International Journal of Agricultural and Life Sciences

ISSN: 2454-6127(Online) Journal homepage: http://www.skyfox.co

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To cite this article : Bharathi, J., & Hazeena Begum, V. (2019). Anti-Inflammatory and antioxidant efficacy of Herbal Mixture on Anti-Inflammatory Paracetamol Induced hepatotoxicity. Int J Agr Life Sci, 5(1), 252-258. doi: 10.22573/spg.ijals.019.s12200093.

To link to this article : https://doi.org/10.22573/spg.ijals.019.s12200093

Data Availability Statement : All relevant data are within the paper and its Supporting Information files.

Funding : The author(s) received no specific funding for this work.

Competing Interests : The authors have declared that no competing interests exist.

Published online: 31 Mar 2019.

International Journal of Agricultural and Life Sciences- IJALS (2019), Volume 5 (1) pp.252-258
http://dx.doi.org/10.22573/spg.ijals.019.s12200093
Available online at http://www.skyfox.co
ISSN: 2454-6127

RESEARCH ARTICLE

Anti-Inflammatory and antioxidant efficacy of Herbal Mixture on Anti-Inflammatory Paracetamol Induced hepatotoxicity

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Received: March 2019 / Accepted: March 2019 / Published: March 2019

ABSTRACT: The present study was carried out to investigate hepatoprotective effects of herbal mixture in female albino rats, in the paracetamol (PCM) induced hepatotoxicity model. Treatment of rats with herbal mixture at dose of (2g /kg for 4 weeks) significantly prevented the serum biomarker enzymes (AST and ALT). Herbal mixture further reduced PCM induced oxidative stress and inflammation in the rat liver by inhibiting lipid peroxidation and restoring the levels of antioxidant enzymes (PCO, LPO, MPO SOD, CAT, GSH, and HO-1). Our results indicate that herbal mixture can prevent hepatic injury with PCM induced hepatotoxicity by eliciting therapeutic protection against free radical stress and inflammation.
Keywords: Herbal Mixture, Glutathione, Hemeoxygenase (HO), Hepatotoxicity, Paracetamol (PCM)

INTRODUCTION

The liver as a vital organ in the body is primarily responsible for the metabolism of endogenous and exogenous agents. It plays an important role in drug elimination and detoxification and liver damage may be caused by xenobiotics, alcohol consumption, malnutrition, infection, anemia and medications (Mroueh *et al*., 2004). Paracetamol is widely used an analgesic and antipyretic, but produces acute liver damage at higher doses. The hepatotoxicity of paracetamol has been attributed to the formation of toxic highly reactive metabolite n-acetyl parabenzoquineimine (NAPQI). Despite the fact that hepatic problems are responsible for a significant number of liver transplantations and deaths recorded worldwide, available pharmacotherapeutics options for liver diseases are very limited and there is a great demand for the development of new effective drugs (Akindele *et al*., 2010). Conventional or synthetic drugs used in the treatment of liver diseases are inadequate and can have serious adverse effects. So there is a worldwide trend to go back to traditional medicinal plants. Many natural products of herbal origin are in use for the treatment of liver ailments (Mitra *et al*., 2000).

Herbs play a major role in the management of various liver disorders. A number of plants possess hepatoprotective property Mansour *et al*. (2006). Herbal mixture contains a number of chemical constituents which are reported to possess antioxidant activities (Khalafetal, 2004). Also, herbal mixture formulation is known for its medicinal properties as liver protective agent, antioxidant, hypoglycemic, hypotensive, anti-inflammatory, antimicrobial, antitumor and as an immune system stimulant Said *et al*. (2006). The aim of this study was to evaluate the hepatoprotective activities of extracts of herbal mixture paracetamol induced hepatotoxicity in rats. The hepatoprotective activity of the herbal mixture extracts were compared with a standard hepatoprotective drug, silymarin.

