First year progress report of the

Project entitled

"Scientific Validation of Safety, Protective and Curative Efficacy of a Patented Folklore Medicine "Savliv" Developed for the Management of Hepatic Disorders- A Preclinical Study"

Submitted to

Drugs and Pharmaceuticals Research Programme of

Technology Development and Transfer Division
Department of Science and Technology

Govt. of India, New Delhi
By

M/s. Harshul Ayur Pharma

Bambagher, Ramnagar -244715

(Nanital) Uttrakhand, India

&

Centre for

Toxicology and Developmental Research (CEFT)

Sri Ramachandra University

Chennai

July'2013

ACKNOWLEDGEMENT

I express our sincere thanks for the expert committee members, **Department of Science and Technology**, Government of India, who had reviewed our proposal and recommended for the support. I shall strive hard to fulfil the objectives of the proposal and to gratify the confidence of the expert committee in the forthcoming years.

I express our sincere thanks to **Dr. G. J. Samathanam**, Advisor and Head, Technology Development and Transfer, Drugs and Pharmaceuticals Research Programme, Department of

Science and Technology, Govt. of India, New Delhi, for creating an inspiration in us in the validation of herbal formulations. I shall try hard to deliver our best possible to accomplish the vision of Drugs and Pharmaceutical Research programme.

Mere words are inadequate to express our sincere and deepest feelings of gratitude originating from the innermost core of the heart for our Chancellor, **Shri. V.R. Venkataachalam,** for his valuable unflinching interest, relentless efforts and moreover, motherly attitude in developing research in our University.

I am extremely grateful to **Dr. J.S.N. Murthy**, Vice-Chancellor, for his constant support and stimulation in maintaining the consistency in our research

I am indebted to Prof. **Dr.K.V.Somasundaram**, Dean of Faculties, for his encouragement and support in shaping each and every step of the present research project.

I also express our sincere gratitude to **Prof. S.P.Thyagarajan**, Professor of Eminence and Dean (Research) for his constant support to complete the first year of research successfully.

I am extremely thankful to **Shri.Vimal Kumar**, Managing Director, M/s. Harshul Ayur Pharma, an entrepreneur who desires to bring world-class and quality healthcare to enlarge the society.

I owe or deep sense of thanks to **Thiru N. Natarajan**, Registrar, SRU, **Thiru V. Swaminathan**, Additonal Registrar (Law), **Mr. Ravi Shankar**, Chief Finance Officer, **Mr. Lakshminarayanan**, Manger, Finance and Accounts, SRU, **Mr. Gnanakumar**, Purchase Officer, SRU, **Mr. M. Mani**, Supervisor, CEFT, SRU, **Mr. S. Jeevanandham**, Preclinical technician - CEFT, SRU and all other staff for supporting us in successful completion of the first year of the project. I also extend our thanks to our faculties at CEFT who had co-operated and assisted in all the stages of the project work.

C. Saravana Babu

(Principal Investigator)

Summary

Further to signing of tripartite ‘Memorandum of Understanding’ between Department of Science and Technology, Govt of India, M/s Harshul Ayur Pharma and Sri Ramachandra University on July’2012, staff recruitment, chemicals and consumables were purchased to carry out the research. M/s. Harshul Ayur Pharma, Uttrakhand, prepared and supplied the test drug, Savliv, to carry out the research.

Savliv was tested for sugars, glycosides, steroids, alkaloids, saponins, phenols, tannins, flavones, triterpenoids, and anthraquinones by preliminary phytochemical analysis. Savliv was also subjected to quantitative estimation of total phenols, flavonoids, tannins, Vit-E equivalent, Vitamin C and Citric acid content. The test drug was also evaluated for *in vitro* anti-oxidant capacity in various models such as DPPH radical scavenging assay, super oxide radical scavenging assay, lipid peroxidation inhibitory assay, nitric oxide scavenging assay

and H_2O_2 radical scavenging assay. The HPTLC fingerprint of Savliv was also developed

Savliv was tested for heavy metals such as lead, mercury, arsenic and cadmium. Accelerated stability study of Savliv was also performed to establish the stability of Savliv and the data were documented.

Acute oral toxicity study of Savliv was completed in female Swiss albino mice following OECD guideline 423.

Currently, 28 day repeated oral toxicity of Savliv was initiated following OECD test guideline 407.

5. Introduction

Hepatic metabolism plays a key role in the regulation of whole-body energy status since liver is the major site for multiple interrelated functions. It persuades the uptake, storage, metabolism and release of nutrients such as amino acids, carbohydrates, lipids, vitamins and minerals which are essential for growth. It promotes the synthesis of bile salts from cholesterol and their secretion to assist with fatty acid absorption and digestion. It also concerned with synthesis and secretion of plasma proteins necessary for blood clotting and transport of molecules through the circulation. Apart from these functions, it detoxifies the drugs, hormones and the end-products of metabolism.

Over the last 30 years, scientists have witnessed major progress in the knowledge and management of liver disease, yet approximately more than 29 million people globally still suffer from chronic liver conditions. According to WHO data, liver disease deaths in India reached 2.31% of total deaths and India is ranked 27 in the world for prevalence of liver disease.

Liver disease

Liver disease refers to any disorder of the liver and includes the following:

- Steatosis or fatty deposits in liver

Fibrosis or scarring of liver

Hepatitis or inflammation of liver

- Cirrhosis where scarring and inflammation spread through the liver and irreversibly disrupt its shape or function causing permanent cell damage and ultimately liver failure

Alcoholic hepatitis results as a cause of chronic intake of alcohol. It is usually found in association with hepatosteatosis, an early stage of alcoholic liver disease, and may contribute to the progression of fibrosis, leading to cirrhosis. Symptoms of alcoholic include jaundice, ascites (fluid accumulation in the abdominal cavity), fatigue and hepatic encephalopathy

(brain dysfunction due to liver failure). Mild cases are self-limiting, but severe cases have a high risk of death (McCullough *et al*., 1998).

> **Non-alcoholic fatty liver disease (NAFLD)** is now widely recognised as the leading cause of liver dysfunction in affluent societies and is the most common cause of chronic liver disease worldwide. NAFLD is associated with obesity, metabolic syndrome, insulin resistance and type 2 diabetes. As such, it is better described as obesity-induced liver disease. NAFLD is a spectrum of disease, ranging from hepatosteatosis and non-alcoholic steatohepatitis (NASH), to cirrhosis and hepatocellular carcinoma (Ekstedt M *et al*., 2006). It is projected that within a decade or so, a greater number of patients will receive a transplant for endstage NAFLD than for end stage liver disease secondary to chronic hepatitis C (Charlton M, 2004).

Drug-induced hepatotoxicity is one of the frequent causes of liver injury. The predominant clinical presentation is acute hepatitis and/or cholestasis, although almost any clinical pathological pattern of acute or chronic liver disease can occur. The pathogenesis of drug-induced liver disease usually involves the participation of the parent drug or metabolites that either directly affect the cell biochemistry or elicit an immune response. Each hepatotoxin is associated with a characteristic signature regarding the pattern of injury and latency. Susceptibility to drug-induced hepatotoxicity is also influenced by genetic and environmental risk factors. Unpredictable, low-frequency, idiosyncratic reactions often occur on a background of a higher rate of mild asymptomatic liver injury although it is difficult to predict. Recent and future advances in toxicogenomics and proteomics should improve the identification of risk factors and the understanding of idiosyncratic hepatotoxicity (Neil, 2004).

Signs and Symptoms

The clinical presentations of hepatotoxicity that are most readily distinguished are acute hepatocellular injury and cholestatic liver disease. Acute hepatocellular injury often is associated with symptoms of malaise, abdominal pain, and jaundice. The alanine aminotransferase level is markedly elevated, with minimal elevations in the alkaline phosphatase level. The combination of jaundice, impaired hepatic function (indicated by an increased prothrombin time or its INR), and encephalopathy indicates particularly severe liver injury. The development of these signs less than 26 weeks after the onset of illness in a patient without pre-existing cirrhosis is the hallmark of acute liver failure. This syndrome has a poor prognosis without liver transplantation and is a problem of great concern.

Prevention and Management

A better understanding of hepatotoxicity is a major challenge in near future. Unfortunately,

scientific data also show that socio-economically lowered sections over the population are now the dominant victims of liver diseases and its risk factors. Therefore, there is a need to propose novel therapeutic strategy to forestall this crucial warning of increasing mortality due to hepatic damage. Life style management, smoking cessation, a sensible diet for patients with limitation of alcohol intake will reduce the increased risk of liver disease. Several approaches have been proposed to counter progress the liver disease, but there still seems no consensus. However, some patients cannot tolerate the adverse effects transplantation and oral pharmacological drugs on long term use. As a consequence, there is continuous high demand for newer therapeutic regimen. Prevention and management of liver disorders without any side effects is still a challenge to the medical system.

In India, traditional system of medicine is in vogue since time immemorial. Currently poly herbal preparations are gaining preference compared to single herb preparation. Although many herbs and herbal based products are claimed for their hepatoprotecive effect, they are overlooked or remain undesirable due to lack of standardization of the herbal drugs, ignorance on active ingredient(s)/principles(s) present, limited or no controlled clinical trials (RCTs) and less indication of toxicological profile of herbal drugs.

Savliv, **a herbomineral formulation was developed by M/s. Harshul Ayur Pharma, Uttrakhand, India, for the management/treatment of various liver diseases such as liver cirrhosis, hepatitis 'B', fatty liver and etc. In the traditional practice of M/s. Harshul Ayur Pharma, Uttrakhand, Savliv had shown significant curative effect in patients with liver diseases such as jaundice, cirrhosis.** ***Savliv*** **was also patented for its processing technique (Patent No: 239637, CGPDTM, Ministry of commerce and Industry, Govt of India). The present project was undertaken with objectives to scientifically validate the safety of Savliv following OECD test guidelines for toxicological studies, standardise the formulation and to evaluate its shelf life through accelerated stability study.**

OBJECTIVES OF THE PROJECT

- Standardisation of ***Savliv*** by HPTLC fingerprinting

To analyse the **heavy metals** contents in ***Savliv***
To determine the stability of ***Savliv*** by **accelerated stability studies**.
To establish the **toxicology** profile of ***Savliv*** by oral **acute, sub-acute** (repeated 28 days) and **chronic** (repeated 90 days) studies in rats

PROJECT DURATION WITH MILESTONE OF PHASES - 2 YEARS

Proposed Research		1st Year			
		A	B	C	D
Phase I	Literature review				
	Equipment Purchase & installation				
	Formulation				
	Standardisation & Heavy metals analysis				
Phase II	Stability studies				
Phase III	Acute, sub-acute & Chronic toxicity				
Phase IV	Updated literature, Documentation and Reporting				

First year Research commitments

(Jul'2012-July'2013)

S No	Commitments	Status
	Phase I	
	Standardisation	
1	To perform qualitative phytochemical analysis of Savliv	Completed
2	To determine Secondary metabolite contents of Savliv	Completed
3	To determine Vitamin C and Citric acid content of Saviv	Completed
4	To develop HPTLC fingerprint of Savliv	Completed
5	To perform *In vitro* anti-oxidant activity of Savliv	Completed
6	To analyse Heavy metals (Pb, Cd, Hg, As) contents in Savliv	Completed
	Phase II	
7	To evaluate the stability of Savliv through accelerated stability study	Completed
	Phase III	
8	To carry out the acute oral toxicity of Savliv	Completed
9	To carry out the sub-acute oral toxicity of Savliv	On-going

8. METHODOLOGY

8.1. Standardisation of Savliv

In the present study, Savliv was standardised using phytochemical analysis, Vitamin E, Vitamin C, Citric acid estimation, HPTLC fingerprinting and *in vitro* antioxidant studies.

8.1.1. Qualitative phytochemical analysis of Savliv

8.1.1.1. Detection of phenolic compounds

To few ml of Savliv, a few drops of 1:10 diluted Folin's phenol reagent and few drops of 10% sodium hydroxide were added. The formation of bluish green or bluish black colour will indicate the presence of phenolic compounds.

