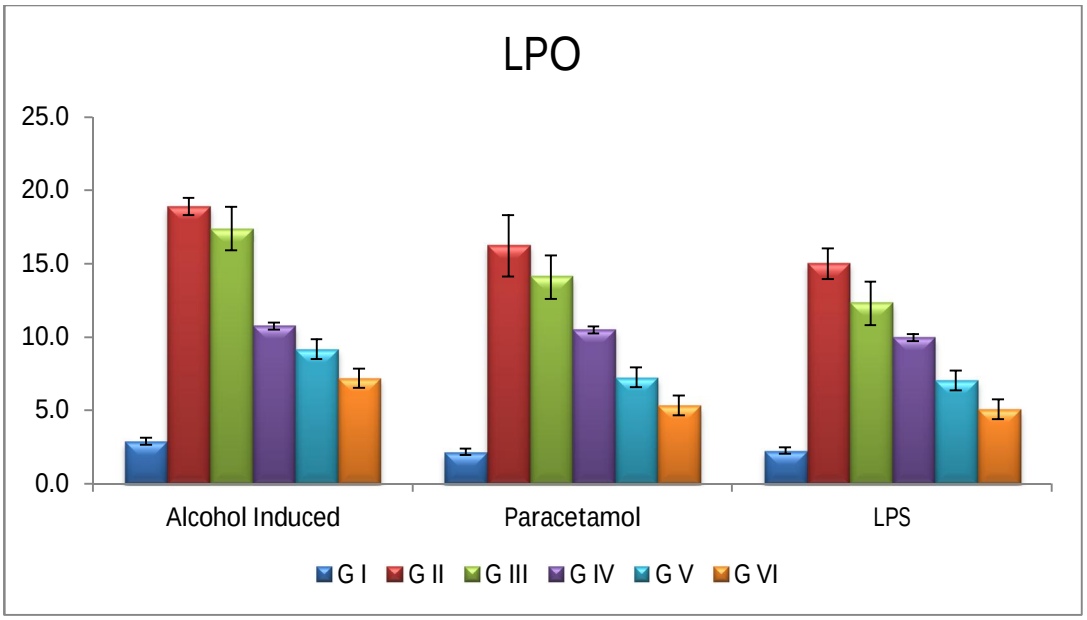
Groups	Estimation of GSH mg/dl		
	64.a Alcohol Model	64.b Paracetamol Model	64.c LPS- d Galn Model
Group I	52.5±2.36	50.83±1.79	51.67±2.19
GroupII	26.5±1.96	12.67±1.80	29.5±1.56
Group III	28.83±1.4	18±0..97	31.33±2.32
Group IV	34±1.34*	26.33±1.20**	35.67±1.76
Group V	38±1.84**	34.5±1.11**	40±1.24**
Group VI	47.67±2.89**	43.17±1.58**	44±2.37**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 65: Effect of SSM on Estimation of LPO in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

Groups	Estimation of LPO mg/dl		
	65.a Alcohol Model	65.b Paracetamol Model	65.c LPS- d Galn Model
Group I	2.9±0.16	2.17±0.26	2.27±0.28
GroupII	18.9±0.59	16.23±2.09	15±1.04
Group III	17.38±0.76	14.1±0.43	12.32±0.55
Group IV	10.75±0.40**	10.48±0.33**	9.97±0.35**
Group V	9.17±0.67**	7.25±0.14**	7.03±0.08**
Group VI	7.2±0.22**	5.33±0.20**	5.08±0.19**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

8. DISCUSSION

In Siddha formulary the drugs of herbal origin metals, minerals and animals are used liberally for therapeutic purposes. The usage of metals and minerals in this traditional system of medicine is the most controversial issue of the present day. Enormous data has been generated during the past century about the serious toxicity produced by the metal based products. However there are standard operating procedures (SOP) for all existing drugs for universal acceptance.Hence there is a vital need for the standardization of these ancient drugs[97]. Standardization and quality control of herbal drugs is the present day demand of WHO. This includes all aspects from selection and handling of crude material, safety and efficacy to the stability assessment of final product and also includes the provision of product information to the consumer[98].

In Traditional medicine numerous process have been mentioned to use toxic metals as its therapeutic compound form on a prescribed dosage.This study on SSM involved the standardization and testing of the safety and efficacy profile the Siddha herbo-mineral formulation for the treatment of liver disorders in experimental animal models and the results were analysed.

The herbal and mineral ingredients used for the preparation of the Siddha formulation *SSM* were identified and authenticated through their physical appearance, botanical features such as plant anatomical structure to confirm the peculiarity of raw material. The identified and authenticated raw materials were purified as per Siddha text and the purification methods were standardized using three batches of ingredients to assure the unique chemical nature of its own and to avoid drug to drug variation in the final product[99].

The results of Table-1 shows the quantity of ingredients required for standardizing the purification process of Pooram and its analyses reveals the exact time duration required for the complete process of purification of Pooram. Though the Siddha literature mentions about the ratio of ingredients that are required to

purify Pooram, it lacks the prescribed time required for the evaporation of 1/4th of the liquid medium.

The study reveals that time required for purification of Pooram was 1hour 30 minutes to 1 hour 40 minutes. This process of purification of Pooram by knotting the raw Pooram in the cloth and dipping in the liquid medium containing betel leaves and pepper potentiates the minerals and removes the physical and chemical impurities. This process makes the minerals brittle, reduces the particle size and evaporates the toxic ingredients into the liquid medium making the minerals to become less toxic. Table 2 & 3 shows the percentage of weight loss before and after purification of Pooram and Vengaram. The results show that the weights of Pooram and Vengaram before and after purification were almost similar in all the three samples. Whereas on comparing the reduction of weight, it was more than 50% in Vengaram due to the frying process in which de-hydration occurs and the crystalline structure of Vengaram is changed into light and puffed up structure indicating the evaporation of water[100].

The amount of lemon juice that was required during summer for preparing the three samples of SSM A, B and C (Pooram 100 gm, Vengaram-50 gm and Manjal-300 gm) was 1350ml, 1330ml and 1320ml respectively. The above preparation was prepared traditionally as per ancient texts by using manual grinding (kalvam). Therefore the three batches of purification process and the prepared medicine shows that there is not much variation in the three purified samples and the three batches of final product (SSM). The prepared samples of SSM A, B and C were then subjected to physicochemical variation for standardization purpose.

The organoleptic characters of SSM were studied in all the three samples and all of them were found to be dark red in colour and sour in taste with turmeric odour. The loss on drying test is designed to measure the moisture content of a sample on drying it under specific conditions[101].Minimal moisture content is always desirable for good stability of drugs and to prevent the drug degradation[102]. The loss on drying of the three samples of SSM at 105^{0}C contains 3.53%– 3.62% of moisture, ensuring that it is well protected against microbial contamination and humid atmosphere. SSM contained 11.27% -11.32% of total ash value and 8.19% -9.50 of acid

insoluble ash value as mentioned in Table-5. The sulphated ash value was found to be 8.12% -9.50%. Hence these parameters determine the strength of this formulation and its purity[103].

The disintegration time of SSM was found to be 2.32-2.42 hours and the solubility was analyzed to be 15 hours and 30 minutes. This is because the formulation is insoluble in water. However as per Siddha literature on SSM, it is advised to administer the powdered form of this tablet mixed with the adjuvant honey. It has been claimed that honey helps in the active transport of the drug across the membrane and facilitates its entry into the blood stream[104].Hence these parameters, disintegration and solubility does not apply to this kind of classical formulation.

The prevalence and concentration of heavy metals in SSM samples were done using AAS and the concentration of lead, cadmium; mercury and arsenic were found to be below the detectable limit. Further work has been done by using sophisticated instrument analysis to confirm the safer limits of heavy metals. The presence of microbial contaminants indicate the poor quality of production and harvesting of raw materials and poor handling by persons during manufacture process as per WHO guidelines for assessing the quality of herbal medicines[105]. SSM was found to contain the permissible limits of bacterial and fungal count. The other microorganisms such as Enterobacteriaceae, E.coli, Salmonella sp and Staphylococus aureus were absent in the test sample indicating that there is no favorable moisture content for bacterial and fungal growth. Similarly no significant aflatoxin was identified for contamination. Since aflatoxins are reported as nephro-toxic, hepato-toxic and carcinogenic causing nervous disorder the study confirms that SSM is free from such harmful hazards[106]. Pesticide residues were not detected in the tested samples of SSM indicating the quality assurance of the final product[107]. pH value of SSM is 4.6-4.8 indicating that it is a weak acid and its diffusion occurs in the gastric mucosa[108]. Weight of the SSM pills varied from 60-90mg and the size of the SSM pills as measured by vernier caliper was found to be 4mm-6mm which is approximately equal to that of pepper size(6mm).Variations of pill size and weight are due to manual preparations which needs much caution to avoid alteration in dosage level.

The final product of the three SSM samples was studied for the qualitative and quantitative estimation of the active component curcumin. The High performance Thin layer chromatography (HPTLC)is the most affordable gold standard method that is simple and accurate.This study analysed and quantified the "Curcumin" content as 0.6589 % in SSM-A, 0.6884 %in SSM-B and 0.7104 % inSSM-C[109].The significant compounds curcumin present in curcuma longa which is the major ingredient of SSM was further analysed by UV spectroscopy.

This study reveals the presence of Curcumin in SSM and was calculated as SSM A -1.15%, SSM B-1.19%, and SSM C-1.23% .The above studies justifies that the three samples of SSM do not significantly differ from each other as documented by the reproducibility and repeatability of the method. The process of standardization thus adapted can help in the large scale manufacturing and processing of this formulation.

TGA analysis showed that there is a weight loss of 77% whichcould be due to the burning of attached organic matter[110]. The presence of organic matter along with mineral drug suggests that organic matter can act as coating material just as the concept of novel drug delivery system developed in recent years. TGA analysis showed that major content in final product is organic.

Raman spectroscopy showed the presence of Mercurous chloride in both raw and purified Pooram and also the presence of hydroxyl group in raw, purified Vengaram and prepared SSM but no possible conclusion could be related with its physico-chemical nature. Therefore IR spectroscopy was performed which is a significant tool for the identification of chemical molecules that are actively present.

The FTIR spectrum obtained in this study drug SSM (O-H str at 3500-3200 cm-1, C=C str at 1600 cm-1, C-O str at 1620 cm-1 and intermolecular H-bonding) was found to be in match with the FTIR spectrum of curcumin found in the literature[111]. There was increased inter molecular H bonding in SSM and the shift of peak in the range of 1000-4000cm-1 supports the formation of organo-mineral complex. The complex formation may be formed due to the interaction occurring

between C=C, C=O, -OH, aromatic ring, etc. present in curcumin which is supported by shift in the C=O stretching of the complex.

Though FTIR and Raman analysis showed the presence of organo-mineral complex, the exact form of organo-mineral complex is still unknown. Based on the raw materials added there is a chance for formation of 2 forms of complexes. Either compounds of turmeric can form complex with boron or it can form complex with purified pooram. There is also chance for formation of both types of complexes depending on the environmental conditions.

After that, XRD Analysis was performed for Pooram, Vengaram and the final product SSM in order to understand the characterization of compounds in their raw form before purification and their change in chemical structure after purifying those using organic compounds and by heating process. XRD graph of raw Vengaram showed a change in structure from rhombohedral ($Na_2B_4O_7$ $10H_2O$) to orthorhombic ($Na_2B_4O_7$ $5H_2O$) form when purified. This change is due to the evaporation of water molecules during the frying process of purification. Similar results are also obtained from previous study by Srinivasulu et al., 2012[113].

Further the tetragonal crystalline structure of raw Pooram also showed a change in its crystalline structure after purification process (P1) although the exact structural change could not be established.The final product SSM showed similar peaks corresponding to P1, indicating that Hg_2Cl_2 from P1 is intact. The increase in intensity of 44.5 peak decrease in intensity of 46.44 peak in SSM was compared with P1. The presence of organo-metallic complex was still ambiguous, as part of the P1 could have formed complex with compounds of turmeric and the rest would have remained unreacted leading to the XRD signatures. Moreover, signatures of V1 are completely absent in SSM. This signifies that, $Na_2B_4O_7$ from V1 is not found in crystalline form and had formed complex with compound in turmeric in SSM

Although SSM has been indicated for liver disorders in Siddha texts, and there is a fairly extensive data on its herbal and mineral ingredients there is a biological necessity to analyze the toxicological profile of this herbo mineral drug since insufficient data exist for most herbal drugs including Siddha preparations to

guarantee their quality, efficacy and safety[114]. According to WHO, the use of herbal medicine should be substantiated by the affordability, knowledge of medicinal plants and the belief of safety[115].Hence to optimize the beneficial use and safety of SSM, a comprehensive data has been generated on the toxicological profiling of SSM.

The Acute oral toxicity study indicated that 2000 mg/ kg body weight of SSM given orally to rats did not induce mortality and there was no abnormal behavioural signs noted during the 14 days of study period suggesting the safety of the preparation (Table-13). The given dosage is 100 times more than the therapeutic dosage. Therefore the drug can be assured that it is well tolerable. Repeated dose 28 days study (sub acute toxicity) of low (25mg/kg), mid (125mg/Kg) and high (250mg/Kg) dose reveals that there was no alteration in food and water consumption and there was an obvious weight gain in the SSM treated animals when compared to the control groups of the experimental animals, therefore it is crystal clear that there was no catabolism and the metabolic process of the rats remain conserved[87].

No significant changes were observed in the hematological parameters (Figures 30a,b & 31c,d) which have a higher prediction value towards the toxicity of a drug. The results therefore indicate that SSM is a safe drug which shows no noticeable toxic change in terms of haematopoesis and leukopoesis which is the production and circulation of blood cell components such as RBC, WBC and Platelets[116].

