Section A-Research paper
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In vivo Antimicrobial activity of N-Acetyl D-Glucosamine against *Salmonella typhimurium* induced infection in Experimental *Wistar* Rats-An Experimental Research

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Abstract

AIM: To test N-Acetyl-D-Glucosamine *in vivo* antimicrobial activity against *Salmonella typhimurium* -induced infectious rat model.

Methods: A *Salmonella typhimurium*-infected rat model was used in the present study to evaluate the anti-microbial activity of NAG. The effects of NAG on infected Wistar rats were studied by using standard methods. Changes in various parameters such as weight gain, water intake, hematological parameters, lipid profile, and histopathology upon administration of NAG were studied.

Results: It has been demonstrated that N-Acetyl-D-Glucosamine has a significant effect on the number of viable *Salmonella typhimurium* recovered from feces and could be able to stop salmonellosis after 8 to 10 days of treatment in male Wistar rats. When compared to the positive control group, NAG had a significant effect on restoring healthy body weight, individual organ weights, hematological parameters, and histopathological changes in liver tissue.

Conclusion: The overall results demonstrated that NAG has the potential to provide an alternative effective treatment against salmonellosis, including typhoid.

Keywords: N-Acetyl-D-Glucosamine, *Salmonella typhimurium*, salmonellosis, typhoid, antimicrobial, Wistar rats.

1. Introduction:

Typhoid is a febrile disease caused by a dangerous bacterium called *Salmonella typhi. S. typhi* is a gram- negative, obligate anaerobe, and rod-shaped bacillus that affects the g.i.t of infected patients. It is transmitted by the fecal-oral route through contaminated food and water. Endo toxins released by gram-negative bacteria are responsible for the pyrogenic activity that occurs upon entry of S. typhi[1]. The two important public health issues are universally caused by systemic infection with the bacterium *Salmonella typhi* and *paratyphi*, respectively, and they are the major causes of morbidity and mortality in developing countries[2]. Typhoid and paratyphoid fevers are acute febrile illnesses that share symptoms such as a gradual onset of sustained fever, chills, hepatosplenomegaly, gastrointestinal hemorrhage, intestinal perforation, abdominal pain, rashes, nausea, anorexia, diarrhea or constipation, headache, vomiting, bradycardia, and unconsciousness [3].

Typhoid fever is considered a disease in developing countries, with the highest annual incidences being in Papua New Guinea (1208 cases per 100,000 population) and Indonesia (> 1000 cases per 100,000 population). In highly endemic areas, typhoid affects mainly the young in Indonesia [4].Although typhoid is curable with available antibiotics, the emergence of antibiotic resistance necessitates the development of new antimicrobial agents against Salmonella species. As the cost of available antibiotics for the treatment of typhoid is costly, it requires a natural, cost-effective antimicrobial agent for the treatment of typhoid.

The most abundant amino polysaccharide in nature is chitin poly (β-(1-4)-N-acetyl-D-glucosamine) [5]. Chitin is a widely available natural hetero polymer of N-Acetyl glucosamine (GlcNAc) and D-glucosamine, which is the second most abundant polysaccharide after cellulose, and it is commonly present in the shells of crustaceans, cuticles of insects, and cell walls of fungi [6]. Chitin and its Chitosan derivatives are both biocompatible, biodegradable, and non-toxic, which makes Chitosan suitable for use in agriculture, cosmetics, water treatment, and medicine [7]. They are also used as antimicrobial and hydrating agents [8].

Chitin, present in shrimp shells, is tightly entangled with proteins and minerals. The low solubility of chitin limits its applications [9]. It is widely used in the food, cosmetics, and pharmaceutical industries. Commercially, it is produced from the acid hydrolysis of chitin, one of the most renewable polymers on earth [10]. Derivatives of chitin are used for various purposes, like immune regulatory [11], antioxidant [12], anti-tumor [13], anti-bacterial [14], antihypertensive [15], and anti-diabetic [16] activities. Various studies on typhoid have revealed that the salmonella infection has occurred only in human hosts, which limits the studies on *S. typhi*. So, the current study was done using *Salmonella typhimurium* instead of *Salmonella typhi*, but *Salmonella typhimurium* can be able to cause similar infectious symptoms as *Salmonella typhi* in rats.

2. Materials and methods

2.1. Drugs and chemicals

N. Acetyl D-Glucosamine test drug, 1% acetic acid used as a solvent to dissolve test drug, Ciprofloxacin, and cyclophosphamide were used as standard antibiotics and immunosuppressor agents were obtained from Sigma Aldrich, Bangalore.

2.2. Test bacterial strains

S. typhimurium was used as an infectious agent in the present study. It was provided by the Indian Institute of Chemical Technology, Hyderabad. The test strain was maintained on an agar slant and stored at 4 °C. Sub-cultures were done just 24 h before antimicrobial testing using a fresh appropriate agar plate, i.e., Salmonella-Shigella agar. The same medium can be used for *in vivo* assays in rats for bacterial counts and identification.

2.3. Culture medium

In this study, Salmonella-Shigella agar was used for the sub-culturing of test bacteria and also for the study of *in vivo* parameters like identification and counting of bacterial cells in the experimental animals.