MATERIALS AND METHODS

PLANT PREPARATION FOR HERBAL MIXTURE

The herbal mixture plant such as *Eclipta alba (Leaf), Spheranthus (whole plant), Nelumbonucifera (Flower), Occinum sanctum (Leaves), Aeglemarmelous (Leaf) and Centellaasiatica (Leaf)* was collected from Tamil university campus. All the plants were shade dried powdered and mixed thoroughly in different proportions and crude fine powder used for experimental studies. The herbal mixture crude power was added to ethanolic for 18h at room temperature. The extract was filtered and used for enzymatic analysis and antioxidant activity.

EXPERIMENTAL DESIGN

This study was performed on Female albino rats of weighing about 150-200g body weight. The experiments were carried out following the guidelines of ethical committee. The rats were divided into four groups of six animals each. At the end of the experiment, animals were sacrificed and the blood serum was separated for enzyme analysis.

Group1	**:**	**Served as a control. Received distilled water orally.**
Group2	:	Received Paracetamol (2g/kg p.o) single dose.
Group3	:	Received Paracetamol (2g/kg p.o) and herbal mixture (2g/kg P.O) for four weeks.

International Journal of Agricultural and Life Sciences- IJALS (2019), Volume 5 (1) pp.252-258
http://dx.doi.org/10.22573/spg.ijals.019.s12200093
Available online at http://www.skyfox.co
ISSN: 2454-6127

Group4 : Received Paracetamol (2g/kg p.o) single dose and Silymarin (100mg/kg P.O) for four weeks.

BIOCHEMICAL ANALYSIS OF MARKER ENZYMES

The serum AST and ALT were assayed according to the Standard method Kit of Riteman&Frankann, 1957.

ENZYME ASSAYS

Superoxide dismutase (SOD) activity was determined by the modified of Kakkar, *et al*., (1984). 0.5 ml of tissue homogenate was mixed with ethanol: chloroform mixture and centrifuged. To the supernatant, assay mixture (sodium pyrophosphate buffer [0.025 M, pH8.3], phenazinemethosulphate, nitro blue tetrazolium and reduced nicotinamide adenine dinucleotide (NADH) was added and incubated at 30c 90s. The reaction was stopped by the addition of glacial acetic acid and mixed with n-butanol. The intensity of the chromogen in the butanol was measured at 560nm.A single unit of enzyme was expressed as 50% inhibition of NBT (nitrobluetetrazolium) reduction 1 min/mg protein.

Catalase (CAT) was assayed colorimetrically as described by Sinha (1972). The reaction mixture contained phosphate buffer (0.01M, pH 7.0) tissue homogenate and 2 M H2O2. The reaction was stopped by the addition of dichromate acetic acid reagents (5% potassium dichromate and glacial acetic acid were mixed in a ratio of 1:3). The intensity was measured at 620 nm and the amount of hydrogen peroxide hydrolysed was calculated for the catalase activity. A single unit of enzyme was expressed as 1 mol of H2O2 consumed/min/mg protein.

GSH was determined by the method of Ellman (1959). To the homogenate, 10% TCA was added and centrifuged. 1.0 ml of the supernatant was treated with 0.5 ml of Ellans reagent (19.8 mg of 5,5 –dithiobisnitrobenzoic acid in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 421nm. The absorbance was read at 412nm. Glutathione peroxidase activity was expressed as µg of GSH consumed/min/mg protein and reduced glutathione as mg/100g of tissue.

Protein carbonyl (PCO) content was determined by Levino *et al*. (1994). 100ml of tissue homogenate was incubated with 0.5ml DNPH for 60min. subquently, the protein was precipitated from the solution with the use of 20% TCA the pellet was washed after centrifugation with ethyl acetate and ethanol (1:1v/v) mixture three times to remove excess DNPH. The final protein pellet was dissolved in 1.5ml of 6 M guanidine hydrochloride. The carbonyl content read in a spectrometer at 370nm. Standard of bovine albumin was included to determine protein carbonyl content. The results were expressed as nmoles of carbonyl per mg protein.

