https://doi.org/10.47552/ijam.v16i2.5530 ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 16 (2), 2025; 483-492

In-Vivo Anti Oxidant Effect of *Mukta Pishti* against Paracetamol Induced Hepatic Damage in Wistar Albino Rats

Research Article

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Abstract

Mukta Pishti is a unique Herbo-mineral calcium containing formulation in *Ayurveda*. It is widely practiced as anti-ulcerative and antacid but traditionally it has mentioned as *Ayushya*, *Vrishya, Rasayani, Balya kara* etc. which has broad spectrum use to enhance our quality of life. This study aimed to evaluate the antioxidant activity of *Mukta Pishti* in Wistar albino rats, particularly its protective effects against paracetamol (PCM)-induced hepatic damage by oxidative stress. *Mukta Pishti* was prepared through a traditional Ayurvedic process, including *shodhana* with *jayanti patra swarasa* and *bhavana* with *Gulab arka* over 21 days. A total of 30 Wistar albino rats were divided into five groups, for a 14-day treatment protocol: vehicle control, negative control (PCM), standard (silymarin), and two *Mukta Pishti* treatment groups (100 mg/kg and 200 mg/kg) body weight. Biochemical analyses of serum and liver tissues were conducted post-treatment to assess oxidative stress markers, including glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA). *Mukta Pishti* administration significantly reduced MDA levels ($p<0.001$) and increased GSH ($p<0.001$), SOD ($p<0.0001$), and CAT levels ($p<0.0001$) in serum, indicating strong antioxidant activity, particularly at the 200 mg/kg dose. Liver homogenate results varied, suggesting enzyme interactions affecting oxidative stress markers. *Mukta Pishti* exhibits significant antioxidant properties, potentially through mechanisms involving cell membrane stabilization and activation of antioxidant enzymes. These findings support its traditional use in treating oxidative stress-related conditions and highlight its therapeutic potential in liver protection against toxic agents. Further studies are needed to elucidate the specific pathways involved in its protective effects.

Keywords: *Mukta Pishti,* Cultured pearl, Paracetamol, Oxidative stress, Free radical scavenging activity, Anti-oxidant.

Introduction

The ancient intricate art of Rasashastra, holds the utmost important place in Ayurveda which deals with various metals, and minerals. Among them, pearl is a unique aquatic gem which is having various qualities to enhance the quality of life.

Recent advances in our understanding of free radicals and reactive oxygen species (ROS) are driving a transformation in the field of medicine, paving the way for new approaches to health and disease management. (1) Interestingly, while oxygen is essential for life, it can also have harmful effects on the body under certain conditions. (2) Free radical productions have increased in today's world due to the use of addictive foods and modern diets. These cytotoxic free radicals have an enormous impact on immune system function and raise an individual's susceptibility to a variety of serious illnesses in addition to increasing oxidative stress. The immune system creates free radicals to prevent disease. Therefore, not all free radicals are dangerous to people. Problems arise when the body is unable to deal with the overabundance of free radicals. As a result, they could gravely damage our bodies.

In addition to the normal chemical reactions that occur within the body, certain environmental variables such as radiation, cigarette smoke, contaminated air, processed foods, and industrial chemicals may increase the production of free radicals.

Antioxidants stop additional oxidation events from occurring by breaking the chain reactions of oxidation. Free radicals, which are produced by oxidation reactions, can set off a chain reaction that harms cells. Antioxidants are necessary to eliminate free radical intermediates and fight these free radicals in our systems. It functions on several levels, including repair, radical scavenging, and prevention. (3)

Mukta, freshwater cultured pearl, was selected for the study due to its properties, which include *ayushya, vrishya, pushtikarana, (4) virya-balya-buddhi vardhanam, medhya*, and *hridya* (5) effect. Additionally, *Mukta* has therapeutic indications for *kshaya, agnimandya, shwasa, kasa, (4) asthishosha,*

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jeernajwara, (5) *visha vikara, rajayakshma,* (6) and so on. Despite having a wide range of medicinal applications, it is rarely used. Hence, it inspired us to carry out the current study, which explains *Mukta Pishti's* likely cellular mode of action and provide a scientific confirmation for its anti-oxidant activity.

Materials and Methods

Drug Preparation

The drug Mukta Pishti was prepared according to *Rasatantrasara & Siddhaprayoga samgraha* by giving 21 days *bhavana* with Gulaba arka and stored in air tight container. Prior to that *shodhana* was done with *Jayanti patra swarasa* (*Sesbania sesban* (L) merr. leaves juice) in dola yantra for 3 hours followed by *Prakshalana* (washing) with hot water. (7,8,9, &10)

Chemicals

All the chemicals and reagents were of analytical grade.

Experimental animals

Wistar albino rats (150-200 g) of either sex were used. Animals were procured from Maratha Mandal NGH Institute of Dental Sciences and Research Centre (CRL), Reg. No. 607|PO| ReBiBt|S|02|CPCSEA. They were housed in clean polypropylene cages (6 rats per cage) containing sterile paddy husk as bedding (replaced every 48 hours) under standard conditions (temperature: 25 ± 2 ºC, humidity: 55 ± 5%, and 12 h light/dark cycles) and fed with a standard pellet diet and water ad libitum. Animals were habituated to laboratory conditions for 48 hours prior to experimental protocol to minimize if any of the non-specific stress. All animals were handled with humane care. Experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (BMK/IAEC/Res.No.23/2022-04) of KAHER's Shri B. M. K. Ayurveda Mahavidyalaya, Belagavi.

Animal protocol/ design for anti-oxidant study:

A total 30 rats both sexes were randomly divided into five groups having 6 animals in each group and kept in the cages for 14 days.

