

# The properties of nefopam as analgesic co-administration with caffeine as adjuvant in induced pain in mice

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**ABSTRACT:** This study aimed to evaluate nefopam's analgesic properties, explore caffeine's potential adjuvant effects, and examine its impact on renal and hepatic function. The study involved 56 female albino mice, divided into seven groups: Group A, as the control group, received distilled water; Group B received 0.75 mg per body weight of nefopam orally; Group C received 0.71 mg/B.W. of nefopam and 0.125 mg/B.W. of caffeine; Group D received 0.67 mg/B.W. of nefopam and 0.25 mg/B.W. of caffeine; and Group E received 0.63 mg/B.W. of nefopam and 0.37 mg/B.W. of caffeine. Group F received 0.6 mg/B.W. nefopam and 0.5 mg/B.W. caffeine orally; Group G received 0.56 mg/B.W. of nefopam and 0.62 mg/B.W. of caffeine. Several experimental models have been utilised to investigate the analgesic advantages. The hot plate test is a method used to assess the response time to thermal stimuli. Group D produced the most prolonged response duration to thermal stimuli, namely thermal pain, while the writhing test involves the injection of acetic acid, resulting in a significant decrease in the frequency of writhing observed in Group D. In conclusion, Nefopam demonstrated analgesic properties in all experimental groups, even when augmented with caffeine, without any observed changes in renal or liver function.

**KEYWORDS:** Analgesic; Nefopam; Caffeine; Hot Plate; Acetic acid; Renal test; Hepatic test.

## 1. INTRODUCTION

Pain is a sensory and affective sensation resulting from or related to bodily harm or expressed in connection with such harm. Pain can be categorized into nociception, perception, suffering, and pain behaviors, based on anatomical, physiological, and psychological factors. Pain is widely recognized as one of the most significant challenges [1]. The urgency of recall depends on the intensity of the pain [2]. Healthcare providers must possess a thorough understanding of pain to conduct a comprehensive pain evaluation [3].

An analgesic is a pain reliever that targets pain without disrupting nerve signal transmission, sensory experience, or awareness levels. These entities are primarily associated with nociception and pain perception, which are interconnected and contribute to the overall experience of pain. Around 50% of patients requiring surgical intervention received analgesic medications [4]. Analgesia is often experienced after laboratory experiments [5]. The individual is experiencing significant discomfort [6]. Nefopam, a nonopioid analgesic, inhibits the reuptake of dopamine, noradrenaline, and serotonin in the spinal and supraspinal regions. This benzoazocine drug is commonly used as an alternative to opioid analgesics to alleviate moderate-to-severe pain [7]. Nefopam is a drug that has pain-relieving effects similar to those of aspirin, dextropropoxyphene, and pentazocine when taken by mouth. Its effects are also similar to those of moderate amounts of morphine, pethidine, and pentazocine when given intravenously. Excessive nefopam use can cause symptoms like convulsions, restlessness, perceptual changes, reduced urine output, dilated pupils, diminished reflexes, and heightened reflexes [8]. Caffeine is a potent stimulant that stimulates the central nervous system, enhancing brain activity. Caffeine is present in coffee, tea, and soft drinks [9]. Caffeine acts as an antagonist at adenosine receptors, which are believed to be responsible for modulating and transmitting nociceptive signals. Caffeinated drugs are commonly used as standalone treatments or in combination with other therapeutic methods. Nefopam, is a medication that may cause issues in individuals

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with impaired renal function, hence proper oral administration is crucial for optimal therapeutic outcomes [10]. This study aims to explore strategies for enhancing pain management while concurrently mitigating the potential adverse effects associated with nefopam. Furthermore, the research examines the viability of employing a co-administration method to reduce the oral dosages of nefopam, presenting a unique medication combination for pain control, and incorporating caffeine as an adjuvant to enhance pain relief.