Data analysis of the biochemical parameters of the test drug indicates that it does not cause any significant rise in the transaminases enzymes AST and ALT that are “biomarkers” of liver injury and drug toxicity[117]. ALT in particular is used to assess the liver injury risk for the purpose of drug approvals and drug development and has been regarded as the “Gold standard biomarker for liver injury”[118]. These Proteins leak into the circulation when there is hepatocellular injury showing their elevation in serum levels. The enzyme ALP increases due to hepatic excretion defects. Bilirubin which is the breakdown product of haem provides the assessment of better liver function. Albumin and other proteins are synthesized by liver cells and there is a decrease in these plasma proteins in extensive liver damage[119]. The

study reveals that the test drug does not produce injury to the liver parenchyma cells as there were no significant changes as observed in fig 35.c.1.

Evaluation of renal functions are analysed by renal parameters such as Serum electrolytes, urea and creatinine which are sensitive indicators of negative renal function[120]. Urea which is the main product of protein metabolism has an important diagnostic value indicating renal impairment[121].The blood levels of creatinine depends on its production and excretion[122,123]. No significant change was observed in the renal parameters whencompared with the control. The observed data suggest that there was no serious pathological implications and does not indicate any serious toxic effects.Also the relative organ weight of brain, heart, liver, kidney, spleen, testis and ovaries did not show any remarkable change indicating that the drug does not cause any tissue damage.

Evaluation of sub chronic toxicity study over longer time for 90 days of study period showed no serious behavioural abnormality and mortality. Mild laxative action on high dose (250mg/kg) which was observed may be due to the laxative action of the ingredient pooram[124]. The significant increase in food and water intake also suggest that the drug did not produce any loss of appetite which may be due to the presence of flavanoids in lemon, which are known to stimulate the digestive system and increase secretion of digestive juices thereby enhancing the appetite.[125]

The haematological parameters show some statistically significant results in subchronic toxicity study. Analysis of haematological parameters predicts relevant risk for drug toxicity. Through the study it was revealed that SSM cause significant increase in the packed cell volume and neutrophil count in both male and female rats at high dose when compared with the control an increase in RBC count was also observed in male rats when compared to the control. And the results were within the physiological limits. The increase in erythrocytes may be due to the presence of vitamin C in lemon juice and also due to the vehicle honey which was also found to enhance hematocrit and immune response in a previous study by mykola et al.,[126,127,128]

The biochemical parameters such as glucose, cholesterol, LDL, HDL, triglycerides showed no significant changes. The liver marker enzyme AST which is an indicator of hepatic damage was significantly reduced within the physiological limits in High dose treated male rats indicating healthy liver function. Bilirubin level which is a confirmation of varying liver function also showed no significant change when compared to control group.

Plasma protein is an indicator for assessing synthetic capacity of liver cells, since hepatocytes are responsible for synthesis of proteins, decrease in plasma protein indicates chronic liver damage. The study showed that there was increase in total proteins in low dose of SSM within the physiological limits when compared to the control. But no significant changes were observed in mid dose and high dose indicating that the drug SSM did not cause any change in the hepatic parameters when compared to the control. Both subacute and subchronic renal parameters urea, BUN and creatinine were within the normal limits with no significant change suggesting no renal damage.In post retrieval group the haematological and biochemical parameters were almost restored to that of the control indicating the safety of the drug SSM on long term administration.

Subacute histopathological analyses of internal organs did not exhibit any significant pathological changes when compared with the control. There was no significant change observed in the organ weights in both high dose and post retrieval group when compared with control group.

Subchronic toxicity study revealed the presence of mild periportal necrosis associated with dilated sinusoidal space and presence of minimal renal tubular degeneration in kidney at a high dose of 250mg/kg of SSM but in post retrieval group there was normal hepatocellular architecture present in liver and kidneys showed normal renal parenchyma with normal glomeruli. This confirms that there is no accumulation of metals in the kidney indicating complete restoration of hepatocytes and renal tissues.

Moreover, there was no elevation of liver enzymes (AST, ALT) and renal parameters (Urea, creatinine).These findings support that the test drug SSM did not cause any toxic manifestations..

No microscopic significant change was observed in spleen indicating the normal microstructure of reticuloendothelial system.Also other organs such as brain, lung, heart and stomach showed no histopathological changes. No pathological change observed in the testis, uterus and ovaries indicating no toxic effects in reproductive organs of high dose and post retrieval group.

Further the safety profile of SSM was confirmed by ICP-MS to analyse the presence of mercury content in high dose and recovery group. Trace of mercury was present in the ICP-MS analysis in high dose group. But at the same time, there occurred an interesting fact that the levels of mercury was below detectable limit (BDL) in the post retrieval group after 90 days of treatment indicating that it is not being accumulated in renal tissues and is completely eliminated from the kidney. Hence the mere perception of toxicity based on the concentration levels of mercury can be misleading as the toxicity of a substance depends on its bioavailability, its chemical structure and the biochemical reaction in which it participates[129]. The ICP-MS study therefore confirms that the bioaccumulation of mercury which is the cause of toxicity could not be established in SSM.

The toxicologically analysed herbomineral formulation SSM after being found to be in safer margin was then subjected to efficacy studies. The In-vivo pharmacological studies includedEthanol induced liver damage in mice, Paracetamol-induced liver injury, LPS +D –galactosamine induced hepatoxicity which may mimic alchohol induced liver damage, acute liver damage and viral hepatitis respectively.These studies are essential since the main target of toxicity of several drugs and compounds is liver and most of the substances undergo first-pass metabolism here[130].

Ethanol induced liver injury contributes to the pathogenesis of chronic liver disease.Previous studies also indicate that ethanol induced liver injury exhibited significant increase elevations of Serum liver markers such as AST,ALT, and ROS

production. The study results showed that the Siddha formulation SSM significantly decreased these hepatic biomarkers inboth low and high dose groups almost equal to the standard drug Liv52.

Ethanol can induce the formation of gamma glutamyl transpeptidase which is a known marker of hepatic injury resulting in loss of cellular integrity and structure and also enhances the formation of malondialdehyde which inturn facilitates the generation of free radicals and accentuates the lipid peroxidation (LPO) or the formation of acetaldehyde resulting in the reduction of antioxidant enzymes such as Super oxide dismutase (SOD), Catalase, Glutathione (GSH), Glutathione peroxidase(GPx).[131,132]Ethanol inhibited glutathione peroxidase, decreases the activity of catalase, super oxide dismuthase levels in liver. The data (Table-26) suggest that test drug SSM counteracts with the markers of hepatic injury in Group IVat dose level of (25 mg/Kg) and significantly restores these antioxidant enzymes to near normal when compared to the induced group. Results also confirm that the study drug SSM in high dose grouphas almost equal effect as that of the standard drug Liv 52.

Anindepth morphometric analysis of liver sections and scoring was assessed by considering the degree of inflammation and histopathologic damage with reference to hepatocellular architecture, necrosis, inflammation, mononuclear cell infiltration, Kupffer cells presence, arrangement of sinusoids, vacuolization and vascular congestion was expressed within each liver section classified on a scale of 0–3 (0, absent; 1, mild; 2, moderate; 3, severe)

The study data reveals that in ethanol induced group (GII) the scoring was 3 which indicates severe liver damage of more than 50% with presence of balloon degeneration with more binucleated cells associated with lymphatic infilteration in the portal area and marginal necrotic changes. Mild difference was noted in GroupIII (score-2±0.57) which is almost equal to that of Group II. Group IV showed moderate reduction in inflammation (score-2±0). In group V treated with high dose SSM, the score was decreased (1.33±0.33) as that of the standard drugLiv 52(score-1±0) with restoration of hepatocyte structure back to normal.

Following the morphometric analysis, the thick sections of liver samples were subjected to Masson's trichrome staining in Alcohol Induced liver injury in mice. The Group II showed characteristic feature of collagen fibres present in central vein zone. Group IV, V treated with low (25mg/kg) and high dose (125mg/kg) of SSMshows reduced level of fibrosis with restored hepato cellular architecture in treated mice when compared to the Group II. Group VI (Liv 52) showed normal hepatocytes.The histopathological study reports suggest that the study drug SSM in high dose (125mg/kg) has drastically reduced the liver injury as almost equal to the standard drug liv 52.

Acetaminophen (APAP, also called paracetamol) is an extensively used analgesic and antipyretic drug. It is metabolized by hepatic cytochrome P450 system, especially CYP2E1, which leads to the overproduction of reactive free radicals and n-acetyl-p-benzoquinoneimine (NAPQI)[134]. Injury to the liver, whether acute or chronic, eventually results in an increase in serum concentrations of aminotransferases. AST and ALT [135] The results obtained from the present study reveals that mice treated with paracetamol (2gm/kg) shows significant increase in serum levels of AST, ALT, ALP, urea, bilirubin and decrease in the level of total proteins.

Treatment with SSM at both the dose level of 25 and 125 mg/kg significantly decreases the levels of AST, ALT, ALP, Urea, bilirubin and increase in total protein when compare to that of paracetamol (2mg/kg) treated group. Similar pattern of reduction in the level of serum hepatic enzymes AST, ALT, ALP including urea and bilirubin was observed in standard drug (Liv 52 (2.6ml/kg)) treated group.Mice treated with vehicle control honey 40mg/kg shown marked reduction in the level of serum hepatic enzymes including urea, bilirubin and increase in serum total protein level when compared to that of disease control group.

It is well known that high doses of paracetamol depletes the level of hepatic anti-oxidant enzymes such as SOD, CAT, GRD, GPx in turn enhances oxidation stress by increasing the level of lipid peroxidation, impairs liver functions, causes hepatocyte necrosis, and even promotes liver failure or death [136,137]

Oxidative stress is animportant mechanism for the development of paracetamol induced hepatotoxicity. Paracetamol causes enormous alteration of antioxidant defense systems resulting in hepatotoxicity [138]. In the present study, it was found that decreased level of enzymes such as SOD, CAT, GRD, GPx and increased level of lipid peroxidation in the liver of mice treated with 2gm/kg of paracetamol. Treatment with SSM at both the dose level of 25 and 125 mg/kg significantly increases the levels of SOD, CAT, GRD, GPxand decreasesthe level of lipid peroxidation when compare to that of paracetamol(2mg/kg) treated group. Similar pattern on increase in liver antioxidant enzyme and decrease in LPO level was observed in mice treated with Liv 52 at the dose of 2.6ml/kg.Mice treated with vehicle control honey 40mg/kg shown very mild increase in hepatic antioxidant profile of treated animals.

Morphometric analysis of Acetaminophen induced rats were observed as shown in Fig-). Group I showed 0 score as they were normal hepatocytes. In Induced group II, the scoring was 2.67±0.33 which indicates severe liver damage of more than 50% with clustered hepatocytes, increased sinusoidal space and derangement of portal triad. Group III (score-2.33±0.33) almost mimicks the features of GroupII. Group IV showed dilated portal vein with moderate inflammatory changes with rejuvenating hepatocytes emerging from necrotizing zone.(score-2±0.58). In group V treated with high dose SSM, the score was decreased (1.67±0.33) as that of the standard drugLiv 52(score-0.67±0.33) with reduced signs of inflammation and necrosis.The architecture of hepatocytes was retored back to normal.

In Masson's trichrome staining in paracetamol induced liver injury in mice the Liver section shows that the level of fibrosis and deposition of collagen was greatly controlled in trichrome stained liver of group IV, V treated with low (25mg/kg) and high dose (125mg/kg) of SSM while the paracetamol induced group showed increased deposition of collagen fibres around the congested central vein indicating severe fibrosis. Marginal restoration of hepato architecture with trace deposition of fibrous tissue was observed in group VI treated with standard drug Liv 52.

The histopathological study reports suggest that the study drug SSM has considerably reduced the paracetamol induced liver injury as almost equal to the standard control in both low (25mg/kg) and high dose (125mg/kg).

Further to confirm the hepatoprotective activity of SSM, it was investigated whether SSM offers hepatoprotection against D-Galactosamine which is a well established hepatotoxicant to cause acute liver injury.The specificity of Galactosamine is its ability to disrupt the synthesis of uridylate nucleotides in the hepatocytes. Depletion of these nucleotides in turn impairs the synthesis of protein and glycoprotein leading to sequential damage of cellular membranes and leakage of enzymes.

The study result data suggests that administration of Galactosamine significantly elevated all the hepatic enzyme levels AST, ALT, ALP, urea, bilirubin and decrease in the level of total proteins. However SSM treatment attenuated the increased levels of all the enzymes at both low dose(25mg/kg) and high dose (125mg/kg) and the enzymes were subsequently restored towards normalization. The findings suggest that the hepatoprotective activity of SSM may be due to its ability to prevent maintaining the stability of cell membrane of hepatocyte by preventing the loss of cellular functional integrity and leakage[139].

The liver injury induced by D-Galactosamine is associated with reactive oxygen species and oxidative stress. In the present study D-Galactosamine induced liver injury was evident from the salient features of increase in lipid peroxidation and decreased activities of enzymatic antioxidants (SOD, CAT, and Glutathione peroxidase and glutathione reductase). The present study results clearly exemplify that SSM treated Group IV (25mg/Kg) and Group V (125mg/kg) showed highly significant increase in the antioxidant enzyme with significant decline in the LPO as almost equal to that of the standard drug Silymarin (200mg/Kg)[140].