- **Vehicle control:** Animals received 1 % Carboxy Methyl Cellulose (CMC) dissolved in 0.9% NaCl solution at 0.5 ml/rat with normal diet.
- **Negative control (Paracetamol induced):** Animals received paracetamol alone at 640 mg/kg BW (p.o.) dissolved in the vehicle. (Vehicle is mentioned previously in vehicle control)
- **Standard:** Animals received silymarin at a dose of 100 mg/kg BW (p.o.) and paracetamol (640 mg/kg BW, p.o.) dissolved in the vehicle.
- **Treatment 1:** Animals received *Mukta Pishti* at a dose of 100 mg/kg BW (p.o.) and paracetamol (640 mg/kg BW, p.o.) dissolved in the vehicle.
- **Treatment 2:** Animals received *Mukta Pishti* at a dose of 200 mg/kg BW (p.o.) and paracetamol (640 mg/kg BW, p.o.) dissolved in the vehicle.

CMC was dissolved in NS prior night. Everyday drug was prepared with the vehicle prior to administration. Paracetamol (PCM) was dissolved in Normal saline before administration.

Based on an earlier study, doses of *Mukta Pishti* were administered to rats, whereas the Paracetamol dose was designated according to the findings of Janbaz and Gilani. (11) Oral administrations were given in the morning between 08:30 am and 10:30 am and continued for 14 consecutive days. The oral administration of PCM was performed one and half hours prior to the administration of the treatment drug except for group I (vehicle control). During experimentation, the rats were observed daily for any unusual behaviour and death. Body weight (BW) changes were observed on every 4th day and the given doses were also calculated according to the body weight. After 14 days of treatment, euthanasia of animals was performed using diethyl ether, followed by confirming the death by checking the respiration and heartbeat. Afterward, blood samples and liver tissues were collected for anti-oxidant assay as indicated below. (12)

Serum and liver tissue homogenate preparations

By using a heparinized syringe, blood samples (approximately 3-4 ml) were drained out from the inferior vena cava of rats in each group. Then, the blood samples were placed into EDTA vials and kept at room temperature before centrifugation at 3000 rpm for 10 minutes, to yield the serum needed for subsequent biochemical analysis (13). At the same time, liver tissues were flensed immediately from the surrounding tissues and washed with cold saline. Subsequently, liver samples were homogenized with phosphate buffer saline (25 mM, pH 7.4) to produce an approximately 10% (w/v) homogenate. Centrifugation was then done at 1700 rpm for 10 min, and the supernatant was collected prior to storage at - 18 to - 20 ◦C until analysis.

Parameters

Lipid peroxidation (LPO) assay

Malondialdehyde (MDA) is renowned as an indicator of lipid peroxidation which was determined by standard method. (13) Briefly, homogenate tissue (0.2 ml) was mixed with 8.1% sodium dodecyl sulfate (0.2 ml), 20% acetic acid (1.5 ml), and 8% thiobarbituric acid (1.5 ml). Subsequently, distilled water (4 ml) was added, and the mixture was heated at 95 ◦C in a water bath for 60 minutes. After finishing the heat, the mixture was then allowed to cool to room temperature, and the final volume was increased to 5 ml. The mixture of butanol: pyridine (15:1) was added, and the contents were allowed to vortex for 2 minutes. The mixture was then centrifuged at 3000 rpm for 10 minutes, the upper organic layer was extracted, and its absorbance was determined at 532 nm against a blank. Levels of MDA were expressed as nmol of thiobarbituric acid reactive substances (TBARS) per mg of protein.

https://doi.org/10.47552/ijam.v16i2.5530 ISSN No: 0976-5921

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The level of lipid peroxidation was determined by assessing the MDA concentration present in the sample. The assay was performed manually using the principle of adduct formation between TBA and MDA a product of lipid peroxidation. The reaction yields a red MDA-TBA adduct, which forms a pink complex.

Evaluation of reduced glutathione (GSH) levels

Reduced glutathione (GSH) was determined using Ellman's reagent (14 & 15) to measure glutathione (GSH) levels in serum. The same way liver homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5′ 5-dithiobis (2 nitrobenzoic acid) (DTNB), and 0.4 ml of double-distilled water was added. The mixture was vortexed by vortex mixture and absorbance was taken at 412 nm within 15 minutes using UV–VIS spectrophotometer. The concentration of reduced glutathione was expressed as nmol/mg of protein.

Evaluation of superoxide dismutase (SOD) levels

According to Nandi and Chatterjee method, superoxide dismutase (SOD) level was assessed. (16) In brief, 2.860 ml of tris-buffer (50 mM and pH 8.5) mixed with 0.1 ml of EDTA solution. After that, 0.02 ml of homogenate sample and 0.02–0.08 ml of pyrogallol were added into the reaction mixture. The absorbance was recorded by a spectrophotometer at 420 nm against the blank. The results were articulated in units per mg of protein.

The activity of SOD enzyme in liver homogenate was determined based on the activation of riboflavin by a protein which further oxidizes an electron donor which is the EDTA which further reduces the riboflavin to a semi-quinone state that leads to the reduction of oxygen to O_2 which further reacts with NBT to form a purple colour. The formed chromogenic then measured spectrophotometrically.

Evaluation of catalase (CAT) levels

The activity of catalase (CAT) enzyme was accomplished using hydrogen peroxide as a substrate following the method of Aebi. (17) The assay was done by taking 0.1 ml of homogenate sample and 1.9 mL of PBS pH 7.0 in a 3 mL cuvette and the reaction was started by the addition of 1 ml of H^2O^2 (30 mM/L). The absorbance was documented at 240 nm by using UV–VIS spectrophotometer. The activity of catalase was expressed in units per mg of protein.

