IJPSR (2022), Volume 13, Issue 7 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 05 October 2021; received in revised form, 28 December 2021; accepted, 05 January 2022; published 01 July 2022

HEPATOPROTECTIVE ACTIVITY OF EXTRACTED FLAVONOIDS FROM *ORTHOSIPHON STAMINEUS* AGAINST HALOTHANE INDUCED HEPATOTOXICITY IN RATS

Narsapuram Bharathi, Ganesh Akula *, Santhosh Suddagoni and M. Venkataramana

Department of Chemistry, Surabhi Dayakar Rao College of Pharmacy, Rimmanaguda, Gajwel, Siddipet - 502312, Telangana, India.

Keywords:

Orthosiphon stamineus, Hepatoprotective, Hypouricemic, Methanolic extract, Biochemical markers

Correspondence to Author:
Dr. Ganesh Akula

Associate professor,
Department of Chemistry, Surabhi Dayakar Rao College of Pharmacy, Rimmanaguda, Gajwel, Siddipet - 502312, Telangana, India.

E-mail: akulaganesh@gmail.com

ABSTRACT: Usually, medicine includes the use of herbal medicines, animal parts, and minerals. However, herbal medicines are the most widely used of the three. The general guidelines for policies on Research and Evaluation of Traditional Medicine cover a wide range of issues and are intended to meet the different states in various countries and regions of the world. *Orthosiphon stamineus* is an herb that is widely grown in tropical areas. It is also known as *Orthosiphon aristaeus.* The plant can be identified by its white or elaborate colored flowers that resemble cat whiskers. It was found that *O. stamineus* exhibits diuretic, hypouricemic and renal caring, antioxidant, and anti-inflammatory, hepatoprotective, gastroprotective, anti-hypertensive, anti-diabetic, anti-hyperlipidemia, anti-microbial, and anorexic activities. Liver failure is continually associated with cellular necrosis, increased tissue lipid peroxidation and depletion in the flesh GSH levels. In addition, serum levels of many biochemical markers like SGOT, SGPT, triglycerides, cholesterol, bilirubin, alkaline phosphatase are elevated. Hence in the present study, the best effort is made to evaluate the caring effect of methanolic extract of *Orthosiphon stamineus* leaves against various hepatotoxic agents in validated rat models.

INTRODUCTION: Medicine includes the use of herbal medicines, animal parts, and minerals [1]. The general guidelines for policies on Research and Evaluation of Traditional Medicine cover a wide range of issues and are intended to meet the different states that exist in various countries and regions of the world. More than 13,000 plants have been studied through the last 5 year period [2, 3]. *Orthosiphon stamineus*, family Euphorbiaceae [4, 5]. It is used as an antioxidant for ghee, plant oils used to create hair fixers, and ointments. Seed oil is used in paints and seed cake as manur [19-21]. The liver is one of the vital organs endowed with several important homeostatic responsibilities. It has got its own importance in the physiological system [6, 7].

One of the liver's primary functions is to aid in the metabolism of ingested substances, including food, dietary supplements, alcohol, and the majority of medications. However, several reasons are known to cause moderate to severe hepatic complications. Liver failure is continually associated with cellular necrosis, an increase in tissue lipid peroxidation, and depletion in the flesh GSH levels. In addition, serum levels of many biochemical markers like SGOT, SGPT, triglycerides, cholesterol, bilirubin,

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DOI: 10.13040/IJPSR.0975-8232.13(7).2844-55

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.13(7).2844-55

alkaline phosphatase are elevated [8, 9, 10]. In spite of the phenomenal growth of modern medicine, there are few synthetic drugs available for the action of hepatic disorders. However, so many herbs claimed to have possessed beneficial activity in treating hepatic illnesses. But they need to be validated in the light of science to ensure their ability to conserve therapeutic effectiveness in the design form [11, 12]. Hence, in the present study, the best effort is made to evaluate the caring effect of methanolic extract of *Orthosiphon stamineus* leaves against various hepatotoxic agents/drugs in validated rat models [13].

FIG. 1: *ORTHOSIPHON STAMINEUS* PLANT

FIG. 2: *ORTHOSIPHON STAMINEUS* LEAVES

MATERIALS & METHODS:

Collection of Plant Materials: The leaves of *Orthosiphon stamineus* used for the current studies were collected from the Ooty district of Tamil Nadu. The plant was known, confirmed, and authenticated by comparing with voucher specimen available at Review of medicinal plants & collection unit, Department of AYUSH, Ministry of Health & Family Welfare, Govt. of India. The plants were cut into small pieces and shade dried. The dried physical was then pulverized separately into coarse powder by a mechanical grinder. The resulting powder was then used for extraction.

Preparation of Methanolic Extract: The powdered drug was dry and packed well in Soxhlet apparatus and removed with 1500 ml of methanol for seven days. The extract was focused and dried using a Rotary flash evaporator. It was kept in desiccator until used.

Experimental Animals: Albino rats (Wistar strain) of either sex weighing among 150-200g, the creatures were adjusted for seven days under laboratory conditions. The animals were fed with a commercially available rat pelleted diet. Water was allowed *ad libitum* under strict clean conditions. The experiment was approved by the IAEC of the college and done following the norms of CPCSEA (CPCSEA registration number 13/5H/SDRCP/IAEC)

The methanolic extract of *Orthosiphon stamineus* leaves was endangered to the following investigations:

Pharmacological Activities:

1. Toxicity studies (LD_{50}).
2. Hepatoprotective activity.

Determination of acute toxicity (LD_{50}):

14 Days Single-Dose Oral Acute Toxicity and Gross Behavioral Study:

Preparation of Dose: Methanolic extract of *Orthosiphon stamineus* leaves was suspended in 3% CMC, to prepare a dose of 2000mg/kg body weight of the animal, and ran 1ml/100gmbody weight of the animal.