8.1.1.2. Detection of tannins

To few ml of Savliv, few drops of 1:10 diluted Folin's phenol reagent and few drops of sodium carbonate (dissolved in water) were added. The appearance of bluish green colour will indicate the presence of tannins.

8.1.1.3. Detection of saponins

Few ml of Savliv was shaken well with few ml of water in the test tube. Copious lather formation will reflect the presence of saponins.

8.1.1.4. Detection of flavones (Shinoda test)

Few ml of Savliv was mixed with alcohol. A few magnesium turnings and a few drops of concentrated hydrochloric acid were added to the tube and boiled for few minutes. The appearance of red colour will indicate the presence of flavones.

8.1.1.5. Detection of alkaloids

Few ml of Savliv was mixed with few drops of concentrated hydrochloric acid. To this acidic mixture, 1ml of Dragendorff's reagent was added. The appearance of orange or red precipitate will indicate the presence of alkaloids.

8.1.1.6. Detection of Carbohydrates

Fehling's Test

To few ml of Savliv, equal volume of Fehling's A and B reagents were added and heated. Appearance of red colour will indicate the presence of carbohydrates.

8.1.1.7. Detection of triterpenoids

a. Few ml of Savliv was added to 1ml of chloroform; 1 ml of acetic anhydride and 2ml of conc.H_2SO_4. Formation of reddish violet colour will indicate the presence of triterpenoids.

Salkowski test

Savliv was warmed with tin granules and thionyl chloride. Pink color formation will indicate the presence of triterpenoids.

8.1.1.8. Detection of glycosides

Savliv was hydrolyzed with hydrochloric acid. To the hydrolysate, 1ml of pyridine and few drops of sodium nitroprusside were added and then made alkaline with sodium hydroxide. Appearance of pink to red colour will show the presence of glycosides.

8.1.1.9. Detection of anthraquinones

5 ml of Savliv was hydrolyzed with diluted sulphuric acid and 1ml of diluted ammonia was added to it. Appearance of pink coloration will indicate the presence of anthraquinones.

8.1.1.10. Detection of quinones

Savliv was mixed with 1ml of 10% sodium hydroxide and shaken vigorously. Blue, green or red coloration will indicate the presence of quinones.

8.1.1.11. Detection of steroids

Few ml of the Savliv was treated with equal volume of chloroform and concentrated sulphuric acid. Turning of upper layer to red and the sulphuric acid layer to yellow with green fluorescence will

indicate the presence of steroids.

8.1.2. Determination of Secondary metabolites contents in Savliv

8.1.2.1. Total phenolic compounds

Total phenolic content was analyzed using Folin's Ciocalteau reagent (McDonald *et al*., 2001). Savliv (250, 500 and 1000 µl), 1.25 ml of 1:10 diluted Folin's Ciocalteau reagent and 1 ml of 7.5% Na_2CO_3 were mixed and allowed to stand for 30 min at 37°C and the blue colour developed was measured at 640 nm in a Multiskan spectrophotometer. A calibration curve was plotted using gallic acid as standard (concentration range: 20-100 µg). The results were expressed in µg/ml Savliv

8.1.2.2. Tannins

Total tannin content was determined as per Schanderl *et al*., (1970) method. Savliv was added at volume ranging from 250, 500 and 1000 µl and made up to 1.0 ml with methanol. 0.25 ml of Folin's phenol reagent and 2.5 ml of 1% sodium carbonate were pipetted into all the tubes. The tubes were incubated for 5 minutes at room temperature. 0.25 ml of Folin's phenol and 2.5 ml of 1% sodium carbonate served as blank. The blue colour developed was measured at 640 nm. A calibration curve was plotted using gallic acid as standard (Concentration range: 20-100 µg).

The results were expressed in µg/ml Savliv

8.1.2.3. Flavonoids

Total flavonoid content was determined by aluminium chloride colorimetric method (Chang *et al*., 2002). Savliv was added at volume ranging from 250, 500 and 1000 µl and the final volume was made up to 2.5 ml with methanol. 0.1ml of 10% aluminum chloride, 0.1 ml of 1M sodium acetate and 2.8ml of distilled water were added to all the tubes. All the above reagents excluding the Savliv served as blank. The tubes were incubated at room temperature for 30 min. The absorbance was measured at 415 nm in Multiskan spectrum. A calibration curve was plotted using quercetin as standard (Concentration range: 20-100 µg). The results were expressed in µg/ml Savliv.

8.1.2.4. Vitamin- E equivalent (total antioxidant capacity)

Total antioxidant capacity of Savliv was evaluated by the method of Prieto *et al*., (1999). 500 µl of Savliv, 0.5ml of ethanol and 1.0ml of Vitamin E reagent were added to the tubes. In case of blank, ethanol was added in spite of the Savliv. The tubes were incubated at 60˚C for 30min in a boiling water bath. Samples were cooled to room temperature and the absorbance was measured at 695nm against the blank in Thermo Scientific multiskan spectrophotometer, USA. A standard calibration curve was plotted using vitamin E in the concentration range of 100µg to 500µg. The results were expressed in ml equivalent to 1 mg of Vitamin E.

8.1.3. Vitamin C – UV/Visible Multiskan spectrophotometer

Vitamin C was estimated in Savliv according to the method described by Oyaizu (1986). 500 µl of Savliv was taken in triplicates, 0.5 ml of 5% TCA (trichloro acetic aicd) and 0.1 ml of DTC reagent (Dinitro Phenyl Hydrazine, Thiourea, Copper sulphate) were added to the tubes, incubated at 37˚C for 3 h. After the incubation, 0.75 ml of 85% sulphuric acid was added and tubes were incubated for 30 min at room temperature. The absorbance was measured at 540 nm against blank in UV/Visible Multiskan spectrophotometer, USA. A calibration curve was plotted using vitamin C as standard (Concentration range: 20-100 µg). The results were expressed in mg/ml Savliv

8.1.4. Citric acid content – Volumetric analysis

Citric acid content in savliv was estimated as per Oremusova *et al*., (2007). Standardisation of sodium hydroxide was performed with 0.1 N of standard oxalic acid. 0.1 N solution of standardized NaOH was taken in the burette. Approximately 3 drops of phenolphthalein indicator was added to 10 ml of 1:10 diluted Savliv in a conical flask. NaOH was titrated against the test drug till the colour of solution changes from colourless to pale pink. The indicator colour must remain stable for 30 seconds and be light pink when viewed over a white background. 1ml consumption of NaOH is equivalent to 0.64 mg of acid content. The results were expressed in mg/ml Savliv

8.1.5. HPTLC Fingerprinting - Savliv

High Pressure Thin Layer Liquid Chromatography (HPTLC) was performed on a 10x10 cm pre activated silica gel 60F 254 plate. The plates were pre-washed by methanol and activated at 600 C for 5 minutes prior to chromatography. The slit dimension was kept at 5 minutes x 0.45 minutes and 20 minutes scanning speed was employed. Savliv was applied to the plate as 6mm wide band with an automatic TLC applicator Linomat 5 with

N2 flow (CAMAG, Switzerland), 8 mm from the bottom. 5µl sample was spotted into a silica plate using Linomat 5 applicator. It was allowed to develop in a twin trough chamber, 20 x 10 cm at 25°C which were already saturated with solvent system n hexane:ethyl acetate (7:2) in an ascending mode manner under room temperature. The solvent system was selected on trial and error manner to elute the maximum number of peaks. After development, solvent front was marked using pencil and then the plates were allowed to dry using a hair dryer. The developed plates were scanned for densitometric scanning at appropriate wavelength (254 and 366 nm) using scanner 3 and photo-documented using CAMAG Reprostar 3.

8.1.6. *In vitro* antioxidant potential of Savliv

In vitro anti-oxidant assays were performed to investigate the free radical scavenging activity of Savliv. Based on the specific gravity of savliv (1.037), concentrations were fixed.

8.1.6.1. DPPH radical scavenging assay

DPPH radical scavenging assay was performed as described by Koleva II *et al*., (2002). 10 µL each concentration (1.95 – 1000 µg /0.1 ml) of Savliv was added to 190 µL ethanolic DPPH (150 µM) solution. After vortexing, the mixture was incubated for 30 min at 37°C. Control blank contains solvent without Savliv. The decrease in absorbance of test mixture (due to quenching of DPPH free radicals) was measured at 517 nm in Multiskan spectrophotometer, USA and the percentage inhibition was calculated using the formula

$$\text{Percentage inhibition} = [(\text{control} - \text{test})/\text{control}]*100$$

8.1.6.2. Superoxide radical scavenging assay (Kakkar *et al*., 1984)

To 0.1 ml of Savliv (concentrations: 1.95-1000 µg / 0.1 ml), 0.25 ml of sodium pyrophosphate buffer (0.025 M), 0.025 ml of PMS (186 µM) and 0.075 ml of NBT (300 µM) were added. The reaction was triggered by the addition of 0.075 ml of NADH (780 µM). After incubation at 30˚C for 90s, the reaction was terminated by addition of 0.25 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 2.0 ml of n-butanol. The mixture was allowed to stand for 10 min and centrifuged. n-butanol alone served as blank. The colour intensity was read at 560nm in Multiskan spectrophotometer, USA. All analyses were run in duplicate and mean values were calculated. IC_{50} value (the concentration required to scavenge 50% of superoxide radical) was calculated. The percentage of superoxide radical scavenging activity was calculated using the formula

$$\text{Percentage inhibition} = [(\text{control} - \text{test})/\text{control}]*100$$

8.1.6.3. Lipid peroxidation inhibitory assay

Chick liver tissue was obtained from local slaughter house and 10% liver homogenate was prepared using ice-cold KCl (0.15M) in a Teflon tissue homogenizer. Homogenate was incubated with Savliv at different concentrations (1.95-1000 μg / ml). In the control tubes, 1 ml of tissue homogenate was added and the lipid peroxidation was initiated by the addition of 0.1 ml of $FeSO_4$ (25 μM), 0.1 ml of ascorbate (100 μM) and 0.1 ml of KH_2PO_4 (10 mM) and the volume was made up to 3 ml with distilled water and incubated at 37˚C for 1 h. After incubation, 1 ml of 5% TCA and 1 ml of 0.375% TBA were added to the reaction mixture and the tubes were boiled for 30 min in a boiling water bath. Tubes were then centrifuged at 3500 rpm for 10 min. Control tubes contains all other reagents except Savliv and the extent of lipid peroxidation inhibition was determined in terms of thiobarbituric acid reactive substances (TBARS) level by measuring the optical density at 532 nm in Multiskan spectrophotometer, USA (Ohkawa *et al*., 1979). All analyses were run in duplicate and mean values were calculated. IC50 value (the concentration required to scavenge 50% Lipid peroxide radical) was calculated. The percentage inhibition of lipid peroxidation was calculated using the formula

Percentage inhibition = [(control – test)/control]*100

8.1.6.4. Nitric oxide radical scavenging assay

Aqueous sodium nitroprusside at physiological pH generates nitric oxide (NO) spontaneously, which interacts with oxygen to produce nitrite. Nitrite, thus formed can be estimated using Greiss reagent. The reaction mixture contains 0.5 ml of Savliv (1.95-1000μg/0.5ml) and 1.25 ml of sodium nitroprusside (5 mM) in phosphate buffer which in turn is allowed to react with 1.25 ml Greiss reagent after the incubation period of 2½ h at 37˚C. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm (Green *et al*., 1982) in Multiskan spectrophotometer, USA. All analyses were run in duplicate and mean values were calculated. IC_{50} value (the concentration required to scavenge 50% nitric oxide radical) was calculated. The percentage of nitric oxide radical scavenging activity was calculated using the formula

Percentage inhibition = [(control – test)/control]*100

8.1.6.5. H_2O_2 radical scavenging assay

H_2O_2 radical scavenging assay was used for determining the ability of the Savliv to scavenge H_2O_2 radicals. The reaction mixture contained H_2O_2 (2 mM) with different concentrations of Savliv ranging from 1.95-

1000µg/0.1ml. Dichromate acetic acid reagent (5% Potassium dichromate and Glacial Acetic Acid in 1:3 v/v) was added to the reaction mixture after the incubation period of 5 min at 37ºC and absorbance was measured at 570 nm (Sinha., 1972) in Multiskan spectrophotometer, USA. 2 ml of dichromate acetic acid reagent alone served as blank whereas the reaction mixture without substance served as control. All analyses were run in duplicate and mean values were calculated. IC_{50} value (the concentration required to scavenge 50% H_2O_2 radical) was calculated.