2.4. Experimental animals

Rats of *Wistar Albino* of the male sex, weighing 150-200 g and aged 7-8 weeks, were purchased from local approved breeders and fed *libitum* in ambient conditions of 12 hr dark and 12 hr light cycle at 23 2 °C. They were acclimatized to experimental conditions at least 1 week before the commencement of study as per CPCSEA guidelines in a registered animal facility (**Registration No:** 2134/PO/Re/S/21/CPCSEA) with a protocol I.D-SPCP/2021-22/Col/04.

2.5. Study design

This study was reported in line with the ARRIVE criteria [17].The study was registered and approved by the institutional animal ethical committee of St.Pauls College of Pharmacy (Registration No: 2134/PO/Re/S/21/CPCSEA) with a protocol I.D-SPCP/2021-22/Col/04.Wistar Albino rats of the male sex were used for the study. The animals which found to abnormal behavior or any pathological signs were excluded from the study. Total of 72 Wistar Albino Rats were taken, randomly grouped into 6, consisting of 12 animals in each. Each rat was kept individually in a separate cage to reduce the risk of cross- infection.

Salmonellosis was induced by the following method:

Initially an immune suppressor drug, Cyclophosphamide, at a dose of 40 mg/kg was administered through the intraperitoneal route to all the rats except control group 1, which served as normal control, receiving only distilled water (neither infected nor treated) to facilitate fast occurrence of infection [18]. On the third day of immunosuppressant administration, all groups of rats fasted overnight and administered with 1.5 ×108 CFU of Salmonella typhimurium in 1ml of 0.9% NaCl by oral route, except for group 1.Group 2 animals were infected but not treated, and served as a negative control. Group 3 received ciprofloxacin as a positive control. To confirm whether the infection had occurred or not, the feces of the animals were collected and examined for bacterial load before one day and after for four days of infection. The establishment of infection has confirmed by a steady increase in the bacterial load during the first four days. Groups 4, 5, and 6 were administered with graded doses of N-acetyl D-glucosamine at 100, 300, and 500 mg/kg, respectively, from day1 to 2 weeks by using oral gavage.

Group1-Normal control-administered with distilled water

Group2-Negative control-administered only with the inducing agent

Group3-Positive control, administered with inducing agent + Ciprofloxacin (14.3mg/kg)

Group4-inducing agent +N-acetyl D-glucosamine 100mg/kg

Group5-inducing agent +N-acetyl D-glucosamine300mg/kg

Group6-inducing agent +N-acetyl D-glucosamine500mg/kg

During the entire study duration, the animals were provided with water, *ad libitum* and maintained at room temperature 23 ± 2 °C with a 12 h light-dark cycle**.** Fecal samples were collected every day following the administration of test inoculums, and the number of bacteria present per gram of feces was determined.

1 g of feces was dissolved in 2 mL of saline (0.9% NaCl) to make a suspension. From that serial dilutions were made to get 100 µL aliquots of faecal suspension in saline distilled water (0.9% NaCl) and then plated on fresh *Salmonella-Shigella* agar plates. Then the plates were incubated overnight at 37 °C and observed for the development of colonies after incubation. By the standard plate count method, the number of colonies developed on *Salmonella-Shigella* agar plates was determined [19].

At the end of the treatment period, the following parameters were evaluated by using standard methods and kits.

2.6. Body weight

All of the animals' body weights were recorded before, during, and after infection. Relative changes in body weight were noted and compared.

2.7. Daily food intake, water intake and percentage of weight gain

Daily food and water intake were measured daily from the amount of food and water supplied. The weight gain or weight loss of animals was measured daily and the percentage of weight gain was calculated.

2.8 Collection of blood and hematological assays

At the end of the treatment, blood was collected from all the groups of rats under chloroform anaesthesia by cardiac puncture in two different tubes, i.e., one tube containing EDTA and the other without EDTA. Blood without EDTA is used for the preparation of serum. Whereas blood collected in the tube with EDTA was used for the determination of haematological parameters.

2.9. Hematological assays

Haematological parameters like white blood cell (WBC) count, red blood cell (RBC) count, haematocrit (Ht) and haemoglobin (Hb) concentrations were determined by using an automatic cell analyser (Bharat Biotech) [20].

For the separation of serum, the blood was allowed to stand for 1 h at room temperature until it formed a clot, then it was refrigerated for another 1 h. Then the mixture was centrifuged at 1,058 rpm for 10 min. The supernatant was separated and stored at −30 °C until used for analysis [21].

2.10. Biochemical assays

The above-mentioned sera were used to measure total cholesterol (TC), high-density lipoprotein (HDL), serum triglycerides (TG), low-density lipoprotein (LDL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) [22].

$$LDL = TC - HDL - (TG/5)$$

2.11. Relative organ weight

After collecting the blood from each animal, the abdominal cavity was cut open and individual organs such as the heart, liver, lungs, spleen, and kidneys were removed, cleaned thoroughly with ice -cold saline, weighed, and stored in paraffin solutions at −80 °C. Changes in the organ weight of all the animals were compared [23].

2.12. Preparation of homogenates

Tissue homogenates of various organs were prepared by grinding 500 mg of each organ in a mortar containing 3.34 mL of saline (0.9% NaCl) and placed on a block of ice. Then the homogenates were centrifuged at 1 058 rpm for 15 min. The supernatants were collected and used for protein assays [24].

2.13. Percentage weight gain, daily feed, and water intake measurement

Daily, the amount of feed and water taken was measured from the quantity of feed and water supplied and the amount remaining after 24 h. The weight increase or weight loss of animals was observed daily and the percentage weight gain was determined [25].