Procedure: The procedure was separated into Phase I (observation made on day one) and Phase II (observed the animals for the next 14 days). Two sets of healthy female rats (each set of 3 rats) were used for the experiment. First set creatures were divided and fasted for 18 hours deprived of food, water reserved before 4 hours of the dosing, body weights were noted before and after dosing with methanolic leaf extract of *Orthosiphon stamineus* (200mg/kg) orally. Individually animals were observed for 4 hours to see any clinical symptoms, change in behaviour, or death. 6 h post-dosing again body weights noted.

From the next day onwards, each day 1 hour, the behavioural change, clinical symptoms or mortality was observed in the same creatures for the next 14 days and animal body weights were recorded on 8th and 14th day. The same process was repeated with another set of creatures to nullify the errors.

Selection and Preparation of Dose for Pharmacological Screening: The methanolic extract was suspended in 0.3% CMC solution to make the animals' two dose levels, 100 and 200mg/kg body bulk.

Hepatoprotective Activity:

Halothane Induced Hepatotoxicity:

Group A: Normal control (10 ml/kg distilled water, p. o).

Group B: Toxicant (halothane 600mg/kg, p.o).

Group C: Standard (halothane+Silymarin25 mg/kg, p.o).

Group D: Halothane + Methanolic extract of *Orthosiphon stamineus.* Leaves (100mg/kg, twice daily, p. o).

Group E: Halothane +Methanolic extract of *Orthosiphon stamineus*. Leaves (200mg/kg, twice daily, p. o).

Experimental Procedure: Wistar rats of either sex considering between 150-200 g were divided into five groups of six rats each. Group A (control), Group B (Halothane treated), Group C (Halothane +Silymarin (25 mg/kg p.o), Groups D (Halothane+Methanolic extract of *Orthosiphon stamineus* leaves (100mg/ kg) and Group E (Halothane+Methanolic extract of *Orthosiphon stamineus* leaves (200mg/kg). For the first seven days of study Group, A & B were fed with normal lab feed and water. Group C animals were treated with Silymarin (25mg/kg), and Group D&E animals were treated vocally with methanolic extract of *Orthosiphon stamineus* leaves (100mg/kg & 200mg/kg) respectively for seven days. On the seventh and eighth days, animals of Group B, C, D & E were administered orally with a single dose of Halothane with 5% acacia combination (600mg/kg/ day). After thirty minutes of Halothane administration Group C, D & E rats were treated with MLMP 100mg/kg, MLMP 200mg/kg, and Silymarin 25 mg/kg correspondingly. On day 8, thiopentone sodium (40 mg/kg, i.p) was injected, and the sleeping time was recorded in all the animals. The same animals were then under anesthetic ether, 1 hour after complete recovery from thiopentone sodium effect and blood calm by retro-orbital puncture and biochemical parameters like ALT, AST, ALP, Direct Bilirubin, Total Bilirubin, Triglycerides, Cholesterol, Total Proteins, and Albumin were estimated. The animals were sacrificed by overdose of ether and autopsied. Livers from all animals were removed, washed with ice-cold saline, considered, and measured the wet liver volume. Small pieces of liver tissue were collected and preserved in 10% formalin solution for histopathological training. Livers of some animals were homogenized with ice-chilled 10% KCl soln and centrifuged at 2000 rpm for 10 minutes. Then collect the supernatant liquid and the antioxidant parameters like Catalase, Superoxide Dismutase, and Thio barbiturate were estimated.

A) Physical Parameters:

Determination of Wet Liver Weight: Animals were forgoing, and livers were isolated and washed with saline and weights determined by using an electronic balance. The liver weights were spoken with respect to its body weight *i.e.,* gm/100gm.

Determination of Wet Liver Volume: After recording the weight, all the livers were dropped individually in a measuring cylinder containing a fixed volume of refined water or saline, and the volume displaced was recorded.

B) Functional Parameters: On the last day, Thiopentone sodium (40 mg/kg, i.p) was injected and the asleep time was recorded in all the animals.

C) Biochemical Parameters: The biochemical limits were estimated as per the standard procedure prescribed by the manufacturer's instruction manual provided in the kit. (Coral clinical systems, Verna Goa, India) using Semi Autoanalyser (ARTOS).

1) Estimation of Serum SGPT (UV- Kinetic Method):

Principle: SGPT catalyzes the transfer of amino group from L-Alanine to 2-oxo glutarate with the formation of pyruvate and L-glutamate. The pyruvate so formed is allowed to react with NADH to produce L-lactate.

The amount of this reaction is monitored by an indicator reaction coupled with LDL in the presence of NADH (nicotinamide adenine dinucleotide). The oxidation of NADH in this reaction is measured as a reduction in the absorbance of NADH at 340 nm, which is proportional to SGPT activity.

Procedure:

Pipette	Sample (µl)
Working reagent	1000
Sample	100

Mix well and aspirate.

2) Estimation of Serum SGOT (UV- kinetic Method):

Principle: SG0T catalyzes the transfer of amino group from L- Aspartate to 2-oxo glutarate with the formation of oxaloacetate and L-glutamate. The rate of this response is monitored by an indicator reaction coupled with malate dehydrogenase (MDL).

The oxaloacetate formed is converted to malate ion in the presence of NADH (nicotinamide adenine dinucleotide). The rust of NADH in this reaction is measured as a decrease in the absorbance of NADH at 340 nm, which is proportional to SGOT action.

Procedure:

Pipette	Sample (µl)
Working reagent	1000
Sample	100

Mix well and aspirate.

3) Estimation of Serum Alkaline Phosphatase (ALP):

Principle: Estimation of Serumalkaline phosphatase hydrolyses p-nitrophenyl phosphate in the presence of oxidizing agent Mg^{+2}. This reaction is measured as absorbance is proportional to the ALP activity.