Lipid peroxide assayed was measured by method of Nichans and Samuelson (1968). The measurement of lipid peroxide in serum, brain, liver heat and kidney by a colorimetric reaction with thiobarbituric acid was done as described methods. The determined lipid peroxide is referred to as Malondialdhyde. Briefly tissue were homogenized in 50 MM phosphate buffer (pH 7) using an electronic homogenizer to prepare 10% w/v homogenate. The aliquot of homogenate was taken in a test tube 2.5 ml of 20% trichloroaceticacid solution were added to the samples. The colour of thiobarbituric acid pigment was developed in a 38 water bath at 100 c for 30 min. After cooling with tap water to room temperature, 4 ml n- butanol was added and shaken vigorously after centrifugation the colour of butanol layer was measured at 350nm.

Myeloperoxidase (MPO) assayed was measured by method of (Bradley *et al*., 1982).The buffer containing 0.5% hexadecyltrimethylammonium bromide,10 mM EDTA and 50nm phosphate (pH 6.0) at 25°C for 1 min. The homogenies were then centrifuged at 17,000g at 1Cfor 15 min and MPO activity in the supernatant measured as 450nm.

STATISTICAL ANALYSIS

All the values were expressed as mean ± SEM. The data was statistically analyzed by one way analysis of variance (ANOVA) followed by Dunnett"s, t"-test and values $p< 0.05$was performed using the statistical package for social science (SPSS) version 9 to compare all treated groups. Differences were considered to be significant when ($p< 0.05$).

RESULTS AND DISCUSSION

Results reported in Table 1,shown that levels of serum SGOT and SGPT were markedly elevated in paracetamol treated animal groups compared to control group, indicating liver injury. Administration of herbal mixture as well as standard drug silymarin reversed these altered levels.

The present study revealed the hepatoprotective activity of the herbal mixture against well-known hepatotoxins produced by paracetamol. The liver is the most sensitive organ for peroxidative damage because it is rich in oxidizable substances. The improvement in the oxidative stress of liver cells and the consequently decrease in the antioxidant ability of the cells caused an aggressive cellular damage in those cells in which destruction of membranes occurred and enzymes were released into the blood stream. More severe liver damage releases the higher amount of liver enzymes (El-Khayat *et al*., 2009). The increased levels of serum enzyme such as SGOT and SGPT indicated the increased permeability and damage or necrosis of hepatocytes (Pari & Suresh, 2008).

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ISSN: 2454-6127

In the present study, in paracetamol intoxicated animals, elevated levels of serum enzymes such as SGOT and SGPT which are indicative of cellular leakage and loss of functional integrity of cell membrane in liver were observed. Administration of herbal mixture had restored the markers SGOT and SGPT suggesting the possibility of normalization of the hepatocytes, protecting the membrane integrity against paracetamol induced leakage of marker enzymes into the circulation. These biochemical reaction restorations may be due to the inhibitory effects on cytochrome p450 or promotion of its glucuronidation.

This supports the view of transaminase to normal with the healing of hepatic parenchyma and the regulation of hepotocyte. Our finding is also supported by the earlier work with VasaguduchyadiKwatha (herbal mixture) which had definite hepatoprotective effect (Kalpu Kotecha *et al*., 2015). The possible mechanism responsible for the protection of the paracetamol induced liver damage by the herbal mixture may be as a result of the extract acting as a free radical scavenger by intercepting the radicals involved in paracetamol metabolism by microsomal enzymes or herbal mixture contain phytochemicals because a number of scientific reports indicates the role of certain flavonoids, triterpenoids and steroids in hepatoprotection against hepatotoxins.

Results reported in Table 2, shown that levels of anti-inflammatory (MPO, LPO and PCO) were markedly elevated in paracetamol treated animal groups compared to control group, indicating liver injury. Administration of herbal mixture as well as standard drug silymarin reversed these altered levels.

Myeloperoxidase (MPO) is an enzyme found in neutrophil and its activity is directly proportional to the number of neutrophils. It is reported that assessment of Myeloperoxidase activity is an index to quality cellular inflammation and leukocytes accumulation.