2.14. Histopathological analysis

Small sections of liver tissue were made after sacrificing the animals and were fixed in 10% formalin solutions. The tissue was embedded in paraffin wax and made into 5 micrometer thick sections using a rotary microtome. Then they were stained with haematoxylin and eosin (H&E stain). Then the sections were examined under a light microscope at 100X magnification and the photographs were taken [26].

2.15. Statistical analysis

The collected data were expressed in terms of mean ± SD. One-way ANOVA was used to statistically analyze the data. When the groups were compared using the one-way ANOVA non-parametric test, a P value < 0.05 was considered statistically significant when using the software Graph Pad prism 9.

3. Results

3.1. Antibacterial activity of NAG in rats

In vivo anti-salmonella activity was determined by collecting the faces after induction of infection. The number of viable S. *typhimurium* found in the faecal matter during the first day following the infection was increased. Based on the results obtained, it was found that administration of NAG reduced the number of viable bacteria. The groups 4, 5, and 6 showed no reduction in the number of viable bacteria from the eighth to the tenth day of treatment, whereas the standard drug group showed no reduction in the number of viable salmonella bacteria during the fifth and sixth days of treatment. The number of viable bacteria also decreased in the case of infected and untreated control group animals, but this was observed only three to four days after that of the treated animals.

Figure 1 shows the effect of NAG on faecal shedding. NAG at a dose of 500mg/kg has shown a significant effect (P<0.05) when compared to the standard drug administered group (group3).

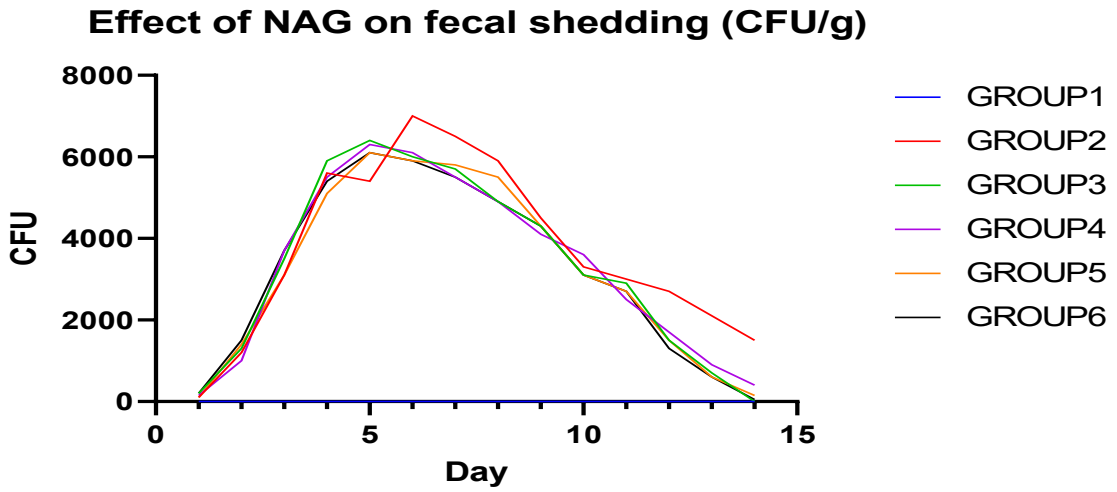

Figure1.Effect of NAG on fecal shedding of Salmonella Typhimurium (CFU/g)

Data expressed as Mean ± SD. Data analyzed by one way ANOVA, P<0.05 considered statistically significant.

3.2. Effect of NAG on the body weight of animals

It was found that there was a reduction in body weight of animals administered with only inducer during the first and second week of the treatment period. The animals administered with different doses of NAG reversed the weight loss observed in the second group of animals. The animals administered with standard drugs also improved the weight gain when compared to the second group.

There was no significant change in food intake during the first week of treatment. A significant change ($P<0.05$) was observed in the intake of food in group 6 when compared to group 3 during the second week of treatment (Table 1).

Table 1: Effect of NAG on food intake in rats during *in vivo* studies

Group	food intake	
	Week 1	Week 2
Group1	113.3±9.86	116.6±9.42
Group2	75.8±6.71	92.3 ±7.88
Group3	110±9.57	113.3±11.05
Group4	83.3±12.13	95.5±11.95
Group5	95±10.80	103.3±3.72
Group6	103.3±10.6	109.1±7.3***

Values are expressed as Mean± SD; all the groups were compared by one way ANOVA non parametric test. $P<0.05$ is considered statistically significant.