Statistical analysis

All the data are presented as mean ± standard error mean (SEM). The statistical analysis of the results was carried out with Microsoft Excel 2019. Data were subjected to one-way analysis of variance (ANOVA), and statistical analysis was performed with the aid of Tukey's multiple comparison to analyse data sets by using Graph Pad Prism (version: 8.0), considering significant at $p < 0.05$.

Results

Free radical scavenging (antioxidant) activity results

CAT analysis results in blood serum

Table 1: Comparison of five groups (1, 2, 3, 4 & 5) of CAT analysis in blood serum with the mean value by one-way ANOVA

Groups	Mean	SEM
Group 1(Normal)	0.0316	0.001632653
Group 2 (Disease)	0.0337	0.001632653
Group 3 (Standard)	0.04	0.002857143
Group 4 (Treatment 1)	0.033	0.002857143
Group 5 (Treatment 2)	0.0424	0.002040816
F-value	4.380	
P-value	0.0081**	

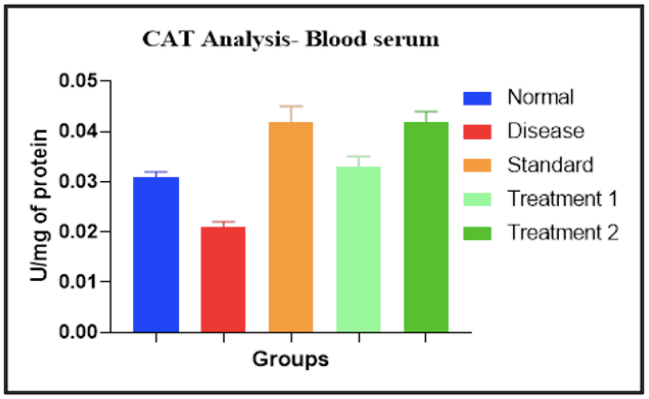
**p<0.0, (SEM- standard error of mean)

Table 2: Pair-wise comparison of five groups (1, 2, 3, 4 & 5) with mean Blood serum value according to CAT analysis by Tukey's multiple comparisons test

Group Test details	P value	Significant (Yes/No)
Normal vs. Disease	0.0102	Yes*
Normal vs. Standard	0.0042	Yes**
Normal vs. Treatment 1	0.9485	No
Normal vs. Treatment 2	0.0042	Yes**
Disease vs. Standard	<0.0001	Yes@
Disease vs. Treatment 1	0.0017	Yes**
Disease vs. Treatment 2	<0.0001	Yes@
Standard vs. Treatment 1	0.024	Yes*
Standard vs. Treatment 2	>0.9999	No
Treatment 1 vs. Treatment 2	0.024	Yes*

*p<0.05, **p<0.01, @ p<0.0001

Figure 1: Effect of *Mukta Pishti* on CAT analysis in PCM-induced oxidative stress



There is a significant difference between the disease group vs. the standard treatment group ($p < 0.0001$) and the disease group vs. Treatment 2 (200 mg/kg b. w) ($p < 0.0001$). This suggests that Treatment 2 is also effective in altering the disease condition. There is no significant difference between the standard treatment group and Treatment 2 ($p > 0.9999$). This implies that Treatment 2 does not differ significantly from the standard treatment. There is a significant difference between Treatment 1 (100 mg/kg b. w) and Treatment 2 ($p = 0.024$). This suggests that the effect is dose dependent.

LPO analysis results in blood serum

Table No. 03: Comparison of five groups (1, 2, 3, 4& 5) of LPO analysis in blood serum with the mean value by one way ANOVA

Groups	Mean	SEM
Group 1(Normal)	4.51	0.16
Group 2 (Disease)	8.13	0.23
Group 3 (Standard)	5.01	0.11
Group 4 (Treatment 1)	7.36	0.12
Group 5 (Treatment 2)	6.77	0.11
F-value	95.6	
P-value	<0.001 #	

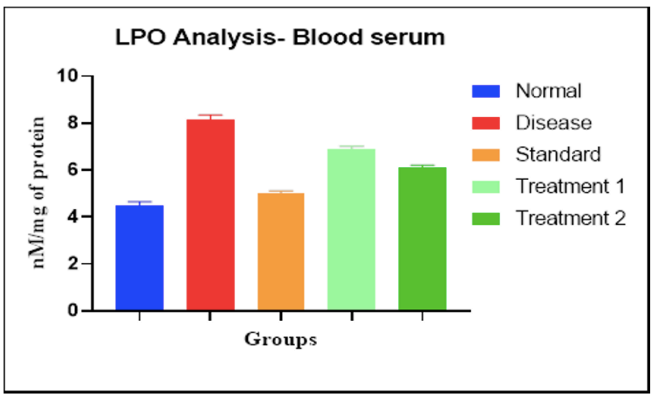
#p<0.001 (SEM- standard error of mean)

Table 4: Pair-wise comparison of five groups (1, 2, 3, 4 & 5) with mean Blood serum value according to LPO analysis by Tukey's multiple comparisons test

Group Test details	P value	Significant (Yes/No)
Normal vs. Disease	<0.001	Yes#
Normal vs. Standard	0.16	No
Normal vs. Treatment 1	<0.001	Yes#
Normal vs. Treatment 2	<0.001	Yes#
Disease vs. Standard	<0.001	Yes#
Disease vs. Treatment 1	<0.001	Yes#
Disease vs. Treatment 2	<0.001	Yes#
Standard vs. Treatment 1	<0.001	Yes#
Standard vs. Treatment 2	<0.001	Yes#
Treatment 1 vs. Treatment 2	0.007	Yes**

**p<0.01, #p<0.001

Figure 2: Effect of *Mukta Pishti* on LPO analysis in PCM-induced oxidative stress in rat's blood serum



There is a significant decrease in LPO levels in the silymarin treatment group compared to the disease group (p<0.0001), indicating that silymarin significantly reduces lipid peroxidation in PCM-induced conditions. There is also a significant decrease in LPO levels in the high-dose Mukta Pishti group compared to the disease group, indicating that the high dose of Mukta Pishti significantly reduces lipid peroxidation in PCM-induced conditions, though still elevated compared to the normal group. There is a significant difference in LPO levels between the silymarin group and the high-dose Mukta Pishti group, with silymarin being more effective, though the high-dose Mukta Pishti also shows a significant reduction. There is a significant difference in LPO levels between the low-dose and high-dose Mukta Pishti groups (p<0.05), indicating that the high dose is more effective in reducing lipid peroxidation compared to the low dose.