Myeloperoxidase is related to the infiltration inflammatory response in the liver cell. In PCM treated female rats the MPO activity was found increased in liver tissue. Herbal mixture treatment reduced the inflammation marker enzyme, MPO. Myeloperoxidase related with neutrophil infiltration during oxidative stress induced inflammation in the cells. Decrease in the activity of MPO suggest the hepatoprotective effect through the inflammation. It was reported earlier that major terpene components had effective suppressive activity. (Hori *et al*., 1995). Our finding also be suggest above possible mechanism of anti-inflammatory activity.

Lipid peroxidation (LPO) has been a frequently invoked mechanism in ROS- induced cell death and liver injury. Common initiators of the peroxidation process are HO and HOO, which can be generated through the Fenton reaction. In addition LOP can be caused by peroxynitrite and heme dependent lipid peroxide decomposition. The abstraction of H from lipid molecules produces fatty acid radicals. This initiates a free radical chain reaction that triggers the peroxidation of a large number of target molecules, which would ultimately severely destroy the integrity of cell membrane damage the function of membrane bound enzymes and even impaired nuclear DNA.

Furthermore, it has been suggested that LPO may contribute to the development of hepatic toxicity. In this study, it was observed that treatment with herbal mixture in paracetamol induced toxicity confers significant protection to the liver from LPO, as evidenced by reduced levels in liver tissues. Galal *et al*., (2012) reported the important role played by honey in preventing the formation of hydroxyl radicals and in maintaining cellular integrity and functions. Interestingly, it has been reported that components isolated from honey can promote the production of inflammatory cytokines such as interleukins and tumor necrosis factors (Tonks *et al*., 2007). Similarly, it has also been revealed that herbal mixture can stimulate the production of inflammatory cytokines important in tissue repair by human monocytic cells.

Protein CO is used as a biomarker of oxidative stress. The usage of protein CO groups as biomarkers of oxidative stress is advantageous compared to the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins. We found that enhanced level of protein CO as well as level of lipid peroxidation in hepatic tissues of herbal mixture treated rats.

In our present study Myeloperoxidase, Lipid peroxidation and Protein Carbonyl levels were found increased in the tissues of liver in paracetamol intoxicated rats. Herbal mixture administration decreased MPO, LPO and PCO levels, similarly standardsilymarin also showed inhibition of peroxidative factors. The elevation the lipid peroxide and protein carbonyl in the liver tissues indicate the excessive free radical generation that leads to severe damage to tissues. The level of peroxide in a measure of membrane damage and alteration in structure and function of cellular membrane.

Results reported in Table 3,shown that antioxidant enzymes reduced (SOD, CAT, GSH) were as Hemeoxygenase (HO) markedly elevated in paracetamol treated animal groups compared to control group, indicating liver injury. Administration of herbal mixture as well as standard drug silymarin reversed these altered levels.

Superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in live injury. SOD has been one of the most important enzymatic in the antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. In herbal mixture drug causes a significant increase in hepatic SOD activity and thus reduces reactive free radical induced oxidative damage to liver. (Chuma *et al*., 2017)

Catalase enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver. Catalase an antioxidant protective enzyme that scavenge the free radicals produced during toxicity. Increase in OH- production in subcellular region inactivates enzyme

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ISSN: 2454-6127

activity and thereby causes tissue damage by lipid peroxidation.

The antioxidant enzyme SOD and catalase evidence have defensive antioxidant potential of this herbal mixture by modulating the enzymatic natural antioxidant in hepatic cells. Thus our studies reveals hepatoprotective efficacy of herbal mixture by the antioxidative mechanism. The standard available Silymarin had revealed protective mechanism against free radical and antioxidant enzymes and biochemical parameters evidences of hepatoprotective efficacy. SOD and CAT was found decrease in the PCM intoxicated of female rats. Therapeutic treatments with herbal mixture were found to shown increase the hepatic SOD and catalase activity.