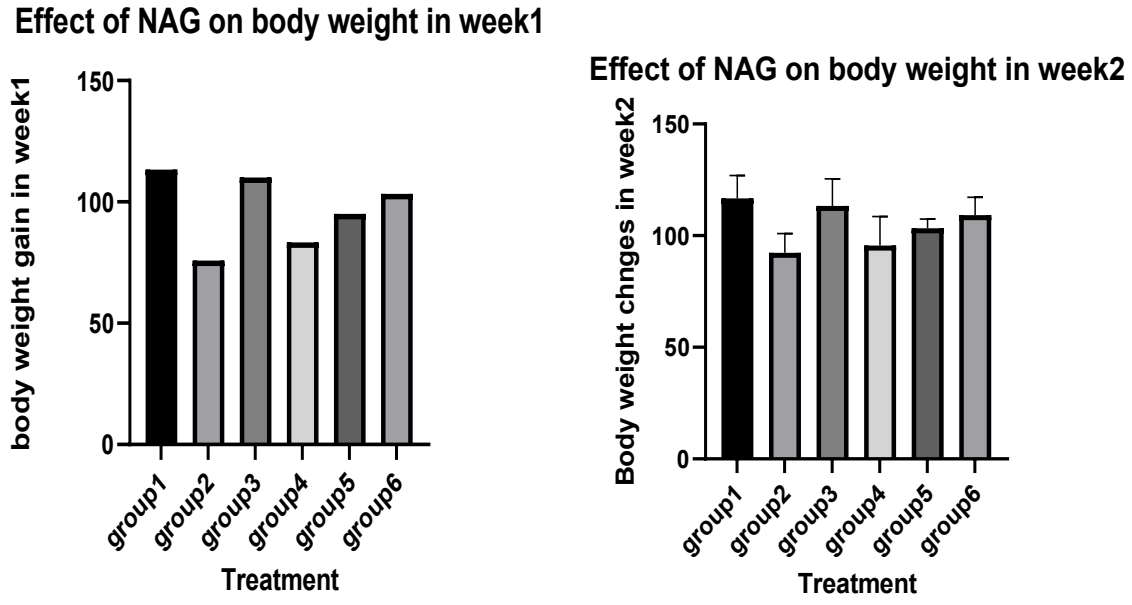

Figure 2. Effect of NAG on food intake in rats during study period (2 weeks)

3.3. Effect of NAG on water intake in rats

There was a significant (P<0.05) change in water intake was observed in the group administered with NAG at a dose of 500 mg/kg during the study period.

Table 2: Effect of NAG on water intake in rats during *in vivo* studies

Groups	Water intake	
	Week1	Week2
Group1	94.5±3.54	98.3±5.52
Group2	84.3±3.49	89.6±4.18
Group3	94.3±3.49	98±4.65
Group4	86.6±2.87	92.6±6.77
Group5	92.6±6.99	93.3±5.52
Group6	94.3±3.49**	95.83±5.33***

Values are expressed as Mean ± SD, all the groups were compared by One way ANOVA non parametric test. P<0.05 is considered statistically significant.

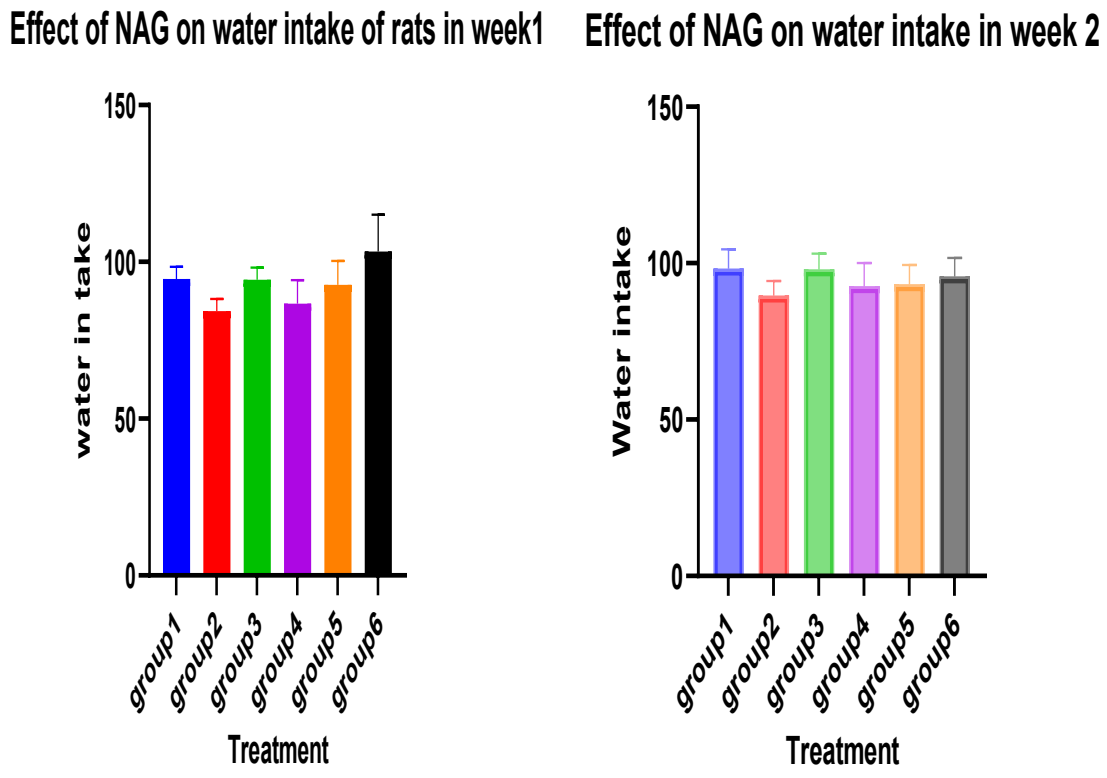

Figure 3. Effect of NAG on water intake in rats during study period (2 weeks)

3.4. Effect of NAG on the relative organ weights

After two weeks of treatment, all the group animals were sacrificed and the weight of individual organs was measured. Administration of different doses of NAG has not shown any significant (P<0.05) effect on weight gain of individual organs except the lungs when compared to a group of animals administered with the standard drug. The effect is greater in animals administered with 500 mg/kg, which shows an effect similar to the standard.