## 2. RESULTS and DISCUSSION

### 2.1. Hot plate test:

The hot plate test showed non-significant difference in response time to thermal stimuli among all groups before treatment, but post-treatment analysis showed a significant increase ( $P \leq 0.05$ ) in response time compared to the control group (Group A). The results support the finding of previous work which indicates that nefopam can limit monoamine uptake in synapses, leading to increased levels of noradrenaline, dopamine, and serotonin [11]. This effect can be linked to the notable increase in reaction time to thermal sensitivity observed in all experimental groups receiving nefopam alone or in co-administration with caffeine. However, Group D received a 0.67mg nefopam and 0.25mg caffeine treatment, resulting in the longest reaction time to heat stimuli, with an average duration of  $31.9 \pm 2.44$  seconds, the significant increase ( $P \leq 0.05$ ). In thermal response duration in cohorts treated with nefopam-caffeine amalgamation compared to nefopam alone, due to the potential use of nefopam as an indirect modulator of the N-methyl-D-aspartate receptor which agreed with prior study [12]. Caffeine is rapidly absorbed by the gastrointestinal tract, achieving a bioavailability of up to 100% [13]. On the other hand, the findings are consistent with the research conducted by Kadi et al., who demonstrated that the administration of caffeine to mice resulted in an increase in reaction time when compared to the control groups that received treatments aimed at improving pain tolerance [14]. In Group E, nefopam was administered at a dosage of 0.63mg per body weight (B.W.) and caffeine at a dosage of 0.37 mg per B.W. This resulted in a reaction time to thermal stimuli of  $31.2 \pm 1.96$  seconds. Conversely, Group F received Nefopam at a dosage of 0.6mg per B.W. and caffeine at a dosage of 0.5mg per B.W., which led to a reaction time to thermal stimuli of  $29.6 \pm 1.75$  seconds.

The effects of blocking adenosine receptors, inhibiting phosphodiesterase enzymes, and mobilizing calcium from the endoplasmic reticulum may vary depending on the dosage administered. The result agreed with a prior study by [15], who observed that the affinity for adenosine receptors was only observed in low dosages, whereas high concentrations did not produce the same impact. which revealed the cause of fluctuations in response time to thermal stimuli following exposure to minimal quantities of caffeine. Moving to Group G, which administered nefopam at a dosage of 0.56 mg per body weight and caffeine at a dosage of 0.62 mg per body weight, as a consequence, their response time to heat stimuli was measured to be  $23.9 \pm 1.24$  seconds. Group C was administered nefopam at a dosage of 0.71 mg per body weight and caffeine at a dosage of 0.125 mg per body weight, which exhibited a reaction time to thermal stimuli of  $21.5 \pm 1.28$  seconds.

The administration of high doses of caffeine has been observed to result in analgesic effects in hot-plate experiments, potentially accounting for the differences in response time to heat stimuli. The plasma half-life of the substance under investigation is said to be 5–6 hours, as documented previously [16]. The potential causes for the observed variability in responsiveness to heat stimuli may be linked to the co-administration of varying dosages of caffeine and its half-life. Lastly, group B, who only received Nefopam at a dose of 0.75 mg/B.W., had the lowest reaction time to thermal stimuli at  $17.4 \pm 1.05$  sec. These results were compared to the control group (group A), as shown in Table 1. According to Eiamcharoenwit et al., the mechanism of action of nefopam may entail the modulation of glutamatergic pathways, namely through the involvement of calcium channels, resulting in pain relief [17].

**Table 1.** The hot plate test comparison between difference groups in Average paw withdrawal & licking & jumping

Group	Mean ± SE	
	Before Average paw withdrawal & licking & jumping (sec.)	After Average paw withdrawal & licking & jumping (sec.)
Group A	10.4 ± 0.67 A b	10.5 ± 0.67 C b
Group B	10.2 ± 0.55 A b	17.4 ± 1.05 B a
Group C	10.4 ± 0.62 A b	21.5 ± 1.28 B a
Group D	10.4 ± 0.69 A b	31.9 ± 2.44 A a
Group E	10.4 ± 0.54 A b	31.2 ± 1.96 A a
Group F	9.8 ± 0.38 A b	29.6 ± 1.75 A a
Group G	10.5 ± 0.72 A b	23.9 ± 1.24 B a
LSD value	5.683 *	

Means with different big letters in the same column and small letter in the same row are significantly different. \* (P ≤ 0.05).