Procedure:

Pipette	Sample (µl)
Working reagent	1000
Sample	20

4) Estimation of Serum Bilirubin:

Principle: Bilirubin reacts with diazotized sulphanilic acid in an acidic medium to form a pink coloured azobilirubin with absorbance directly proportional to bilirubin concentration. Direct bilirubin, being water-soluble directly counter in acidic medium. But, indirect and unconjugated bilirubin is solubilized using a surfactant, and then it reacts similarly to direct bilirubin.

Procedure:

	Blank(µl)	Standard	Test
Working reagent	500	500	500
Distilled water	25	-----	-----
Standard	-----	25	------
Sample	-----	-----	25

Mix well. Incubate for 5 minutes at 37°C temperature for Total bilirubin and direct bilirubin. Read absorbance at 546/630 nm against Mixture blank.

5) Estimation of Serum Cholesterol:

Principle: In the presence of cholesterol esterase, fat esters are dissociated into cholesterol and fatty acids; cholesterol oxidase then changes the cholesterol into hydrogen peroxide and cholesterone. In the presence of peroxidase, hydrogen- bleach reacts with 4-amino antipyrine and phenol to form a quinoneimine dye. The absorbance of quinoneimine measured spectrometric ally at 505nm was proportional to cholesterol concentration in the example.

Procedure:

	Blank (µl)	Standard (µl)	Sample (µl)
Working regent	1000	1000	1000
Distilled water	20	----	----
Standard	----	20	----
Sample	----	----	20

Mix well and incubate at 37°C for 10 min. Aspirate blank followed by standard and tests then measure the absorbance of the sample and standard against total at 510/630nm.

6) Estimation of Serum Triglycerides (Enzymaticmethod): The intensity of chromogen quinoneimine formed is proportional to the triglyceride focus in the sample when measured at 510 nm.

Procedure:

	Blank (µl)	Standard (µl)	Sample (µl)
Working regent	1000	1000	1000
Distilled water	20	----	---
Standard	----	20	---
Sample	----	----	20

Mix well, incubate at 37 °C for 10 minutes. Measure absorbance of standard and sample against blank within one hour.

7) Estimation of Serum Total Proteins:

Principle: The peptide bond of proteins reacts with CU^{+2} ions in alkaline solution to from a blue violet

complex (Biuret reaction), each copper ion complexing with 5 or 6 peptide bonds. Tartrate is added as an additive, while iodine is used to prevent auto reduction of the alkaline copper complex. The color formed is proportional to the protein attention and is measured at 546 nm.

Procedure:

	Blank (µl)	Standard (µl)	Sample (µl)
Working regent	1000	1000	1000
Distilled water	20	----	---
Standard	---	20	---
Sample	----	----	20

Incubate for 10 min. at 37 °C. Read absorbance of standard and each sample at 546 nm against reagent blank.

Livers of the animals were regulated with ice-chilled 10% Kclsoln and centrifuge at 2000 rpm to 10 minutes. Then gather the supernatant liquid and estimate the antioxidant parameters like Catalase, Super oxidase, and lipid peroxidation

4) Antioxidant Parameters:

(1) Catalase:

Principle: In U.V. range, H_2O_2 shows a repeated increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed straight by the decrease in absorbance at 240nm. The change in absorbance per unit is a measure of catalase activity.

Procedure: The liver homogenates containing 5µg total protein were varied separately with 700µl, 5mM hydrogen peroxide and incubated at 37°C. The disappearance of peroxide was experimental at 240nm for 15min. One unit of catalase activity is that which lessens 1µmol of hydrogen peroxide per minute.

Observation: Check absorbance at time interval of (0sec, 15sec, 30sec, 45sec, 60sec, 75sec, 90sec, 105sec and 120sec).

2) Superoxide Dismutase:

Principle: The enzyme is essential for survival in all oxygen-absorbing cells. It is found in the cytosol and intermembrane space of mitochondria of eukaryotic cells. It contains copper and zinc. In normal cells, this essential alone is the precursor of hydrogen peroxide. Superoxide dismutase scavenges the superoxide (O^-_2) and thus provides a first-line defense against free radical injury. SOD'S are an enzyme that catalyzes the dismutation of superoxide anion (O_2) to hydrogen peroxide and molecular oxygen in the following manner. In the erythrocytes, the superoxide anion (O^-_2) networks with peroxides to form hydroxyl radicals ($^-$OH), which causes hemolysis in the absence of SOD activity. SOD measurement was carried out on the ability of SOD to inhibit the natural oxidation of epinephrine to adrenochrome.

Procedure: 2.8 ml of sodium carbonate buffer (0.05 mM) and 0.1 ml of tissue homogenate or sucrose (Blank) were incubated at 30°C for 45 minutes. Then, the absorbance was adjusted to 0 to sample. After that, the reaction was started by adding 10µl of adrenaline solution (9mM). The change in absorbance was recorded at 480nm for 8-12 minutes. Throughout the assay, the temperature was preserved at 30 °C. Similarly, SOD calibration curve was prepared by taking 10 units/ ml as a standard solution. 1 units of SOD produce approximately 50% of inhibition of auto-oxidation of adrenaline. The results are spoken as a unit (U) of SOD activity per mg of tissue.