Table 3: Effect of NAG on weight of individual organs

Groups	Liver	Spleen	Kidney	Heart	Lungs
Group1	40.3±3.65	5.05±0.29	11.16±2.57	3.91±0.38	7.55±0.51
Group2	35.5±1.83	4.06±0.55	12.4±0.51	3.65±0.56	10.1±0.47
Group3	39.6±3.95	5.08±0.39	11.63±0.40	4.03±0.20	7.85±0.55
Group4	37.3±3.91	4.23±0.68	12.1±0.58	3.85±0.44	9.96±0.56
Group5	38.6±2.48	4.65±0.68	11.8±0.64	3.88±0.48	8.91±0.40
Group6	40±3.66	4.9±0.54	11.4±0.19	4.06±0.39	7.76±0.512****

Values are expressed as Mean± SD; all the groups were compared by one way ANOVA non parametric test. P<0.05 is considered statistically significant.

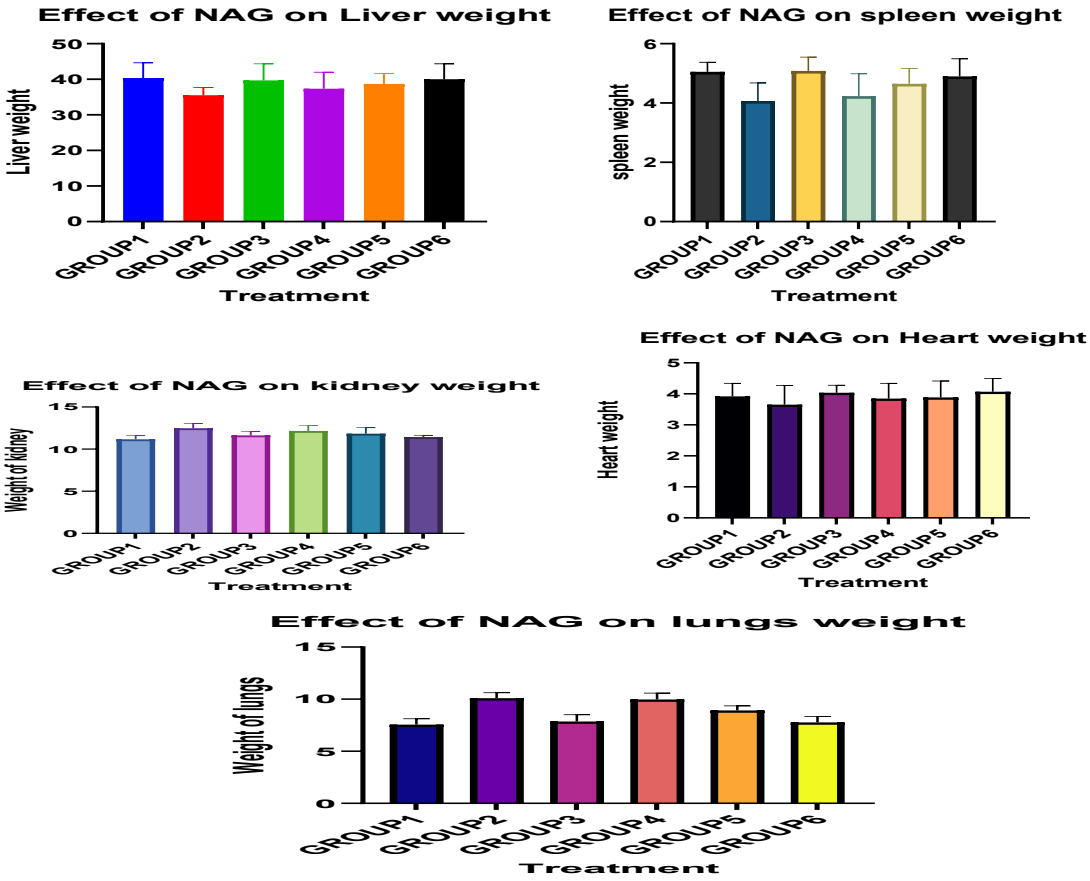

Figure 4: Effect of NAG on weight of individual organs

3.5. Effect of NAG on various hematological parameters

Based on the haematological analysis, it was observed that there was a significant (P<0.05) improvement in the levels of all the haemotological parameters of animals administered with 500mg/kg of NAG compared to animals administered with the standard drug.

Table4. Effect of NAG on hematological parameters followed by 2 weeks of its treatment

Groups	RBC ($\times 10^6/mm^3$)	WBC ($\times 10^6/mm^3$)	Hemoglobin (g/dL)	Hematocrit (%)
Group1	6.28±0.211	5.41±0.24	11±1.29	36.83±1.34
Group2	2.7±0.216	10.08±0.61	8.36±0.99	32.16±1.34
Group3	5.2±0.197	7.16±0.40	11.16±1.06	36±1.15
Group4	4.78±0.26	9.13±0.42	8.71±0.94	29±0.816
Group5	4.98±0.26	6.63±0.60	9±0.86	30.8±1.77
Group6	5.15±0.33****	5.96±0.31****	10.5±1.70**	34±1.29****

Values are expressed as Mean± SD; all the groups were compared by one way ANOVA non parametric test. P<0.05 is considered statistically significant.