Weight of the liver was observed an index of potential drug induced hepatotoxicity. This was a significant increase in the average mean weight of liver in all the three induced group Ethanol induced liver damage in mice, Paracetamol - induced liver injury, LPS +D –galactosamine induced hepatoxicitywhen compared

to that of the control group mice. Treatment with SSM at both the dose level of 25, 125 mg/kg, standard drug Liv 52 (2.6 ml/kg)/Silymarin (200mg/Kg) significantly decreases the liver weight in treated mice.

Further the D-Galactosamine induced rats were subjected to morphometric analysis as shown in Fig 50. Group I showed 0 score as they were normal hepatocytes. In D-Galactosamine induced group II, the scoring was 3 which indicates severe liver damage of more than 50% with clustered infilteration of mononuclear inflammatory cells with increased kupfer cells and dilated sinusoidal space. Also derangement of hepatic architecture was observed. Group III (score-2.5±0.22) almost resembles the features of Group II. Group IV showed mild derangement in the hepatic cord. (Score-2.33±0.21). In group V treated with high dose SSM, the score was decreased (1.33±0.33) as that of the standard drugs Silymarin (score-0.33±0.33) with drastic reduction in the signs of inflammation and restoration of normal hepatic cord and sinusoidal space.

Masson's trichrome staining in Lipopolysaccharide (LPS)/d-galactosamine (d-GalN)induced Group II, the Liver section showed significant level of fibrous tissue in periportal region in hepatocytes indicating extreme liver injury.Moderate accumulation of extracellular matrix proteins around central vein was observed in group III. But in group IV and V treated with low (25mg/kg) and high dose (125mg/kg) of SSM the level of fibrosis and deposition of collagen was greatly reduced. The results indicate that the test drug reversed the Hepatocytic architecture and fibrous tissue formation almost equal to that of standard drug Silymarin in GroupVI.

Thus Siddha formulation Santha santhrothaya mathirai (SSM) has the efficacy to restore the liver enzymes and antioxidants along with ability to renovate the hepatocellular injury by inhibiting the progression of fibrosis and reducing the collagen deposition[141].Therefore it is well established through the above pharmacological studies that the test drug SSM may potentially attenuate alchohol induced liver damage, acute liver damage and viral hepatitis all of which can induce liver injury.

In recent times safety concerns of traditional system of medicines especially that are of mercury based has become a major area of research and public interest. While several studies claim that exposure to heavy metals such as arsenic, cadmium, chromium, copper, lead and mercury to be toxic, very few researches highlight the safety of traditional herbomineral formulations. The above research work was carried out on the safety and efficacy of Siddha herbomineral combination that contains pooram (Mercurous chloride) as one of its ingredient. But the amazing fact behind this research is its high margin of safety despite its higher permissible limit as depicted by its toxicological and pharmacological studies.

Moreover it has been studied that the primary route of mercurial toxicity is through the depletion of glutathione (GSH) and binding to sulfhydrayl group proteins which induces oxidative stress[142].

In this juncture of these existing theories on mercurial toxicity, the reason for the safety of SSM can be attributed due to the specific purification process of pooram which renders it to be compatible with human body to produce a biologically acceptable product. The purified and processed drug SSM on sophisticated instrument analysis was found to have organo metallic complex due to the organic ligands which facilitates the better assimilation of the drug.

Also the HPTLC analysis of this study reveals the presence of “curcumin” that has been studied to offer protection against hepatic damage induced by mercury through its ability to scavenge the free radicals generated by the oxidative stress. This could be possible by the chelating property of curcumin and pectin in lemon juice which is a chemical process in which a substance can bind with heavy metals and facilitates its excretion from the body[143, 144]. Citrus fruits also helps in biosorption is a bioremedial chemical interaction of alternative technology that has developed in 1980’s that may occur between the cellular components such as polysaccharides, proteins lipids and metals in order to remove the heavy metal toxicity[145].

These scientific analytical facts unravel the ancient wisdom of Siddhars to structure this unique combination of herbomineral formulation SSM with suitable ingredients such as Pooram, borax, turmeric and lemon which blend with each other and provide both therapeutic efficacy as well as safety.

Therefore the Classical herbomineral drug SSM can be used in Liver disorders without any harmful effects and the above analytical studies have reassured the safety of mercurial drugs when they are purified and processed as per mentioned in Siddha literature and used in humans as indicated pertaining to the dosage and vehicle.

9. SUMMARY

Santha santhrothaya mathirai (SSM) is a herbo-mineral formulation widely prescribed for hepatic disorders. The ingredients of SSM include mercurous chloride, borax, turmeric and lemon juice all of which are said to possess hepato-protective activity. Although, the medicinal preparations mentioned in Siddha literature are time-tested standard preparations it is need of the hour to document standardization procedures by using sophisticated instrumental analysis to maintain quality control. In the present study the preparation of SSM was standardized initially and then it was scientifically validated by adopting various analytical techniques.Toxicity and pharmacological studies were also performed to understand the safety and efficacy of the traditional drugs.

The raw materials used in the preparation of SSM were identified and authenticated using contemperory accepted practice. The raw materials of mineral origin viz., pooram and vengaram were purified strictly following the classical Siddha texts. The study drug SSM was prepared adopting the method mentioned in the “Siddha Vaithiya Thirattu”[12].

In order to standardize the preparation protocol, SSM was prepared thrice (SSM-A, SSM-B and SSM-C) by following the same protocol and the similarity was demonstrated by examining various physical and chemical nature such as total ash, moisture content. The microbial load, aflatoxins and pesticide levels were analyzed and were found to be within the WHO permissible limits in all the three samples . Quantity of heavy metals (lead, cadmium, mercury and arsenic) in all the 3 samples of SSM were estimated by using atomic absorption spectroscopy (AAS) and were found to be within the permissible limits.

Total curcumin content of SSM was estimated in all the three samples of SSM by HPTLC analysis. The amount of curcumin present in Sample A was 0.6589 %; Sample B was 0.6884 % and Sample C was 0.7104 %.Total curcumin content of SSM - (A-1.15%,B 1.19%, C1.23%) were analysed by UV spectroscopy . There was no significant variation in the curcumin content in all the three samples of SSM.

Since all samples were similar throughout the above analysis one of them was taken for further analytical procedures.

TGA analysis of SSM confirms a weight loss of 77% indicating the presence of large quantity of organic organic material in SSM.

The chemical changes that have occurred during the Siddha methods of purification process were assessed by using Raman spectroscopy, FTIR, XRD and SEM Analysis. The analytical studies confirm the changes in the chemical structure of raw and purified state. The tetragonal crystalline structure of Pooram (Mercurous chloride) showed changes in its crystalline structure though the exact shape could not be established. Similarly Vengaram (Borax) also showed a change in its structure from rhombohedral form to orthorhombic form. Moreover the presence of organo-mineral complex in SSM can be due to the combination of turmeric with purified vengaram, purified pooram or both. SEM Analysis revealed the particle size of 1µm to 500 nm in various magnifications. Also the morphological structure showed variations in purified and prepared medicine SSM when compared to raw samples.

In order to understand the nature of inorganic materials present in SSM, the XRF, ICP-OES and mercury analysis by tituration method was performed. Heavy metals concentration of Mercury, lead, cadmium and arsenic were observed by the ICP-OES. This analysis of raw pooram (P1), raw vengaram (V1) showed mercury level and Sodium level as 123.241ppm and 545.862ppm respectively. There was significant reduction in the content of mercury (55.87ppm) in Pooram and the content of Sodium (203.110ppm) in Vengaram in purified state. Further reduction was observed in the mercury and Sodium level in study drug SSM as 3.154ppm and 125.383 ppm respectively. The other heavy metals such as As, Cd, and Pb were below detectable level (BDL) in all the samples (Raw, Purified and prepared samples).

The concentration of elements in oxide form was analyzed through X-Ray Fluorescence in the Raw, purified and prepared samples. The XRF study revealed the addition and deletion of trace elements and a reduction in the percentage of

Mercury from 87.23% in raw form to 59.59% in purified form and 58.82% in SSM finished sample. From the results of tituration method, mercury content was also reduced from raw state of ingredients to purified and finished state of SSM.

These analytical studies are essential since the raw materials for herbo-mineral preparations are often sourced from various regions and during various seasons. The present study ensures the quality control of the drug which is essential for the prevention of adulteration, reproducibility, assessment of finished product, estimation of active principle and global acceptance.

SSM was accessed for its safety on short-term and long-term administration by performing acute, sub acute, subchronic toxicity in animal models. In acute toxicity study, *SSM* did not produce any mortality or exhibit any abnormal signs for 14 days even upto the dose of 2000mg/kg, on single oral administration.Gross necropsy of different organs revealed no abnormalities after 14 days.In subacute toxicity study, oral administration for a period of 28 days did not showany behavioural abnormality and mortality even at the dose of 250 mg/kg. Gross pathological examination and histopathological analysis of the various organs such as heart, liver, kidney, lung, spleen, stomach, brain did not reveal any lesions in any of the groups.

In subchronic toxicity study, there was no behavioural abnormality and mortality throughout the study period of 90 days except mild laxative effect in high dose group. Few changes were observed in haematological and biochemical parameters of SSM treated animals when compared to control groups but were within the physiological limits. Moreover the reduction of liver parameters ALT and AST reveals the hepatoprotective effect of SSM. Histopathological examinations of the tissues revealed mild inflammatory changes in liver and kidney with no significant changes in AST, ALT, urea and creatinine which are biomarkers of hepatic and renal damage. Recovery group was maintained to assess all the above haematological, biochemical and histopathalogical parameters and were found to have no significant toxicological changes. ICP-MS analysis of renal tissue indicates that the study drug SSM does not cause any cumulative toxic effect in renal tissues as the levels of mercury was below the detectable limit in post retrieval group. The results of the toxicity study confirms the safety of SSM towards human consumption.

The pharmacological activity of SSM was accessed using three different animal model that represents Paracetamol - induced liver injury, LPS +D–galactosamine induced hepatoxicity which may mimic alchohol induced liver damage, acute liver damage and viral hepatitis respectively. In all the three models, SSM was found to reduce various liver function parameters such as AST, ALT, ALP, Urea and bilirubin that were elevated due to disease induction. Moreover, SSM also increases the total proteins in all the three pharmacological models. Furthermore, treatment with SSM at the dose levels of 25 and 125mg/Kg significantly increases the hepatic antioxidant enzymes such as SOD, CAT, GRD, and GPx and decreases the level of lipid peroxidation when compared to the disease control groups.

Histopathology analysis of liver showed that SSM at Low (25mg/kg) and high (125mg/Kg) doses greatly reduced the level of fibrosis and deposition of collagen fibres in alchohol induced liver injury and paracetamol induced liver injury as almost equal to the standard drug Liv 52 and the levels of fibrosis and restoration of collagen fibres in D-Galactosamine induced liver injury were almost similar to that of the standard drug Silymarin.

Over all SSM was found to contain notable quantity of mercury in the form of organometallic complex. But it was found to be effective in the management of liver disorders and was experimentally very safe even at very high doses. This study clearly demonstrated the traditional mercury containing drugs are safe for oral administration plausibly may be due to the traditional purification and preparation protocols employed during the process of formulation.

10. CONCLUSION

It is concluded that the trial drug SSM a classical Siddha formulation, was well formulated ensuring the quality by complying with the GMP and GLP standards at every step right from procurement, authentication ,purification to final product analysis. The toxicological profiling of SSM reveals that it has a large margin of safety on acute, sub acute and sub chronic toxicity in experimental rats. Also the pharmacological studies on SSM has proved it to be hepatoprotective and efficacious in the therapeutic dosage for treating liver diseases.

The reverse pharmacological work to re-validate the traditional formulation, SSM confirms the safety profile and effectiveness by adopting the modern scientific and acceptable methods to satisfy the present day demands.Hence through this work, an effort has been made to bring out SSM as evidence based safe Siddha medicine for Liver disorders.

11. RECOMMENDATIONS

- Standardization can be done in large number of samples using the ingredients collected from various geographical regions to assess the quality of raw materials and to prevent the batch to batch variation when manufactured at a large scale.

- Further more studies warranted on the toxicity studies such as carcinogenicity, teratogenicity and mutagenicity can be done to assess the safety profile at genetic level.

- In-vitro studies and molecular biological studies of SSM can be done in future to evaluate the efficacy of the drug at cellular level.

- Pharmacodynamic and pharmacokinetic action of SSM to study the molecular targets of SSM is also warranted.

- Finally extensive clinical trial documentation of this traditional herbomineral formulation is necessary for further scientific validation of SSM.