3) Lipid Peroxidation:

Procedure: Thiobarbituric acid sensitive substances (TBARS), the last product in lipid peroxidation pathway, were measured using the modified Method of Esterbauer and Cheeceman, 1990. Liver tissue (200mg) was standardized in 10 volumes of ice-cold 50mM Phosphate buffer (pH 7.4) and the homogenates were centrifuged at 12,000rpm for 15min at 4°C. The supernatant was used for the assay. Protein attentions of different homogenates were measured according to the method of Bradford. Protein (1mg) was incubated at 37°C for 1h and then 1ml 20% TCA and 2ml 0.67% TBA was added and animated for 30min at 100°C. The residue was removed by centrifugation at 1000*g* for 10min. The absorbance of the samples was stately at 535nm against a blank that contains all the reagents except the sample. TBARS concentrations of the samples were calculated using the extinction coefficient of MDA, which is $1.56\times10^5 mmol^{-1}cm^{-1}$ as 99% of TBARS is MDA.

Histopathological Studies:

Processing of Isolated Liver: The animals were forwent, and the liver of each animal was isolated and was cut into small pieces, preserved, and fixed

in 10% formalin for two days. Then the liver piece was eroded in running water for about 12 hours to remove the formalin and was followed by dehydration with isopropyl alcohol of swelling strength (70%, 80%, and 90%) for 12 hours each. Then finally, dehydration is done using absolute alcohol with about three changes for 12 hours each. Dehydration was performed to remove all traces of water. Further alcohol was removed by using chloroform, and chloroform was removed by paraffin access. The clearing was done by using chloroform with two vagaries for 15 to 20 minutes each. After paraffin infiltration, the liver bits were subjected to an automatic tissue processing unit.

Embedding in Paraffin Vacuum: Hard paraffin was melted, and the hot paraffin was poured into L-shaped blocks. The liver pieces were then released into the molten paraffin quickly and allow to cool.

Sectioning: The blocks were cut using microtome to get sections of the thickness of 5µ. The sections were taken on a micro slide on which egg albumin *i.e.*, sticking substance, was applied. The units were allowed to continue in an oven at 60°C for 1 hour. Paraffin melts, and egg albumin denatures, thereby fixing tissue to slide.

Staining: Eosin is an acid stain; hence it stains all the cell constituents pink, which are basic in nature *i.e.*, cytoplasm. Hematoxylin, a basic stain which stains all the acid cell components blue *i.e*., DNA in the nucleus.

RESULTS:

Toxicity Study: In the present education, the Methanolic extract of *Orthosiphon stamineus* trees was exposed for toxicity studies. For the LD_{50} dose determination, Methanolic extract was administered upto dose 2 gm/kg body weight, and extract did not food any mortality, thus $1/10^{th}$, $1/20^{th}$ of maximum dose tested were particular for the present study. LD_{50} of extracts of *Orthosiphon stamineus* was intended and found to be as follows: Methanolic extract – 2000 mg/kg.

Hepatoprotective Activity:

Halothane Induced Toxicity:

A) Physical Parameters:

Wet liver Weight and Wet Liver Volume: Halothane treatment in rats caused in enlargement of liver which was plain by increase in the wet liver weight and volume.

The groups were treated with Silymarin, and methanolic extract of *Orthosiphon stamineus* leaves showed important restoration of wet liver weight and wet liver volume faster to normal.

TABLE 1: EFFECT OF METHANOLIC EXTRACT OF *ORTHOSIPHON STAMINEUS* ON WET LIVER WEIGHT & WET LIVER VOLUMES IN HALOTHANE INDUCED HEPATOTOXIC RATS

Group	Treatment	Dose	Wet Liver weight (gm/100gm) (Mean ± SEM)	Liver volumes (ml/100gm) (Mean ±SEM)
A	Normal control	10ml/kg p.o	2.875 ± 0.375	2.75±0.10
B	Toxicant Control	Halothane-600 mg/kg, p.o.	5.35± 0.150	4.4±0.15
C	Standard	100mg/kg, p.o + Halothane	3.55 ± 0.05**	3.0±0.05**
D	MPME	100mg/kg, p.o + Halothane	4.15± 0.10*	3.665±0.015*
E	MPME	200mg/kg, p.o + Halothane	3.675 ± 0.125**	3.40±0.150**

Values are mean ± SEM (n=6) one way ANOVA followed by Tukey-Kramer's test. Where, * represents significant at $p<0.05$, ** signifies highly significant at $p< 0.01$, *** represents very significant at $p<0.001$.

B) Bio-chemical Parameters:

Effect of Methanolic Extract of *Orthosiphon stamineus* Leaves on Biochemical Parameters in Halothane Induced Hepatotoxic Rats:

A) Effect of Serum Marker Enzymes: Rats treated with Halothane advanced significant hepatic damage observed as elevated serum levels of hepato specific enzymes like SGPT, SGOT and SALP when compared to usual control. Pretreatment with Silymarin, methanolic extract presented good protection against Halothane-induced liver toxicity.

Tukey- Kramer's test indicates an important reduction in elevated serum enzymes levels with extract frozen animals compared to toxicant change animals.

TABLE 2: EFFECT OF METHANOLIC EXTRACT OF *ORTHOSIPHON STAMINEUS* LEAVES ON SGPT, SGOT, ALP LEVELS IN HALOTHANE INDUCED HEPATOTOXIC RATS

Group	Treatment	Dose	SGPT levels (U/L) (Mean ± Sem)	SGOT levels (U/L) (Mean ± Sem)	ALP level (mg/dl) (Mean ± Sem)
A	Normal control	10ml/kg p.o	28.175±0.325	34.05±4.5	33.0±0.50
B	Toxicant Control	Halothane-600 mg/kg, p.o.	108±2.50	188.05 ±2.50	92.95±0.550
C	Standard	100mg/kg, p.o + CCl_4	37.5±1.0***	51.5±1.0**	40.05±0.55**
D	MPME	100mg/kg, p.o + CCl_4	87.5±2.0*	143.75±8.750*	81.2±0.30*
E	MPME	200mg/kg, p.o + CCl_4	53.9±1.30**	88.5±3.0**	51.75±0.250**

Values are mean ± SEM (n=6) one-way ANOVA followed by Tukey-kramer's test. Where, * represents significant at p<0.05, ** represents very significant at p< 0.01, and *** represents very important at p<0.001.