Glutathione (GSH) is the main intercellular non-protein sulfhydryl and it plays an important role in the maintains of cellular proteins and lipids, in their functional states. When GSH is lowered, the toxic effects of oxidative stress are increased by cellular damage. At this point other protein and non-protein sulfhydryl group present in the cell provide an important alternate protection (fraga and oteiza 2003). Decline in the GSH content in the liver of PCM intoxicated rats and it subsequent return towards the near normally in herbal mixture administered groups also reveal anti-lipid per oxidative effects.

A significantly higher content GSH in liver would afford the tissue a better protection against antioxidative stress, thus contributing to the abolishment of paracetamol induced hepatotoxicity. Therefore herbal mixture is a promising hepatoprotective agent. The hepatoprotective action combined with antioxidant activity has a synergistic effect to prevent the process of initiation and progress of hepatocellular damage.

HO-1is a highly attractive and interesting candidate stress-response protein, which may play key role(s) in mediating protection against oxidant-mediated damages. These effects are completed by the intriguing biological activities of the catalytic byproducts, carbon monoxide (CO), iron, and bilirubin. In fact, it has demonstrated that overproduction of biliverdin and bilirubin acts as an antioxidative defense mechanism such as LDL oxidation inhibition. An important role of the HO reaction in regulating liver function is to generate CO, which activating the soluble guanylatecyclase triggers an intracellular cyclic guanylyl mono-phosphate (cGMP) increase similar to that attributable to nitric oxide (NO). It was demonstrated that CO, by its inflammatory effects, involves the MAP kinase signaling pathway and acts as an endogenous regulator that is necessary for maintaining microvascular blood flow in the liver. Many studies showed that increase of HO-1 activity and expression results in the reversal of oxidative stress and decrease in liver damage. (Abraham and Kappas 2008)

Reports available that HO1 induction results in the down regulation of cytokine synthesis. The potential pro oxidant consequences of HO activity was highlighted by (Sass *et al*., 2008). The HO reaction releases iron, which could be involved in deleterious reactions that compete with iron reutilization and se-quest ration pathways. The induction of HO activity may have both pro- and anti-oxidant sequelae depending on cellular redox potential, and the metabolic fate of the heme iron.

HISTOPATHOLOGICAL RESULTS

The hepatoprotective effect of herbal mixture was confirmed by histopathological examination of the liver tissue of control and treated animals: Fig1: Liver section of normal control rats (Group I) showing normal liver lobular architecture with well brought out central vein and prominent nucleus and nucleolus. Histopathological study of the liver showed in paracetamol induced intoxication of hepatocytes. Fig 2: Paracetamol (2g/kg) intoxicated rats (Group II) showing severe toxicity with congested blood vessels with inflammatory cell collection and endothelial cell swelling. Our finding is also similar to earlier work with vasaguduchayadikwatha decoction thus suggesting hepatoprotective potential of herbal mixture. (Kalpu *et al*., 2015). Fig 3: Herbal mixture (2g/kg) treated rats (Group III) showing only moderate inflammation around portal tract and the maximum protection against hepatic damage was achieved. Produced mild degenerative changes and absence of necrosis, sinusoidal dilation, and central vein congestion. Fig 4: Standard drug (Silymarin100mg/kg) treated rats (Group IV) showed almost normal hepatic architecture. It is reported that silymarin had prevented hepatic cell necrosis in 87.5% of animals subjected to paracetamol induced hepatotoxicity. Thus evidencing that silymarin showed histopathogical evidence of hepatoprotection by preventing hepatic cell necrosis or by hepatic cell regeneration. (Kotechakalpu et *al*., 2012)

The histopathological observation of the liver of rats treated with herbal mixture showed a more or less normal architecture of the liver having reversed to large extent, the hepatic lesions produced by paracetamol almost comparable to the normal groups.