Percentage inhibition = [(control – test)/control]*100

8.2. Determination of heavy metal content in Savliv

Herbal medicines are gaining importance in various disciplines of natural health care system. However there exist a common misperception that medicines of natural substances cannot be toxic, but according to the advanced researches it has been documented that plants not only contain toxic secondary metabolites, but they are also contaminated with environmental pollutants specially heavy metals, which pose a great health risks to all living organisms upon long term exposure (Arpadjan *et al*., 2008; Lasisi *et al*., 2006). Hence, in the present study we estimated heavy metals such as lead [Pb], Cadmium [Cd], Mercury [Hg], Arsenic [As] in Savliv using a hydride generator attached to Atomic Absorption Spectrophotometer (AAS).

8.2.1. Digestion and preparation of sample

Few ml of Savliv was treated with 5 ml HNO_3. The flasks were covered with watch glass and heated to reflux on an electric hot plate at 80 to 100°C. Following one hour heating, the contents of flasks were treated again with 5 ml of HNO_3 followed by 2 ml of 30% H_2O_2 and the heating at gentle reflux was continued till clear solution was obtained. This solution was diluted in deionized water, filtered through a whatman # 42 paper into volumetric flasks and was marked as sample solution.

8.2.2. Elemental Analysis using Atomic Absorption Spectrophotometer

The elemental analysis of digested samples was determined by Atomic Absorption Spectrophotometer (AAS model 400 Perkin Elmer). In this method the sample was introduced into flame where thermal and chemical reactions create “free” atoms capable of absorbing, emitting or fluorescing at characteristic wavelengths. A light source emitting a narrow spectral line of the characteristic energy is used to excite the free atoms formed in the flame. The decrease in energy (absorption) is then measured.

The absorption is proportional to the concentration of free atoms in the flame, given by the Lambert-Beer law.

$$\textbf{ABSORBANCE} = \log_{10} I_0/I_t = K * C * L$$

Where, I_0 = Intensity of incident radiation emitted by the light source

I_t = Intensity of transmitted radiation

C = Concentration of sample (free atoms)

K = Consent (can be determined experimentally)

L = path length

8.3. Determination of stability of Savliv by accelerated stability study

Stability study is mainly to determine the period of storage at a specified conditions in which the drugs meet its established specification. The stability study of Savliv was conducted according to Good Manufacturing Practices for Finished Pharmaceuticals, Testing and Release for Distribution, as per ICH guidelines. The stability study was conducted with accelerated storage condition (Table 1)

Table.1. Storage conditions and test schedule for stability study

Type of stability study	**Test Sample**	**Optimum Temperature/Relative humidity (RH)**	**Duration (months)**	
Accelerated temperature storage	Savliv Stored in a closed container	40°C± 2°C 75%± 5% RH	Day 0	3
			3 months	

Table.2. Test conditions for stability studies

Test Conditions	40°C/75% RH Months
Physiochemical	
Appearance, colour and odour	0,3
pH	0,3
Specific gravity	0,3
Chemical	
Secondary Metabolite	0,3
Citric acid	0,3
Vitamin C	0,3
Microbial Load	0,3

8.3.1. Analytical Methods

Stability study was performed in triplicates with given batch. Sample was placed in the above specified temperatures in controlled chambers. Day 0 analysis is the date on which sample was put on station within the designated storage chambers. Day 0 analysis was completed within 15 days of preparation date. Following analysis, the sample was stored at ambient conditions.

Appropriate validation studies were performed following approved protocol to demonstrate that the test methods were stability-indicating and suitable for their intended use. The analytical testing methods were summarized below.

8.3.1.1. Organoleptic analysis

8.3.1.1.1. Appearance , Colour and Odour

Color indicated the nature of the formulation. Appearance and Odour were extremely sensitive criteria. Appearance and color was determined by visual examination.

8.3.1.1.2. pH

pH of Savliv was determined by using Digital pH meter in accordance to the prescribed standard method in Indian Pharmacopoeia. One gram of Savliv was dissolved in 100 ml of distilled water (1% w/v) and stored for

two hours and were used for pH measurement.

8.3.1.1.3. Specific Gravity

Specific gravity of Savliv was determined using the formula

$$\text{Specific Gravity} = \frac{\text{Density of Liquid (g/mL)}}{\text{Density of water (g/mL)}}$$

8.3.2. Chemical Analysis

8.3.2.1. Secondary Metabolites, Citric acid and Vitamin C

Secondary metabolite content such as tannins, Total phenols flavanoids and Vitamin C in Savliv were determined.

8.3.2.1.1. **Total Phenol**

Total phenolic contents was analysed using Folin's Ciocalteau reagent (McDonald *et al.*, 2001).

8.3.2.1.2. Tannins

Total tannin content was determined as per Schanderl *et al.*, (1970) method.

8.3.2.1.3. **Flavonoids**

Total flavonoid content was determined by aluminium chloride colorimetric method (Chang *et al.*, 2002).

8.3.2.1.4. **Vitamin C**

Vitamin C content was determined by Oyaizu (1986).

8.3.2.1.5. Citric acid

Citric acid content in savliv was estimated as per Oremusova *et al.*, 2007.

8.3.2.2. Determination of Microbial Load

8.3.2.2.1. **Determination of bacterial and fungal contamination in Savliv**

Bacterial load is of primary importance as this may alter the physicochemical characteristics of the formulation. Suitable culture analysis was performed in order to identify the major contaminants in herbal preparations such as *E.coli, Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus*. Streak culture was done to identify aerobic spore barriers, which are non-pathogenic. Total microbial count was performed at Department of microbiology, SRU as per the standard protocol. 100µg of Savliv (v/w) was dissolved in 100µl of peptone water. Approximately 0.01ml of the test solution was inoculated in blood agar and McConkey agar plates. The plates were incubated at 37°C overnight and the colonies were counted. The organisms were identified using basic biochemical tests such as Indole test, Triple sugar iron (TSI), Urease, Citrate, Mannitol Motility, and Phenyl pyruvic acid.

8.4. Acute Oral Toxicity Study of Savliv (HAPLIV) In Female Swiss Albino Mice

Savliv was coded as HAPLIV in acute toxicity study.

8.4.1. Test Guideline

The study was performed according to the OECD guideline 423 (Acute Oral Toxicity - Acute toxic class method) adopted in December 2001.

8.4.2. Experimental Design

Institutional animal ethical committee (IAEC), Sri Ramachandra University, Chennai approved that study protocol (IAEC/XXXI/SRU/236/2012). Young healthy adult Swiss albino female (Nulliparous and Non-pregnant) mice weighing 18-22g b. wt., were divided into two groups (3 animals/group). Animals were housed individually in a well ventilated polypropylene cage with dedusted and autoclaved husk as bedding material. Cages and bedding material were changed on alternate days. A 12-h light/12-h dark artificial photoperiod was maintained. Room temperature 22°C (±3°) and relative humidity 50–70% were maintained in the room. Animals had free access to pelleted feed. (Provimi Animal Nutrition India Pvt. Ltd, India) and Reverse osmosis (Rios, USA) purified water ad libitum.

Animals were acclimatized for a period of 5 days to the laboratory conditions prior to initiation of the experiment. Animals showing no visible signs of illness were used for the study. Animals were marked with permanent marker. Test item was prepared with water for injection prior to administration. Animals received a single dose of HAP-LIV at 2000 mg/kg body weight by oral route after being fasted 3 to 4 hours for Step 1 and 2 with free access to water. Food was provided again at approximately 3 to 4 hours after dosing for all the steps. The dose volume was 10 mL/kg body weight. Three female mice were received a single dose of the HAP-LIV at

2000 mg/kg body weight (Step I) by oral administration. Since, mortality was found in first 72 hours, another three females were dosed at 2000 mg/kg body weight (Step II) after 72 hours. Following 72h of observations such as lethality and abnormal clinical signs to group I, a confirmatory step was performed to group II as stated above. Animal number allotment were as follows:**Animal number allotment**

Groups / Steps	**Number of animals per step (female)**	**Animal number**		
Step –1	3	1	-	3
Step – 2	3	4	-	6

8.4.3. Observation

Body Weight

Body weight was measured on day 0 (prior to administration), 7 and 14.

Mortality

Animals were observed for mortality at 30 minutes, 1, 2 and 4 hours following drug administration on day 0 and twice a day for 1-14 days.

Clinical Signs

Animals were observed for the clinical signs (moribund, repetitive circling, convulsion, tremor, Ataxia, walking backward, piloerection, hunched posture, vocalization, self mutilation, skin erythema, skin swelling, Exophthalmos, lacrimation, chromodacyorrhea, dyspnea, nasal discharge, salivation, bleeding from external orifices, diarrhoea etc) at 30 minutes, 1, 2 and 4 hours following drug administration on day 0 and once a day for days 1- 14.

Pathology - Necropsy

All the survived animals were euthanized for gross necropsy using overdose of CO_2 for necropsy. Organs such as external orifices, skin with mammary gland, lymph nodes, trachea, thyroid with parathyroid, heart, lungs, eyes, brain, stomach, large and small intestine (with payer's patches), spleen, liver, adrenals, kidneys, urinary bladder, ovaries and uterus with cervix were observed for gross lesions in all experimental animals.

8.4.4. Data Analysis

Data was analysed according to the decision tree of OECD- test guideline-423: Acute toxic class method, adopted 17th December 2001. No statistical analysis of data was performed.

9. Results

c. Standardisation of Savliv

Standardisation of herbal drugs plays vital role in safety and efficacy of the preparation. In the present study, Savliv was standardised by following methods and the results are as follows:

iv. Qualitative phytochemical analysis of Savliv

Savliv was subjected to qualitative phytochemical analysis and results are shown in **Table 9.1.**

Table.9.1. Phytochemicals of Savliv

S.No.	Phytochemicals	Inference*
1	Phenolic compounds	+++
2	Tannins	+++
3	Saponins	-
4	Glycosides	+
5	Sugars	++
6	Alkaloids	+
7	Quinones	-
8	Anthroquinones	-
9	Flavones	++
10	Triterpenoids	-
11	Steroids	-

*- + low, ++ mid, +++ high, - absent

v. Determination of Secondary metabolites contents in Savliv

There is an intense interest in plant polyphenols as witnessed by the numerous papers devoted to various therapeutic benefits aspects of these class of compounds . Phenolic compounds possess antioxidant properties, which in turn aids them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Pietta, 2000). Earlier reports suggest that plant derived metabolites like flavonoids, tannins, anthocyanins and other phenolic constituents present in food are potent antioxidants (Salah *et al*., 1995).

6. Total Phenol

Total phenol content in the Savliv was found to be 440.74 ± 18.11 µg/ml Savliv (**Figure 9.1.)**

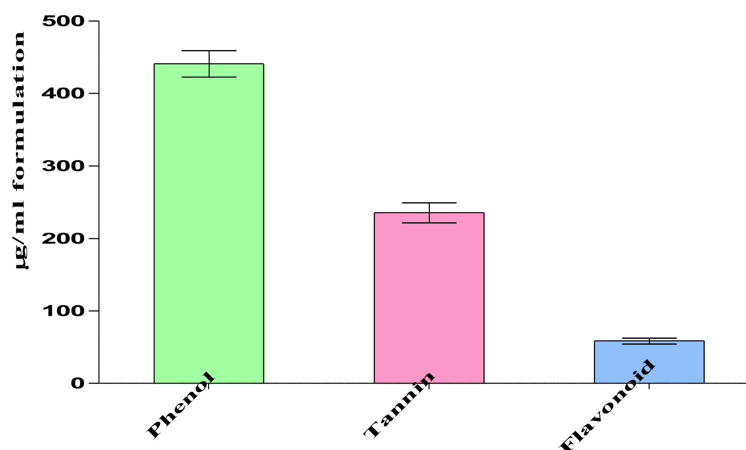
7. Total Flavonoid

Total flavonoid content in the Savliv was found to be 58.57 ± 4.03 µg/ml Savliv (**Figure 9.1).**

8. Total Tannin

Savliv was found to contain tannin content of 235.37 ± 13.70 µg/ml Savliv (**Figure 9.1**).