SOD analysis results in blood serum

Table 5: Comparison of five groups (1, 2, 3, 4& 5) of SOD analysis in blood serum with the mean value by one way ANOVA

Groups	Mean	SEM
Group 1(Normal)	13.07	0.08
Group 2 (Disease)	5.16	0.16
Group 3 (Standard)	11.96	0.22
Group 4 (Treatment 1)	7.39	0.19
Group 5 (Treatment 2)	8.95	0.12
F-value	406.2	
P-value	<0.0001 @	

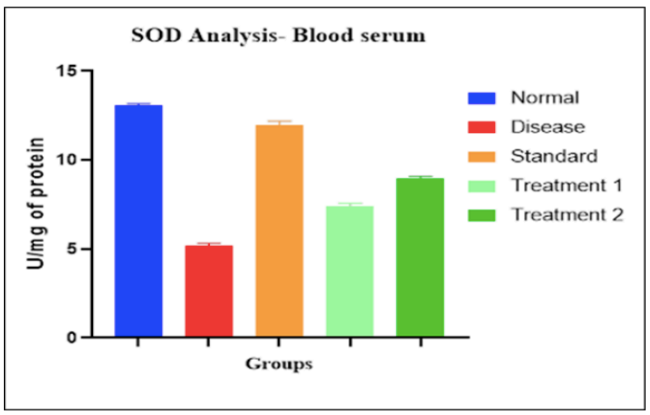
@p<0.0001 (SEM- standard error of mean)

Table 6: Pair-wise comparison of five groups (1, 2, 3, 4 & 5) with mean Blood serum value according to SOD Analysis by Tukey's multiple comparisons test

Group Test details	P value	Significant (Yes/No)
Normal vs. Disease	<0.0001	Yes@
Normal vs. Standard	0.0005	Yes#
Normal vs. Treatment 1	<0.0001	Yes@
Normal vs. Treatment 2	<0.0001	Yes@
Disease vs. Standard	<0.0001	Yes@
Disease vs. Treatment 1	<0.0001	Yes@
Disease vs. Treatment 2	<0.0001	Yes@
Standard vs. Treatment 1	<0.0001	Yes@
Standard vs. Treatment 2	<0.0001	Yes@
Treatment 1 vs. Treatment 2	<0.0001	Yes@

#p<0.001, @p<0.0001

Figure 3: Effect of *Mukta Pishti* on SOD analysis in PCM-induced oxidative stress in rat's blood serum



There is a significant increase in SOD levels in the silymarin treatment group compared to the disease group (p<0.0001), indicating that silymarin significantly restores SOD activity in PCM-induced conditions.

https://doi.org/10.47552/ijam.v16i2.5530 ISSN No: 0976-5921

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There is also a significant increase in SOD levels in the high-dose Mukta Pishti group compared to the disease group, indicating that the high dose of Mukta Pishti significantly restores SOD activity in PCM-induced conditions, though still reduced compared to the normal group. There is a significant difference in SOD levels between the silymarin group and the high-dose Mukta Pishti group ($p<0.0001$), with silymarin being more effective, though the high-dose Mukta Pishti also shows a significant restoration.

GSH analysis results in blood serum

Table 7: Comparison of five groups (1, 2, 3, 4 & 5) of GSH analysis in blood serum value with the mean value by one-way ANOVA

Groups	Mean	SEM
Group 1(Normal)	531.11	4.85
Group 2 (Disease)	348.93	2.56
Group 3 (Standard)	524.78	6.85
Group 4 (Treatment 1)	364.1	5.41
Group 5 (Treatment 2)	442.59	8.8
F-value	201	
P-value	<0.001#	

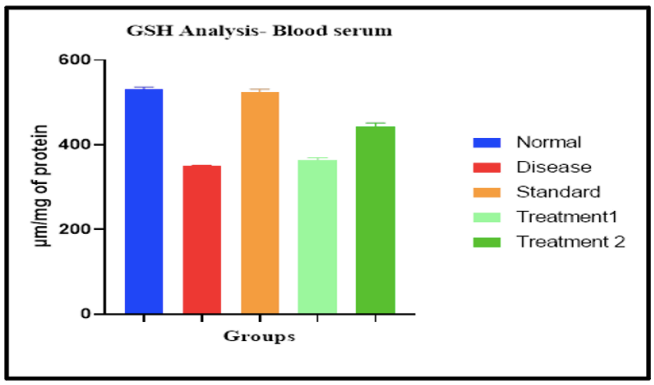
#p<0.001, (SEM- standard error of mean)

Table 8: Pair-wise comparison of five groups (1, 2, 3, 4 & 5) with mean Blood serum value according to GSH analysis by Tukey's multiple comparisons test

Group Test details	P value	Significant (Yes/No)
Normal vs. Disease	<0.001	Yes#
Normal vs. Standard	0.95	No
Normal vs. Treatment 1	<0.001	Yes#
Normal vs. Treatment 2	<0.001	Yes#
Disease vs. Standard	<0.001	Yes#
Disease vs. Treatment 1	0.41	No
Disease vs. Treatment 2	<0.001	Yes#
Standard vs. Treatment 1	<0.001	Yes#
Standard vs. Treatment 2	<0.001	Yes#
Treatment 1 vs. Treatment 2	<0.001	Yes#

#p<0.001

Figure 4: Effect of *Mukta Pishti* on GSH analysis in PCM-induced oxidative stress in rat's blood serum



There is a significant difference between the disease group vs the silymarin treatment group and the disease group vs the high-dose Mukta Pishti group ($p<0.001$).