CONCLUSION

The results of screening on hepatoprotective activity and antioxidant property indicated that the herbal mixture possesses significant hepatoprotective activity and antioxidant property. Liver antioxidant and biomarkers were elevated, while the serum and lipid parameters were maintained at normal levels compared to control groups. Histopathological examination of the liver showed that silymarin have a protective role over the toxicity of paracetamol. The herbal mixture contain presence of some important phenolic, flavonoids and terpenoids compounds which could be responsible for imparting protection to the liver of rats. Hence herbal mixture could be hepatoprotective potential and possible mechanism for their hepatoprotective activity may be their membrane stabilizing activity, healing of hepatic parenchyma and the regeneration of hepatocytes and reduction in reactive free radical induced oxidative damage to liver antioxidant activity.

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http://dx.doi.org/10.22573/spg.ijals.019.s12200093
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ISSN: 2454-6127

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Table. 1. Effects of Herbal mixture on the activities of serum enzymes

Groups	SGOT	SGPT
Group I Normal	32.38±1.20[a]	43.56±1.38[a]
Group II PCM intoxicated	87.58±0.52[ab]	90.92±0.98[ab]
Group III Herbal Mixture	41.54±1.36[bc]	42.34±0.80[bc]
Group IV Sylimarin	35.12±0.81[bd]	46.01±0.86[bd]

The results are presented as means ± SE of six rats. [a]significant from normal group, [b]significant from Herbal mixture group and [c]significant from paracetamol group. [bd]significant from sliymarin group Level of significance is at P <0.05.

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ISSN: 2454-6127

Table. 2. Effects of Herbal mixture on the activities of anti-inflammatory parameters

Groups	MPO	LPO	PCO
Group I Normal	1.57±0.15[a]	0.11±0.02[a]	2.38±0.03[a]
Group II PCM intoxicated	2.66±0.09[ab]	0.24±0.01[ab]	3.548±0.03[ab]
Group III Herbal Mixture	1.40±0.09[bc]	0.08±0.01[bc]	2.20±0.15[bc]
Group IV Syilmarin	1.55±0.04[bd]	0.16±0.01[bd]	2.54±0.27[bd]

The results are presented as means ± SE of six rats. [a]significant from normal group, [b]significant from Herbal mixture group and [c]significant from paracetamol group. [bd]significant from sliymarin group Level of significance is at P <0.05.

Table. 3. Effects of herbal mixture on antioxidants in liver tissue

Groups	SOD	CAT	GSH	HO
Group I Normal	2.22±0.19a	11.40±0.31a	6.22±0.19a	22.32±0.54a
GroupII PCM intoxicated	1.4±0.56ab	14.32±0.23ab	2.22±0.36ab	33.28±0.27ab
Group III Herbal Mixture	2.28±0.27bc	10.58±0.25bc	6.56±0.23bc	13.62±0.31bc
Group IV Syilmarin	1.76±0.74bd	12.60±0.49bd	6.10±0.15bd	10.50±0.27bd

The results are presented as means ± SE of six rats. [a]significant from normal group, [b]significant from Herbal mixture group and [c]significant from paracetamol group. [bd]significant from sliymarin group Level of significance is at P <0.05.

HISTOPATHOLOGICAL PROFILE

Fig 1

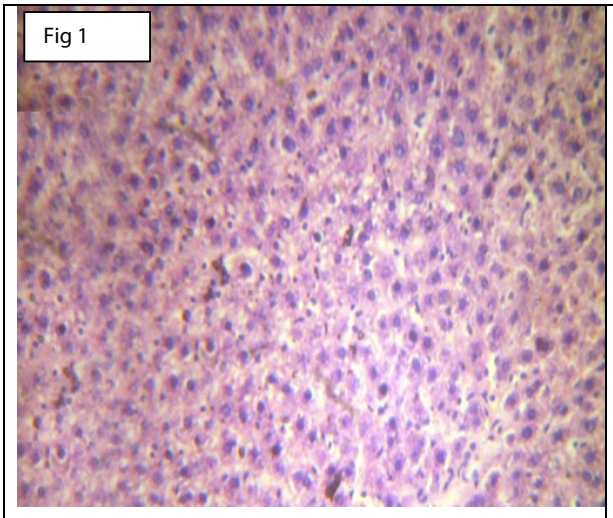

Fig 1- Representative sections of liver of albino rats from normal group showing Hc- Hepatic cell, S-Sinusoid and normal cyto architecture.