Serum Direct Bilirubin and Total Bilirubin: Raise of direct and total bilirubin levels after the direction of Halothane indicate its hepatotoxicity. Pretreatment with Silymarin, Methanolic extract significantly summary levels of direct and total bilirubin levels when compared to toxic control group indicating Hepatoprotective effect of Methanolic cutting of *Orthosiphon stamineus* leaves.

TABLE 3: EFFECT OF METHANOLIC EXTRACT OF *ORTHOSIPHON STAMINEUS* LEAVES ON DIRECT BILIRUBIN, DIRECT BILIRUBIN LEVELS IN HALOTHANE INDUCED HEPATOTOXICITY RATS

Group	Treatment	Dose	Direct bilirubin levels (mg/dl) (Mean ± SEM)	Total bilirubin levels (mg/dl) (Mean ± SEM)
A	Normal control	10ml/kg p.o	0.173±0.0120	0.293±0.029
B	Toxicant Control	Halothane-600 mg/kg, p.o.	1.679±0.099	1.8±0.005
C	Standard	100mg/kg, p.o + CCl_4	0.256±0.012***	0.463±0.049***
D	MPME	100mg/kg, p.o + CCl_4	1.26±0.032**	1.33±0.059**
E	MPME	200mg/kg, p.o + CCl_4	0.50±0.040**	0.736±0.1105**

Values are mean ± SEM (n=6) one way ANOVA followed by Tukey-kramer's test. Where, * represents significant at p<0.05, ** signifies highly significant at p< 0.01, and *** signifies very significant at p<0.001.

Serum Total Protein Levels: Halothane action considerably reduced serum total protein levels. Pretreatment with Silymarin and methanolic extract of *Orthosiphon stamineus* leaves showed a significant surge in total protein levels as likened with the toxicant control group.

Serum Cholesterol and Triglycerides: From the results, it was found that rats treated with Halothane showed a marked increase in lipid and triglycerides levels when compared to the normal control group.

In rats pretreated with Silymarin and Methanolic extract of *Orthosiphon stamineus* leaves, the serum fat and triglycerides levels had knowingly educed when compared to the toxicant control group.

TABLE 4: EFFECT OF METHANOLIC EXTRACT OF ORTHOSIPHON STAMINEUS LEAVES ON SERUM TOTAL PROTEIN, TOTAL CHOLESTEROL & TRIGLYCERIDE LEVELS IN HALOTHANE INDUCED HEPATOTOXIC RATS

Group	Treatment	Dose	Total protein levels (gm/dl) (Mean ± SEM)	Total Cholesterol levels (mg/dl) (Mean ± SEM)	Triglyceride levels (mg/dl) (Mean ± SEM)
A	Normal control	10ml/kg p.o	5.45±0.07	135.6±4.59	0.515±0.0126
B	Toxicant Control	Halothane-600 mg/kg, p.o.	2.89±0.25	324.05±10.5	2.38±0.0879
C	Standard	100mg/kg, p.o + CCl_4	4.86±0.06***	165±3.46***	0.890±0.0382***
D	MPME	100mg/kg, p.o + CCl_4	3.52±0.78**	284.5±4.53**	2.015±0.0182**
E	MPME	200mg/kg, p.o + CCl_4	4.45±0.12***	214±5.50***	1.08±0.0694***

Values are mean ± SEM (n=6) one-way ANOVA followed by Tukey-Kramer's test. Where, * represents significant at p<0.05, ** represents highly significant at p< 0.01, *** represents very significant at p<0.001.

C) Antioxidant Parameters:

TABLE 5: EFFECT OF METHANOLIC EXTRACT OF ORTHOSIPHON STAMINEUS LEAVES ON CATALASE, SUPEROXIDE DISMUTASE AND LIPID PEROXIDATION IN HALOTHANE INDUCED HEPATOTOXICITY

Group	Treatment	Dose	CAT (Mean ± SEM)	SOD (Mean ± SEM)	LPO (Mean ± SEM)
A	Normal control	10ml/kg p.o	91.8±3.412	14.5±0.5774	3.83±0.60
B	Toxicant Control	Halothane- 600 mg/kg, p.o.	22.3±0.8819	3.23±0.088	89.26±0.1856
C	Standard	100mg/kg, p.o +CCl_4	83.05±0.622***	11.0±0.352***	6.53±0.202***
D	MPME	100mg/kg, p.o+ CCl_4	36.4±0.585**	5.8±0.152**	7.86±0.03**
E	MPME	200mg/kg, p.o+ CCl_4	51.03±1.093***	7.7±0.2517***	7.26±0.0667**

a= nmole of MDA/mg of protein. b= Units/mg of protein c=µmole of H2O2consumed/min/mg of protein, d= µ g/mg of protein. Values are mean ± SEM (n=6) one-way ANOVA trailed by Tukey-Kramer's test. Where, * signifies significant at p<0.05, ** represents highly significant at p< 0.01, *** signifies very significant at p<0.001.

D) Functional parameters:

Thiopentone Induced Sleeping time: From the results, it was found that rats treated with Halothane have shown a marked reduction in onset of sleep and increase in the duration of asleep time when compared against a normal control group. The onset of sleep had significantly increased in the rats pretreated with Silymarin and Methanolic extract of *Orthosiphon stamineus* leaves, while the length of sleeping time had significantly decreased when compared to the toxicant group.