CAT analysis results in liver homogenate

Table 9: Comparison of five groups (1, 2, 3, 4 & 5) of CAT analysis in liver serum with the mean value by one-way ANOVA

Groups	Mean	SEM
Group 1(Normal)	0.037801748	0.001304
Group 2 (Disease)	0.033777496	0.003568
Group 3 (Standard)	0.06001551	0.005749
Group 4 (Treatment 1)	0.033492668	0.003454
Group 5 (Treatment 2)	0.049224478	0.001391
F-value	10.81	
P-value	<0.0001 @	

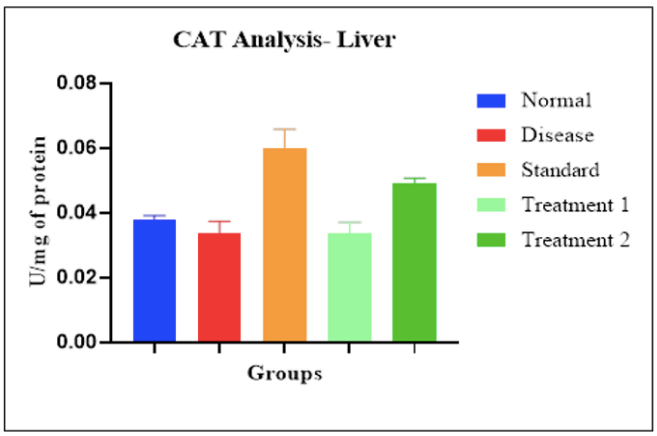
@ p<0.0001

Table 10: Pair-wise comparison of five groups (1, 2, 3, 4 & 5) with mean liver serum value according to CAT analysis by Tukey's multiple comparisons test

Group Test details	P value	Significant (Yes/No)
Normal vs. Disease	0.9244	No
Normal vs. Standard	0.0012	Yes**
Normal vs. Treatment 1	0.9052	No
Normal vs. Treatment 2	0.1763	No
Disease vs. Standard	0.0002	Yes#
Disease vs. Treatment 1	>0.9999	No
Disease vs. Treatment 2	0.0335	Yes*
Standard vs. Treatment 1	0.0001	Yes@
Standard vs. Treatment 2	0.2204	No
Treatment 1 vs. Treatment 2	0.0294	Yes*

*p<0.05, **p<0.01, #P<0.001, @ p<0.0001

Figure 5: Effect of *Mukta Pishti* on CAT analysis in PCM-induced oxidative stress in rat's liver homogenate



There is a significant difference between the disease group and the silymarin treatment group, suggesting that silymarin significantly alters the measured parameter compared to the disease condition, indicating its effectiveness. There is also a significant difference between the disease group and the high dose of Mukta Pishti group (p-0.0335). There is no significant difference between the silymarin group and the high-dose Mukta Pishti group, indicating that these two treatments have similar effects. There is a significant difference between the low-dose and high-dose Mukta Pishti groups, indicating that the two doses have significantly different effects (p-0.0294).

LPO analysis results in liver homogenate

Table 11: Comparison of five groups (1, 2, 3, 4 & 5) of LPO analysis in liver serum with the mean value by one-way ANOVA

Groups	Mean	SEM
Group 1(Normal)	5.230873	0.094401
Group 2 (Disease)	8.94626	0.177289
Group 3 (Standard)	5.157543	0.082121
Group 4 (Treatment 1)	7.504103	0.098154
Group 5 (Treatment 2)	5.560858	0.051764
F-value	237.5	
P-value	<0.0001 @	

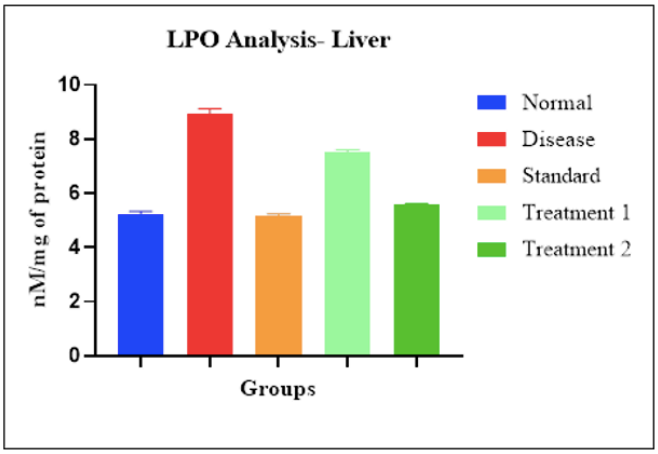
@ p<0.0001

Table 12: Pair-wise comparison of five groups (1, 2, 3, 4 & 5) with mean liver serum value according to LPO analysis by Tukey's multiple comparisons test

Group Test details	P value	Significant (Yes/No)
Normal vs. Disease	<0.0001	Yes@
Normal vs. Standard	0.9889	No
Normal vs. Treatment 1	<0.0001	Yes@
Normal vs. Treatment 2	0.2349	No
Disease vs. Standard	<0.0001	Yes@
Disease vs. Treatment 1	<0.0001	Yes@
Disease vs. Treatment 2	<0.0001	Yes@
Standard vs. Treatment 1	<0.0001	Yes@
Standard vs. Treatment 2	0.0976	No
Treatment 1 vs. Treatment 2	<0.0001	Yes@