Fig 2

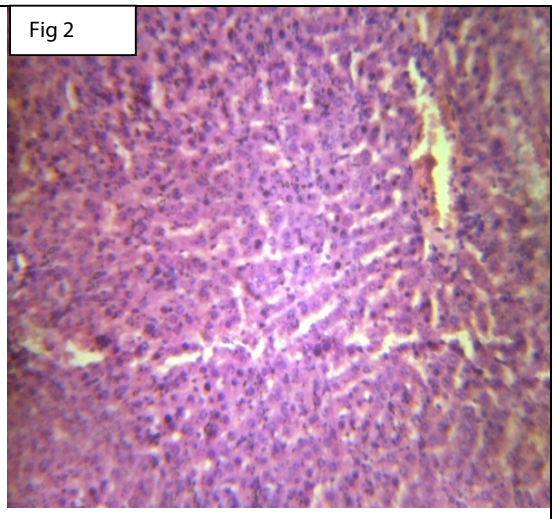

Fig 2-Representative sections of liver of albino rats from paracetamol control group showing Hc- Hepatic cell,S-Sinusoid periportal, CI- Cell infiltration necrosis

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ISSN: 2454-6127

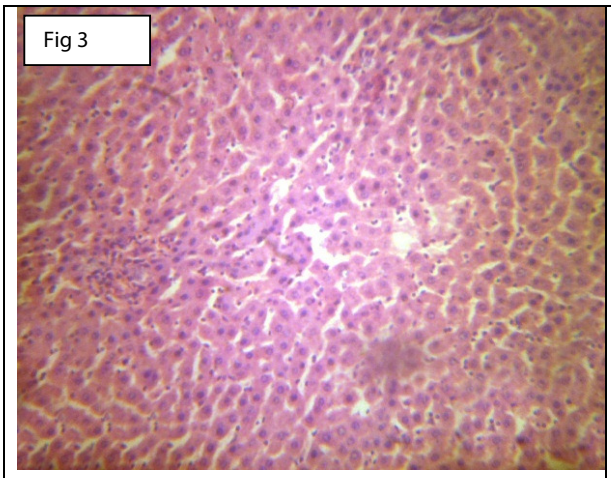

Fig 3- Representative sections of liver of albino rats from paracetamol and Herbal mixture group showing almost normal cytoarchitecture.

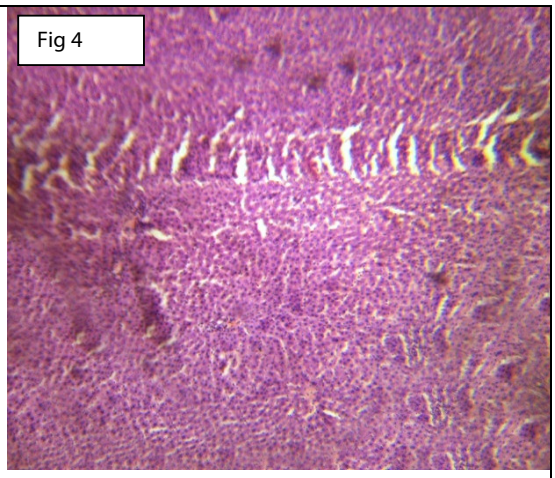

Fig 4- Representative sections of liver of albino rats from paracetamol and silymarin group showing almost normal architecture.

How to cite this article
Bharathi, J., & Hazeena Begum, V. (2019). Anti-Inflammatory and antioxidant efficacy of Herbal Mixture on Anti-Inflammatory Paracetamol Induced hepatotoxicity. Int J Agr Life Sci, 5(1), 252-258. doi: 10.22573/spg.ijals.019.s12200093.

CONFLICTS OF INTEREST

"The authors declare no conflict of interest".