TABLE 6: EFFECT OF METHANOLIC EXTRACT OF *ORTHOSIPHON STAMINEUS* LEAVES ON ONSET OF SLEEP& DURATION OF SLEEP IN HALOTHANE INDUCED HEPATOTOXICITY RATS

Group	Treatment	Dose	Onset of time(sec) (Mean ± SEM)	Duration of sleeping(min) (Mean ± SEM)
A	Normal control	10ml/kg p.o	177.5±2.5	96.75±3.775
B	Toxicant Control	Halothane- 600 mg/kg, p.o.	65±10.0	225.6±7.68
C	Standard	100mg/kg, p.o +CCl_4	157.5±7.5***	115±2.74***
D	MPME	100mg/kg, p.o+ CCl_4	101.5±3.5*	192.83±3.712**
E	MPME	200mg/kg, p.o+ CCl_4	130±5.0**	152.33±3.84***

Values are mean ± SEM (n=6) one way ANOVA followed by Tukey-Kramer's test. Where, * represents significant at p<0.05, ** represents highly significant at p< 0.01, *** and represents very significant at p<0.001.

E) Histopathological Studies of the Liver in Halothane Induced Hepatotoxicity:

Normal Control Group: The section studied shows liver parenchyma with intact architecture. Some of the hepatocytes show mild nuclear pleomorphism with prominent nucleoli. There are seen scattered mononuclear inflammatory infiltrations within all the zones.

Halothane Treated Group: Section calculated shows liver parenchyma with effaced architecture. All the zones show areas of hemorrhage, necrosis, micro vesicular steatosis, macrovesicular steatosis, degenerative hepatocytes. There are seen totals of mixed inflammatory infiltration within all the zones.

Silymarin + Halothane Treated Group: Section willful shows liver parenchyma with focal eroded architecture. Few of the perivenular hepatocytes and focal midzonal hepatocytes show macrosteatosis and microsteatosis. Some of the vital veins and sinusoids show dilatation with focal congestion. Also seen are mild stromal inflammatory infiltration comprising of lymphocytes and macrophages.

Methanolic extract (100mg/kg) + Halothane: The section studied displays liver parenchyma with partially destroyed architecture. Some of the perivenular hepatocytes and focal midzonal hepatocytes show necrosis. At the same time, some of the hepatocytes show macrosteatosis and microsteatosis. There are seen moderate mixed fiery infiltration comprising of neutrophils and lymphocytes.

Methanolic extract (200mg/kg) +CCl_4: Section studied displays liver parenchyma with the partial effaced building. Few of the perivenular hepatocytes and focal midzonal hepatocytes show macrosteatosis and microsteatosis. There are seen modest mononuclear inflammatory penetrations within the perivenular hepatocytes and also in midzonal hepatocytes.

Plate 1: Histopathology of Liver:

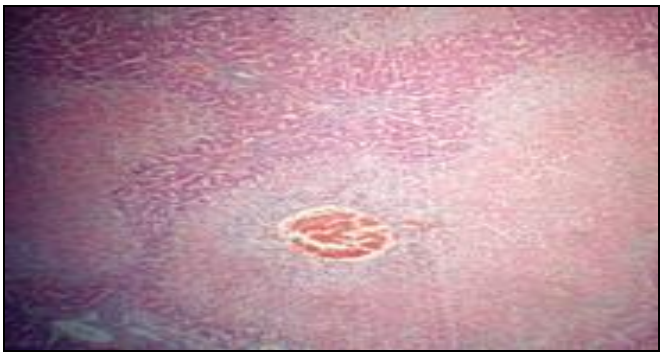

FIG. 3: CONTROL GROUP

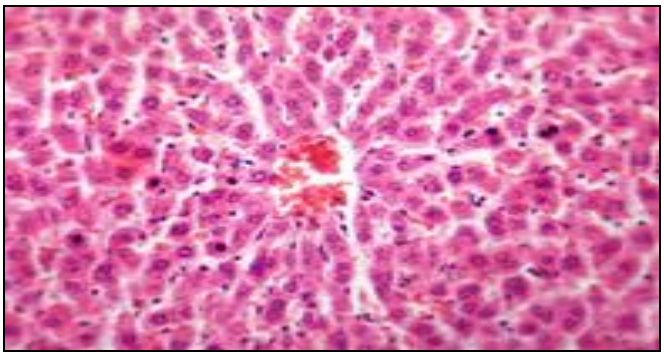

FIG. 4: HALOTHANE TREATED GROUP

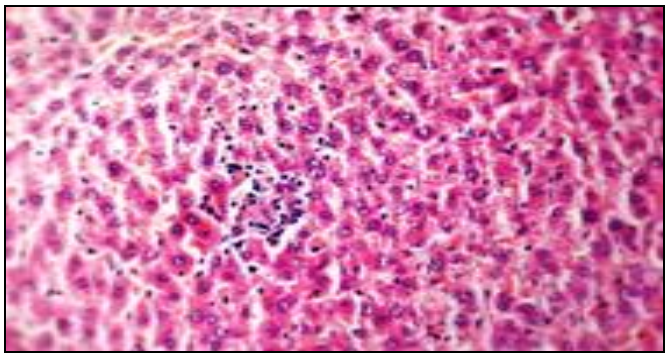

FIG. 5: STANDARD GROUP

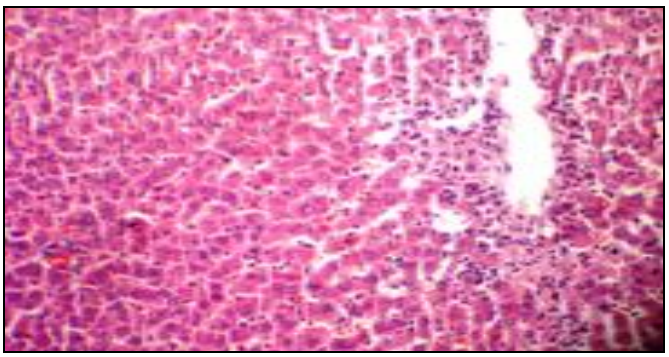

FIG. 6: TEST GROUP-I

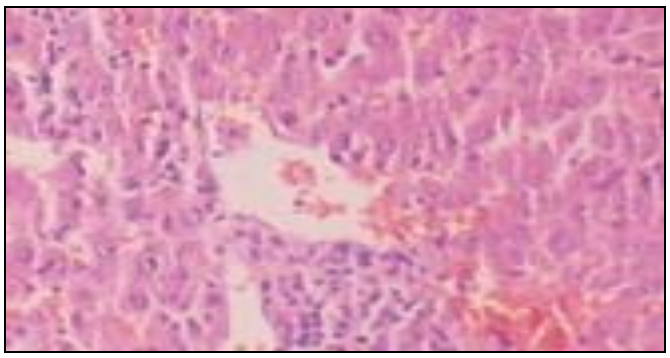

FIG. 7: TEST GROUP-II

DISCUSSION:

Hepatoprotective Activity: The liver participates in a change of metabolic activities, possibly by the presence of number of enzymes, and thus may self-expose too many toxicants, elements, and drugs that could injure it. In our hepatoprotective study, Halothane is used as a hepatotoxicant to induce liver injury since these agents are used/ agreed frequently to human lives for medicinal or non-medical purposes [15].

Mechanism of Halothane-induced Hepato-toxicity: The Halothane is absorbed by CYP 450 enzyme system to trichloromethyl radical ($CCl_3^{\bullet}$). This in turn reacts with molecular oxygen and gets converted to trichloromethyl peroxy radical. This essential forms covalent bonds with sulfydryl group of several membrane molecules like GSH leading to their reduction and causing lipid peroxidation. The lipid peroxidation initiates a cascade of responses leading to tissue necrosis.

Physical Parameters:

Wet Liver Weight and Wet Liver Volume: In the case of toxic liver, Wet liver weight and Wet liver volumes are enlarged. Toxicants induced hepatotoxicity crop fatty changes, and also it is observed that there is a fall in serum lipids in another series of experiments. In this case, water is retained in the cytoplasm of hepatocytes, leading to the enlargement of liver cells, resulting in increased total liver mass and volume. It is reported that liver mass and volume are important parameters in ascertaining the hepatoprotective effect of the

medications. Action with methanolic extract of the leaves of *Orthosiphon stamineus* significantly reduced the wet liver weight and wet liver volumes of creatures, and hence it possesses statistically important hepatoprotective activity.

Biochemical Parameters:

Estimation of Serum Marker Enzymes: Hepatotoxin gets converted into activists in the liver by the action of enzymes & these attacks the unsaturated fatty acids of skins in the presence of oxygen to give lipid peroxides thus. The functional integrity of hepatic mitochondria is different, leading to liver damage. During hepatic damage, cellular enzymes like AST, ALT, and ALP present in the liver cells leak into the serum, resultant in increased concentrations. Halothane was running for 25 days significantly amplified all these serum enzymes. SGPT is a cytosolic enzyme mainly present in the liver. The level of SGPT in serum increases due to the escape of this cellular enzyme into plasma by toxicants induced hepatic injury. Serum levels of SGPT can increase due to injury of the tissues producing acute hepatic necrosis, such as virus-related hepatitis and acute cholestasis.

Alcoholic liver damage and cirrhosis also can associate with mild to moderate elevation of transaminases. In the current study, treating rats with methanolic extract of the leaves of Orthosiphon stamineus significantly reduced the levels of SGPT in serum, which is an indication of hepatoprotective activity. SGOT is a mitochondrial enzyme unconfined from the heart, liver, skeletal muscle, and kidney. Liver toxicity elevated the SGOT levels in serum due to the damage to the tissues producing acute necrosis, such as plain viral hepatitis & acute cholestasis.

Alcoholic liver injury and cirrhosis can also associate with mild to moderate elevation of transaminases. In the current study, treating animals with the methanolic cutting of Orthosiphon stamineus leaves significantly diminished the levels of SGOT in serum, indicative of hepatoprotective activity [15, 16]. In the toxic liver, alkaline phosphatase levels are very high, which may be due to defective hepatic excretion or by amplified production of ALP by hepatic parenchymal or duct cells. In the current study, treatment of animals with methanolic extract of *Orthosiphon stamineus* leaves significantly lessened the levels of ALP in serum as a sign of hepatoprotective activity.

Direct and Total Serum Bilirubin: In the case of deadly liver, bilirubin levels are elevated. Hyperbilirubinemia can result from impaired hepatic uptake of unconjugated bilirubin. Such a state can occur in generalized liver cell injury. Certain drugs (*e.g.,* rifampin and probenecid) interfere with the net acceptance of bilirubin by the liver cell and may produce a mild unconjugated hyperbilirubinemia. Bilirubin level rises in viruses of hepatocytes, obstruction to biliary excretion into the duodenum, in haemolysis, and defects of hepatic uptake and conjugation of bilirubin pigment such as in Gilbert's disease. In the present study, treating animals with methanolic extract of Orthosiphon stamineus leaves significantly reduces the levels of bilirubin (direct and total) in serum, which indicates hepatoprotective activity [17, 18].

Cholesterol and Triglycerides: Toxicant induces hypercholesteremia and hypertriglyceridemia, which may be due to the activation of enzyme HMG CoA reductase, the rate-limiting step in fat biosynthesis. The increased serum triglyceride level in Halothane-treated rats may be due to the reduced activity of lipoprotein lipase, which is complex in the uptake of triglyceride-rich lipoprotein by the extrahepatic skins. Pretreatment with methanolic extract of *Orthosiphon stamineus* leaves reduced the elevated fat and triglyceride levels, suggesting that the extracts prevented ethanol-induced hyperlipidemia may be due to their hepatoprotective activity. Treatment with MPME significantly reduced the levels of fat and triglycerides in Halothane induced hepatotoxic animals, signifying the hepatoprotection.