Figure.9.1. Secondary metabolites of Savliv



ix. **Vitamin E (Total anti-oxidant capacity)**

Vitamin E is well-known primary fat-soluble antioxidant in mammalian systems. A balanced diet with sufficient vitamin E and other plant origin antioxidants is desirable for optimal health and, probably, contributes to increased longevity (Friedrich W, *et al*., 1988). In the present tested conditions, it was found that **3.30±0.04** ml Savliv was equivalent to 1mg Vitamin E.

x. Standardisation of Savliv for Vitamin C and citric acid Content

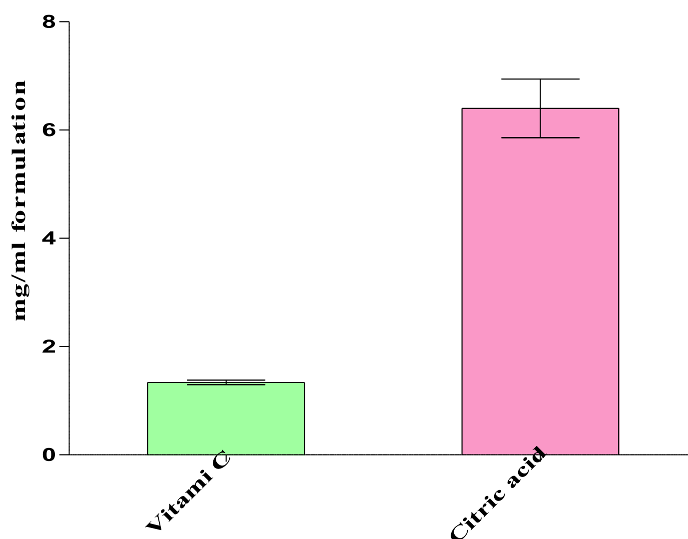
11. **Vitamin C content**

Vitamin C was reported to play prominent role in maintaining the physiological redox equilibrium (Katsuoka*et al*., 2005). Vitamin C content in Savliv was found to be **1.34±0.04 mg/ml (Figure. 9.2.)**.

9.1.4.2. Citric acid content

Citrate content in Savliv was found to be **6.4 ±0.54 mg/ml (Figure. 9.2.)**.

Figure. 9.2.Vitamin C and citric acid of Savliv



l. HPTLC profile of Savliv

Development of HPTLC Fingerprint serves as one of the sophisticated techniques in quality control of herbal drug. The HPTLC finger-print profile establishes the identity and purity of the raw drug used (Gupta and Mittal, 2010). The HPTLC finger print of Savliv was done in a trial and error manner using CAMAG Linomat V. Savliv was applied to the HPTLC plate, and allowed to develop in n hexane: ethyl acetate: as mobile phase, this separated the components of the test material based on the polarity. The developed chromatogram was scanned using a Scanner. The chromatogram developed using HPTLC for Savliv at 254 nm is shown in Fig 9.3. The details of the peak table are tabulated in Table 9.2. Savliv has 11 peaks at 254 nm. Out of 11 peaks, the area of peaks at Rf 0.48, 0.63 and 0.79 were found to be maximum compare to others and they were 45030.8, 22081.4 and 12539.7 respectively. They may an active principle in Savliv which might boost its activity.

Table 9.2: Peak table of HPTLC profile at 254 nm

Peak no.	Start R_f	Start height	Max R_f	Max height	Height %	End R_f	End heigh t	Area	Area %
1.	0.06	2.6	0.07	177.3	7.40	0.08	79.5	2554.5	2.66
2	0.09	81.6	0.10	177.2	7.40	0.11	0.1	2283.9	2.38

3	0.13	3.3	0.15	20.4	0.85	0.17	13.2	459.4	0.48
4	0.17	13.3	0.20	42.5	1.77	0.21	40.3	1029.6	1.07
5	0.21	40.6	0.26	249.4	10.41	0.31	6.4	8343.9	8.69
6	0.32	6.5	0.34	27.1	1.13	0.38	3.4	753.9	0.79
7	0.40	0.1	0.44	16.6	0.69	0.47	8.2	575.6	0.60
8	0.48	4.4	0.59	773.3	32.28	0.63	239.5	45030.8	46.89
9	0.63	240.0	0.66	605.6	25.28	0.71	0.2	22081.4	22.99
10	0.75	2.4	0.78	19.9	0.83	0.79	17.7	382.1	0.40
11	0.79	17.9	0.86	286.1	11.94	0.93	0.1	12539.7	13.06

Figure 9.3 HPTLC profile of Savliv

Peak Area

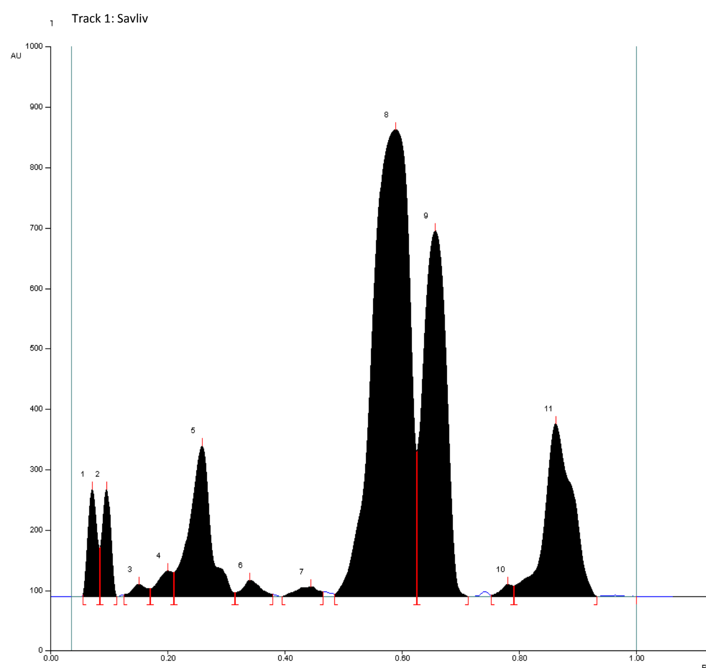
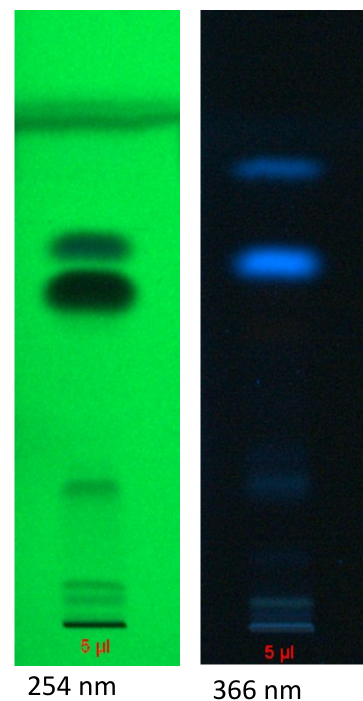


Photo Documentation



m. ***In vitro* antioxidant potential of Savliv**

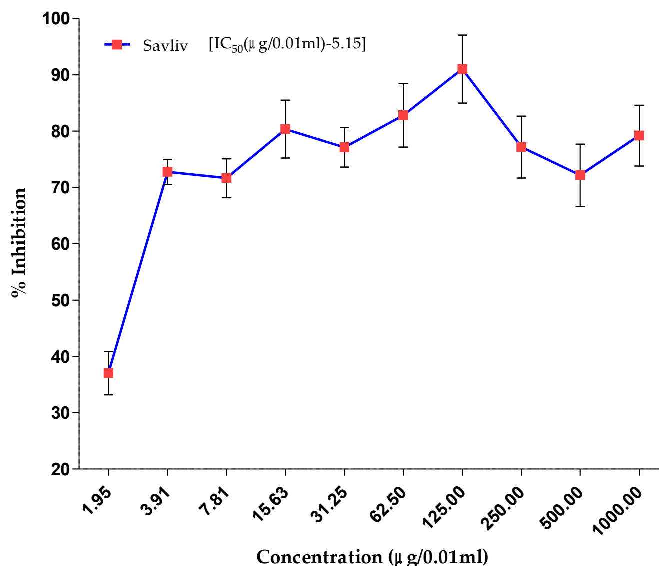
In vitro anti-oxidant assays were performed with standard methods. Based on the specific gravity of savliv (1.037), concentrations were fixed for the *in vitro* assays.

xiv. DPPH radical scavenging assay

Interference of DPPH (stable free radical) with antioxidants, results in transfer of electrons or hydrogen

atoms to DPPH, thus neutralising free radical nature (Naik*et al*., 2003). Results from the current study suggest that Savliv exhibited potent [**IC_{50} of 5.15 µg/0.01 ml**] DPPH radical scavenging activity (**Figure 9.4.**).

Figure 9.4.DPPH radical scavenging activity of Savliv



xv. Superoxide radical scavenging assay

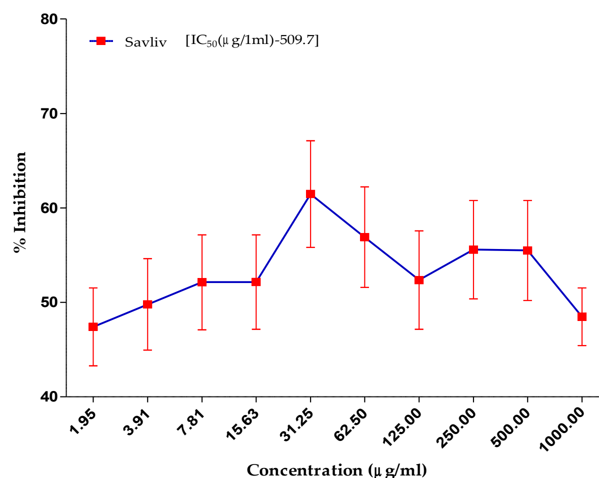
The superoxide radical ($O2^{\cdot}$) is a highly toxic species which is generated by numerous biological and photochemical reactions. Savliv was found to be an effective [**IC_{50} of 45.78µg/0.1ml**] superoxide radical's scavenger (**Figure 9.5**)

Figure 9.5. Superoxide radical scavenging activity of Savliv

xvi. Lipid peroxidation inhibitory assay

Effect of Savliv on non-enzymatic peroxidation of lipids when incubated with ferrous sulphate is shown in **Figure. 9.6.** The normal cellular physiology depends on the intactness of the plasma membrane and any damage including oxidative stress modulates signal transduction pathways that may subsequently affect various downstream processes (Santanam*et al*., 1998). Savliv shows potential lipid peroxidation inhibitory effect with an **IC_{50} of 509.7 µg/1ml.**

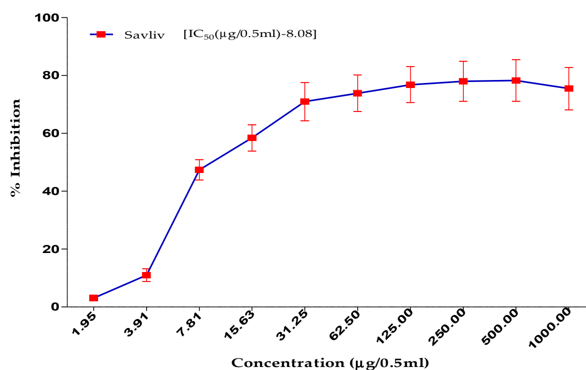
Figure.9.6. Lipid peroxidation inhibitory activity of Savliv



xvii. **Nitric oxide radical scavenging assay**

Reactive oxygen species (ROS) like $O_2\bullet^-$ reacts with nitric oxide (NO) and give rise to various other reactive nitrogen species (RNS) such as NO_2, N_2O_4, peroxynitrite. Both ROS and RNS together induce damages various cellular molecules (Pacifici and Davies, 1991). Savliv was found to be a potent [**IC_{50} of 8.08µg/0.5ml**] nitric oxide scavenger (**Figure. 9.7**).