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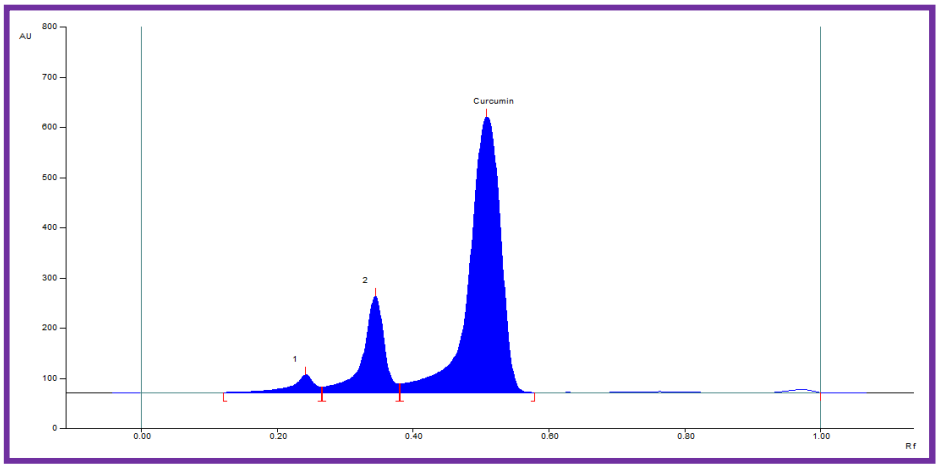
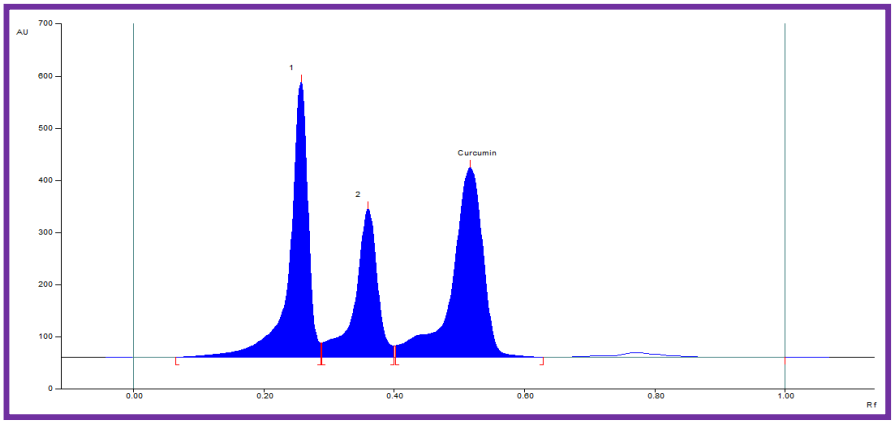
Fig.9. HPTLC finger print of the standard solution of Curcumin



Rf values of the HPTLC finger print of the standard solution of Curcumin

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.18 Rf	1.9 AU	0.25 Rf	17.3 AU	3.76 %	0.27 Rf	6.2 AU	496.6 AU	3.27 %
2	0.27 Rf	6.3 AU	0.35 Rf	91.7 AU	19.97 %	0.38 Rf	9.4 AU	2416.3 AU	15.90 %
3	0.38 Rf	9.4 AU	0.51 Rf	350.3 AU	76.26 %	0.58 Rf	0.2 AU	12280.3 AU	80.83 %

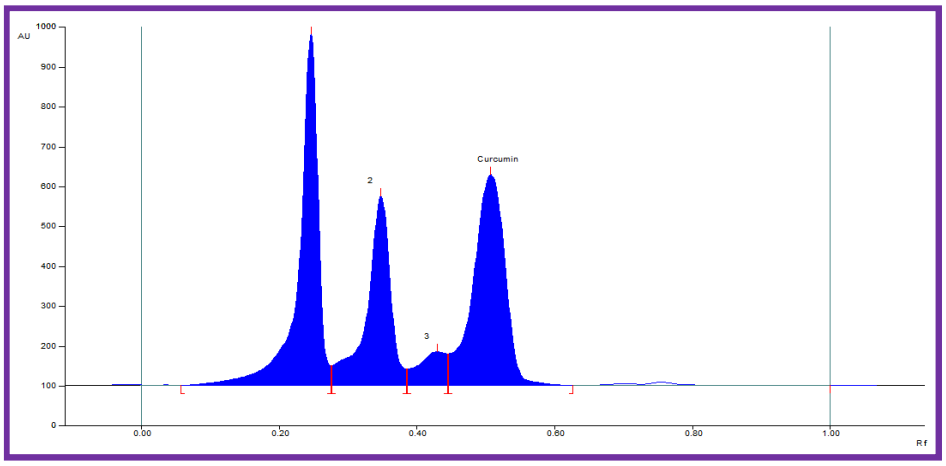
Figure-10. HPTLC finger print of the test solution of sample –A



Rf values of the HPTLC finger print of the test solution of sample –A

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.07 Rf	0.0 AU	0.26 Rf	528.5 AU	44.89 %	0.29 Rf	27.5 AU	12439.3 AU	35.70 %
2	0.29 Rf	27.6 AU	0.36 Rf	284.9 AU	24.20 %	0.40 Rf	22.1 AU	8043.3 AU	23.08 %
3	0.40 Rf	22.2 AU	0.52 Rf	363.9 AU	30.91 %	0.63 Rf	0.1 AU	14364.2 AU	41.22 %

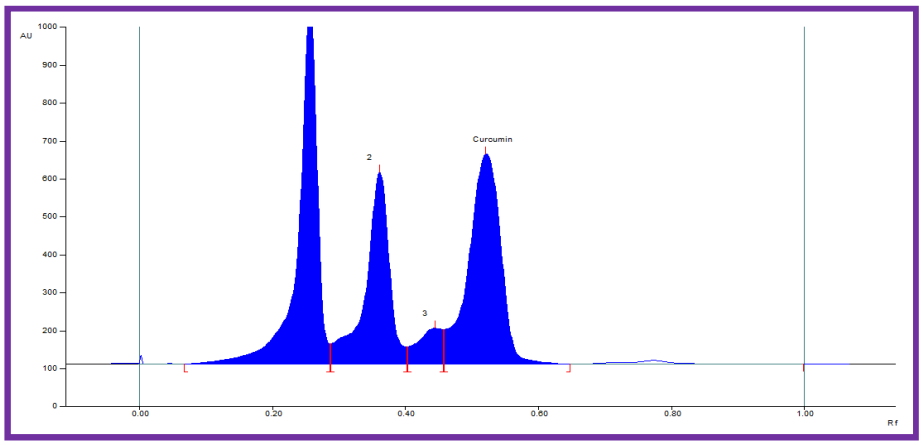
Fig.11. HPTLC finger print of the test solution of SSM –B



Rf values of the HPTLC finger print of the test solution of SSM –B

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.06 Rf	0.0 AU	0.25 Rf	882.8 AU	44.76 %	0.28 Rf	49.0 AU	20410.9 AU	35.68 %
2	0.28 Rf	49.4 AU	0.35 Rf	476.2 AU	24.15 %	0.39 Rf	41.3 AU	13568.0 AU	23.72 %
3	0.39 Rf	41.3 AU	0.43 Rf	84.4 AU	4.28 %	0.45 Rf	79.2 AU	2856.0 AU	4.99 %
4	0.45 Rf	79.3 AU	0.51 Rf	528.7 AU	26.81 %	0.63 Rf	0.0 AU	20366.0 AU	35.60 %

Fig.12. HPTLC finger print of the test solution of SSM – C



Rf values of the HPTLC finger print of the test solution of sample –C

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.07 Rf	0.1 AU	0.26 Rf	935.5 AU	44.76 %	0.29 Rf	53.1 AU	22529.4 AU	35.79 %
2	0.29 Rf	53.4 AU	0.36 Rf	506.6 AU	24.24 %	0.40 Rf	45.9 AU	15112.3 AU	24.01 %
3	0.40 Rf	46.1 AU	0.45 Rf	94.4 AU	4.52 %	0.46 Rf	91.3 AU	2978.0 AU	4.73 %
4	0.46 Rf	91.5 AU	0.52 Rf	553.5 AU	26.48 %	0.65 Rf	0.1 AU	22322.8 AU	35.47 %

Ingredients of Santha Santhrothaya Mathirai

Pooram – Before purification

Pooram – After purification

Vengaram – Before purification

Vengaram – After purification

Kappu Manjal

Powdered Kappu Manjal

Elumichai

Juice of elumichai

Santha Santhrothaya Mathirai

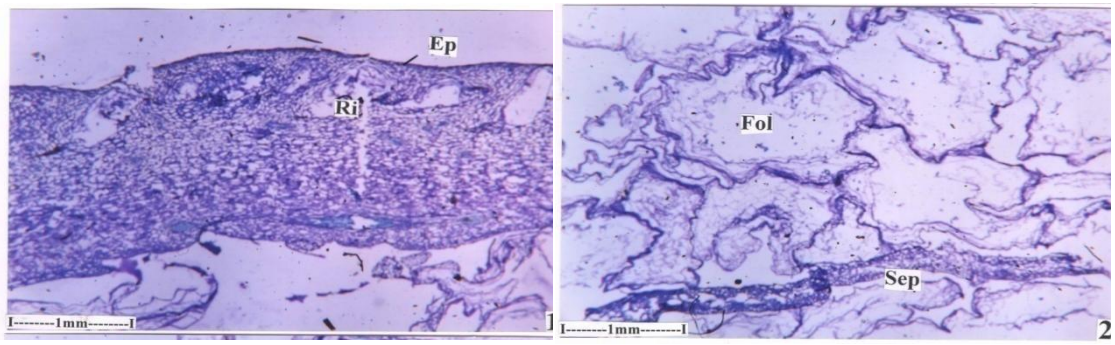
Process of purification

Ingredients of purification

Vetrilai	Milagu
	
Vetrilai milagu karkam	Pooram purification process
	
Vengaram purification	Preparation of SSM
	

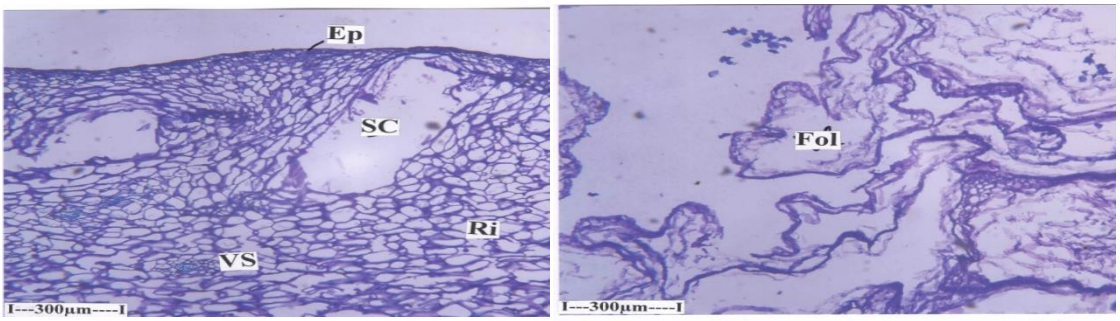
Plant anatomy of citrus fruit

Fig1.1 TS of rind of Citrus fruit **Fig 1.2 Folicles in the fruit**



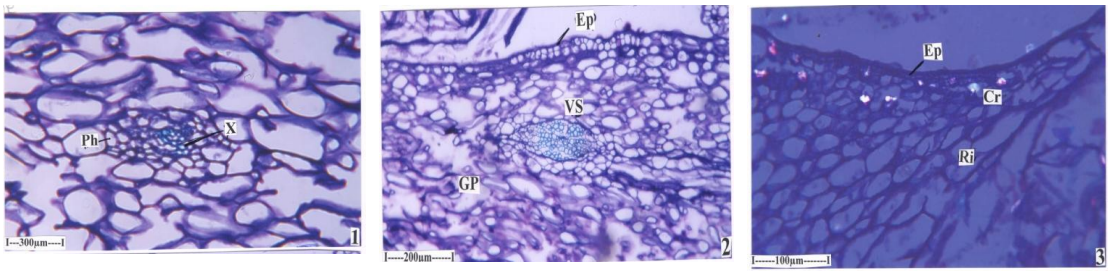
(Ep – Epidermis, Fol – Follicle, Ri – Rind, Sep – Septum)

Fig.2.1 Rind – Outer portion **Fig 2.2 Follicles**



(Ep – Epidermis, Fol –Follicle, Ri – Rind, Sc – Sceretory cavity,VS –Vascular strand)

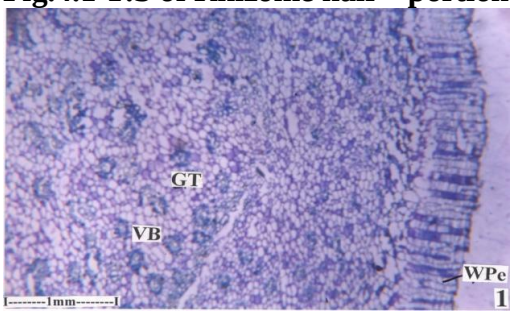
Fig 3.1 Citrus rind – inner vascular bundle **3.2 Septal wall - Vascular bundle** **3.3 Crystals in the Rind**



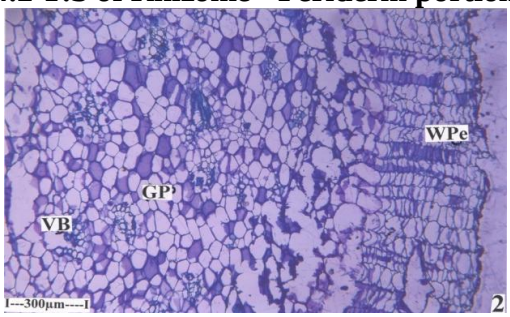
(Cr – Crystals, Ep – Epidermis, GP – Ground Parenchyma, Ri – Rind, Ph – Phloem. VS – Vascular Strand, X – Xylem)

Plant anatomy of curcuma longa

Fig.4.1 T.S of Rhizome half – portion



4.2 T.S of Rhizome - Periderm portion



(GP – Ground Parenchyma, VB – Vascular Bundle, W Pe – Wound Periderm)

Fig 5.1 Periderm zone enlarged

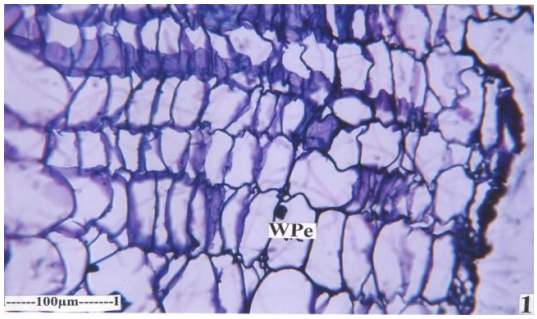
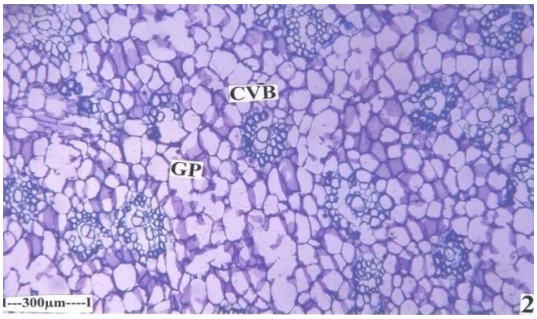