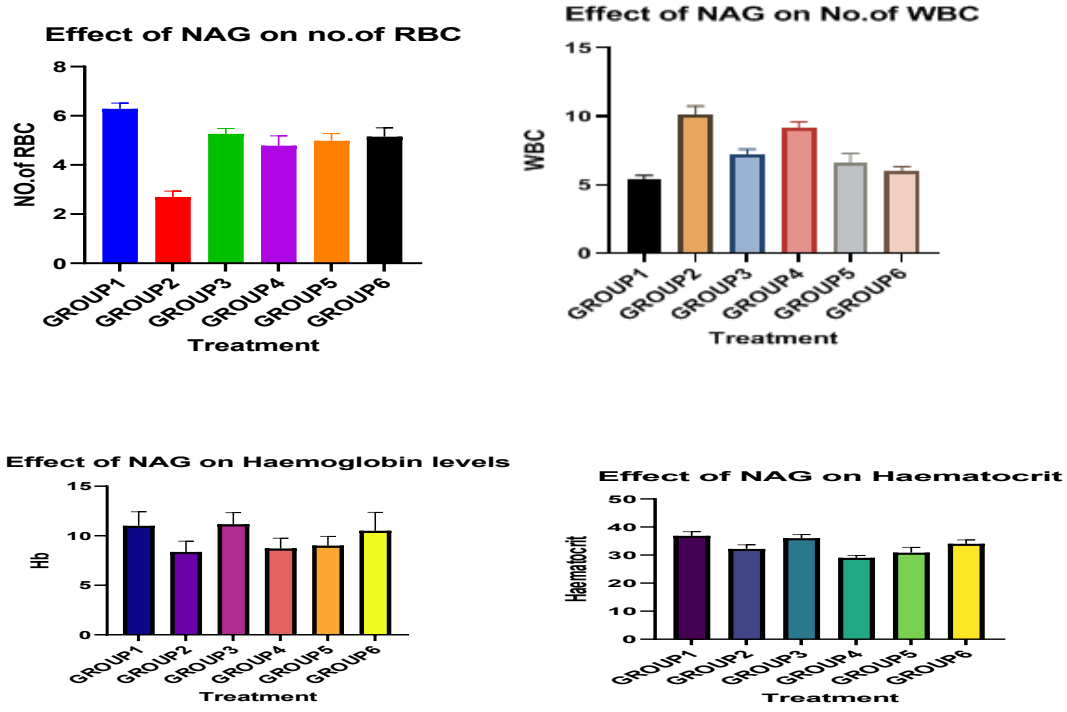

Figure 5: Effect of NAG on hematological parameters followed by 2 weeks of its treatment

3.6. Effect of NAG on serum TC, HDL, LDL and TG

The results of the effect of NAG on the lipid profile after 14 days of treatment demonstrated that there was a rise in the levels of total cholesterol, low density lipoproteins and triglycerides. Administration of NAG has a protective effect on the lipid profile. NAG at 500 mg/kg showed a significant ($P<0.05$) effect on total cholesterol, HDL and triglyceride levels, but there was no significant effect ($P>0.05$) on bad cholesterol (LDL).

Table 5. Effect of NAG on lipid profile followed by 2 weeks of its treatment

Groups	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)
Group1	129±6.71	37.5±9.46	45.6±5.21	76.1±3.67
Group2	85±10.8	31.1±6.33	52.6±8.43	69±2.56
Group3	97.6±8.59	43.1±8.39	43.1±8.27	75.6±5.37
Group4	112.5±8.53	35.5±4.89	51.5±8.03	73.8±4.0
Group5	105.8±9.73	38.6±5.24	50.6±4.22	76±3.62
Group6	103.3±14.33****	40.6±6.47****	48.6±7.40	78±3.41***

Values are expressed as Mean± SD; all the groups were compared by one way ANOVA non parametric test. $P<0.05$ is considered statistically significant.