@p<0.0001

Figure 6: Effect of *Mukta Pishti* on LPO analysis in PCM-induced oxidative stress in rat's liver homogenate



There is a significant decrease in LPO levels in the silymarin treatment group compared to the disease group (p<0.0001), indicating that silymarin significantly reduces lipid peroxidation in PCM-induced conditions. There is a significant decrease in LPO levels in the high-dose Mukta Pishti group compared to the disease group, indicating that the high-dose of Mukta Pishti significantly reduces lipid peroxidation in PCM-induced conditions. There is no significant difference in LPO levels between the silymarin group and the high dose Mukta Pishti group, suggesting similar effectiveness in reducing lipid peroxidation. There is a significant difference in LPO levels between the low-dose and high-dose Mukta Pishti groups (<0.0001), indicating that the high dose is more effective in reducing lipid peroxidation.

SOD analysis results in liver homogenate

Table 13: Comparison of five groups (1, 2, 3, 4 & 5) of SOD analysis in liver serum with the mean value by one-way ANOVA

Groups	Mean	SEM
Group 1(Normal)	116.9935	5.104738
Group 2 (Disease)	63.39869	5.675371
Group 3 (Standard)	118.9542	2.614379
Group 4 (Treatment 1)	86.9281	2.356569
Group 5 (Treatment 2)	94.77124	1.205169
F-value	36.65	
P-value	<0.0001@	

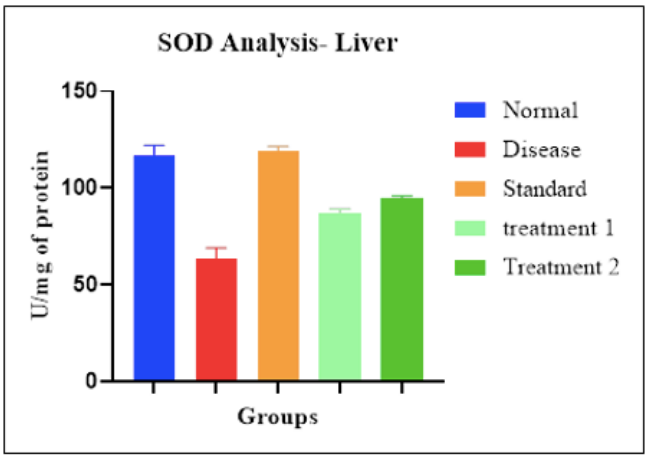
@p<0.0001

Table 14: Pair-wise comparison of five groups (1, 2, 3, 4 & 5) with mean liver serum value according to SOD analysis by Tukey's multiple comparisons test

Group Test details	P value	Significant (Yes/No)
Normal vs. Disease	<0.0001	Yes@
Normal vs. Standard	0.996	No
Normal vs. Treatment 1	<0.0001	Yes@
Normal vs. Treatment 2	0.0029	Yes**
Disease vs. Standard	<0.0001	Yes@
Disease vs. Treatment 1	0.0016	Yes
Disease vs. Treatment 2	<0.0001	Yes@
Standard vs. Treatment 1	<0.0001	Yes@
Standard vs. Treatment 2	0.0012	Yes**
Treatment 1 vs. Treatment 2	0.5963	No

**p<0.01, @ p<0.0001

Figure 7: Effect of *Mukta Pishti* on SOD analysis in PCM-induced oxidative stress in rat's liver homogenate



There is a significant increase in SOD levels in the high-dose Mukta Pishti group compared to the disease group (p<0.0001), indicating that the high dose Mukta Pishti significantly restores SOD activity in PCM-induced conditions, though still reduced compared to the normal group. There is a significant

https://doi.org/10.47552/ijam.v16i2.5530 ISSN No: 0976-5921
International Journal of Ayurvedic Medicine, Vol 16 (2), 2025; 483-492

increase in SOD levels in the silymarin treatment group compared to the disease group. There is no significant difference in SOD levels between the low-dose and high-dose Mukta Pishti groups (p-0.5963), indicating that while both doses have an effect, the difference in their effectiveness is not significant.

GSH analysis results in liver homogenate

Table 15: Comparison of five groups (1, 2, 3, 4 & 5) of GSH analysis in liver serum with the mean value by one-way ANOVA

Groups	Mean	SEM
Group 1(Normal)	525.1733333	13.27336
Group 2 (Disease)	612.8983333	50.80054
Group 3 (Standard)	524.645	62.07731483
Group 4 (Treatment 1)	391.0033333	28.19257
Group 5 (Treatment 2)	546.6666667	11.14951917
F-value	4.321	
P-value	0.0086	

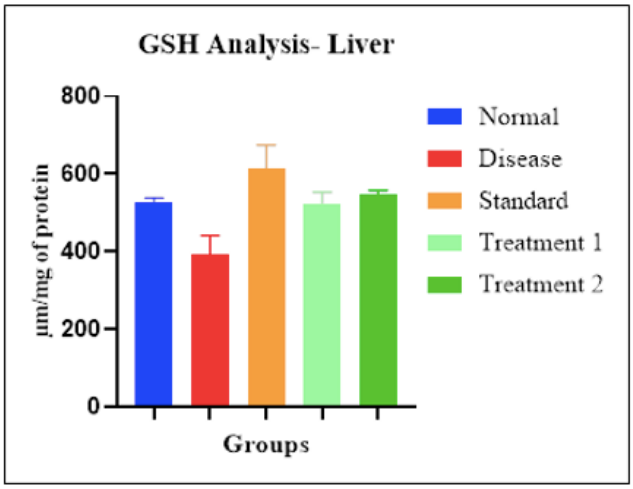
**p<0.01

Table 16: Pair-wise comparison of five groups (1, 2, 3, 4 & 5) with mean liver serum value according to GSH analysis by Tukey's multiple comparisons test