Fig 5.2 Vascular bundles in the rhizome



(GP – Ground Parenchyma, CVB – Central vascular bundle, W Pe – Wound Periderm)

Fig 6.1 Outer Vascular bundle

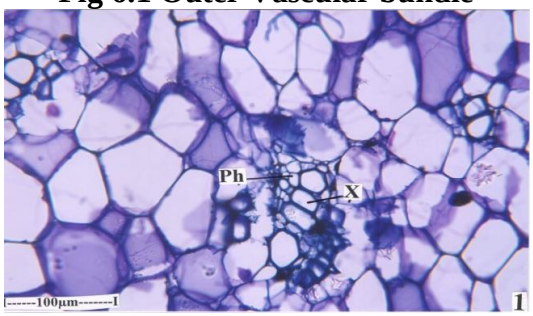
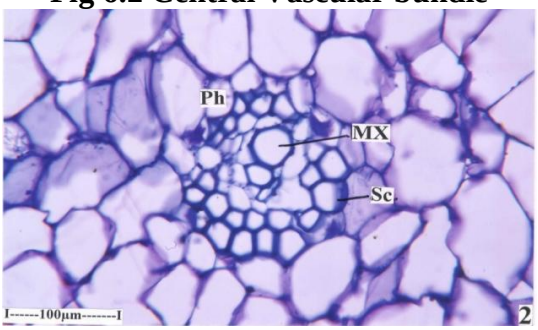


Fig 6.2 Central Vascular bundle



(MX – Metaxylem, Ph-Phloem, Sc- Sclerenchyma, X – Xylem)

Fig 7.1 Crystals in the Rhizomes-(Cr – Crystals)

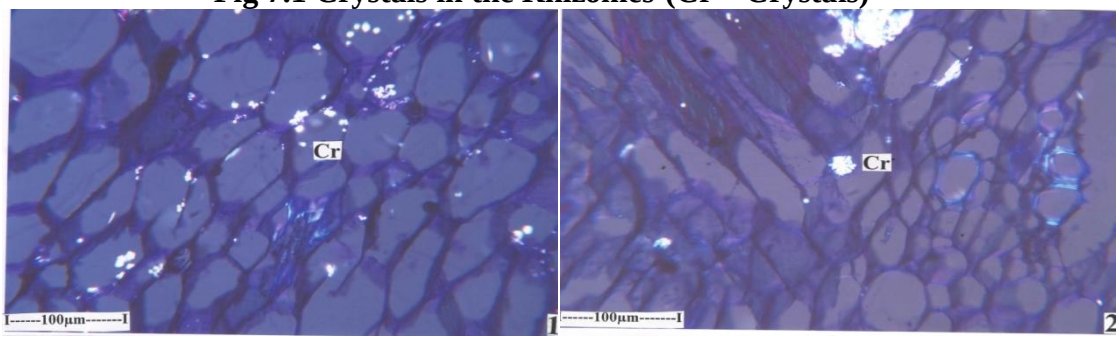
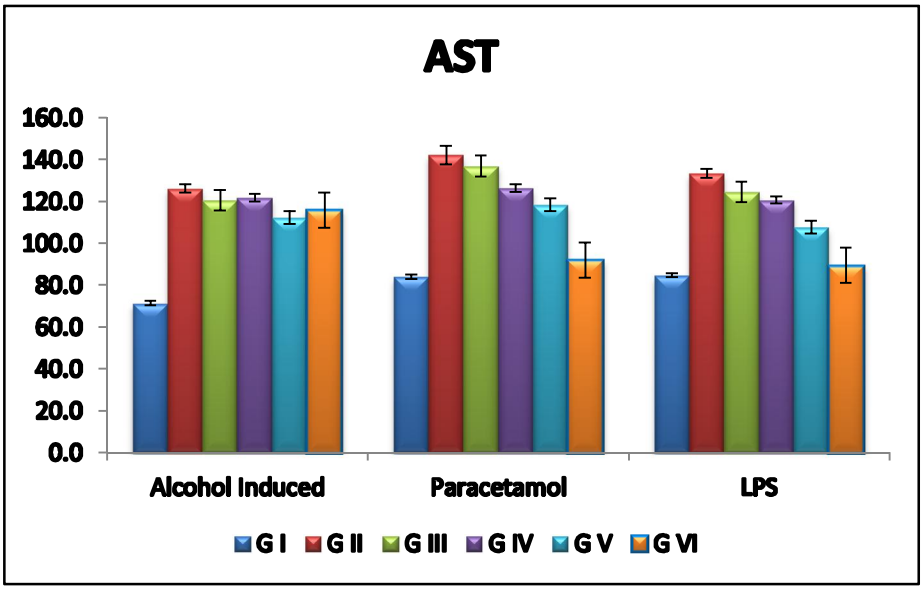


Figure 52: Effect of SSM on estimation of AST in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

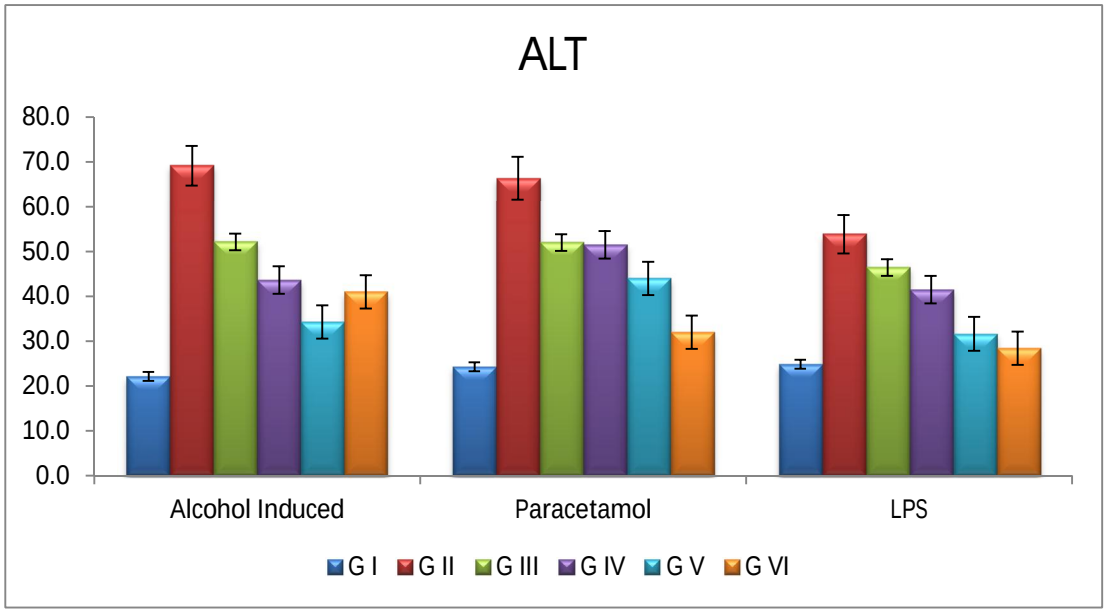
Groups	Total AST/(IU/ml)		
	52.a Alcohol Model	52.b Paracetamol Model	52.c LPS- d Galn Model
Group I	71.33±4.02	84±5.09	84.67 ± 3.45
GroupII	126.2± 1.96	142.2± 4.41	133.3 ± 2.08
Group III	120.5±1.2	136.8 ± 1.58	124.5 ± 1.72
Group IV	121.7±1.76	126.3 ± 1.8**	120.7 ± 1.54**
Group V	112.3±2.11**	118.3 ± 1.96**	107.7 ± 2.43**
Group VI	115.8±1.78**	92± 1.44**	89.33 ± 3.87**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 53: Effect of SSM on estimation of ALT in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

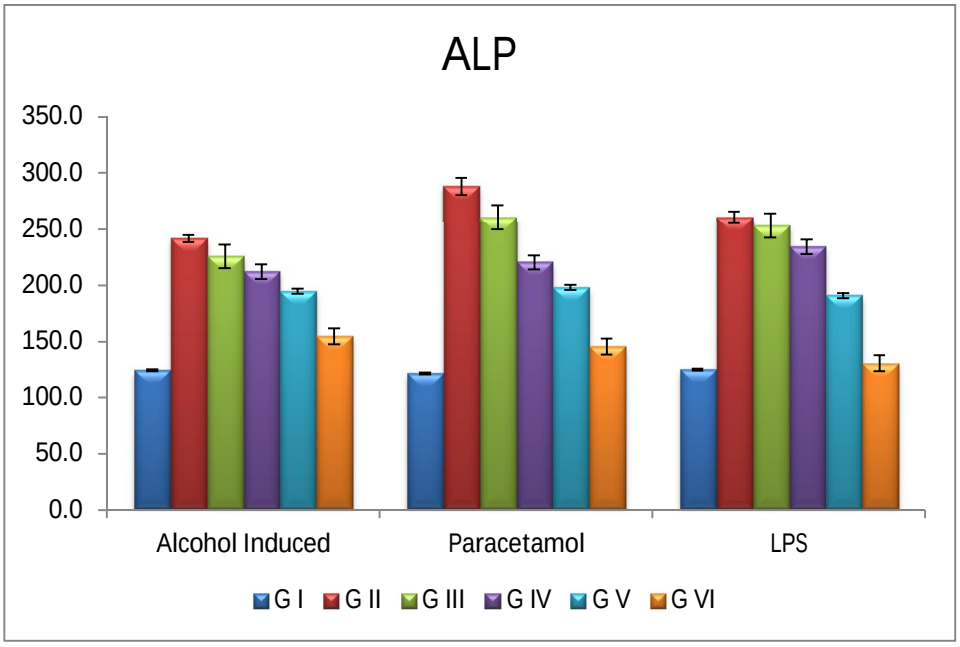
Groups	ALT		
	53.a Alcohol Model	53.b Paracetamol Model	53.c LPS- d Galn Model
Group I	22.17± 3.43	21± 2.6	24.83 ± 1.92
GroupII	69.17± 4.4	66.33± 4.77	53.83 ± 4.25
Group III	52.17± 1.2**	52 ± 1.61**	46.5 ± 2.28
Group IV	43.67± 1.31**	51.5 ± 2.05**	41.5 ± 3.27*
Group V	34.33± 1.5**	44± 2.1**	31.67 ± 1.53**
Group VI	41± 2.85 **	32± 1.71**	28.5 ± 2.26**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 54: Effect of SSM on estimation of ALP in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

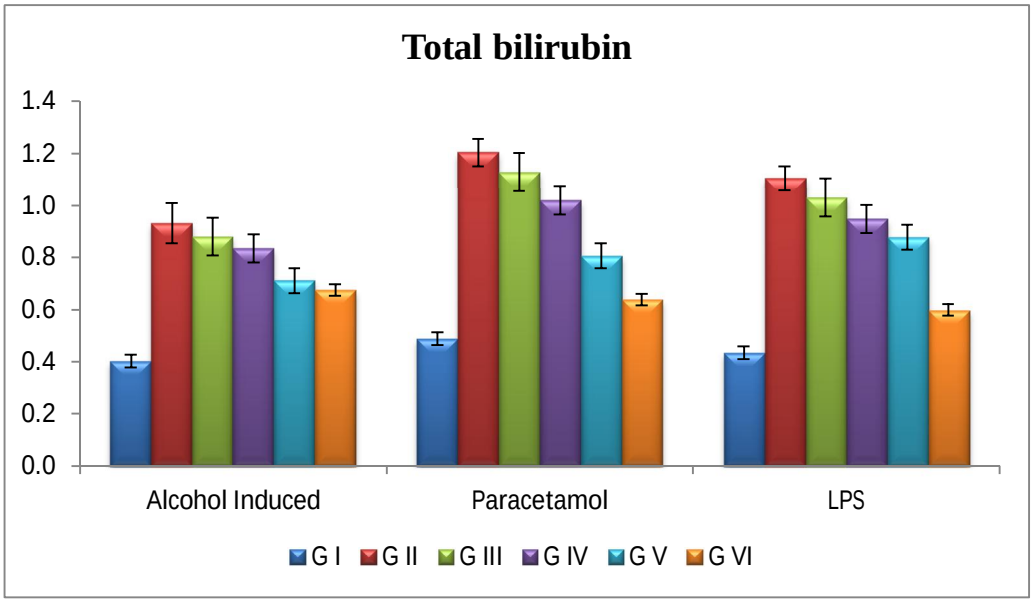
Groups	ALP		
	54.a Alcohol Model	54.b Paracetamol Model	54.c LPS- d Galn Model
Group I	124.3± 3.18	121.5± 3.09	124.8± 3.4
GroupII	241.8± 3.36	288.2± 7.61	260.5± 4.72
Group III	226 ± 2.0**	260.8± 2.02**	253.3± 2.6
Group IV	212.3 ± 3.25**	220.5± 3.91**	234.5± 2.5**
Group V	194.7± 2.31**	198.2± 4.47**	190.8± 2.39**
Group VI	154.8± 2.46**	145.3± 2.91**	130.3± 1.41**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 55: Effect of SSM on estimation of TOTAL BILIRUBIN in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