## 2.2. Acetic acid

The acetic acid test demonstrated that the control group (group A) had the highest number of writhings (average of abdominal contraction, elongation of body, turning of truck, and full extension of hind paw) ( $21.4 \pm 1.03$ ) per 10-minute duration, which was statistically significant ( $P \leq 0.05$ ) compared to the other treated groups. In contrast, group D, treated with nefopam at a dose of 0.67 mg/B.W. and caffeine at a dose of 0.25 mg/B.W., exhibited the lowest number of writhing episodes ( $12.2 \pm 0.62$ ), representing a significant decrease when compared to group B, treated with nefopam alone at a dose of 0.75 mg/B.W., which recorded a number of writhing episodes of ( $17.2 \pm 0.82$ ), as well as group A (the control group) with a number of writhing episodes of ( $21.4 \pm 1.03$ ).

The findings of this study were consistent with a prior investigation which provided evidence that larger dosages of caffeine can induce intrinsic antinociception [18]. According to results illustrated previously, the plasma-protein binding of caffeine is approximately 10–30%, and its half-life is estimated to be approximately 4 hours [19]. The potential explanation for the observed reduction in writhing in Group D, as opposed to the group treated alone with nefopam and the control group, may be attributed to the strong binding affinity of caffeine to blood proteins and its relatively long half-life.

In contrast, Group B administered nefopam at a dosage of 0.75mg per body weight, resulting in a noteworthy reduction in the number of writhing episodes ( $17.2 \pm 0.82$ ), in comparison to Group A (the control group), which exhibited a higher number of writhing episodes ( $21.4 \pm 1.03$ ). The observed impact of endogenous histamine on mice's writhing antinociception, as supported by prior work [20], can be attributed to nefopam's limited binding affinity for histamine H3 receptors. This observation may perhaps elucidate the notable decline in Group B nefopam, particularly in comparison to the other groups.

In the study, the administration of nefopam at a dose of 0.67mg per body weight and caffeine at a dose of 0.25mg per body weight in group D resulted in a notable reduction in the number of writhings ( $12.2 \pm 0.62$ ) as compared to group B and the control group (group A). As reported previously, serotonergic 5-HT1B receptor antagonists have the potential to disrupt the antinociceptive properties of nefopam in the context of the writhing test [21]. The effects of caffeine may vary depending on the dose administered, as it can block adenosine receptors, inhibit phosphodiesterase enzymes, and induce the release of calcium from the endoplasmic reticulum. A study conducted by Lewandrowski and his co-workers, demonstrated that the affinity for adenosine receptors was detected exclusively at lower levels, while larger doses did not exhibit the same affinity [22]. This observation could perhaps account for the notable reduction in the incidence of writhing observed in Group D as compared to the administration of nefopam alone.

Group C received nefopam at a dose of 0.71 mg per body weight (B.W.) and caffeine at a dose of 0.125mg per B.W., resulting in a recorded number of writhing of  $16.6 \pm 0.59$ . G. On the other hand, Group E was treated with nefopam at a dose of 0.63 mg per B.W. and caffeine at a dose of 0.37mg per B.W., which led to a recorded number of writhing of  $14.6 \pm 0.55$ . Similarly, group F received nefopam at a dose of 0.6 mg per B.W. and caffeine at a dose of 0.5mg per B.W., resulting in a recorded number of writhing of  $13.2 \pm 0.58$ . Lastly, group G was treated with nefopam at a dose of 0.56mg per B.W. and Caffeine at a dose of 0.62 mg per B.W., which led to a recorded number of writhing of  $16.0 \pm 0.61$ , as presented in Table 2. The observable impacts of inhibiting phosphodiesterases (PDEs) are primarily evident at doses much exceeding the current therapeutic levels, primarily due to caffeine's limited affinity for PDEs [23], This observation may potentially elucidate the insignificant difference observed in the groups (E, F, and G) that administered large dosages of caffeine in comparison to the other treated groups.