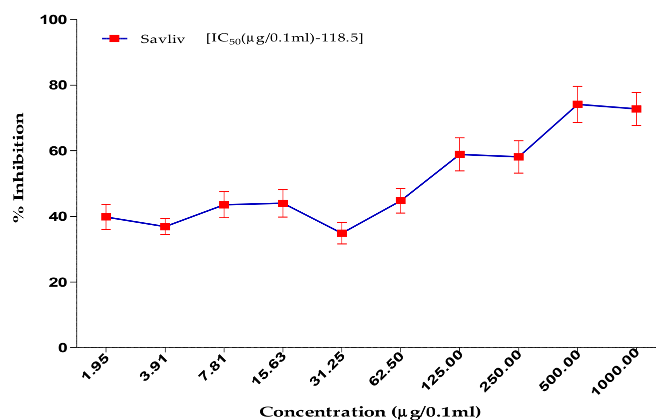
Figure 9.7. Nitric oxide radical scavenging activity of Savliv



xviii. **H_2O_2 radical scavenging assay**

The main oxygen species responsible for oxidative stress in biological system are hydrogen peroxide (H_2O_2), the free radical superoxide anion ($O2^-$) and the hydroxyl radical (OH^-). In the current study, Savliv exhibited potential [**IC_{50} of 118.50µg/0.1ml**] H_2O_2 radical scavenging activity (**Figure. 9.8**)

Figure.9.8. H_2O_2 radical scavenging activity of Savliv



s. **Determination of heavy metal content in Savliv**

Savliv was subjected to heavy metal analysis using atomic absorption spectrophotometer. The heavy metals such as lead [Pb], Cadmium [Cd], Mercury [Hg], Arsenic [As] contents were measured and the results are shown in **Table 9.3.**The heavy metals content of Savliv was found to be well within the limits recommended in the AYUSH guidelines for herbal drugs.

Table 9.3. Heavy metal content in Savliv

S. No.	Heavy metals	Permissible Limit (AYUSH)	Savliv
1	**Lead**	10 ppm	ND
2	**Cadmium**	0.3 ppm	ND
3	**Mercury**	1 ppm	ND
4	**Arsenic**	3 ppm	ND

ND: Not detected

t. **Determination of stability of Savliv by accelerated stability study**

The parameters fixed to evaluate the stability are the physical and chemical changes that occur over time under specified storage conditions.

xxi. **Organoleptic properties of Savliv**

9.4.1.1. Appearance and colour was confirmed by visual examination and results are shown in **Table 9.4.**

Table 9.4 Appearance, odourand color of Savliv

Sample	Storage Condition	Appearance, Color and odour	
		Initial	3rd month
Savliv 001 - 1	40°C/75% RH	Liquid, Greenish Brown,	Liquid, Greennish Brown,

		lime odour	lime odour
Savliv 001 - 2		Liquid, Greenish Brown, lime odour	Liquid, Greenish Brown, lime odour
Savliv 001 - 3		Liquid, Greenish Brown, lime odour	Liquid, Greenish Brown, lime odour

9.4.1.2. The pH of Savliv was determined by using Digital pH meter and the values are given in **Table 9.5.**

Table 9.5.pH of Savliv

Sample	**Storage Condition**	**pH**	
		Initial	**3rd month**
Savliv 001 - 1	**40ºC/75% RH**	3.40	3.41
Savliv 001 - 2		3.43	3.40
Savliv 001 - 3		3.41	3.40

9.4.1.3. Specific gravity of sample was determined and the values are given in **Table 9.6.**

Table 9.6. Specific gravity of Savliv

Sample	**Storage Condition**	**Specific gravity**	
		Initial	**3rd month**
Savliv 001 - 1	**40ºC/75% RH**	1.037	1.035
Savliv 001 - 2		1.037	1.036
Savliv 001 - 3		1.036	1.036

xxii. Chemical properties

23. **Secondary metabolites, Citric acidand Vitamin C content**

Amount of secondary metabolites, Citric acidand Vitamin C present in Savliv were measured using

standard protocols and results are given in **Table 9.7 a and b.**

Table 9.7a.Secondary metabolites and Vitamin C of SAVLIV at storage condition 40ºC/75% RH

Test Duration	Tannin (µg/ml extract)		
	Savliv 001-1	Savliv 001-2	Savliv 001-3
Initial	230.11 ± 11.22	233.43 ± 11.09	236.22 ± 11.27
3rd month	228.71 ± 14.98	236.98 ± 15.02	233.31 ± 13.02

Values are expressed in Mean ± SEM (n=3)

Table 9.7b . Vitamin C and Citric acid of SAVLIV at storage condition 40ºC/75% RH

Test Duration	Vitamin C (mg/ml extract)		
	Savliv 001- 1	Savliv 001- 2	Savliv 001- 3
Initial	1.51±0.12	1.43±0.11	1.48±0.08
3rd month	1.37±0.06	1.38±0.13	1.41±0.12

Values are expressed in Mean ± SEM (n=3)

XXIV. Microbial Load

Bacterial and fungal load was examined in Savliv and the results are given in **Table 9.8.**

Table 9.8. Bacterial and fungal load of Savliv at Storage Condition: 40ºC/75% RH

Test Interval	Sample	Total Bacterial count AYUSH limit- 10^5 - 10^7/g	Total Fungal AYUSH limit -10^3/g	Microbial load in Savliv
Initial	**Savliv 001**	22,000 CFU/g	Absent	Absent
	Savliv 001	19,000 CFU/g	Absent	Absent

	Savliv 001	20,000 CFU/g	Absent	Absent
3rd month	Savliv 001	21,000 CFU/g	Absent	Absent
	Savliv 001	20,000 CFU/g	Absent	Absent
	Savliv 001	21,000 CFU/g	Absent	Absent

x. **Acute Oral Toxicity Study of Savliv (HAPLIV) In Female Swiss Albino Mice**

xxv. **Body weight**

All the survived animals showed gain in body weight on day 7 and 14 in comparison to their day 0 body weight (Table: 9.9 and fig 9.9.).

xxvi. **Mortality**

One animal was found dead in experimental group Step 1. The gross pathological changes could not be examined as all the visceral organs were autolysed. Since there was no death in the step 2 treatment, this death was not considered to be test item related (Table: 9.10).

xxvii. **Clinical signs**

All the animals in Step 1 and Step 2 appeared normal at 30 minutes, 1, 2 and 4 hours observation and from day 1 to 14 observations, following HAP-LIV administration (Table: 9.10).

xxviii. **Gross pathology**

No gross lesions were recorded in all the experimental animals of Step 1 and Step 2 during necropsy (Table: 9.11).

xxix. **Conclusion**

Based on the above observations, the LD50 value of "HAP-LIV" was found to be greater than 2000mg/kg body weight and classified as Category-5 or unclassified based on Globally Harmonised Classification System (GHS) for Chemical Substances and Mixtures.

Table 9.9.Body weight of the experimental animals

Group/Step	Treatment	Body weight (g)		
		Day 0	Day 7	Day 14
I	Savliv	21.67±1.52	25.00±1.0*	25.00±4.12*

	(2000mg/kg b.wt.)			
II	Savliv (2000mg//kg b.wt.)	23.33±1.53	25.00±1.0	26.03±1.0

Values expressed in mean±SD; n=3; * n=2 One animal was found dead in experimental group Step 1

Figure 9.9. Body weight of the experimental animals

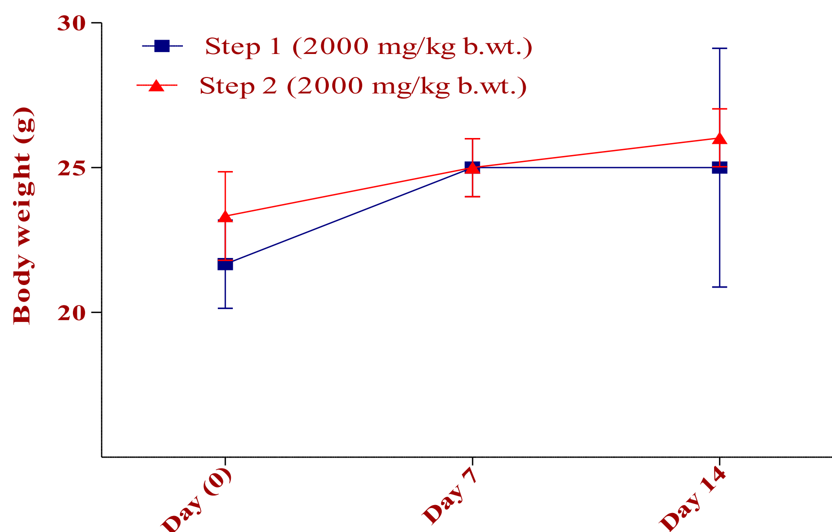


Table: 9.10 Mortality/Clinical Signs of Individual animals

Step	Dose mg/kg b.wt	Animal No.	Sex	Mortality	Clinical observations (Test days)																	
					0				1	2	3	4	5	6	7	8	9	10	11	12	13	14
					0.5h	1h	2h	4h														
1	2000	01	F	X	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
		02	F	√	N	N	N	N	N	N	D	-										
		03	F	X	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2	2000	04	F	X	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
		05	F	X	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
		06	F	X	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Key: D= Dead, N = Normal; h= hours, X= Absence, √= Presence ;Clinical examination was performed after test item administration.; No clinical signs were observed during acclimatization

Table 9.11 Macroscopic Findings

Step	Dose (mg/kg. b. wt.)	Animal No.	Sex	Mode of death	External	Internal
1	2000	01	F	TS	NAD	NAD
		02	F	Found dead	*	
		03	F	TS	NAD	NAD
2	2000	04	F	TS	NAD	NAD
		05	F	TS	NAD	NAD
		06	F	TS	NAD	NAD

Key: TS = Terminal Sacrifice, NAD = No Abnormality detected, * = Autolysed

10. Conclusion

- ✓ Phytochemical analysis revealed the presence of phenolic compounds, tannins, glycosides, sugars, alkaloids and flavones in Savliv.
- ✓ Savliv was found to contain 440.74±18.11 µg/ml of total phenol, 58.57±4.03 µg/ml Savliv of flavonoid and 235.37±13.70 µg/ml of tannin.
- ✓ Savliv was found to contain Vitamin C (1.34±0.04 mg/ml) and Citric acid (6.4 ±0.54 mg/ml).
- ✓ HPTLC finger printing of Savliv showed 11 peaks in the tested chromatographic condition.
- ✓ Savliv was found to be potent scavenger of DPPH, super oxide, lipid peroxide, nitric oxide and H_2O_2 radicals.
- ✓ Savliv is free from heavy metals such as lead [Pb], Cadmium [Cd], Mercury [Hg] and Arsenic [As].
- ✓ Savliv was found to be stable for a period of three months even at accelerated stability conditions (40°C± 2°C 75%± 5% RH).

From acute oral toxicity study, the LD50 of Savliv was greater than 2000mg/kg b.wt. Hence, Savliv was found to be safe when administered once orally at a dose of 2000mg/kg b.wt. in mice.