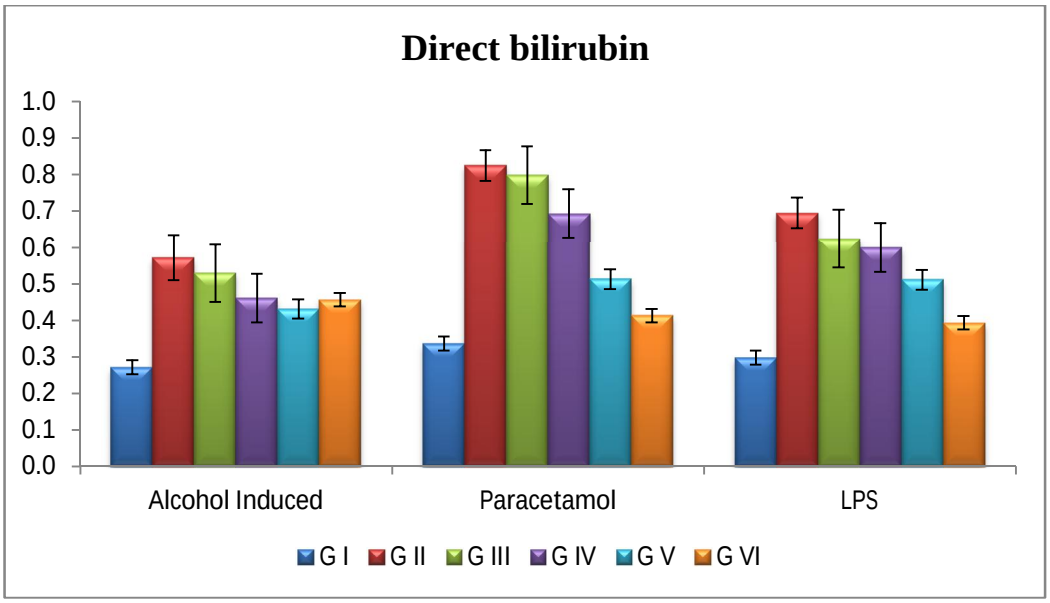
Groups	TOTAL BILIRUBIN		
	55.a Alcohol Model	55.b Paracetamol Model	55.c LPS- d Galn Model
Group I	0.4 ± 0.02	0.49 ± 0.03	0.43 ± 0.03
GroupII	0.93 ± 0.07	1.2 ± 0.05	1.11 ± 0.05
Group III	0.88 ± 0.02	1.13 ± 0.05	1.03 ± 0.03
Group IV	0.84 ± 0.03	1.02 ± 0.03*	0.95 ± 0.05*
Group V	0.71 ± 0.06*	0.81 ± 0.03**	0.88 ± 0.05 **
Group VI	0.68 ± 0.05**	0.64 ± 0.03**	0.6 ± 0.02**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 56: Effect of SSM on estimation of Direct bilirubin in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

Groups	Direct bilirubin mg/dl		
	56.a Alcohol Model	56.b Paracetamol Model	56.c LPS- d Galn Model
Group I	0.27 ± 0.02	0.34 ± 0.02	0.29 ± 0.02
GroupII	0.57 ± 0.06	0.83 ± 0.04	0.7 ± 0.04
Group III	0.53 ± 0.01	0.8 ± 0.04	0.63 ± 0.04
Group IV	0.46 ± 0.02	0.69 ± 0.03*	0.6 ± 0.03
Group V	0.43 ± 0.03*	0.51 ± 0.02**	0.51 ± 0.03**
Group VI	0.46 ± 0.04	0.41 ± 0.03**	0.39 ± 0.04**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 57. Effect of SSM on estimation of Indirect bilirubin in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

Groups	Indirect bilirubin mg/dl		
	57.a Alcohol Model	57.b Paracetamol Model	57.c LPS- d Galn Model
Group I	0.13 ± 0.01	0.15 ± 0.02	0.13 ± 0.02
GroupII	0.36 ± 0.03	0.37 ± 0.03	0.41 ± 0.02
Group III	0.35 ± 0.0	0.33 ± 0.04	0.41 ± 0.03
Group IV	0.37 ± 0.02	0.32 ± 0.05	0.34 ± 0.03
Group V	0.28 ± 0.03	0.29 ± 0.02	0.37 ± 0.03
Group VI	0.21 ± 0.02**	0.22 ± 0.03*	0.2 ± 0.02**

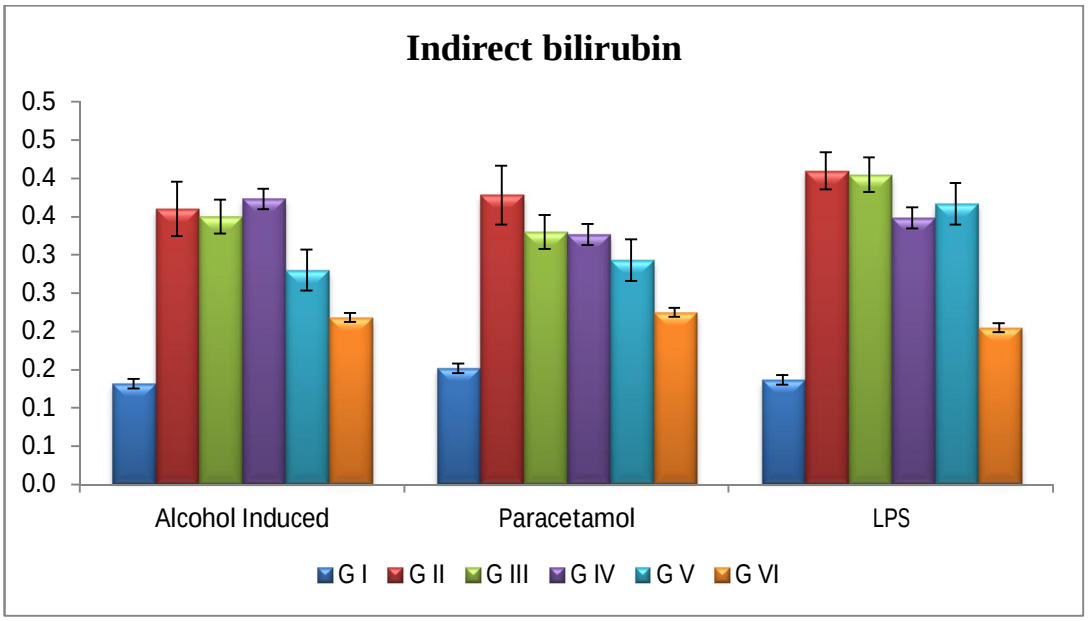


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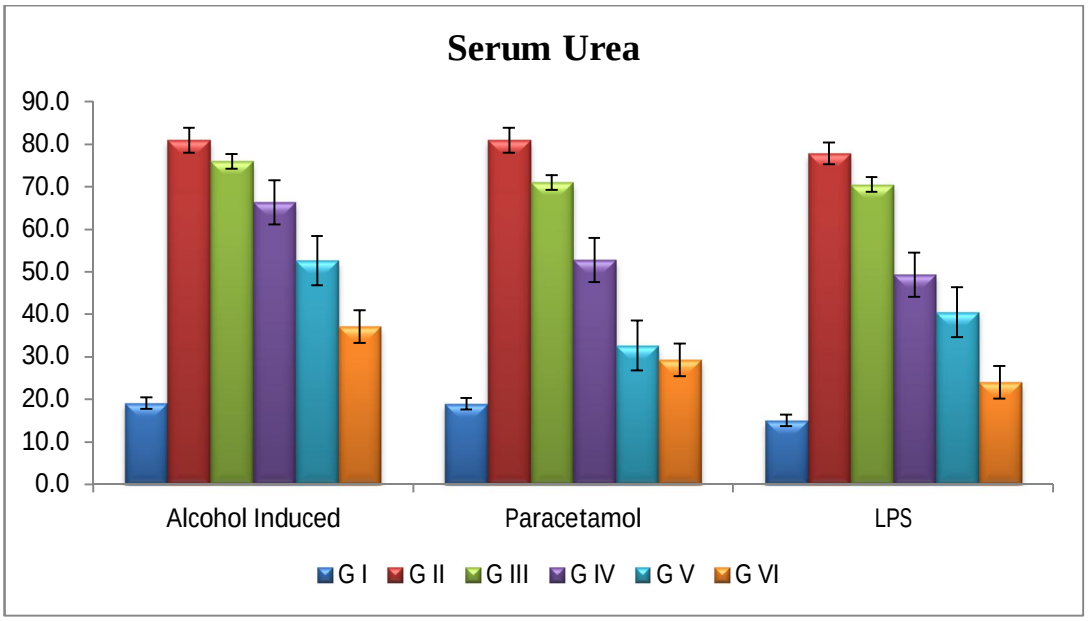
Figure 58: Effect of SSM on estimation of Serum Urea in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

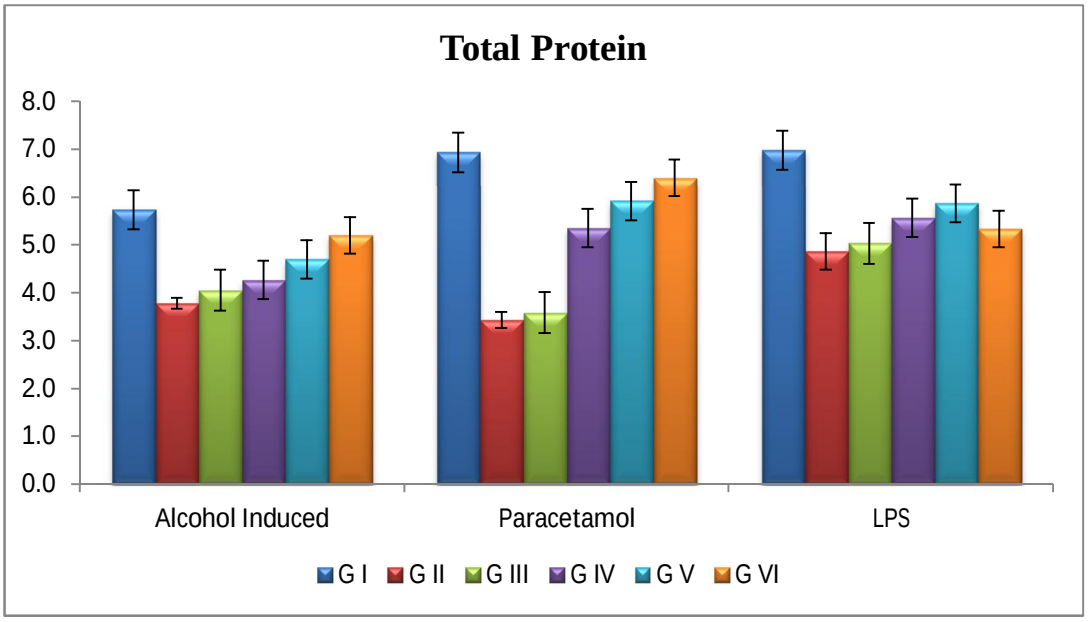
Groups	Serum Urea mg/dl		
	58.a Alcohol Model	58.b Paracetamol Model	58.c LPS- d Galn Model
Group I	19.17 ± 1.3	19 ± 1.18	15 ± 1.24
GroupII	81 ± 2.94	81 ± .94	77.83 ± 2.57
Group III	76 ± 2.2	71 ± 2.15	70.5 ± 1.18
Group IV	66.33 ± 1.65**	52.83 ± 2.24**	49.33 ± 2.22**
Group V	52.67 ± 1.73**	32.67 ± 1.5**	40.5 ± 1.9**
Group VI	37.17 ± 2.02 **	29.33 ± 2.04 **	24 ± 1.53**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 59: Effect of SSM on estimation of Total Protein in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

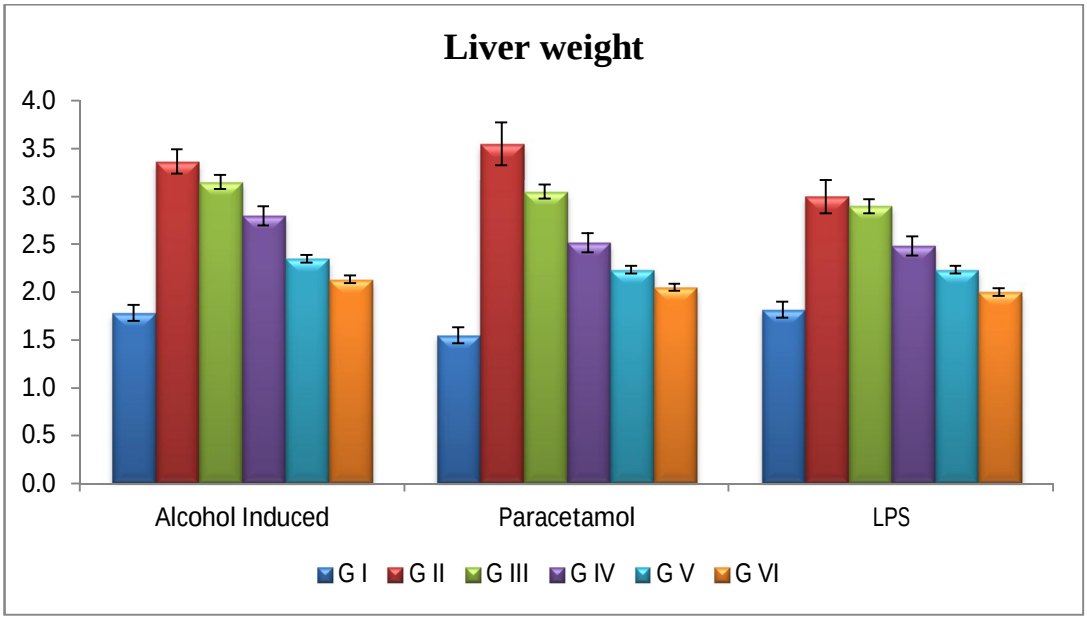
Groups	Total Protein mg/dl		
	59.a Alcohol Model	59.b Paracetamol Model	59.c LPS- d Galn Model
Group I	5.73±0.26	6.93± 0.23	6.98± 0.24
GroupII	3.78±0.11	3.43± 0.16	4.86± 0.38
Group III	4.05±0.17	3.58± 0.29	5.03± 0.12
Group IV	4.26±0.24	5.35± 0.18**	5.56± 0.22
Group V	4.7±0.17**	5.91± 0.22**	5.86± 0.33
Group VI	5.2±0.11**	6.4± 0.21**	5.33± 0.16