**Table 2.** Comparison between difference groups in number of writhing (average of abdominal contraction and elongation of body and turning of truck and full extension of hind paw)

Group	Number of writhing(average of abdominal contraction and elongation of body and turning of truck and full extension of hind paw)
Group A	21.4 ±1.03 A
Group B	17.2 ±0.82 B
Group C	16.6 ±0.59 BC
Group D	12.2 ±0.62 C
Group E	14.6 ±0.55 BC
Group F	13.2 ±0.58 BC
Group G	16.0 ±0.61 BC
LSD value	4.877 *

+Means with different letters in the same column are significantly different. \* (P≤0.05).

### 2.3. The Effect of Nefopam-Caffeine combination in Comparison between different groups on serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline enzyme activity of (ALP), Albumin, Total protein and Bilirubin.

The findings indicated that there were non-significant differences ( $P > 0.05$ ) seen in the levels of ALT, AST, and ALP among all groups when compared to the control group, as represented in Table 3. According to previous work, these findings may provide an explanation for the insignificant differences observed in the groups receiving nefopam as a standalone treatment versus those receiving nefopam in conjunction with caffeine to [24]. The results of this study are consistent with the research conducted previously, which also examined the multiple differences in serum ALP levels among groups receiving caffeine administration [25]. The results indicated that there was a non-significant difference between the control group and the groups receiving caffeine. Caffeine undergoes metabolism inside hepatocytes, and the findings indicate that the group administered caffeine did not exhibit any significant alterations in AST and ALT levels. This observation may be attributed to the administration of therapeutic dosages of caffeine, which effectively counteracted any detrimental effects on hepatocytes, hence preventing severe modifications as agreed with prior studies [26].

**Table 3.** The effect of nefopam caffeine co-administration in comparison between different groups in liver enzymes (ALT, AST and ALP).

Groups of mice	Mean ± SE		
	ALT(mu/ml)	AST(mu/ml))	ALP(mu/ml))
Group A	23.91 ±1.22	31.37 ±1.67	67.79 ±2.62
Group B	23.42 ±1.37	31.43 ±1.87	68.56 ±3.17
Group C	23.32 ±1.42	31.22 ±2.07	67.32 ±2.96
GroupD	22.45 ±0.97	31.23 ±1.86	67.21 ±2.68
Group E	22.13 ±1.25	30.26 ±1.65	66.85 ±3.06
Group F	22.51 ±1.03	30.28 ±2.19	66.41 ±2.91
Group G	21.32 ±0.78	30.09 ±1.78	66.23 ±3.76
LSD value	4.378 NS	3.052 NS	6.902 NS

NS: Non-Significant. N=5

These findings were consistent with the recently published research [27]. It has been suggested that the administration of nefopam for a duration of one week leads to improvements in liver function markers such as ALT, AST, ALP, and bilirubin. Additionally, it is known that caffeine does not promote liver damage, which may explain the lack of significant differences observed between the group treated with nefopam alone and the group treated with a combination of nefopam and caffeine. The findings indicated an insignificant difference ( $P > 0.05$ ) in the levels of albumin, total protein, and bilirubin across all groups, as demonstrated in Table 4.

**Table 4.** Effect of nefopam-caffeine co-administration in comparison between difference groups in albumin, total protein and bilirubin.

Group	Mean ± SE		
	Albumin(mg/dl)	Total protein(mg/dl)	Direct Bilirubin(mg/dl)
Group A	4.06 ±0.08	4.17 ±0.07	0.153 ±0.02
Group B	4.56 ±0.06	4.54 ±0.11	0.183 ±0.04
Group C	4.35 ±0.06	4.58 ±0.09	0.189 ±0.04
Group D	4.58 ±0.11	4.61 ±0.09	0.162 ±0.03
Group E	4.50 ±0.08	4.50 ±0.11	0.16 ±0.05
Group F	4.44 ±0.10	4.68 ±0.14	0.158 ±0.02
Group G	4.65 ±0.13	4.52 ±0.08	0.181 ±0.04
LSD value	1.084 NS	0.933 NS	0.056 NS