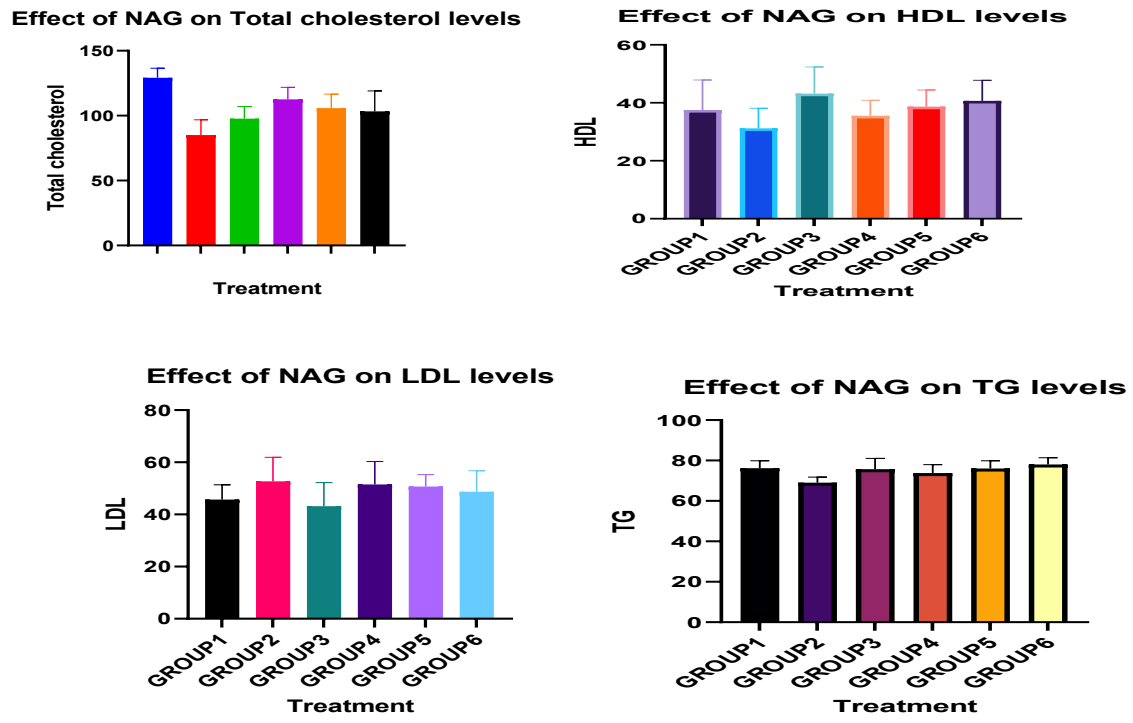

Figure 6: Effect of NAG on lipid profile followed by 2 weeks of its treatment

3.7. Effect of NAG on serum transaminases

Levels of serum transaminases (ALT and AST) after 2 weeks of treatment revealed that AST levels were significantly (P<0.05) elevated in the infected and untreated control group. ALT activity was significantly (P<0.05) increased in groups administered with different doses of NAG and standard drugs.

Table 6. Effect of NAG on levels of ALT and AST in serum samples

Groups	ALT(IU/L)	AST(IU/L)
Group1	34.8±2.67	83.3±1.97
Group2	42.1±1.57	93±3.05
Group3	37.3±1.10	85±2.23
Group4	41.3±2.35	90±3.41
Group5	40.8±1.63	88±3.41
Group6	38±1.63****	87±3.10****

Values are expressed as Mean± SD; all the groups were compared by one way ANOVA non parametric test. P<0.05 is considered statistically significant

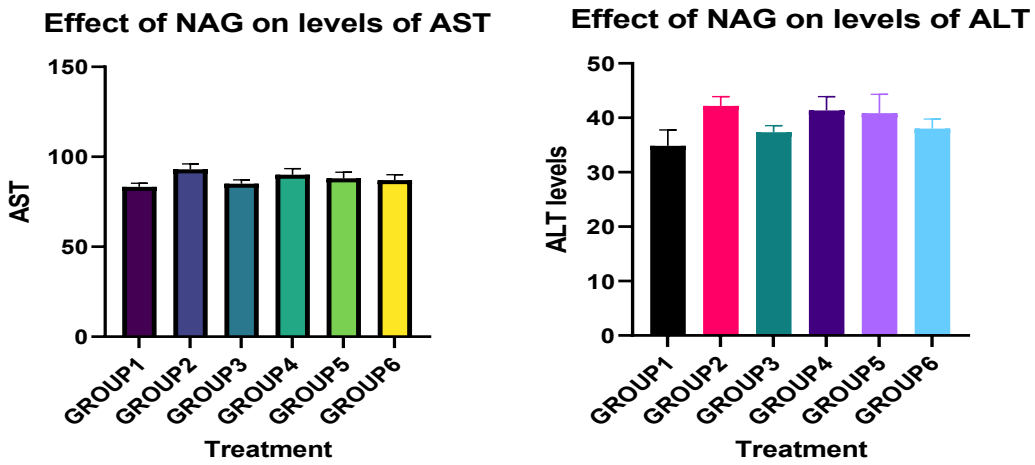

Figure 7: Effect of NAG on levels of ALT and AST in serum samples

3.8. Effect of NAG on histopathology of the liver

Histopathological studies of liver sections have revealed that there was a normal appearance of the liver tissue in animals administered with 500 mg/kg of NAG as a normal control. Histology of group 2, i.e., infected and untreated control group animals, showed dilation of the sinusoid, inflammation of the parenchyma and the portal space. Liver sections of groups treated with 150mg/kg and 300mg/kg of NAG showed slight inflammation of the liver parenchyma and vascular congestion.