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 60: Effect of SSM on estimation of Weight of Liver in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

Groups	Weight of Liver mg/dl		
	60.a Alcohol Model	60.b Paracetamol Model	60.c LPS- d Galn Model
Group I	1.78 ± 0.19	1.55 ± 0.09	1.81 ± 0.21
GroupII	3.37 ± 0.13	3.55 ± 0.22	3 ± 0.17
Group III	3.15 ± 0.16	3.05 ± 0.11	2.9 ± 0.17
Group IV	2.8 ± 0.13 *	2.51 ± 0.18**	2.48 ± 0.14
Group V	2.35 ± 0.16**	2.23 ± 0.15**	2.23 ± 0.12 **
Group VI	2.13 ± 0.10 **	2.05 ± 0.17**	2 ± 0.18 **



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 61: Effect of SSM on Estimation of SOD in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

Groups	Estimation of SOD mg/dl		
	61.a Alcohol Model	61.b Paracetamol Model	61.c LPS- d Galn Model
Group I	9.9 ±0.49	9.03 ±0.51	10.92 ±0.81
Group II	4.9 ±0.16	2.07 ±0.13	4.27 ±0.32
Group III	5.21 ±0.15	3 ±0.12	5.85 ±0.35
Group IV	5.91 ±0.24*	5.47 ±0.22**	5.92 ±0.3**
Group V	6.3 ±0.19**	6.4 ±0.26**	6.79 ±0.17**
Group VI	7.57 ±0.33**	8.55 ±0.40**	7.23 ±0.35**

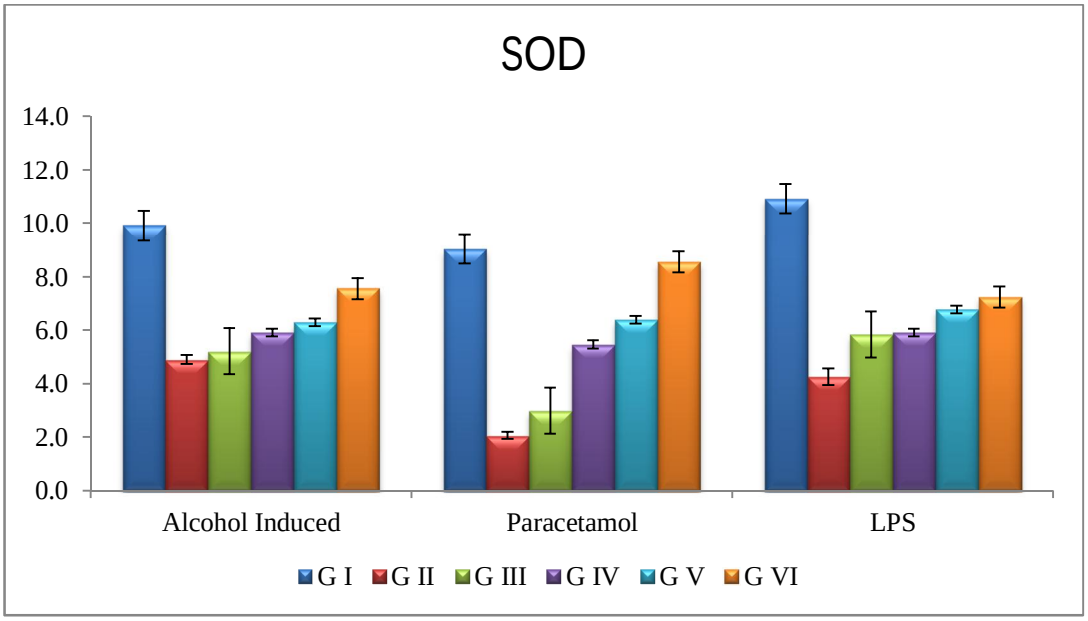


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Figure 62: Effect of SSM on Estimation of CATALASE in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

Groups	Estimation of CATALASE mg/dl		
	62.a Alcohol Model	62.b Paracetamol Model	62.c LPS- d Galn Model
Group I	3.12 ±0.19	2.77 ±0.21	3.02 ±0.27
Group II	1.28 ±0.1	1.36 ±0.14	1.55 ±0.11
Group III	1.31 ±0.11	1.58 ±0.11	1.65 ±0.12
Group IV	1.73 ±0.09	2.11 ±0.18*	1.9 ±0.13
Group V	1.93 ±0.15**	2.33 ±0.14**	2.25 ±0.21*
Group VI	2.42 ±0.15**	2.58 ±0.26**	2.33 ±0.15**

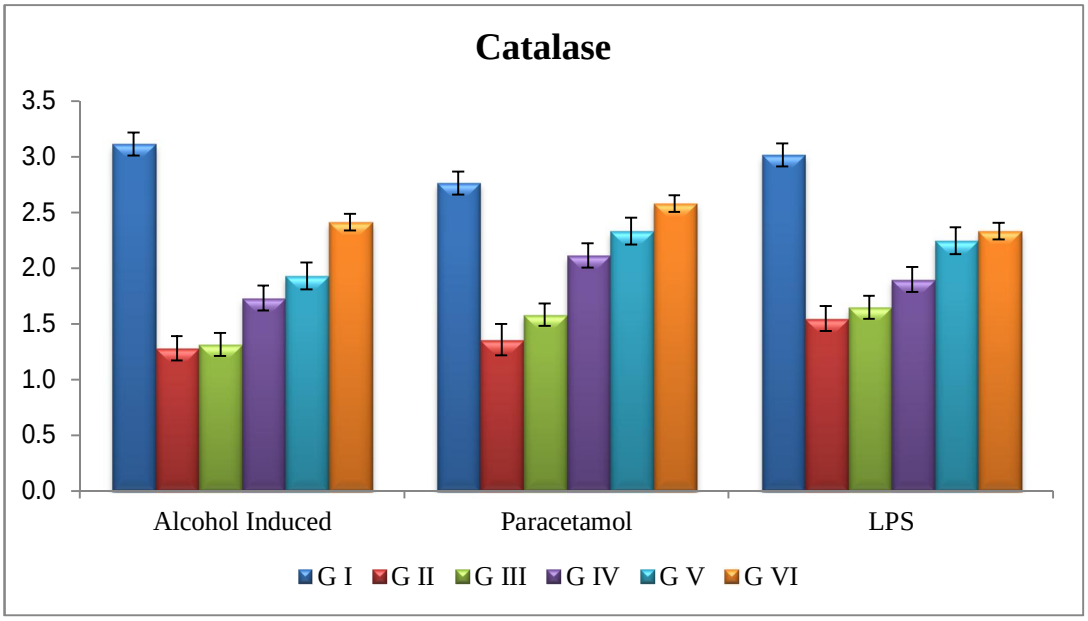

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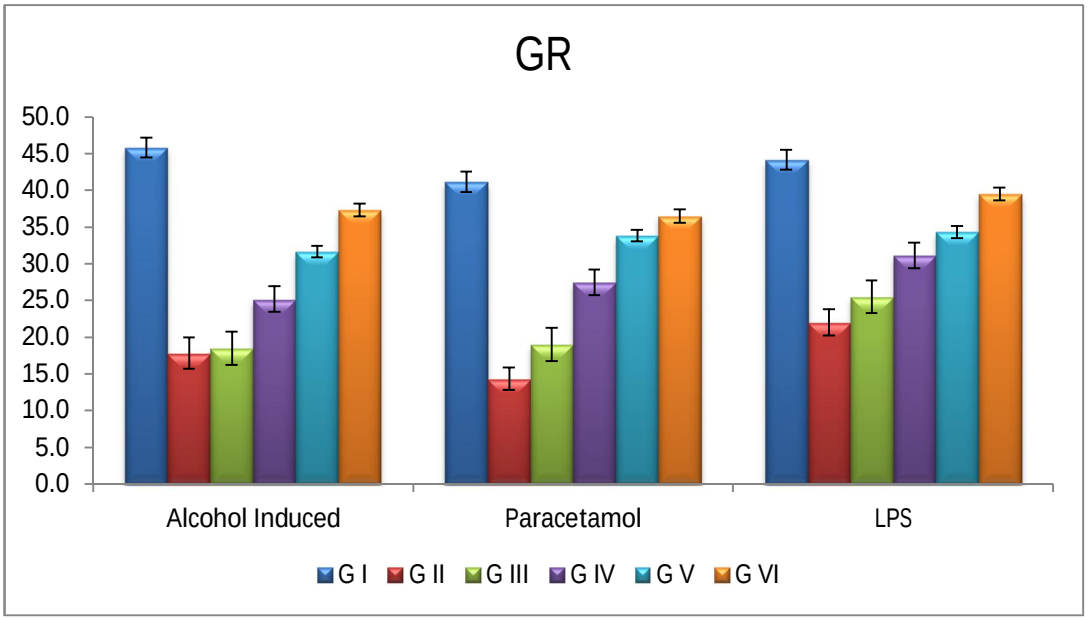
Figure 63: Effect of SSM on Estimation of GR in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

Groups	Estimation of GR mg/dl		
	63.a Alcohol Model	63.b Paracetamol Model	63.c LPS- d Galn Model
Group I	45.83 ±2.39	41.17 ±1.99	44.17 ±2.55
GroupII	17.83 ±2.12	14.33 ±1.59	22 ±1.79
Group III	18.5 ±0.85	19 ±1.59	25.5±0.85
Group IV	25.17 ±2.04*	27.5 ±3.33**	31.17 ±1.30**
Group V	31.67 ±1.76**	33.83 ±1.30**	34.33 ±1.41**
Group VI	37.33 ±2.10**	36.5 ±2.11**	39.5±2.14**



Values are expressed as mean ± S.E.M. (n=6) ,comparisons were made between: a Group II (negative control) vs Group III (Honey40 mg/kg), Group IV (SSM 25 mg/kg), Group V (SSM125 mg/kg) and Group VI Liv 52 (2.6 ml/kg)/Silymarin (200 mg/kg).Symbols represent statistical significance: *p<0.05, **p<0.01, ***p<0.001. One way ANOVA followed by Dunnett's test

Figure 64: Effect of SSM on Estimation of GSH in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

Groups	Estimation of GSH mg/dl		
	64.a Alcohol Model	64.b Paracetamol Model	64.c LPS- d Galn Model
Group I	52.5±2.36	50.83±1.79	51.67±2.19
GroupII	26.5±1.96	12.67±1.80	29.5±1.56
Group III	28.83±1.4	18±0..97	31.33±2.32
Group IV	34±1.34*	26.33±1.20**	35.67±1.76
Group V	38±1.84**	34.5±1.11**	40±1.24**
Group VI	47.67±2.89**	43.17±1.58**	44±2.37**