NS: Non-Significant.N=5

The results were in line with previously published research which reported that there is no statistical significant changes in albumin levels following caffeine use by [28]. The results obtained by Pasutharnchat et al., supported the results of the current research, which suggested that using a minimally effective dosage in conjunction with a brief treatment period could potentially mitigate the likelihood of undesirable effects with unfavorable outcomes [29]. Because of this, the small amount of nefopam used and the short length of the trial may explain why there weren't any clear differences in how well the different groups of people were able to deal with pain. Furthermore, it is possible that the prevalence of adverse effects associated with analgesics may have been underestimated due to the limited timeframe of side effect assessment, which was restricted to a 48-hour period. Hence, it is possible that the occurrence of transient side effects that arose may have been overlooked, thereby accounting for the insignificant variation in liver enzyme levels. This can be attributed to the fact that our nefopam doses were administered within the therapeutic range for a brief period, thereby posing no damage to the liver.

The findings supported previous research, which suggested that the rational approach to pain management involves the combination of analgesic medications [30]. The potential advantages of combining analgesics are evident, including improved patient adherence, increased effectiveness, and the possibility of minimizing adverse effects. There was no significant difference between the groups that received nefopam and caffeine and the control group in the levels of liver enzymes (AST, ALT, ALP, total protein, albumin, and bilirubin).

The findings were consistent with the prior research which showed that caffeine has the potential to mitigate liver damage by preserving the structural integrity of the plasma membrane [31]. This preservation mechanism effectively inhibits the release of enzymes across membranes, hence demonstrating protective effects on the liver. The restoration of marker enzyme activities may be attributed to the introduction of caffeine. A statistical analysis was conducted to evaluate liver function test results among all groups under study. The findings indicated that there was no significant difference between the caffeine group and the control group. This suggests that the treated groups, which received a combination of nefopam and caffeine, also exhibited minor differences when compared to the control group.

#### 2.4 The effect of Nefopam-Caffeine combination in comparison between different groups in kidney function test (Urea, Creatinine).

The findings indicated insignificant differences ( $P>0.05$ ) in the levels of urea and creatinine across all groups, as presented in Table 5, as compared to the control group. Protein catabolism results in the production of urea, while creatine metabolism leads to the formation of creatinine, a byproduct associated with muscular waste. The detection of these substances in the bloodstream serves as an indicator for the existence of renal illness, which is agreed with previous work [32].

**Table 5.** The effect of nefopam-caffeine co-administration in comparison between different groups in urea and creatinine

Groups of mice	Mean $\pm$ SE	
	Urea(mg/dl)	Creatinine (mg/dl)
Group A	22.62 $\pm$ 1.04	0.55 $\pm$ 0.04
Group B	24.05 $\pm$ 1.33	0.67 $\pm$ 0.07
Group C	24.34 $\pm$ 1.16	0.62 $\pm$ 0.07
Group D	21.66 $\pm$ 1.08	0.54 $\pm$ 0.05
Group E	22.14 $\pm$ 1.27	0.56 $\pm$ 0.04
Group F	20.88 $\pm$ 0.93	0.55 $\pm$ 0.04
Group G	21.53 $\pm$ 1.03	0.59 $\pm$ 0.06
LSD value	5.031 NS	0.185 NS

NS: Non-Significant.N=5

According to a prior study, the administration of nefopam orally results in the attainment of peak plasma concentrations ( $C_{max}$ ) within 1-2 hours after dosage. The drug is then removed from the body, with an apparent terminal half-life ( $t_{1/2}$ ) ranging from 3 to 8 hours [33].

The administration of nefopam at therapeutic dosages in individuals with normal renal function has been investigated [34]. This observation could perhaps account for the small disparities observed in urea and creatinine levels across all groups during nefopam therapy.

The results of the current study were in consistent with a previous one, which showed that laboratory tests measuring creatinine levels did not show that the inclusion of nefopam had an impact on renal function [14] and also in agreement with another research that conducted by Mohammed which indicated that the use of caffeine had an effect, although the observed alterations were not statistically significant [35]. In addition, there were a non-significant alterations observed in the plasma concentrations of urea and creatinine. The results of this study are consistent with previous research, which came to the conclusion that there was no statistically significant difference in creatinine levels after the administration of caffeine compared to the control group [36]. Mahdavi and his colleagues demonstrated that there was a non-significant rise in creatine kinase levels during physical activity which explains the Insignificance may be attributed to the relatively brief length of caffeine exposure, which remained within therapeutic values [37].