Group Test details	P value	Significant (Yes/No)
Normal vs. Disease	0.1364	No
Normal vs. Standard	0.512	No
Normal vs. Treatment 1	>0.9999	No
Normal vs. Treatment 2	0.9947	No
Disease vs. Standard	0.0037	Yes**
Disease vs. Treatment 1	0.1389	No
Disease vs. Treatment 2	0.062	No
Standard vs. Treatment 1	0.5062	No
Standard vs. Treatment 2	0.7475	No
Treatment 1 vs. Treatment 2	0.9942	No

**p<0.01

Figure 08: Effect of *Mukta Pishti* on GSH analysis in PCM-induced oxidative stress in rat's liver homogenate



There is a significant difference in GSH levels between the disease group and the silymarin treatment group, indicating that silymarin significantly improves GSH levels in the PCM-induced condition. There is no significant difference in GSH levels between the disease group and the high-dose Mukta Pishti group, although it is close to significance.

Discussion

Mukta has *ayushya* (vitalizing), *vrishya* (aphrodisiac), *balya* (increase strength), *pushtiprada* (nourishing), *buddhi bardhanam* (increase intellect), *medhya* (nootropic), *hridya* (cardio protective) etc. properties. According to *Atharvaveda*, the drug that gives body freedom from diseases is *Mukta*. However, due to the scarcity and price, using natural pearls in medicine is much less. Thus, cultured pearls were used for the study. Mukta Pishti was prepared according to classics and classical *anagni bhasma pareeksha* was observed. Physicochemical and instrumental analysis were found to be as per standard limits.

Paracetamol (acetaminophen) toxicity affects several key biomarkers of oxidative stress and antioxidant defense, including Glutathione (GSH), Lipid Peroxidation (LPO), Superoxide Dismutase (SOD) and Catalase (CAT).

Silymarin possesses anti-oxidative, anti-inflammatory, anti-lipid peroxidative, membrane stabilizing, and liver regenerating activities. Clinically prescribed for alcoholic liver disease, cirrhosis, drug-induced liver disease, mushroom poisoning, etc. (18)

The hepatoprotection by silymarin occurs in the following ways: Acts against lipid peroxidation as a result of free radical scavenging & ability to increase the cellular content of GSH. Regulates membrane permeability & increases membrane stability in the presence of xenobiotic damage. Inhibits absorption of toxins, prevents them from binding to cell surface & inhibits membrane transport system. (19)

Catalase is an enzyme that catalyzes the conversion of hydrogen peroxide (H_2O_2- free radical) to water and oxygen, playing a vital role in cellular antioxidant defense. In the blood serum CAT activity, Treatments 1 and 2 have shown significant differences compared to the disease condition (p<0.0001), indicating their potential effectiveness. However, Treatment 1 does not differ from the normal condition, suggesting it may restore normalcy effectively. The standard treatment is significantly different from the disease condition, suggesting its effectiveness. It is similar to Treatment 2 but different from Treatment 1, indicating varying efficacy or mechanisms of action. In the liver serum CAT activity, Silymarin and Treatment 2 (p-0.0335) significantly improved the condition compared to the disease state (PCM-induced), suggesting its effectiveness in the treatment.

Lipid Peroxidation is a process in which free radicals attack lipids containing carbon-carbon double bond(s), particularly in cell membranes, leading to cell damage. MDA (malondialdehyde) is the major oxidant product of PUFA (Polly unsaturated fatty acids) & its elevation is an important indicator of lipid peroxidation-induced tissue damage due to the failure of the antioxidant defense mechanism. (20)

The uprising level of MDA is considered as an important biomarker of lipid peroxidation in liver

tissues. (21) It has been demonstrated that the high dose of PCM causes the oxidation of unsaturated fatty acids in the cell membrane, which is correlated with lipid peroxidation (LPO). (22) In this experiment, the PCM-treated group showed a significant increase in MDA, whereas, *Mukta Pishti*-treated group significantly reduced the MDA level. It is well established that defensive response of organisms against oxidative stress is mediated by an endogenous antioxidant system including SOD, CAT and GSH. They keep away excessive production and maintain low levels of reactive oxygen species (ROS). (23) L. Duan et al. showed that intake of pearl powder could significantly reduce serum lipid peroxide products in a mouse model. (24) In both serum (blood & liver) LPO activity suggests that, Silymarin shows the highest effectiveness in reducing LPO levels, followed by the high dose of *Mukta Pishti*. The low dose *Mukta Pishti* shows some effect but is less effective compared to both the high dose *Mukta Pishti* and silymarin.

Glutathione is a non-enzymatic, highly concentrated intracellular antioxidant present in the liver. It appears to be a sensitive indicator of a cell's overall health & its ability to resist toxic challenges. (25) It detoxifies reactive oxygen species (ROS) and maintains cellular redox balance. Paracetamol is metabolized in the liver to a toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). GSH conjugates with NAPQI to detoxify it. Reduction in GSH enhances lipid peroxidation and triggers the process of apoptosis. Higher GSH concentrations are associated with good health. In the blood serum, the data suggest that while silymarin is effective in restoring normalcy, a higher dose of *Mukta Pishti* is also a promising treatment for PCM-induced conditions. The dosage of *Mukta Pishti* is important, as the low dose is not as effective as the high dose. In the liver serum, the data suggest that silymarin is effective in improving GSH levels in PCM-induced conditions, while *Mukta Pishti*, at either dose, did not show significant improvement.

Superoxide dismutase is an enzyme that inhibits oxidation. An increase in the concentration shows reduced cell damage. Superoxide dismutase serves as the first line of defense against damage brought on by reactive oxygen species (ROS).