11. REFERENCES

- Ekstedt, M., Franzen, LE., Mathiesen, UL. 2006. Hepatology. 44, 865-873.
- Charlton, M., 2004. Clinical Gastroenterology Hepatology. 2, 1048-1058.
- McCullough, AJ., O'Connor, JF. 1998. "Alcoholic liver disease: proposed recommendations for the American College of Gastroenterology". American Journal of Gastroenterology. 93, 2022–2036.
- Neil Kaplowitz. 2004. Drug-Induced Liver Injury. Clinical Infectious Diseases. 38 (Supplement 2, S44-S48.
- Leuenberger, H., Sebastian Kaerger, J., Stephen Edge., Robert Price. 1982. The compressibility and compactibility of powder systems. International Journal of Pharmaceutics. 12, 41–55.
- Chang, C., Yang, M., Wen, H., Chern, J. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food Drug Analysis. 10, 178-182.
- Schanderl, SH. 1970. In : Methods in Food Analysis. Academic Press, New York, p 709.
- McDonald, S., Prenzler, PD., Autolovich, M., Robards, K. 2001. Phenolic content and antioxidant activity of olive extracts. Food Chemistry. 73, 73 - 84.
- Oremusova, J. 2007. Manual for laboratory practice in physics for students of pharmacy, Department of Physical Chemistry, Faculty of Pharmacy, Comenius University, Bratislava, Slovak.
- ICH Q1B Photo stability Testing of New Drug Substances and Products
- ICH Q1C Stability Testing for New Dosage Forms
- ICH Q3A Impurities in New Drug Substances
- ICH Q3B Impurities in New Drug Products
- ICH Q5C Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products
- ICH Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances
- ICH Q6B Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Biotechnological/Biological Products.
- Chang, C., Yang, M., Wen, H., Chern, J. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food Drug Analysis. 10, 178-182.
- Duthie, G., Crozier, A. 2000. Plant-derived phenolic antioxidants. Curr Opi Lipidol. 11, 43–47.
- Friedrich, W., Walter de Gruyter. 1988. Vitamin E in Vitamins. Chap-4.

- ✓ Green, LC., Wagner, DA., Glogowski, J., Skipper, PL., Wishnok, JS., Tannenbaum, SR. 1982. Analysis of nitrate and in biological fluids. Analytical Biochemistry. 126, 131.
- ✓ Harbone, JB., 1998. Phytochemical methods. A guide to modern techniques of plant analysis. 3rd Edn, Chapman and Hall Int, New York.
- ✓ Kakkar, P., Das, B., Viswanathan, PNA. 1984. Modified spectrophotometric assay of superoxide dismutase. Indian Journal of Biochemistry and Biophysics. 21, 130-132.
- ✓ Katsuoka, F., Motohashi, H., Ishii, T., Aburatani, H., Engel, JD., Yamamoto, M. 2005. Genetic evidence that small maf proteins are essential for the activation of antioxidant response element-dependent genes. Molecular Cell Biology. 25, 8044–8051.
- ✓ Kokate, CK. 2001. Pharmacognosy. 16th Edn, Nirali Prakasham, Mumbai.
- ✓ Koleva, II., Van Beek, TA., Linssen, JPH., de Groot, A., Evstatieva, LN. 2002. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochemical Analysis. 13, 8-17.
- ✓ McDonald, S., Prenzler, PD., Autolovich, M., Robards, K. 2001. Phenolic content and antioxidant activity of olive extracts. Food Chemistry. 73, 73-84.
- ✓ Naik, GH., Priyadarsini, KI., Satav, JG., Banavalikar, MM., Sohoni, PP., Biyani, MK., Mohan, H. 2003. Comparative antioxidant activity of individual herbal components used in Ayurvedic medicine. Phytochemistry. 63, 97–104.
- ✓ Ohkawa, H., Ohishi, N., Yagi, K. 1979. Assay of lipid peroxides in animal tissues by thio babituric acid reaction. Analytical Biochemistry. 95, 351.
- ✓ Oyaizu, M. 1986. Studies on product of browning reaction prepared from glucose amine. Japan Journal of Nutrition. 44, 307-315.
- ✓ Pacifici, RE., Davies, KJ. 1991. Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited. Gerontology. 37, 166–180.
- ✓ Pietta, PG. 2000. Flavonoids as antioxidants. Journal of Natural Products. 63, 1035–1042.
- ✓ Prieto, P., Pineda, M., Agulilar, M. 1999. Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application of Vitamin E. Analytical Biochemistry. 269, 337-341.
- ✓ Salah, N., Miller, NJ, Paganga, G., Tijburg, L., Bolwell, GP., Rice-Evans, C. 1995. Polyphenolic flavonols as scavenger of aqueous phase radicals and as chain-breaking antioxidants. Archives of Biochemistry Biophysics. 2, 339–346.

- ✓ Santanam, N., Ramachandran, S., Parthasarathy, S. 1998. Oxygen radicals, antioxidants, and lipid peroxidation. Seminar on Reproduction Endocrinology.16, 275–280.
- ✓ Sinha, AK. 1972. Colorimetric assay of catalase. Analytical Biochemistry. 47, 389-394.
- ✓ Toxic metal analysis in Ayurvedic drug systems. XXXII National Systems Conference, 2008, 17-19.
- ✓ OECD, 1998: Hormonised integrated hazard classification system for human health and environmental effects of chemical substances as endorsed by the 28th joint meeting of the chemicals committee and the working party on chemicals in part 2, pp. 11.
- ✓ OECD, Guidelines for the Testing of Chemicals/Section 4: Health Effects Test No. 423: Acute Oral toxicity - Acute Toxic Class Method. Organization for Economic Cooperation and Development, Paris, France, 2002.
  - Kokate, C.K., Purohit, A.P., Gokhale, S.B. 2009. Pharmacognosy, 41st Edn, Nirali prakashan, Mumbai.
  - The Ayurvedic Pharmacopoeia of India, Part- I, Volume – V, Pp 23.
  - Anna SK. 2009; Variation of phenolic acids from herbs and roots of Salsola kali. L. Acta societatis Botanicorum Polaniae

Effect of SAVLIV against CCL4 induced hepatotoxicity in mice - Acute model

Carbon tetra chloride model of hepatotoxicity was carried out as per Yadav et al., (2004) with minor modifications.

Experimental animals

Balb/C mice weighing 20-25g was housed 6/cage and was maintained at 25 ± 2°C in 12-h dark/12-h light cycles, with both standard pelleted diet and water ad libitum in accordance to the CPSCEA guidelines. All the animals will be acclimatized, at least for a period of 7 days, to the laboratory conditions prior to experimentation. The experiments were carried out based on ethical clearance (IAEC/41/SRU/380/2014) from Institutional Animal Ethics Committee of Sri Ramachandra University, Chennai, India.

Experimental design

Animals will be acclimatized for a period of 7 days to the laboratory conditions. Following acclimatization, experimental animals were divided into seven groups of six rats each as given below. Experimental animals

were received appropriate treatment (vehicle / reference / Savliv) for 5 days and on day 5 one hour after the vehicle/drug treatment, CCl_4 (2ml/kg b.wt. i.p.) were administered intraperitoneally. 24 h following CCl_4 administration, blood were collected from retro orbito plexus, serum was separated and processed for biochemical estimation.

Grouping

Mice were divided into six groups of six rats each.

Group	Treatment	No. of animals / group
I	Normal control (Vehicle)	6
II	Positive control (CCl_4 (2ml/kg b.wt. i.p.)+ Vehicle)	6
III	Reference control (CCl_4 + LIV52 – 100mg/kg)	6
IV	Test drug (CCl_4 + SAVLIV – 25mg/kg)	6
V	Test drug (CCl_4 + SAVLIV – 50mg/kg)	6
VI	Test drug (CCl_4 + SAVLIV – 100mg/kg)	6
VII	Drug control (SAVLIV – 100mg/kg)	

Observations

Bodyweight

Body weight of all the animals was noted before experiment initiation.

Biochemical analysis

In plasma – Urea, creatinine, total protein, albumin, SGOT, SGPT, ALP, GGT and Total bilirubin were measured

Histopathology

After a minimum of 24 h fixation, the liver samples will be processed by conventional methods, paraffin blocks will be made and 6 µm paraffin sections will be stained with Hematoxylin and Eosin. They will be examined under a light microscope. All deviations from normal histology will be recorded and compared with the corresponding controls.

Statistical analysis

Results are expressed as mean ± SEM. Mean difference between the groups will be compared by one way analysis of variance (ANOVA) followed by tukey post hoc multiple comparison test. P < 0.05 & 0.01 will be considered to be significant. Statistical analyses are performed using Graphpad prism 4.0 version.

Group	Treatment	T.bilirubin (mg/dl)	UREA (mg/dl)	Creatinine (mg/dl)	Albumin (g/dl)	T.protein (g/dl)	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	GGT (U/L)
I	Normal control (Vehicle)	0.29±0.02	27.3±01.35	0.52±0.04	2.62±0.16	6.58±0.06	68.67±2.61	22.96±2.09	283.31±25.34	4.78±0.48
II	Positive control (CCL4 + Vehicle)	1.15±0.20##	32.07±1.29	0.99±0.18##	2.77±0.05	7.01±0.17	138.66±9.13**	52.63±4.93**	398.83±21.22*	6.69±0.42**
III	Reference control (CCL4 + LIV52)	0.32±0.05**	30.41±1.89	0.44±0.05**	2.49±0.04	6.46±0.25	77.20±7.41**	31.38±1.55**	308.20±22.92	5.06±0.46*
IV	Test drug (CCl4 + SAVLIV - low dose)	0.48±0.06**	28.57±1.60	0.49±0.03**	2.50±0.15	6.07±0.26**	114.92±5.73	33.25±0.95**	352.70±43.61	6.12±0.25
V	Test drug (CCl4 + SAVLIV - mid dose)	0.34±0.03**	28.07±1.31	0.40±0.03**	2.71±0.11	6.29±0.17*	105.24±7.19*	32.30±1.78**	340.65±21.01	5.49±0.41
VI	Test drug (CCl4 + SAVLIV - high dose)	0.34±0.04**	30.84±0.81	0.41±0.06**	2.87±0.15	6.21±0.11*	98.89±14.19**	29.44±2.13**	316.70±16.07	5. 42±0.22
VII	Drug control (SAVLIV – high dose)	0.27±0.02**	29.46±0.80	0.40±0.05**	2.64±0.10	6.42±0.18	64.06±5.65**	24.38±1.92**	297.67±31.03	5.10±0.24*

Results are expressed in mean ± SEM (n =6); Statistical analysis was done using prism 4.0 Version, Tukey multiple comparison test. # (*p*<0.05) &# # (*p*<0.01) compared with Normal control, * (*p*<0.05) & ** (*p*<0.01) compared with Positive control

Effect of SAVLIV against ethanol induced hepatotoxicity in mice - Acute model

Ethanol model of hepatotoxicity was carried out as per Enomoto et al., (2000) with minor modifications.

Experimental animals

Balb/C mice weighing 20-25g was housed 6/cage and was maintained at 25 ± 2°C in 12-h dark/12-h light cycles, with both standard pelleted diet and water ad libitum in accordance to the CPSCEA

guidelines. All the animals will be acclimatized, at least for a period of 7 days, to the laboratory conditions prior to experimentation. The experiments were carried out based on ethical clearance (IAEC/41/SRU/380/2014) from Institutional Animal Ethics Committee of Sri Ramachandra University, Chennai, India.

Experimental design

Animals were acclimatized for a period of 7 days to the laboratory conditions. Following acclimatization, experimental animals were received appropriate treatment (vehicle / reference / Savliv) for 5 days and on day 5 one hour after the vehicle/drug treatment, ethanol (6g/kg b.wt. p.o.) were be administered every 12 hours for a total of 3 doses. At 4h after last induction, blood were collected from retro orbito plexus, serum was separated and processed for biochemical estimation

Grouping

Mice were divided into seven groups of six rats each.

Group	Treatment	No. of animals / group
I	Normal control (Vehicle)	6
II	Positive control (Ethanol + Vehicle)	6
III	Reference control (Ethanol + LIV52)	6
IV	Test drug (Ethanol + SAVLIV - low dose)	6
V	Test drug (Ethanol + SAVLIV - mid dose)	6
VI	Test drug (Ethanol + SAVLIV - high dose)	6
VII	Drug control (SAVLIV – high dose)	6

Observations

Bodyweight

Body weight of all the animals was noted before experiment initiation.

Biochemical analysis

In plasma – Urea, creatinine, total protein, albumin, SGOT, SGPT, ALP, GGT and Total bilirubin were measured

Histopathology

After a minimum of 24 h fixation, the liver samples will be processed by conventional methods, paraffin blocks will be made and 6 µm paraffin sections will be stained with Hematoxylin and Eosin. They will be examined under a light microscope. All deviations from normal histology will be recorded and compared with the corresponding controls.