The findings of the present work were consistent with the prior research that conducted by Mittur [38]. After being administered orally, nefopam exhibits a  $T_{max}$  of 1-2 hours and is removed from the body with an apparent terminal half-life ( $t_{1/2}$ ) ranging from 3 to 8 hours. The maximum plasma concentrations of nefopam are observed at 2 hours, which can account for the insignificant differences in renal function observed in healthy mice. Analgesics are widely used and often misused globally [39].

### 3. CONCLUSION

In conclusion the combination of nefopam and caffeine has been proven to enhance analgesic effects without affecting renal or hepatic function, as per laboratory tests.

### 4. MATERIALS AND METHODS

The animals received veterinarian services [40]. The analgesics that have been authorized for use [41]. The research was granted approval by the institutional animal care and use committee, specifically the College of Veterinary Medicine at the University of Baghdad. The approval, with reference number 2289 and dated October 19, 2023, underwent a thorough review and was subsequently granted approval. The research involved 56 female Swiss albino mice, maintained at the University of Baghdad's College of Veterinary Medicine. The mice were fed a conventional rodent meal, and their conditions were carefully controlled to maintain a temperature of 20°C, a light/dark cycle of 14 hours and 10 hours, and regular air replacement using ventilation vacuums and weekly litter replacement.

#### 4.1. Experimental design

The animals were categorized into seven groups of mice, with each group including eight mice. In this study, as the following :

- Group (A) a control group was administered distilled water.
- Group (B) received 0.75mg of a total dose of 30mg per kg of body weight (B.W.) of nefopam alone at a dosage of 0.75 mg/B.W.

- Group (C) received (95%) a total dose of 30mg per kg B.W. of nefopam [51], at a dosage of 0.71 mg/B.W. along with caffeine at a dosage of 0.125 mg/B.W. of (5%) a total dose of 100mg per kg [52].
- Group(D) received (90%) a total dose of 30mg per kg B.W. of nefopam at a dosage of 0.67 mg/B.W. and caffeine at a dose of 0.25 mg/B.W. of the (10%) total dose of 100mg per kg.
- Group (E), which received nefopam at 0.63mg of the (85%) total dose of 30mg per kg and caffeine at a dose of 0.37 mg/B.W. of the (15%) total dose of 100mg per kg
- Group (F), which received nefopam at 0.6mg of the (80%) total dose of nefopam at a dose of 30mg per kg B.W. and caffeine 0.5mg of the (20%) total dose of caffeine at a dose of 100mg per kg body weight B.W.
- Group (G), which received nefopam 0.56 mg of the (75%) of total dose of 30mg per kg body weight B.W. and caffeine at a dose of 0.62 mg of the (25%) total dose of caffeine at a dose of 100mg per kg of body weight orally.

## 4.2. Preparation of Dosage Solutions and Doses

A 30 mg stock solution was prepared and diluted with 40 ml of distilled water to provide a final dosage of 0.2 ml per 25 grams of mouse body weight [42]. A 100-mg caffeine stock solution was prepared and diluted with 10 ml of distilled water to provide a final dosage of 0.2 mL per 25 g of body weight in mice [43]. A series of dilutions was conducted to create stock solutions of nefopam with varying concentrations. The initial nefopam stock solution was combined with a caffeine stock solution. A 95% nefopam stock solution was prepared by combining 9.5 ml of the initial nefopam stock solution with 0.5 ml of the original caffeine stock solution. A 90% nefopam stock solution was prepared by combining 9 ml of the original nefopam stock solution with 1 ml of the original caffeine stock solution. An 85% nefopam stock solution was prepared by combining 8.5 ml of the original nefopam stock solution with 1.5 ml of the original caffeine stock solution. An 80% nefopam stock solution was prepared by combining 8 ml of the original nefopam stock solution with 2 ml of the original caffeine stock solution. A 75% nefopam stock solution was prepared by combining 7.5 ml of the original nefopam stock solution and 2.5 ml of the original caffeine stock solution.