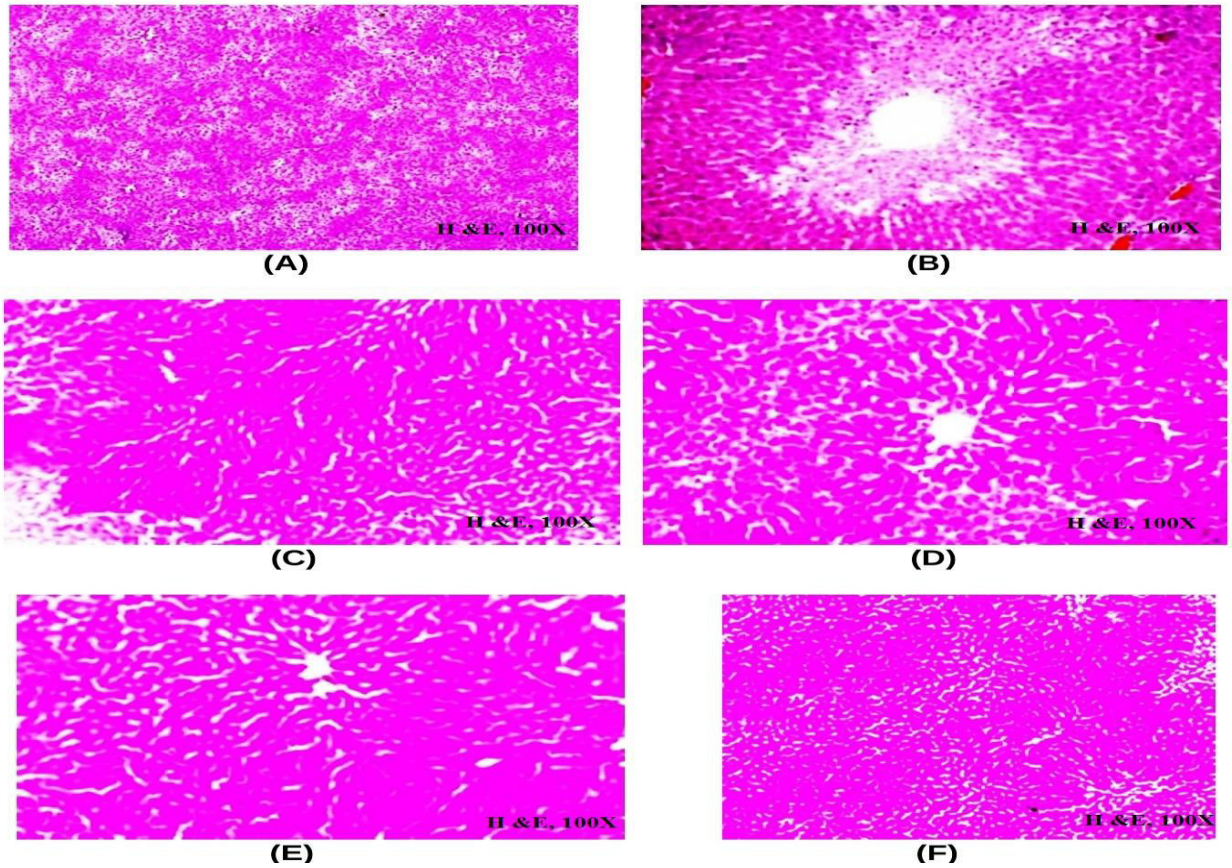

Figure8: Effect of NAG on histopathology of liver tissues

A. Control-Shows normal hepatocytes, **B**. Negative control-shows vacuolated hepatocytes, **C**. Positive control-shows the normal structure ofhepatocyte, D. Infiltrations are moderate when treated with 100mg/kg NAG. **E**-Treated with 300mg/kg NAG shows mild infiltrations, **F**-Treated with 500mg/kg shows the presence of normal hepatocytes.

4. Discussion

Typhoid is a type of enteric fever that affects most of the population every year, causing even deaths. It has become endemic in many areas of the country, requiring the discovery of new antibiotics to combat the gastric infection that occurs due to the feco-oral contamination of food and water. Now a day's polysaccharides are used as Nutraceuticals for the benefit of various ailments. One such polysaccharide is N. Acetyl D-glucosamine, a monomer of polysaccharide chitin found to have anti-microbial properties revealed in traditional uses and *in vitro* studies. In the present study, N. Acetyl D-Glucosamine was subjected to an *in vivo* study to prove its therapeutic efficacy against *salmonella typhimurium* infection. Based on the *in vivo studies,* it has proved that administration of N. Acetyl D-Glucosamine has reduced the number of viable bacteria in the faecal matter during the eighth to tenth day of treatment. This suggests that NAG has a beneficial effect against S. typhimurium infection. It has also been observed that NAG administration has reversed the weight loss observed in a group of animals which are only infected but not treated. A hematological study on blood collected after 14 days of treatment has

revealed that NAG has improved the levels of RBC and haemoglobin but reduced the levels of WBC compared to the disease-induced but not treated group [27].NAG also had a significant impact on the lipid profile, as well as AST and ALT levels. A significant effect on all lipid profiles has been observed at a dose of 500 mg/kg. It also raised the levels of good cholesterol, i.e., HDL.AST and ALT are the liver enzymes, used as markers to detect the presence of hepatic necrosis [28, 29]. Singh et al. [30] found that ALT is present in hepatocytes while the enzyme AST is available in various tissues such as the heart, kidneys, and liver. A rise in the levels of AST and ALT indicates the presence of various diseases [31]. Hepatotoxicity was observed in the group of animals that were infected and not treated. Usually, there is an increase in the activity of serum transaminases in typhoid. This was confirmed by the raised levels of AST, ALT [32,33] and histopathology of liver sections. The administration of NAG has opposed these changes. Histopathology of liver sections of uninfected and untreated control animals (neutral control) and of those infected and treated at doses of 500 mg/kg of NAG showed normal hepatic cell structure with all the prominent cellular organelles. The liver sections of animals, the group infected and untreated control rats, showed dilation of sinusoid, parenchymal inflammation and the portal space due to infection.

5. Conclusion

Based on the above results, it was concluded that N. Acetyl D-Glucosamine has a wide potential to fight against salmonella infection. Administration of NAG has shown a dose-dependent therapeutic effect on an experimentally induced rat model. A significant effect has been observed at a dose of 500mg/kg.

6. Conflict of interest statement

The authors of the present study declare that there is no conflict of interest.

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