Total Protein: Liver toxicity reduces the Total Protein level in serum due to tissue damage. Since the methanolic extract of *Orthosiphon stamineus* leaves shows an increase in Total Protein level in serum of animals, it owns statistically important hepatoprotective activity.

Functional Parameters:

Thiopentone Induced sleeping time: Toxicant also alters the metabolic action of hepatocytes, thereby inducing hepatic damage. Barbiturates are a class of xenobiotics that are extensively absorbed

in the liver. Deranged liver function leads to a delay in the clearance of barbiturates, resulting in a longer duration of hypnotic effect. In the present study, the management of thiopentone sodium to rats pretreated chronically with a toxicant caused an increased duration of thiopentone sleeping time. Pretreatment with methanolic extract of *Orthosiphon stamineus* leaves decreased thiopentone-induced slumber time, an indirect sign of their hepatoprotective effect.

Antioxidant Parameters:

Super Oxide Dismutase and Catalase: Our study further revealed that lingering exposure to ethanol decreased the activities of the ROS scavenging enzymes, *viz.* SOD and CAT. This is in line with the assumption suggested earlier by Sandhir and Gill, that decrease in the action of antioxidant enzymes SOD and CAT next Halothane exposure may be due to the damaging effects of free radicals, or alternatively could be due to a direct effect of acetaldehyde, formed from oxidation of, Halothane on these enzymes. Our studies discloses that MPME could restore the activity of both these antioxidant enzymes and possibly could reduce the group of free radicals and hepatocellular injury.

Lipid Peroxidation: ROS, oxidative pressure, and hepatocellular injury have been implicated in alcoholic liver disease. It has been documented that Kupffer cells are the major sources of ROS during chronic Halothane ingesting, and these are primed and triggered for the enhanced creation of pro-inflammatory factors. Additionally, alcohol-induced liver injury has been associated with an augmented amount of lipid peroxidation. Indeed, MPME supplementation in our study was potentially effective in dampening lipid peroxidation, suggesting that MPME possibly has antioxidant stuff to reduce Halothane-induced membrane lipid peroxidation. The hepatotoxicity of Halothane results from its metabolic conversion to free radical product CCl3 by Cyt P-450. Once CCl_3 has been fashioned, it reacts rapidly with O2 to produce CCl3OO- a much more reactive radical than CCl_3. These free radicals attack microsomal lipids chief to their peroxidation and also covalently bind to microsomal lipids and proteins. This results in the generation of reactive oxygen species (ROS), including the superoxide anion O_2, H_2O_2, and the hydroxyl radical. Although various enzymatic and non-enzymatic systems have stood developed by the cell to cope up with the ROS and other free radicals, when a disorder of oxidative stress establishes, the defense capacities against ROS becomes insufficient ROS also affects the antioxidant defense devices, reduces the intracellular concentration of GSH, and decreases the activity of SOD and CAT. It has also been known to decrease the detoxification system produced by GST. Increasing evidence indicates that oxidative stress causes liver injury, cirrhosis growth, and carcinogens. In our studies, it exposes that MPME could restore the movement of both these antioxidant enzymes.

CONCLUSION: The hepatoprotective result of methanolic extract of *Orthosiphon stamineus* leaves was confirmed by the following measures: The remote livers from the various toxicant treated (Halothane) animals exhibited an increase in their physical limits like wet liver weight and wet liver volume. Indeed, extract frozen animals exhibited a decrease in the values of the above physical parameters as a sign of hepatoprotection.

In the present study, the methanolic extract of *Orthosiphon stamineus* leaves significantly summary the toxicant elevated levels of above-stated serum marker enzymes and increased in the levels of protein. Hence, it is concluded that the methanolic extract of Orthosiphon stamineus leaves possess hepatoprotective activity and significantly reduced the toxicant elevated heights of thiobarbituric acid reactive substances (TBARS) like Malondi aldehyde. Hence, it is decided that the methanolic extract of *Orthosiphon stamineus* leaves possesses hepatoprotective activity. In the present study, management of thiopentone sodium to rats pretreated chronically with toxicant caused in an increased duration of thiopentone sleep time. Pretreatment with methanolic extract of *Orthosiphon stamineus* leaves reduced thiopentone-induced sleep time, an indirect sign of their hepatoprotective effect. Carbon tetrachloride made hepatotoxicity was significantly prevented by pretreatment with methanolic extract of *Orthosiphon stamineus* leaves. Reduced in wet liver weight and wet liver volumes, reduction in elevated biochemical parameter after treatment with methanolic extract of *Orthosiphon stamineus* leaves confirmed the hepatoprotective effect of

excerpt under study. In liver injury models in rats, restoration of hepatic cells with minor fatty vicissitudes and absence of necrosis after extract was observed, representing satisfactory hepatoprotection. Based on improvement in serum indicator enzyme levels, physical parameters, Antioxidant parameters, useful parameters, and histopathological studies, it was concluded that methanolic extract of *Orthosiphon stamineus* leaves possesses significant hepatoprotective action in the amounts used.

ACKNOWLEDGEMENT: Authors are very grateful to the staff of Surabhi Dayakar Rao College of Pharmacy for their technical assistance

CONFLICTS OF INTEREST: The authors state that there is no conflict of interest.

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How to cite this article:
Bharathi N, Akula G, Suddagoni S and Venkataramana M: Hepatoprotective activity of extracted flavonoids from *Orthosiphon stamineus* against halothane induced hepatotoxicity in rats. Int J Pharm Sci & Res 2022; 13(7): 2844-55. doi: 10.13040/IJPSR.0975-8232.13(7).2844-55.