SOD catalyzes the conversion of superoxide radical to H^2O^2. Although H^2O^2 is not radical, it is rapidly converted to a highly reactive hydroxy radical through the activity of CAT. (26) Thus; there is a mutual protective relationship between the two enzymes. When the superoxide radical is produced, it is disabled by CAT, while SOD is inhibited by $H^2O^{2\cdot}$ (27) Therefore, the antioxidant defense mechanism is affected by ROS thereby decrease SOD, CAT, and GSH, leading to hepatic damage. (28) The PCM-treated animal group significantly reduce GSH, SOD, and CAT levels compared with the control group. PCM and *Mukta Pishti* treated group reversed the situation. *Mukta Pishti* treated group at 200 mg/kg dose significantly increases the SOD and CAT level. The depletion of cellular GSH and SOD is thought to be due to excessive NAPQI formation because of PCM toxicity. (29) In both the serum, Silymarin and the high dose of *Mukta Pishti* (treatment 2-200mg/kg b.w) showed the highest effectiveness in restoring SOD activity.

Mukta Pishti treatment activates the nuclear factor erythroid 2-related factor (Nrf) 2, increases antioxidant enzyme activities, and modulates the Calcium/Calmodulin-Dependent Protein Kinases (CaMKs) and MAPK pathway inflammation, and apoptosis. The pathway is yet to be established in treating or preventing PCM-induced liver toxicity (Younis et al., 2020).

Poornima et al., have proved that the *Mukta Bhasma* is also having anti-oxidant potential against CCl^4-induced hepatotoxicity. (30)

Mechanism of calcium in free radical scavenging activity in the human body:

When calcium carbonate dissolves, it releases calcium ions into the biological system, which plays a vital role in maintaining the stability and integrity of cell membranes. A proper balance of calcium is essential for ensuring membrane fluidity and preventing lipid peroxidation. Maintaining intracellular calcium homeostasis is crucial for overall cellular health and functionality.

Superoxide dismutase (SOD) primarily relies on metals such as copper, zinc, manganese, or iron as cofactors, and *Mukta Pishti* contains these elements in trace amounts. Calcium ions can indirectly influence SOD activity through various cellular signaling pathways.

In addition to calcium, *Mukta Pishti* also contains iron (Fe), which acts as a cofactor for the enzyme catalase, helping detoxify hydrogen peroxide by converting it into water and oxygen.

Calcium/Calmodulin-Dependent Protein Kinases (CaMKs):

These kinases can be activated by calcium ions and subsequently phosphorylate transcription factors that regulate the expression of antioxidant enzymes, including SOD, CAT, etc. (31)

Nrf2 Pathway:

The nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is a key regulator of antioxidant response. Calcium ions can trigger signaling cascades that enhance the nuclear translocation of Nrf2, resulting in increased expression of SOD and other antioxidant enzymes. (32)

MAPK Pathway:

The mitogen-activated protein kinase (MAPK) pathway can also be influenced by calcium signaling, leading to altered expression of antioxidant enzymes such as SOD and catalase. (33)

Negative feedback:

However, elevated levels of intracellular calcium can result in mitochondrial dysfunction, increasing reactive oxygen species (ROS) production and promoting lipid peroxidation. The activation of certain

calcium-dependent kinases may induce antioxidant enzymes like SOD and catalase, which help alleviate oxidative stress.

Probable mode of action through Ayurvedic point of view

Mukta possesses *deepaniya* and *agnivriddhikara* properties, despite having *madhura rasa* and *sheeta virya*. As stated, "*sarva roga mandagneya*" hence if the *agni (digestive fire)* is corrected and balanced then the physiological function remains in equilibrium and prevents diseases. Psychological factors like r*aga, krodha (anger), irsha (jealousy), and shoka (grief)*, etc. can lead to mental disturbances (*manodusti*) which in turn may cause physical ailments (*sharira vikara*). *Soumya guna* of *Mukta Pishti* can pacify these psychological factors. As *Mukta* is also having *visopaham* property indicating its efficacy against microorganisms or toxins (*garavisha* etc.). Furthermore, "*Prakritastu balam sleshma*" which means *prakrita sleshma* is a vital force or strength (*bala*), so here *Mukta* helps in balancing *prakrita kapha* due to *madhura rasa, madhura vipaka, sheeta veerya, hima and snigdha guna*. *Mukta* is also added in *Vayasthapanam dashemani* and also has qualities like A*yushya, vrishya, kshayapha, virya-bala-buddhi vardhanam*, and *pusti karanam*, etc. which will help in overall to promote health and well-being synergistically.

Conclusion

The current research aimed to screen the free radical scavenging activity and immune-modulatory effect of *Mukta Pishti*. The results have shown In-Vivo a significant effect of free radical scavenging activity by reduction in the MDA level ($p<0.001$), and increase in CAT ($p<0.0001$), SOD ($p<0.0001$), and GSH level ($p<0.001$) in rat's blood serum, represents good antioxidant activity of *Mukta Pishti* given in both the doses, particularly in the dose of 200mg/kg b.w. However, in liver homogenate slightly different result was found compared to blood serum. This might be because of the other enzymes present in the liver which might have hindered the study parameters taken. The mode of action of *Mukta Pishti* in antioxidant activity against PCM may be due to cell membrane stabilization, hepatic cell regeneration, and activation of antioxidant enzymes such as CAT, SOD, and GSH, and maintaining overall cellular health and functionality.

It is used in treatment of bone metabolic disorders associated with calcium deficiency. *Mukta Bhasma* was evaluated for its antioxidant activity in animals. The experimental paradigms used for antiulcer activity were cold restraint stress induced ulcer model and Diclofenac induced ulcer model in rats.

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