## 4.3. Evaluation of analgesic

### 4.3.1. Thermally induced pain in mice

The study used a hot plate to measure response time in animals. The hot plate was set at  $54\pm 1$  °C, and each animal was placed on a 50-cm-diameter glass beaker. The time interval between placing the animal on the hot plate and the initiation of behaviors like jumping or licking their paws was recorded. To prevent tissue injury, a time limit of 30 seconds was automatically implemented [44].

### 4.3.2. Acetic acid induced writhing in mice

The mice were given acetic acid. The experimental procedure involved injecting a 0.6% acetic acid solution intraperitoneally, which caused writhing (abdominal contraction and elongation of body and turning of trunk and full extension of hind paw). The observation period for counting episodes lasted from 5-10 minutes post-injection [20,45].

### 4.3.3 Collection of Blood Serum Samples

Biochemical studies of blood offer valuable insights by comparing diseased animals with healthy ones<sup>60</sup>. The experiment involved drawn blood samples from the mouse heart using a syringe, placing them in sterile centrifuge tubes, and then transferring them into Eppendorf tubes for biochemical analysis. The samples were then centrifuged at 4000 revolutions per minute for 15 minutes to separate the transparent serum, after which they were transferred into Eppendorf tubes for further analysis [46].

### 4.3.4. Serum Levels Concentration of Alanine Transaminase (ALT), Aspartate Transaminase (AST), and Alkaline Phosphatase (ALP).

#### A: Alanine Transaminase (ALT):

The alanine aminotransferase (ALT) enzyme converts alanine to 2-oxoglutarate, resulting in the synthesis of pyruvate and glutamate. The catalytic concentration is determined using the lactate dehydrogenase (LDH)-linked reaction, using the average NADH reduction value at 340 nm wave length [47].

### **B: Aspartate Transaminase (AST):**

Aspartate Aminotransferase (AST) is an enzyme that transfers amino groups from aspartate to 2-oxoglutarate, resulting in the production of oxalacetate and glutamate. Its catalytic concentration is determined by quantifying the rate of NADH reduction at 340 nm using the malate dehydrogenase reaction [48].

### **C: Alkaline Phosphatase (ALP):**

The enzyme alkaline phosphatase (ALP) converts a phosphate group from 4-nitrophenyl phosphate to diethanolamine (DEA) under alkaline conditions, resulting in the formation of 4-nitrophenol. A spectrophotometric examination at 405 nm is used to estimate the catalyst's concentration [49].

### **D: Estimation of Direct Bilirubin:**

The sample contains direct bilirubin, which has the ability to form a colored complex when it reacts with diazotized sulfanilic acid. This complex can be measured and detected using spectrophotometry. The presence of cetrimide allows for the observation of both direct and indirect interactions between bilirubin and diazo [50].

### **E: Estimation of albumin**

The procedure uses bromocresol green (BCG), an anionic dye, to bind to a protein under acidic conditions, altering its absorption wavelength. The concentration of albumin in the sample is directly linked to the observed coloration intensity, forming a BCG-albumin complex:

$\text{BCG} + \text{Albumin} \rightarrow \text{BCG-albumin complex}$  [51].

### **F: Blood Urea Nitrogen (BUN)**

Urea, a nitrogenous substance, is synthesised in the liver through the urea cycle and protein metabolism. Kidneys eliminate 85% of urea, while the gastrointestinal system handles the remaining 15%. Elevated serum urea levels are seen in renal dysfunction, dehydration, catabolic states, and high-protein diets. Low protein intake, liver disease, and fasting can reduce urea production.

### **G: Creatinine**

Creatinine clearance is a key indicator of glomerular function, used to measure GFR. It's recommended to collect urine for 24 hours or 5-8 hours, using the following equation:

$$C = (U \times V) / P$$

C = clearance, U = urinary concentration, V = urinary flow rate (volume/time i.e. ml/min), and P = plasma concentration

### **H: Total Protein**

Healthcare professionals analyze serum or urine for protein presence, using the serum total protein test to measure albumin and globulin concentrations [52]. Albumin, a protein comprising 50% of blood plasma, is crucial for controlling fluid extravasation from the vasculature by regulating plasma oncotic pressure [53].

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