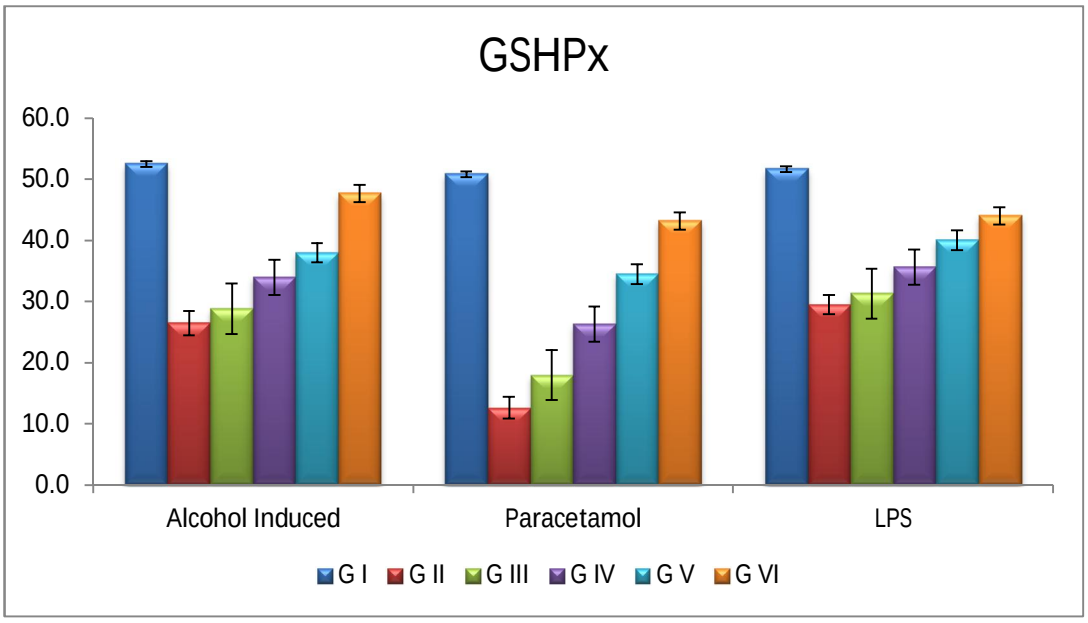


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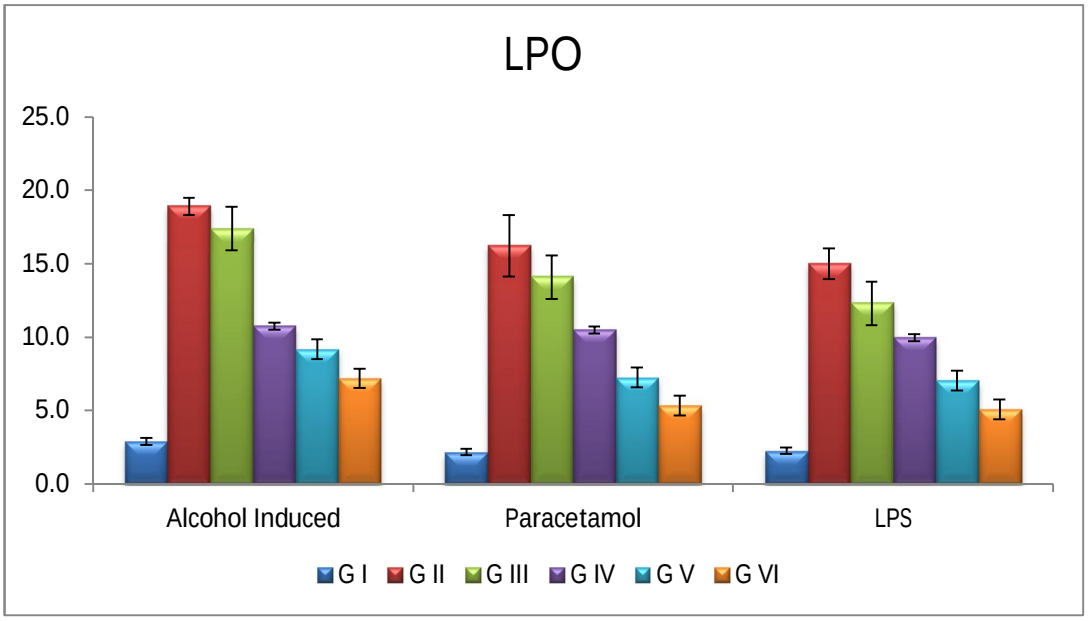
Figure 65: Effect of SSM on Estimation of LPO in Alcohol, Paracetamol and LPS/d-galactosamine (d-GalN) Induced liver injury in mice

Groups	Estimation of LPO mg/dl		
	65.a Alcohol Model	65.b Paracetamol Model	65.c LPS- d Galn Model
Group I	2.9±0.16	2.17±0.26	2.27±0.28
GroupII	18.9±0.59	16.23±2.09	15±1.04
Group III	17.38±0.76	14.1±0.43	12.32±0.55
Group IV	10.75±0.40**	10.48±0.33**	9.97±0.35**
Group V	9.17±0.67**	7.25±0.14**	7.03±0.08**
Group VI	7.2±0.22**	5.33±0.20**	5.08±0.19**



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NATIONAL INSTITUTE OF SIDDHA

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INSTITUTIONAL ETHICS COMMITTEE

Address of Ethics Committee: National Institute of Siddha, Tambaram Sanatorium, Chennai-600047, Tamil Nadu, India	
Principal Investigator: Dr.P.Shanmugapriya,MD(S)-NIS/PPHD/11/0206 Lecturer, National Institute of Siddha	
Protocol title: **A scientific approach on the validation of Santha Santhrothaya Mathirai (SSM) a Siddha Herbo-mineral preparation for its safety and efficacy in the management of hepatic disorders**	
Documents filed	**Yes**
Clinical trial Protocol (others – Specify)	**Preclinical studies - Yes**
Informed consent documents	
Any other documents	**IEC, IAEC Approval**
Date of IEC approval	**23.06.11 NIS/IEC/11/2/05**

(16)

CERTIFICATE

This is certify that the project title... A Scientific approach on the validation of Santha Santhrothaya Mathirai (SSM) a Siddha Herbo-mineral preparation for its Safety and efficacy in the management of hepatic disorders. **has been approved by the IAEC.**

Prof. Dr. K. Manickavasakam, M.D(S)
Name of Chairman/Member Secretary IAEC:

Dr. B. Jayachandran Dare.
Name of CPCSEA nominee:

Signature with date

14/6/11

Chairman/Member Secretary of IAEC:

NATIONAL INSTITUTE OF SIDDHA CHENNAI - 600 047

CPCSEA nominee:

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

KMCH College of Pharmacy, Coimbatore, Tamil Nadu, India.

Committee for the Purpose of control and Supervision of Experiments on Animals (CPCSEA)

Institutional Animal Ethics committee (IAEC)

CERTIFICATE

Title of the Project	:	A scientific approach on the validation of SANTHA SANTHROTHAYA MATHIRAI (SSM) A Siddha herbo-mineral preparation for its safety and efficacy in the management of hepatic disorders			
Proposal Number	:	KMCRET/MD(S)/02/2014-15			
Date received after modification (if any)	:	-NA-			
Date received after second modification	:	-NA-			
Approval date	:	31-01-2015			
Animals	:	**Wistar rats (m)**	**(f)**	**mice (m)**	**Albino mice (m)**
No. of animals sanctioned:	:	25	25	48	96
Expiry date (Termination of the Project)	:	31-01-2017			
Name of IAEC chairperson	:				

Dr. A. Rajasekaran
Signature of IAEC Chairperson

(Shri. Vivek Vinayak Kulkarni)
CPCSEA Inspector & Nominee

(Dr. K.R. Muthusami)
Biological Scientist

(Shri. S. Sengottuvelu)
Biological Scientist

(Mrs. T.R. Muneera)
Social Awareness Member

(Dr. V. Shanmugaraju)
Biological Scientist

(Dr. Anverdeen, M)
Veterinarian

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DR.M.SURESH GANDHI

Assistant Professor
Co-Ordinator, DST Inspire Camp,
Department of Geology

Date : 25.06.2013.

CERTIFICATE OF AUTHENTICATION OF MINERAL SAMPLES

Certified that the minerals submitted for identification by Dr. P.Shanmugapriya, Part time PhD scholar under the guidance of Prof. & H.O.D Dr.M.Murugesan, National Institute of Siddha, Tambaram Sanatorium, Chennai-47 are identified and authenticated as Mercurous Chloride (Calomel) and Borax (Vengaaram) with the microscopic and macroscopic characteristics based on Rutly's and Danas mineral descriptions as given below.

Station:

Date:

M. Suh Gandhi

(Dr. M. Suresh Gandhi)

Dr. M. Suresh Gandhi
Assistant Professor
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Guindy Campus, Chennai-600 025

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(Prof. V. Rammohan)

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Date: 26-06-2013

Botanical Certificate

Certified that the following plant drugs taken up by **Dr.P. Shanmugapriya** M.D.(S) Lecturer, Department of Nanju Nool and Maruthuva Neethi Nool, National Institute of Siddha, Chennai - 600047, for her part time Ph.D. research work is identified and authenticated through Visual inspection, Experience, Education & Training, Morphology, Organoleptic characters, Taxonomical and Microscopical methods as

Piper nigrum Linn. (Piperaceae), Fruit

Piper betle Linn. (Piperaceae), Leaf

Curcuma longa Linn. (Zingiberaceae), Mother rhizome

Citrus limon (Linn.) Osb. (Rutaceae), Fruit

MEDICINAL BOTANY ★ CHENNAI 600 047 ★ National Institute of Siddha

Certificate No: NISMB852013

Reference:

1. D.J.Mabberley, The Plant-Book, A portable dictionary of the vascular plants, Second edition, P. 560, 201, 161
2. Theodore Cooke, The Flora of the Presidency of Bombay, Vol –II, 2006, P. 526, 528, 732.

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IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 15, Issue 4 Ver. XII (Apr. 2016), PP 01-08
www.iosrjournals.org

Toxicological Profiling Of Santha Santhrodhaya Mathirai (*SSM*) A Siddha Herbomineral Formulation in Rats.

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Abstract: *The Siddha system of medicine exhibits its glory and significance since time immemorial. It is essentially concerned about utilizing the flora and fauna of the universe for the benefit of the society. Present day treatment modalities for hepatic disorders involve ready use of herbal drugs. Santha santhrodhaya mathirai(SSM) is the widely used Siddha herbo mineral formulation for the treatment of Pitha suram (Disorders of Liver associated with or without fever) . Currently there is no scientific data available for its safety profile and so the present study is to evaluate the toxicological potential if any of SSM with Wistar albino rat. In acute oral toxicity study, SSM was administered at 2000mg/kg orally and animals were observed for toxic signs at 0.5, 1, 4, 24 h and for 14 days. In repeated dose-28 day toxicity study, SSM was administered at 25,125and 250 mg/kg body weight/day to 3 groups of rats, respectively. The heavy metal analysis of SSM was done at Regional research institute of Unani (RRIU), Chennai. The results showed that neither the acute toxicity study of SSM at the dose level of 2000mg/kg nor the repeated dose study did not produce any toxic sign or mortality during study. In repeated dose toxicity study, no significant changes were observed in the haematological and biochemical parameters, relative organ weight, gross necropsy and histopathological examination with SSM treatment. The Results of the present study suggest that LD_{50} of SSM>2000mg/kg and NOAEL >250mg/kg/day in rats and the heavy metals were within the WHO permissible limits.*

Keywords*: Siddha, Santhasanthrodhayamathirai , Herbomineral, acute oral toxicity, Hepatic disorder .*

I. Introduction

Liver is one of the major metabolic organ and plays a pivotal role in metabolism of food, provision of nutrients to the blood and excretion of toxins. Changes in liver function due to infection or injury due to any cause may alter the metabolic functions and cause irreversible liver diseases.Liver disease deaths in India reached 2.31% of the total deaths according to the latest WHO data published in April 2011.It is estimated that liver diseases are among the top ten killer diseases in India[1]. Liver diseases are caused by toxic chemicals, drugs, viruses (hep A,B,C,D,E), excess alcohol intake etc [2]. The Traditional Siddha system of medicine offers several therapeutic agents like herbal, herbo- mineral, formulations for the treatment of liver diseases. *Santha Santhrodhaya Mathirai(SSM)* is a significant herbo- mineral formulation with a blend of hepatoprotective and anti-inflammatory ingredients for the treatment of *Pitha suram* (biliary disease). Sage Yugi describes the symptoms of *Pitha suram* in Siddha literature as follows, excessive sleep, reddish urine and stools, diarrhoea, vomiting, bitter tastiness, excessive thirst, fatigue, pallor, hiccup[3]which correlates with most of the symptoms of Liver diseases.

The ingredients of *SSM* consist of Mercurous chloride, Borax, Turmeric and Lemon juice all of which are said to have a positive effect on Liver functions. Mercurous chloride is used for the treatment of fever and Jaundice. Borax is reported to have coolant properties and is recommended for indigestion and loss of appetite which are the main symptoms of Liver diseases[4]. Turmeric, is a potent ingredient for treating liver diseases as it has curcumin as its important active principle. It is also an antioxidant and has anti-inflammatory, antiviral, antibacterial, antifungal, and anticancer properties, etc[5]. Lemon is described as a Minister to the 'Minister' pitham and a duke to the duke, in Siddha literature (*Manthirikku manthiri yaai, mannanukku mannanaai*). And it is therefore indicated for the treatment of *Pitham* associated *(Bilious)* disorders[6]. The Citrus nature of lemon has pharmacological significance as it possesses anti-cancer, antimicrobial, antioxidant, antiulcer, anti-inflammatory, and hypolipidemic and hepatoprotective properties[7].

Clinically *SSM* is used as one of the most important herbo-mineral formulations by Siddha practitioners for fevers with Liver diseases. As this formulation contains mercurous chloride, a heavy metal salt as one of the ingredient, this study was conducted to evaluate the safety profile of this herbomineral drug which is the prime step to validate the therapeutic potential of these ancient drugs for further researches.

WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 5, XXX-XXX. Research Article ISSN 2277– 7105

QUALITATIVE ANALYSIS AND QUANTITATIVE DETERMINATION OF "CURCUMIN" IN A SIDDHA HERBO-MINERAL FORMULATION USING HIGH-PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

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Article Received on
09 March 2016,
Revised on 28 March 2016,
Accepted on 19 April 2016
DOI: 10.20959/wjpr20165-6138

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ABSTRACT

Objective: To perform qualitative and quantitative estimation of 'Curcumin' in a Siddha herbo-mineral formulation – 'SanthaSanthrodayamMathirai (SSM)' using High-Performance Thin Layer Chromatography (HPTLC). **Method:** Methanolic extracts of three samples of SSM (A, B, C) prepared using the traditional procedures described in Siddha literature. High Performance Thin Layer Chromatography method was performed for quantification of curcumin in all the three samples by employing chloroform: methanol 9.5:0.5 (v/v) as a mobile phase. **Results:** Curcumin was identified at UV- 366 nm using the Retention factor (R_f) values in the three samples of SSM with reference to standard curcumin. The percentage of curcumin present in sample A, B and C is 0.6589, 0.6884 and 0.7104 respectively. **Conclusion:** Qualitative analysis and quantitative estimation of Curcumin content in SanthaSanthrodayaMathirai (SSM) was successfully done using High-Performance Thin Layer Chromatography (HPTLC). These results suggest that quantification of Curcumin in SSM formulation can be helpful in quality control and the curcumin fingerprints obtained by HPTLC can be helpful in SSM standardization.

KEYWORDS: Curcumin, High-Performance Thin Layer Chromatography, Santha Santhrodaya Mathirai.

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