Statistical analysis

Results are expressed as mean ± SEM. Mean difference between the groups will be compared by one way analysis of variance (ANOVA) followed by tukey post hoc multiple comparison test. $P < 0.05$ & 0.01 will be considered to be significant. Statistical analyses are performed using Graphpad prism 4.0 version.

Group	Treatment	Tbilirubin (mg/dl)	UREA (mg/dl)	Creatinine (mg/dl)	Albumin (g/dl)	Tprotein (g/dl)	SGOT (U/L)	SGPT(U/L)	ALP(U/L)	GGT(U/L)
I	mal control (Veh	0.32±0.02	31.19±2.24	0.51±0.04	2.61±0.16	6.53±0.06	56.67±2.61	23.79±3.23	290.14±20.99	4.76±0.48
II	ontrol (Ethanol +	1.19±0.20$^{\#\#}$	35.75±2.18	0.67±0.10	3.19±0.22	7.12±0.22	126.69±10.29$^{\#\#}$	42.73±4.52$^{\#\#}$	425.28±21.89$^{\#\#}$	6.48±0.32$^{\#\#}$
III	control (Ethanol	0.40±0.10**	31.81±1.10	0.40±0.04**	2.22±0.11**	5.94±0.32**	97.03±8.25	31.33±2.11	311.72±25.09*	5.14±0.34*
IV	hanol + SAVLI\	0.45±0.07**	34.67±2.08	0.48±0.03	2.42±0.10**	6.16±0.24**	106.36±1.5**2	31.31±0.96	341.69±42.05	5.92±0.17
V	hanol + SAVLI\	0.40±0.03**	30.80±1.17	0.38±0.03**	2.63±0.24	5.99±0.15**	81.94±10.83**	30.31±1.60*	324.92±15.30*	5.63±0.46
VI	hanol + SAVLI\	0.35±0.03**	33.31±0.76	0.35±0.07**	2.58±0.15	6.26±0.08*	73.71±7.80**	26.30±1.74**	288.53±26.31**	4.80±0.22**
VII	rol (SAVLIV – 1	0.27±0.01**	32.78±1.01	0.38±0.03**	2.17±0.05**	6.18±0.10**	55.75±5.67**	22.12±1.36**	288.05±20.17**	4.71±0.31**

Results are expressed in mean ± SEM (n =6); Statistical analysis was done using prism 4.0 Version, Tukey multiple comparison test. $^{\#}$ ($p<0.05$) &$^{\#\#}$ ($p<0.01$) compared with Normal control, * ($p<0.05$) & ** ($p<0.01$) compared with Positive control

HEPATOPROTECTIVE EFFECT OF SAVLIV IN PARACETAMOL, ETHANOL AND METHOTREXATE INDUCED CHANG LIVER CELLS

Cell culture and maintenance

Chang liver cells, a human hepatoma cells were obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in Minimum Essential Media (MEM) supplemented with 10% Fetal Bovine Serum (FBS), with 100units/ml penicillin and 100µg/ml streptomycin. Cells were cultured in $75cm^2$ culture flask and incubated at humidified atmosphere with 5% CO_2 at 37°C.

Cell Proliferation Assay or MTT Assay

Cell respiration as an indicator of cell viability and proliferation was determined using a mitochondrial dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5- Diphenyl tetrazolium bromide (MTT) to formazan. Preconfluent chang liver cells were seeded in 96-well plates at a density of 8,000 cells/200µl/well. Cells were treated with different concentrations of the test drug (ranging from $1X10^{-3}$ - $1X10^{6}$ ng) after 24 h following plating and incubated at 37°C for one day. At 20 h following drug exposure, the cells were incubated at 37°C with 0.5 mg/ml MTT for 4 h. At the end of the experiment, the medium was removed, and the insoluble formazan product was dissolved in DMSO (200µl) and kept at least 15 min in dark. The intensity of purple blue colour developed was measured at 570 and 630 nm using Thermoscientific multiscan spectrophotometer, USA. Percentage growth inhibitory rate of the test drug was calculated using the formula

$$\% \text{ Growth inhibitory rate} = ([\text{Control OD} - \text{Test OD}] / \text{Control OD}) * 100$$

Paracetamol induced hepatotoxicity in chang liver cells

Chang liver cells were seeded in 6 well plates at a density of $1X10^{5}$ cells/well and allowed to grow for a period of 24 h. Test drug was administered at a concentration of 0.5, 5 and 50 µg / ml. One hour following test drug exposure, paracetamol dissolved in syringe water (100µg/ml) was added to each well except the control and incubated for a period of 24 h. Cells were then trypsinised for measuring gene expressions of albumin (ALB), uridine glucuronosyl transferase (UGT), and cytochrome P450 (CYP).

Ethanol induced hepatotoxicity in chang liver cells

Chang liver cells were seeded in 6 well plates at a density of $1X10^{5}$ cells/well and allowed to grow for

a period of 24 h. Test drug was administered at a concentration of 0.5, 5 and 50 µg / ml. One hour following test drug exposure, 5% ethanol was added to each well except the control and incubated for a period of 30 min. Cells were then replaced with fresh medium and trypsinised after 24 h after ethanol induction for measuring gene expressions of cytochrome P450 (CYP), caspase-3, BAX and BCL2.

Methotrexate (MTX) induced hepatotoxicity in chang liver cells

Chang liver cells were seeded in 6 well plates at a density of $1X10^5$ cells/well and allowed to grow for a period of 24 h. Test drug was administered at a concentration of 0.5, 5 and 50 µg / ml. One hour following test drug exposure, methotrexate dissolved in DMSO (100mM) was added to each well except the control and incubated for a period of 24 h. Cells were then trypsinised for measuring gene and protein expressions of DHFR, BAX and BCL2.

Reverse transcriptase - Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using TRIzol Reagent (Sigma, USA). After homogenizing the cells with TRIzol reagent, the tubes was incubated for 10 min and centrifuged at 1000 rpm for 5 min. 200 µl of chloroform was added to the supernatant, allowed to incubate for 5min at room temperature and centrifuged at 12000 rcf for 20min. Then 500 µl of isopropyl alcohol was added to the supernatant to precipitate the total RNA and centrifuged at 12000 rcf for 15min following the incubation period of 10 min. The supernatant was decanted carefully; the pellet was washed thrice with 75% ethanol, centrifuged at 12000 rcf for 15 min. The pellet was air dried and re-suspended in 20 µl of RNase free water and stored in -80°C until use. RT-PCR was carried using PCR master cycler gradient (Eppendorf, Germany) and semi-quantified using Bio1D software in gel documentation (Vilber Loumart, France).
Primer sequence used were as follows,

Primer	Forward Primer	Reverse Primer
GAPDH	5’-CGACAGTCAGCCGCATCTT-3’	5’-CCAATACGACCAAATCCGTTG-3’
ALB	5’-CTTGAATGTGCTGATGACAGG-3’	5’-GCAAGTCAGCAGGCATCTCAT-3’
UGT	5′-CCTCTTTCCTATGTCCCCAATGA-3′	5′-CCTTAGTCTCCATGCGCTTTGC-3′
CYP	5′-GTGATGCCCTGGCTGCAG-3′	5′-AATCGAGCTGGATCAAAGTTC-3′
DHFR	5′-CTGTCATGGTTGGTTCGC-3′	5′-AAGCTTTTGGTATTTCCA-3′
BAX	5’-TTTTGCTTCAGGGTTTCATC-3’	5’-GACACTCGCTCAGCTTCTTG-3’
BCL2	5’-ATGTGTGTGGAGAGCGTCAACC-3’	5’-TGAGCAGAGTCTTCAGAGACAGCC-3’
Caspase-3	5’-TCACAGCAAAAGGAGCAGTTT-3’	5’-CGTCAAAGGAAAAGGACTCAA-3’

Data analysis

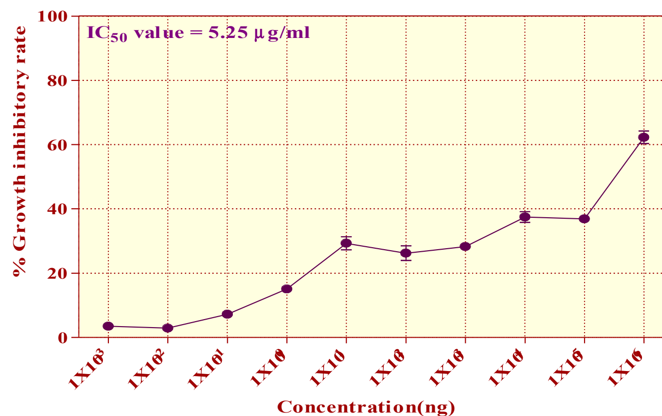
Data were expressed in mean±SEM. Mean difference between the groups were analyzed by one way anova followed by turkey's multiple comparison test using graph pad prism 5.0. $p<0.05$ was considered as statistically significant [#, ## - indicates $p< 0.05$ and 0.01, respectively vs group I; *, ** - indicates $p< 0.05$ and 0.01, respectively vs group II).

Results

Cell Proliferation Assay or MTT Assay

In the present study, SAVLIV exhibited an IC_{50} value 5.25 µg/ml when exposed in chang liver cells for a period of 24 h.

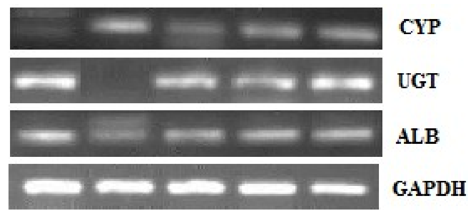
Effect of SAVLIV on growth inhibitory rate in chang liver cell line using MTT assay

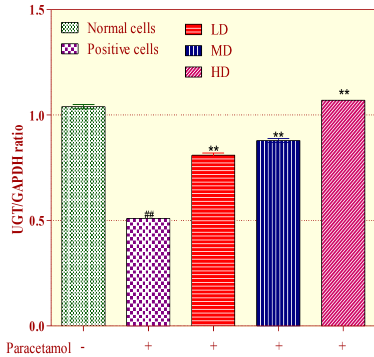


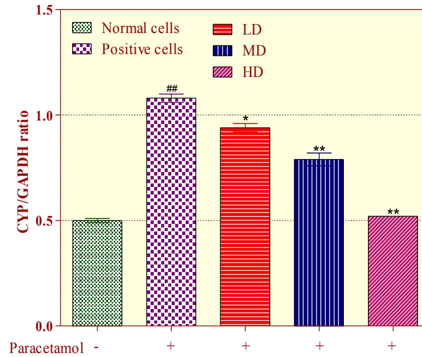
Paracetamol induced hepatotoxicity in chang liver cells

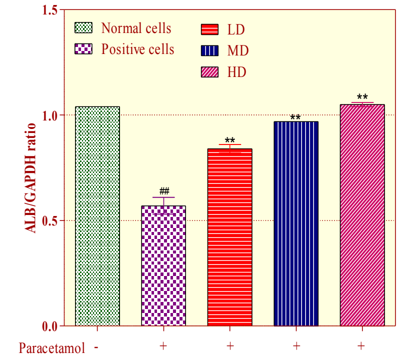
Paracetamol induced chang liver cells showed significant ($p<0.01$) increase in CYP and decrease in UGT and ALB expressions when compared to normal cells. SAVLIV exposure at 0.5, 5 and 50 µg/ml significantly and dose dependently decreased CYP ($p< 0.05$, 0.01 and 0.01, respectively) and increased UGT ($p<0.01$) and ALB ($p<0.01$) expressions in comparison to positive control cells.

Effect of SAVLIV on paracetamol induced hepatotoxicity in chang liver cell line.







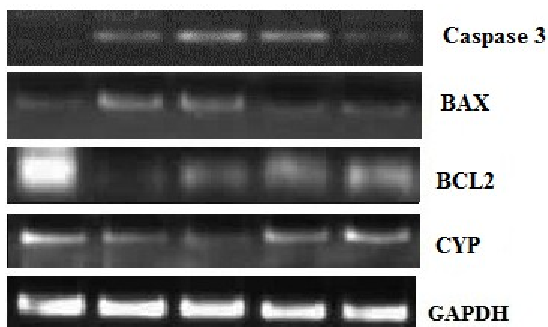


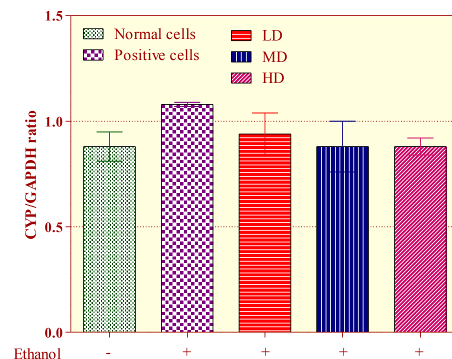
Lane I: Normal control; Lane II: Positive control; Lane III: Low dose; Lane IV: Mid dose; Lane V: High dose. Values were expressed in mean ± SEM; Statistical analysis was performed using one way anova followed by tukey's multiple comparison test in graphpad prism 5.0. $^{\#\#}$ represents p< 0.01 vs Normal control; *, ** represents p < 0.05 and 0.01, respectively vs positive control.

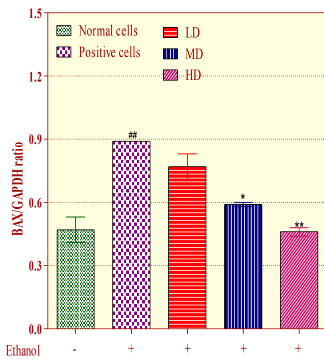
Ethanol induced hepatotoxicity in chang liver cells

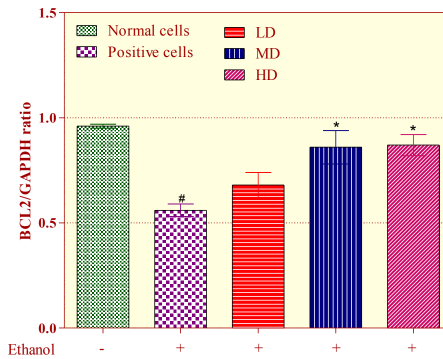
A significant increase in BAX (p<0.01) and caspase 3 (p<0.01) and decrease in BCL2 (p<0.05) gene expressions were observed in ethanol induced chang liver cells when compared to normal cells. Treatment with SAVLIV significantly decreased BAX (p<0.05 and 0.01 at 5 and 50 µg/ml, respectively) and caspase 3 (p<0.01 at 50µg/ml) expressions and increased BCL2 (p<0.05 at 5 and 50 µg/ml) expression in comparison to positive control cells. No significant difference in CYP expression was observed in any of the treatment group.

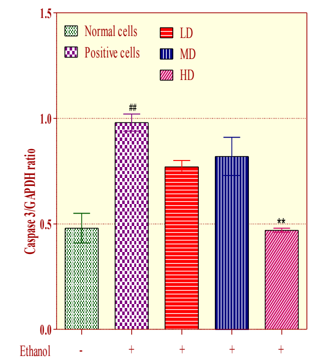
Effect of SAVLIV on ethanol induced hepatotoxicity in chang liver cell line.









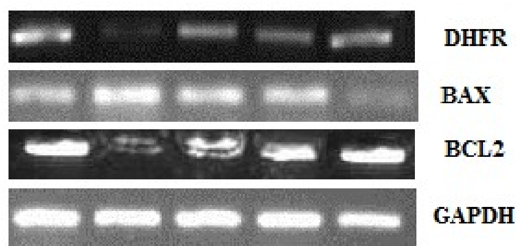


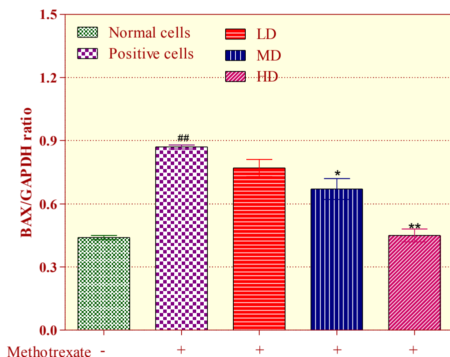
Lane I: Normal control; Lane II: Positive control; Lane III: Low dose; Lane IV: Mid dose; Lane V: High dose. Values were expressed in mean ± SEM; Statistical analysis was performed using one way anova followed by tukey's multiple comparison test in graphpad prism 5.0. $^{\#, \#\#}$ represents p< 0.05 and 0.01, respectively vs Normal control; *, ** represents p < 0.05 and 0.01, respectively vs positive control.

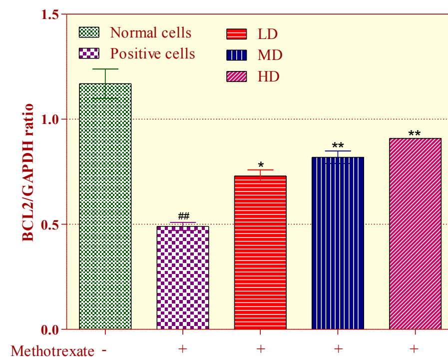
Methotrexate (MTX) induced hepatotoxicity in chang liver cells

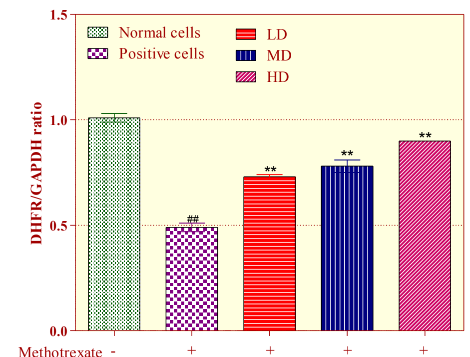
A significant (p<0.01) increase in BAX and decrease in BCL2 and DHFR gene expressions were observed in methotrexate induced chang liver cells when compared to normal cells. Treatment with SAVLIV significantly decreased BAX (p< 0.05 and 0.01 at 5 and 50 µg/ml, respectively) and increased BCL2 (p<0.05, 0.01 and 0.01, respectively) and DHFR (p<0.01) expressions in comparison to positive control cells.

Effect of SAVLIV on methotrexate induced hepatotoxicity in chang liver cell line.









Lane I: Normal control; Lane II: Positive control; Lane III: Low dose; Lane IV: Mid dose; Lane V: High dose. Values were expressed in mean ± SEM; Statistical analysis was performed using one way anova followed by tukey's multiple comparison test in graphpad prism 5.0. $^{##}$ represents p< 0.01 vs Normal control; *, ** represents p < 0.05 and 0.01, respectively vs positive control.

Reference

- Mosmann T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. Journal of lmmunological Methods. 65 (1983); 55-63.
- P. J. Amar and E. R. Schiff, "Acetaminophen safety and hepatotoxicity: where do we go from here?" Expert Opinion on Drug Safety, vol. 6, no. 4, pp. 341–355, 2007.
- Yang L, Wu D, Cederbaum AI. CYP2E1, oxidative stress and MAPK signaling pathways in alcohol-induced hepatotoxicity. Journal of Biochemical and Pharmacological Research. 2014; 2 (2): 74-90.

Effect of SAVLIV against Paracetamol induced hepatotoxicity in mice - Acute model

Acute model of hepatotoxicity was carried out as per Tien LH et al., 1993 with minor modifications.

Experimental animals

Balb/C mice weighing 20-25g was housed 6/cage and was maintained at 25 ± 2°C in 12-h dark/12-h light cycles, with both standard pelleted diet and water ad libitum in accordance to the CPSCEA guidelines. All the animals will be acclimatized, at least for a period of 7 days, to the laboratory conditions prior to experimentation. The experiments were carried out based on ethical clearance (IAEC/41/SRU/380/2014) from Institutional Animal Ethics Committee of Sri Ramachandra University, Chennai, India.

Experimental design

Animals will be acclimatized for a period of 7 days to the laboratory conditions. Following acclimatization, experimental animals were divided into seven groups of six rats each as given below. Experimental animals were received vehicle/ respective test drugs one hour prior to paracetamol (400 mg/kg) induction. Twenty four hours following induction, blood were collected from retro orbito plexus, serum was collected, separated and processed for biochemical estimation.

Grouping

Mice were divided into six groups of six rats each.

Group	**Treatment**	**No. of animals / group**
I	Normal control (Vehicle)	6
II	Positive control (Paracetamol - 400 mg/kg+ Vehicle)	6
III	Reference control (Paracetamol + LIV52 -100 mg/kg)	6
IV	Test drug (Paracetamol + SAVLIV –25mg/kg)	6
V	Test drug (Paracetamol + SAVLIV 50mg/kg)	6
VI	Test drug (Paracetamol + SAVLIV 100mg/kg)	6
VII	Drug control (SAVLIV – 100mg/kg)	6

Observations

Bodyweight

Body weight of all the animals before experiment initiation.

Biochemical analysis

In plasma – Urea, creatinine, total protein, albumin, SGOT, SGPT, ALP, GGT and Total bilirubin were measured

Histopathology

After a minimum of 24 h fixation, the liver samples will be processed by conventional methods, paraffin blocks will be made and 6 µm paraffin sections will be stained with Hematoxylin and Eosin. They will be examined under a light microscope. All deviations from normal histology will be recorded and compared with the corresponding controls.

Statistical analysis

Results are expressed as mean ± SEM. Mean difference between the groups will be compared by one way analysis of variance (ANOVA) followed by tukey post hoc multiple comparison test. $P < 0.05$ & 0.01 will be considered to be significant. Statistical analyses are performed using Graphpad prism 4.0 version.

Group	Treatment	Tbilirubin (mg/dl)	UREA (mg/dl)	Creatinine (mg/dl)	Albumin (g/dl)	Tprotein (g/dl)	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	GGT (U/L)
I	Normal control (Vehicle)	0.26±.02	28.54±.70	0.65±.03	2.68±.14	6.21±0.07	60.72±3.16	23.39±1.31	282.88±14.65	3.92±0.51
II	Positive control (Paracetamol (400 mg/kg) + Vehicle)	1.09±0.19##	34.41±2.80	1.03±0.15##	2.73±0.27	7.08±0.24#	106.28±9.08##	44.79±4.41##	401.40±27.12#	5.67±0.19#
III	Reference control (Paracetamol (400 mg/kg) + LIV52)	0.46±0.12**	26.23±1.91**	0.55±0.04**	2.04±0.04**	6.24±0.28*	82.02±9.21	35.59±2.38*	323.58±18.04	4.98±0.23
IV	Test drug (Paracetamol (400 mg/kg) + SAVLIV – 25 mg/kg)	0.46±0.08**	28.01±1.54	0.59±0.02**	2.38±0.10	5.89±0.29**	105.40±4.13	36.57±0.92	362.95±36.74	5.07±0.48
V	Test drug (Paracetamol (400 mg/kg) + SAVLIV – 50mg/kg)	0.32±0.02**	25.30±1.41**	0.54±0.04**	2.24±0.15	6.15±0.19*	99.27±8.83	33.47±1.78**	337.98±26.84	4.90±0.29
VI	Test drug (Paracetamol (400 mg/kg) + SAVLIV – 100 mg/kg)	0.29±0.03**	26.62±0.67*	0.57±0.06**	2.21±0.07	6.13±0.17*	80.83±7.76	30.90±1.69**	290.87±15.67*	4.60±0.18
VII	Drug control (SAVLIV – 100mg/kg)	0.26±0.01**	25.54±0.76**	0.48±0.03**	2.20±0.08	5.83±0.12**	59.20±4.87**	25.14±1.33**	280.05±28.19**	4.82±0.29

Results are expressed in mean ± SEM (n =6); Statistical analysis was done using prism 4.0 Version, Tukey multiple comparison test. # (*p*<0.05) &# # (*p*<0.01) compared with Normal control, * (*p*<0.05) & ** (*p*<0.01) compared with Positive control